Obligately Anaerobic Alkaliphiles from Kenya Soda Lake Sediments

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by

Gerald G. Owenson B.Sc. (Dundee)

Department of Microbiology and Immunology

University of Leicester

Statement

The work in this thesis was carried out by the author during the period October 1992 to March 1996, under the supervision of Prof. W.D. Grant in the Department of Microbiology and Immunology, University of Leicester. This thesis is submitted for the degree of Doctor of Philosophy at the University of Leicester, and has not been submitted in full or part for any other degree.

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Gerald G. Owenson
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Gerald Owenson

During the month of December 1992, an expedition was undertaken to collect anaerobic sediment samples from the alkaline lakes of the East African Rift Valley in Kenya. During this expedition, eleven samples were collected from the four non-saline, northern lakes (Lake Bogoria, Lake Nakuru, Lake Elmenteita and Lake Sonachi). A further five samples were obtained from the hypersaline Lake Magadi. Utilising an array of media, the isolation of alkaliphilic, obligate anaerobes, representing one of the major undescribed constituents of the trophic network, was attempted.

Extensive investigations into the sulphate-reducing bacteria (SRB) community of the sediment samples failed to provide pure culture isolates. However, successful enrichments utilising a range of substrates were obtained. Ethanol as a substrate resulted in the greatest number of positive enrichments, with representatives from each of the northern lakes visited. These are the first indications that ethanol may be used to enrich for alkaliphilic SRB. Lactate also performed well as a growth substrate, whilst acetate, butyrate, formate and fumarate also resulted in one or more positive enrichments. Contrary to previous findings, these data reveal the presence of alkaliphilic SRB capable of utilising a range of substrates. Although positive enrichments under hypersaline conditions were initially obtained using lactate, these cultures could not be maintained.

Using the substrates betaine, trehalose, starch, carboxymethyl cellulose, xylan and guar gum (galactomannan), a number of other organisms were isolated. Despite being enriched under anaerobic conditions, all the isolates were found to be facultatively anaerobic, although exo-enzyme production appeared to take place only under anaerobic conditions.

Six alkaliphilic, obligate anaerobes were isolated from samples taken from two of the northern lakes (Lake Elmenteita and Lake Bogoria) using a complex medium with glucose. Phenotypic and taxonomic data indicated the presence of five species belonging to the Clostridium spectrum, although they were found to be phylogenetically distinct from previously described isolates. Each organism showed optimal growth at alkaline pH, and tolerated only low concentrations of NaCl (ca. 8% w/v).

Enrichment of three Lake Magadi samples resulted in the isolation of three haloalkaliphilic obligate anaerobes. These organisms had an obligate requirement for high NaCl concentrations (> 16% w/v), and showed a limited pH range for growth in the alkaline region (> pH 9.5). Phylogenetic analysis of 16S rDNA revealed these isolates also clustered within the Clostridium region of the low G+C Gram-positive bacteria, although they were also unrelated to any of the previously described species. Taxonomic proposals for a new genus and several new species are presented.
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Mum and Dad
1 Introduction

1.1 Early investigations

The discovery of microscopic life by Antonie van Leeuwenhoek in the 17th century using a simple, single lens microscope, marked the beginning of the science of Microbiology. During this early work Leeuwenhoek observed extensive unicellular life which we know today as the *protozoa, algae, yeasts, bacteria* and *archaea*. More importantly, the incredible abundance, ubiquity and diversity of microbes was first realised.

Despite this early pioneering work, microbiology was slow to develop due to a lack of technology and understanding. It was not until the 19th century that Louis Pasteur, whilst investigating the fermentation of butyric acid, first noticed that certain organisms were unable to grow in the presence of free oxygen. On further investigation he showed that aeration of the medium completely inhibited butyrate fermentation, disproving the previous hypothesis that all organisms required oxygen to survive. Based on this work, Pasteur was the first to introduce the terms *aerobic* and *anaerobic* growth.

Although Pasteur's investigations on the fermentative processes of bacteria were carried out using mixed microbial communities, he was able to draw correct conclusions on the substrate specificity of certain populations. However, the importance of working with single strains was soon realised, and many techniques were developed in an attempt to obtain *axenic* cultures. Robert Koch developed a number of methods for the isolation of microorganisms using solid media, including *streak* and *pour plate* techniques, and the use of *slant cultures*. This work also saw a transition from gelatin as a
hardening agent in solid media to agar, a compound which is used uniformly in microbiology today.

The development of early microbial isolation techniques was completed by Sergius Winogradsky and Martinus Beijerinck, who pioneered the use of the enrichment culture to select for certain groups of micro-organisms based on their nutritional characteristics. This method was seen as in vitro natural selection based on substrate availability, and enabled the 'isolation of micro-organisms with any desired set of nutrient requirements, provided that such organisms exist in nature'.

Isolating organisms from nature with a desired set of nutritional requirements involves locating an environment capable of supporting such growth. Similarly, to isolate micro-organisms capable of growth at environmental extremes, one would investigate naturally occurring extreme environments. It is naïve to assume that merely because the geophysical characteristics of an environment lie outside those for which we are adapted to, then such habitats are incapable of supporting life. Many apparently harsh locations support abundant populations of Bacteria, Eucarya and Archaea. However, it is the prokaryotes which are found in the most extreme environments, where such organisms not only survive, but actively grow and thrive. Indeed, these extremophiles (extreme-loving organisms) are generally unable to survive in conditions which we accept as normal. So what is an extreme environment? The answer to this question depends on the comparison of an environment to ones own optimal conditions. Hence, the term extremophile is somewhat arbitrary, since it is dependent on the accepted 'normal conditions'.

Investigations into life in extreme environments have led to the discovery of organisms living and growing at over $100^\circ$C and below $0^\circ$C, at greater than pH 11 and under pH 1, in saturated salts and solvents, and at many times
atmospheric pressure. As the number of *extremophiles* increases, the boundaries for the possibility of survival are continually being pushed back.

Extreme environments are found throughout the world, both naturally occurring and man made. Naturally occurring environments are, however, more desirable to the microbiologist as these tend to be more stable than the man-made equivalents. For this reason the alkaline lakes of the East African Rift Valley are the perfect location for the isolation of novel alkaliophilic bacteria.
1.2 Geology of the Kenya Rift Valley

1.2.1 Evolution of the rifts

The genesis of the East African Rift System in Kenya occurred over a long period of time, beginning some 25 million years ago. Before this time, the land was extensively covered with trees, and sloped gently towards the ocean. However, for reasons still not fully understood, the crust of the Kenya plateau began to rise, eventually erupting in huge volcanoes, the stumps of which can still be seen today in the forms of Mounts Kilimanjaro, Kenya and Elgon (Smart et al., 1987). Today, volcanic rocks cover nearly 29.8% of the total land area of Kenya (Ojany and Ogendo, 1973). These geological disturbances continued, with the dome continuing to swell and crack from north to south. Following this, the western area sagged into a great depression, filling with water, creating the inland sea of Lake Victoria. Over millions of years the north - south crack in the dome widened forming the immense sunken East African Rift Valley, which begins in the north at the Afar triple junction, and extends southwards across Ethiopia, Kenya and northern Tanzania (Baker et al., 1972).

The East African Rift System is part of the Afro-Arabian Rift System, which extends 6,500 km from Turkey to Mozambique, and which includes the Dead Sea, the Red Sea, the Gulf of Aden and the West African Rift. The East and West African Rifts join through a broad zone of faults in Tanzania (Baker et al., 1972). The Afro-Arabian Rift System is linked, via a ridge at the eastern end of the Gulf of Aden, to the World Oceanic Rift System, which is an almost continuous system of rift valleys encircling the entire Earth (Ojany and Ogendo, 1973).
The term 'rift valley' was first introduced by Gregory in 1896, who noticed that this tectonic feature was due to faulting (Gregory, 1921). The East African Rift, with an average width of 32-56 km, is divisible into five main sections, namely the North Tanzanian Divergence, the Gregory and Karirondo Rifts, the Turkana Depression, the main Ethiopian Rift, and the Afar Depression (Baker et al., 1972). In Kenya the width varies from 56 km in the Magadi section, to over 322 km at the northern end of Lake Turkana (Figure 1.1). The lowest parts of the rift are at the extreme ends, Lake Magadi being 584m above mean sea level, and Lake Turkana being 375m above mean sea level (Ojany and Ogendo, 1973).

The rift was probably formed as a result of tectonic activity not only in the East African section, but also in the Red Sea and Ethiopian sections, and further north in the Rhine Rift Valley. Gregory first forwarded a theory on the origin of the Rift Valley, postulating that tensional forces on the uparched portion of the Earth caused the formation of the rift. Cloos (1955), however, countered that the rift was in fact the result of distensional forces between the two shifting tectonic plates. A further theory, proposed by Wayland (1929), stated that the rift was formed by compressional forces, whilst a more recent hypothesis involved crustal separation, similar to that found during continental drift (Girdler et al., 1969). Further suggestions have included vertical movements, magmatic pressure and isostatic collapse. In reality it is likely that the rift was formed by a combination of the above forces resulting in a mixture of rifts and faults over a large area. It can also be assumed that the tectonic disturbances did not take place at the same time, and it has been shown that movements in the Kenya section of the East African Rift have continued over the last 20 million years (Ojany and Ogendo, 1973). The rift systems in Zambia, Malawi and parts of Tanzania appear to be slightly older than the main Kenya fault lines, the youngest being the grid faults, many of
Figure 1.1 Outline map of Kenya showing the major rift systems and the approximate location of alkaline lakes.
which have occurred in the last 5,000 years. These more recent rifting movements have tended to rejuvenate old fault lines, and appear to be characteristic in the rifting process.

The Rift Valley in Kenya is not one rift system, but rather a complicated series of faults which are, in places, asymmetrical, and which show differing levels of displacement. This suggests numerous tectonic movements and displacements as opposed to a single catastrophic event causing the rift. The main rift forms a gentle S-curved furrow running from north to south, almost through the centre of the country (Figure 1.1). Many minor rifts are found within the main rift, and these are called Grid or Sykes faults (after L.J. Sykes who first studied them in 1939). These grid rifts, which generally run from north to south, have an average throw of 3-15 metres, whereas the main rift has a displacement in the Central Highlands of between 1800 and 3050 metres (Ojany and Ogendo, 1973).

Geophysical studies have indicated that the East African Rift Valley is underlain by a broad (ca. 1000 km wide) zone of hot, low density upper mantle, which is shallowest beneath the domal uplifts of Kenya and Ethiopia (Frostick et al., 1986). Seismic studies have indicated that, within the Kenya and Ethiopia rifts, the major part of the tensile stress is currently being released by micro-earthquakes along their axial zones where geothermal activity and recent volcanism occur (Fairhead and Stuart, 1983).

The area of interest during the present study is the Gregory Rift in Kenya, which extends from Lake Baringo and the Suguta Grabens in the north to the Lake Magadi / Lake Natron Basin in the south. The rift floor descends from 2,000 metres in central Kenya to 650 metres at Lake Magadi in the south. The main fault scarps range from 300 to 1,600 metres in height. The east side of the rift is stepped, producing the Kinangop and Bahati platforms, climbing finally to the Aberdare mountain range. The western side of the rift is
dominated by the Mau Escarpment, and the Mau mountain range. On both sides of the rift there are sloping 'ramp' structures at major fault offsets (Baker et al., 1972).

1.2.2 The Lakes of the Kenya Rift

Although the way in which the Rift Valley was formed, and the time at which this happened, has been the subject of much controversy, it is generally accepted that the rift took its present form during the Middle Pleistocene (100,000 to 75,000 years before present). Prior to this however, during the Lower and early Middle Pleistocene, a great lake is known to have existed, named Lake Kamasia by Professor Gregory, which stretched from Lake Turkana in the north to Lake Magadi in the south. It has already been observed that, although Lake Naivasha would have sat in the middle of what was Lake Kamasia, this area is at a greater altitude than the lakes at either end. It was proposed that the central area must therefore have been subject to an uplifting process since the Middle Pleistocene. This theory is supported by the fact that the Aberdares, which are over 3,000 metres high, show no signs of having had an ice cap during the Lower Pleistocene whereas other East African mountains were glaciated down to 3,000 metres at this time.

Further geological and sedimentation studies revealed that the lakes were not formed by a gradually dwindling lake since Kamasian times, but rather by lakes which oscillated in depth. The high lake levels corresponded to the increased rainfall, or pluvial periods, whereas low lake levels corresponded to low rainfall, or inter-pluvial periods. Three main pluvial periods have occurred in East Africa, namely the Kageran, Kamasian, and Gamblian pluvials, whilst two minor, post-pluvial wet phases, called the Makalian and Nakuran, followed these. It seems that the pluvials coincided with increased solar radiation, a phenomenon which is still apparent today (e.g. exceptionally
high rainfall in 1947 corresponded to very large sun spots, and therefore increased radiation). Following the Kamasian pluvial, a dry period ensued, during which Lake Victoria became little more than a swamp. This probably coincided with the intense volcanic activity and tectonic movements of the late Middle Pleistocene. The early Upper Pleistocene saw the formation of Gamblian Lake (named after Mr Gamble, the owner of the property near Lake Elmenteita where the lake deposits were found). This lake was created during the Gamblian pluvial and covered the area now occupied by Lakes Nakuru, Elmenteita and Naivasha. It is thought that an outlet from Gamblian Lake flowed through the Njorowa Gorge, cutting a vast gully in what is now Hells Gate National Park.

The Gamblian pluvial was followed by the first post-pluvial wet phase, named Makalian after the river Makalia which flows into Lake Nakuru. The second post-pluvial, named Nakuran, occurred recently during the Neolithic period (ca. 2850 years before present). During this time, the level of Lake Nakuru was known to be 45 metres above the present lake level. However, its level has been steadily receding, with minor fluctuations, indicating more arid conditions in the area, and indeed throughout the whole of the African continent.

**Lake Bogoria**

This lake lies at 0° 20' N and 36° 15' E, 945 metres above mean sea level, and is 16 km long and less than 5 km wide. The lake was originally discovered by the Anglican Bishop James Hannington in 1885, who named it Lake Hannington after himself. This name was changed to Lake Bogoria in the 1970's.

Lake Bogoria is shallow and very alkaline, although it seems to maintain a fairly constant level. The lake is fed by hot springs on its western side, both
on the shore (Figure 1.2) and within the lake itself, and by the only powerful stream in the area, the Wasegess River. This collects many tributaries from the Laikipia Escarpment, and a few from the Bechot Plateau, and curves westwards onto the Baringo Plain at Sandai Gorge. From here the river turns south towards Lake Bogoria, and enters the lake at its northern end. During periods of reduced rainfall the Wasegess River dries up completely (Figure 3.3).

It is now known that Lakes Baringo and Bogoria, now separated by the Loboi Plain, were once joined as one large lake. The Loboi Plain is indeed merely an expanse of silt which was laid down by the original lake.

**Lake Nakuru**

South of the equator, at 0° 22' S, 36° 05' E, Lake Nakuru lies at an altitude of 1759 metres above mean sea level. This lake is 12.9 km long by 6.5 km at its widest point, has a mean depth of only 5.6 metres. The lake level has been receding very rapidly over the past few years, and dried up completely during 1930 - 1940, although since then it has been gradually filling up again (Figure 1.3).

The Rift Valley around Lake Nakuru generally has very poor run off, mainly due to the porous nature of the underlying pumice formations which mantle the older rock surfaces. The lake itself does not receive much water from the rivers which enter it, namely the Njoro, Larmudiac, Makalia, Enderit and Ngosur rivers. Instead the lake is maintained mainly by rainwater which falls on the water surface and immediate surrounding area. The Njoro, Larmudiac, Makalia and Enderit streams drain down the Mau Escarpment towards the lake. The majority of the water in these streams is lost, mainly in the porous or fissured zones, as they reach the valley floor, and thus no great volume of
Figure 1.2 Extensive hot spring complex on the western shore of Lake Bogoria.
Figure 1.3 Photograph of Lake Nakuru taken from Baboon Cliff at the north-west shore. Extensive soda flats can clearly been seen, together with large numbers of flamingoes encircling the lake.
water ever reaches the lake. The Ngosur stream, which is permanent, collects run-off from the Bahati Uplands and then flows across the Bahati Plain, disappearing underground before reaching the lake (Figure 3.2). Man-made inflows have recently developed, originating from the sewage treatment operations serving the town of Nakuru.

Lake Elmenteita

Lake Elmenteita, south-west of Lake Nakuru at 0° 25' S, 36° 15' E, has an altitude of 1775 metres above mean sea level and an area of 16 km². Although a maximum depth of 1.9 metres has been recorded, the level of the lake varies considerably depending on the amount of rainfall (Figure 1.4).

The lake is fed by the Mereroni, Mbaruk and Kariandusi streams, and by the water-table below. Mereroni and Mbaruk streams flow southwards off the Bahati Escarpment towards the lake, losing much of their increment through seepage underground as they reach the valley floor. The volume of water contributed by these ephemeral streams is therefore very low. The Kariandusi stream is however permanent, fed by hot springs at the foot of the Bahati Escarpment. This stream is thought to make a greater contribution to the lake water level (Figure 3.1).

Lake Sonachi (Crater Lake)

Lake Sonachi lies within the crater of an extinct volcano, north-west of Lake Naivasha which is the most elevated of the Kenya Rift Valley lakes. Pea green in colour due to the proliferation of cyanobacteria, this lake is a highly alkaline, stagnant pool (Figure 1.5). It is thought there may be a subterranean link between Lake Sonachi and the freshwater Naivasha / Little Naivasha (Oloiden) lakes, although this has never been proven. However, two points
Figure 1.4 Photograph taken at the north-east shore of Lake Elmenteita. Large numbers of flamingoes can be seen lining the south shore in the distance.
Figure 1.5 Extraction of a core sample from the sedge beds bordering Lake Sonachi. The pea-green lake water can be seen in the background.
give credence to this hypothesis: firstly Lake Sonachi seems to maintain the same water level as that of the main lake; and secondly all three lakes are found in the remains of volcanic craters (Figure 3.4).

Lake Magadi

The most southerly lake in the Kenya Rift Valley is Lake Magadi, at 1° 43' - 2° 00' S, 36° 13' - 36° 18' E. There are in fact 2 lakes in this area, Lake Magadi itself which is 660 metres above mean sea level and has a total surface area of 108 km², and Little Magadi to the west of the most northerly tip of the main lake, which is 683 metres above mean sea level. This lake is characterised by vast deposits of trona (sesquicarbonate of soda: \( \text{Na}_2\text{CO}_3\cdot\text{NaHCO}_3\cdot2\text{H}_2\text{O} \)), and is indeed the largest natural source of trona in the world (Figure 1.6). The lake is being worked to produce sodium chloride and anhydrous sodium carbonate used in glass manufacture (Figure 1.7). Despite this, it has been estimated that the trona is renewed more quickly than it is removed, probably by alkaline waters of a deep seated origin. No surface drainage is observed in the Rift Valley between Lake Naivasha at 1885 metres and Lake Magadi at 660 metres. This is again probably due to the porosity of the upper rocks, through which surface water percolates to the water-table beneath.

The upper beds of the Magadi Basin, dated 10,000 years before present, contain very concentrated brines (ca. 270,000 mg l⁻¹) in equilibrium with thick trona beds. Below these beds lie the Oloronga Beds, dated over 800,000 years before present, which contain less saline brines (ca. 130,000 mg l⁻¹) and which partly find their way up to the hot springs. It appears that the saline waters of the lake are part of a vast hydrological complex and deep circulation within the lava formations.
There are no permanent rivers flowing directly into the lake itself, however rivers close by may contribute to the water-table beneath the lake, and therefore indirectly to the lake itself. The principle flowing river in the area is the Ewaso Ngiro, whose tributaries rise on the south-western side of the Mau Escarpment and the hills of the Loita Plains. The Ewaso Ngiro carries a considerable volume of water during the rains due to its large catchment area, and is one of the few rivers in Kenya to rise outside the Rift Valley and flow into, and end in it. South of the Mau Escarpment, tributaries from the Nkuruman Escarpment join the Ewaso Ngiro. These include the Lenkutoto River, which rises on the Lenkutoto Plateau and descends the Nkuruman Escarpment via spectacular falls over 100 metres high. Further tributaries include the Entosapia and Oloibortoto Rivers, which have cut deep gorges in the Nkuruman Escarpment, and which have many minor falls and rapids in their descent to the valley floor. The Ewasa Ngiro River does not flow towards Lake Magadi, but instead ends in the Ngore Ngiro Marsh west of Shombole, 20 km from the south-western end of the lake.

The Turuka River, east of the main lake, rises south-west of the Kajiado Hills and descends several small escarpments before turning south into the Kabongo depression. This stream flows only during periods of heavy rain, forming a small seasonal lake, called Lake Kabongo, which may persist for many seasons after an extremely wet period. Other ephemeral streams in the area include Ndupa to the north and Olkeiu Ngiro to the east, which rises in the Ololokusale Hills.

The lake is fed mainly by a vast number of alkaline springs which rise about 1 metre above the trona beds, and trickle down the foreshore forming alkaline lagoons at the edge of the lake. The springs commonly issue at the base of fault escarpments bounding the lake, suggesting that the liquors rise in the fault zones and then trickle through the scree at the base of the escarpments,
down towards the lake bed. Little Magadi is fed in the north by a series of hot springs which rise along a dry river bed at the base of an escarpment forming the western edge of the lake. The springs combine, producing a relatively large and rapidly flowing stream of hot water which keeps the level of this lake higher than that found in the main lake (Figure 3.5).
Figure 1.6 Photograph showing the vast trona flats of Lake Magadi.
Figure 1.7 Salt harvesting process taking place at Lake Magadi. Mounds of harvested and cleaned salt can be seen in the background.
1.3 Alkaline environments

1.3.1 Man-made alkaline environments

High pH environments may occur as a result of certain industrial processes producing alkaline waste. Such processes include paper (NaOH) and cement (Ca(OH)$_2$) manufacture, alkaline electroplating, food processing (KOH mediated removal of potato skins) and the treatment of hides to remove hairs (NaOH). Although the alkalinity produced by these processes may be somewhat transient, waste outfalls may have a stable alkaline pH.

A number of these industrial processes are, however, unable to support microbial life due to the presence of toxic compounds in the effluent (Grant and Tindall, 1986). Cement manufacture has been shown to produce a pH of over 12, along with high concentrations of toxic anionic and cationic ions, preventing the establishment of a microbial community (Grant and Tindall, 1986).

1.3.2 Alkalinity as a result of biological activity

Alkalinity produced as a result of biological activity tends to be localised and transient (Grant and Tindall, 1986). These alkaline ‘hot-spots’ may be produced as a result of sulphate reduction (Abd-el-Malek and Rizk, 1963a, b), caused by the production of NaHCO$_3$ and small amounts of Na$_2$CO$_3$. It has been postulated that sulphate reducing bacteria may be partly responsible for the naturally occurring natron (hydrated Na$_2$CO$_3$) in Wadi Natrun, Egypt, produced as a result of the bacterial sulphate reduction of sulphate-rich infiltrating water (Abd-el-Malek and Rizk, 1963c). Further biological processes, including oxygenic photosynthesis and ammonification, have also been implicated in the production of alkaline conditions (Brewer and Goldman, 1976; Langworthy, 1978).
1.3.3 High Ca\(^{2+}\) environments

Groundwaters rich in Ca\(^{2+}\) have been found in a variety of locations, including California (Barnes et al., 1972), Cyprus (Pantazis, 1976), Jordan (Barnes et al., 1982), Turkey (Russel, 1996), Oman and the former Yugoslavia (Barnes et al., 1978), although only limited microbial analyses have been undertaken (Bath et al., 1987). Extremely alkaline environments rich in Ca\(^{2+}\), although rare, have been proposed as model systems for concrete degradation (Bath et al., 1987). An understanding of the processes taking place within cement pore waters is of paramount importance since concrete has been used to construct long-term storage repositories for numerous dangerous materials, including radioactive waste.

The alkalinity of Ca\(^{2+}\) springs is produced as a result of the weathering of olivine (MgFeSiO\(_4\)-CO\(_2\)-H\(_2\)O) and pyroxene (MgCaFeSiO\(_3\)-CO\(_2\)-H\(_2\)O) present in the host rock. These calcium and magnesium silicates decompose on exposure to CO\(_2\)-rich surface waters, resulting in the release of Ca\(^{2+}\) and OH\(^-\) into solution. During this process, Mg\(^{2+}\) is removed from solution by immobilisation as serpentine (Mg\(_3\)Si\(_2\)O\(_5\)[OH]\(_4\)) or by precipitation as brucite (Mg[OH]\(_2\)), magnesite (MgCO\(_3\)), dolomite (CaMg[CO\(_3\)]\(_2\)) or huntite (Mg\(_3\)Ca[CO\(_3\)]\(_4\)). Carbonate present in these groundwaters quickly precipitates as calcite (CaCO\(_3\)), leading to a Ca(OH)\(_2\) ↔ Ca\(^{2+}\) + OH\(^-\) equilibrium, producing an extremely alkaline (ca. pH 11.5) brine. This process also results in highly reducing conditions due to the release of Fe\(^{2+}\) and the production of hydrogen by the oxidation of transient metal hydroxides.

Although the waters of Ca\(^{2+}\) rich environments are extremely nitrogen, phosphorous and carbon limited, it has been possible to isolate a small number of micro-organisms (Bath et al., 1987). Most of the organisms isolated from alkaline groundwaters in Oman were found to be facultative anaerobes, reflecting the low redox potential of these environments (\(E_h < -350\)),
mV). However, very few of the organisms were obligate alkaliphiles, suggesting allochthonous rather than autochthonous origin. Based on these findings, Bath et al. postulated that populations within these environments were similar to those found in less extreme conditions.

These data have indicated a need for research into the microbiology of high Ca\(^{2+}\) environments. Further work is required to elucidate the role of microorganisms in the decomposition and nutrient cycling within these industrially important extreme environments.

### 1.3.4 The soda lake environment

Soda lakes and deserts represent the most stable, naturally occurring alkaline environments found throughout the world (Table 1.1). The distinguishing feature of soda lakes is the reduced availability of the divalent cations Mg\(^+\) and Ca\(^+\). These environments are also characterised by high concentrations of sodium carbonate, present as Na\(_2\)CO\(_3\)·10H\(_2\)O and NaHCO\(_3\)·2H\(_2\)O. The presence of this carbonate confers great buffering capacity to the lake waters, which remain at pH 10 - 11.5 despite fluctuating seasonal rainfall (Grant et al., 1990). During periods of reduced rainfall and increased temperature, other salts, notably sodium chloride, are concentrated by evaporative concentration, leading to the development of alkaline and saline conditions. Under certain conditions, significant amounts of NaCl may precipitate causing hypersaline conditions as found at Lake Magadi and the lakes of the Wadi Natrūn.

The genesis of alkalinity in the soda lake environment is extremely complex and dependent on a combination of geographical, climatic and topographical conditions. Firstly, the lakes must lie within a closed drainage basin with little or no outfall. However, the basin must have sufficient inflow to sustain a body of standing water where rates of evaporation exceed the rate of inflow, leading to evaporative concentration of salts (Grant and Tindall, 1986).
Table 1.1 World-wide locations of soda lakes and deserts. Data from Tindall (1988), Grant and Tindall (1986) and Te-Pang (1890).

<table>
<thead>
<tr>
<th>Continent</th>
<th>Country</th>
<th>Location</th>
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<tr>
<td>North America</td>
<td>Canada</td>
<td>Manito</td>
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<td></td>
<td>USA</td>
<td>Alkali Valley, Albert Lake, Lake Lenore, Soap</td>
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<td></td>
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<td>Lake, Big Soda Lake, Owens Lake, Borax Lake,</td>
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<td></td>
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<td>Mono Lake, Searles Lake, Deep Springs, Rhodes</td>
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<td></td>
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<td>Marsh, Harney Lake, Summer Lake, Surprise Valley, Pyramid Lake, Walker</td>
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<td>Lake, Union Pacific Lakes (Green River), Ragtown Soda Lakes</td>
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<tr>
<td>Central America</td>
<td>Mexico</td>
<td>Lake Texcoco</td>
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<td>South America</td>
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<td>Former Yugoslavia</td>
<td>Pecena Slatina</td>
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<td>Asia</td>
<td>Siberia</td>
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<td></td>
<td>Armenia</td>
<td>Barnaul, Slavgerod, Lake Baikal region, Lake Khatyn</td>
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<td></td>
<td>Turkey</td>
<td>Lake Van, Lake Salda</td>
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<td></td>
<td>India</td>
<td>Lake Looner, Lake Sambhar</td>
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<td>China</td>
<td>Outer Mongolia: Various “nors”</td>
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<td>Sui-Yuan: Cha-Han-Nor, Na-Lin-Nor</td>
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<td>Heilungkiang: Hailar, Tsitsihar</td>
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<td>Kirin: Fu-U-Hsein, Taboos-Nor</td>
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<td>Liao-Ning: Tao-Nan Hsein</td>
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<td></td>
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<td>Jehol: various soda lakes</td>
</tr>
</tbody>
</table>
Introduction

Tibet: Alkaline deserts
Chahar: Cheng-Lang-Chi
Shansi: U-Tsu-Hsein
Shensi: Shen-Hsia-Hsein
Kansu: Ning-Hsia-Hsein
Qinhgai Hu

Africa

Libya
Lake Fezzan

Egypt
Wadi Natrūn

Ethiopia
Lake Aranguadi, Lake Kilotes, Lake Abiata, Lake Shala, Lake Chilu, Lake Hertale, Lake Metahara

Sudan
Dariba Lakes

Kenya
Lake Bogoria, Lake Nakuru, Lake Elmenteita, Lake Magadi, Lake Simbi, Crater Lake (Lake Sonachi)

Tanzania
Lake Natron, Lake Embagi, Lake Magad, Lake Manyara, Lake Balangida, Basotu Crater Lake, Lake Kusare, Lake Tulusia, El Kekhooito, Momela Lakes, Lake Lekandiro, Lake Reshitani, Lake Lgarya, Lake Nduu, Lake Rukwa North

Uganda
Lake Katwe, Lake Mahenga, Lake Kikorongo, Lake Nyamunukula, Lake Munyanyange, Lake Murumuli, Lake Bunyampaka

Chad
Lake Bodu, Lake Rombou, Lake Dijikare, Lake Momboio, Lake Yoan

Australia
Lake Corangamite, Red Rock Lake, Lake Werowrap, Lake Chidnup
Typically, soda lakes are found in tropical and subtropical areas, overlying basal rocks consisting of alkaline trachyte lavas. These basal rocks are characterised by low levels of \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \), and high levels of \( \text{Na}^+ \). Such conditions lead to the precipitation of \( \text{Mg}^{2+} \) (as magnesite, \( \text{MgCO}_3 \)) and \( \text{Ca}^{2+} \) (as calcite, \( \text{CaCO}_3 \)), leaving \( \text{Na}^+ \), \( \text{Cl}^- \) and \( \text{HCO}_3^- / \text{CO}_3^{2-} \) as the major ions in solution (Grant and Tindall, 1986; Jones et al., 1994). Alkalinity develops due to a shift in the \( \text{CO}_2 / \text{HCO}_3^- / \text{CO}_3^{2-} \) equilibrium as shown:

\[
2\text{HCO}_3^- \rightarrow \text{CO}_3^{2-} + \text{CO}_2 \uparrow + \text{H}_2\text{O}
\]

Alkalinity evolves concomitant with the precipitation of other ions, most notably \( \text{Na}^+ \) and \( \text{Cl}^- \), leading to the development of alkaline and saline conditions. The relative salinity of any one lake is intrinsically dependent on the localised geological and climatic conditions, resulting in alkaline lakes with salinities ranging from 1% to > 30% (Baker, 1958; Talling and Talling, 1965). Lakes of lower salinity usually have a \( \text{CO}_3^{2-} \) concentration which exceeds that of the \( \text{Cl}^- \) ion, whereas brines of higher salinity have a \( \text{Cl}^- \) concentration greater than that of \( \text{CO}_3^{2-} \) (Grant and Tindall, 1986).

Microbial activity has been implicated in the crystallisation of minerals within saline and alkaline environments (Norton and Grant, 1988). Evidence for this phenomenon is exemplified by the development of a thick trona (\( \text{NaHCO}_3 \cdot \text{Na}_2\text{CO}_3 \cdot 2\text{H}_2\text{O} \)) crust at the hypersaline, alkaline Lake Magadi. If rapid evaporation were solely responsible for the precipitation at Lake Magadi, then thermonatrite (\( \text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O} \)) or natron (\( \text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O} \)) would precipitate in preference to trona (Jones et al., 1994). Therefore, it has been postulated that the addition of \( \text{CO}_2 \) as a result of microbial activity would probably result in the preferential crystallisation of trona. Microbial activity has also been implicated in the precipitation of calcium (calcite and aragonite) and magnesium (magnesite) at Lake Van and Lake Salda in Turkey (Kempe et al., 1991; Russel, 1996). Development of enormous microbialites (ca. 40 m
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high), which are stromatolite-like reefs, are thought to have involved cyanobacteria belonging to the *Pleurocapsa* group.
1.4 Surviving the alkaline environment

Under normal conditions, oxidations via the respiratory chain lead to an extrusion of protons across the bacterial membrane. This movement of protons establishes two gradients across the cell membrane; the trans-membrane gradient of protons ($\Delta p\text{H}$, acid outside); and the trans-membrane electrical charge gradient ($\Delta \Psi$, positive outside). The sum of these two gradients is termed the electrochemical gradient of protons, or proton-motive force ($\Delta \mu_{\text{H}^+}$) where $\Delta \mu_{\text{H}^+} = \Delta \Psi - Z\Delta p\text{H}$ ($Z = -2.3 \frac{RT}{F} = -60 \text{ mV @ 30 } ^\circ\text{C}$ and $\Delta$ values are expressed as $X_{\text{in}} - X_{\text{out}}$) (Padan et al., 1981). Mitchell’s chemiosmotic hypothesis states that $\Delta \mu_{\text{H}^+}$ is generated by respiration-linked proton extrusion, and this in turn energises membrane-associated processes, including ATP synthesis, solute transport and motility (Mitchell, 1966).

Organisms growing at extremes of pH (acidophiles and alkaliophiles) have been shown to maintain an internal pH close to neutrality, two or more units above or below that of the external milieu (Padan et al., 1981; Krulwich and Guffanti, 1983; Booth, 1985; Matin, 1990). Under neutral and acid conditions, this produces $\Delta p\text{H}$ (acid out) and $\Delta \Psi$ (positive out). However, under alkaline conditions, the $\Delta p\text{H}$ is reversed from $\Delta p\text{H}$ (acid out) to $\Delta p\text{H}$ (acid in), together with an increase in $\Delta \Psi$ (still positive out). This raises the question, do alkaliophiles generate an enormous $\Delta \Psi$, compensating for the adverse $\Delta p\text{H}$ and allowing the total $\Delta \mu_{\text{H}^+}$ to reach normal levels, or are mechanisms present allowing ATP synthesis, motility and solute transport to operate under conditions of low total $\Delta \mu_{\text{H}^+}$.
1.4.1 pH homeostasis in alkaliphilic micro-organisms

Na⁺-requirement of extreme alkaliphiles

The majority of extremely alkaliphilic organisms have been found to have an obligate requirement for Na⁺ ions (Krulwich et al., 1981; McLaggan et al., 1984; Krulwich et al., 1988; Ivey et al., 1992). For example Exiguobacterium aurantiacum has an increasing requirement for Na⁺ as a result of an elevation in the external pH (Booth, 1985). The beneficial effects of Na⁺ have also been reported with Bacillus firmus strain RAB, where the addition of Na⁺ resulted in a sevenfold increase in viability at pH 10.0. This was thought to be due to the influence of Na⁺ on maintaining a low cytoplasmic pH (Kitada et al., 1982). Na⁺ dependency has also been demonstrated with the alkaliphilic Bacillus firmus OF4, which was found to have Na⁺-specific pH homeostasis mechanisms (Krulwich et al., 1994). K⁺ was found to be ineffective for the maintenance of internal pH with this organism.

The specificity for Na⁺ over K⁺ ions has also been demonstrated with Bacillus strain YN-2000, where K⁺ actually had a deleterious effect on growth (Koyama et al., 1987). This may have been due in part to the cytoplasmic K⁺ / Na⁺ ratio, the maintenance of which prevents Na⁺ toxicity, but it may also have been due to the Na⁺ specificity of trans-membrane solute transport mechanisms.

Na⁺ / H⁺ antiporter and Na⁺ / solute symporter mechanisms

Evidence suggests that alkaliphiles depend on Na⁺ / H⁺ antiporter systems to maintain intracellular pH levels in the region of pH 7.0 to 9.0, with the usual respiration-coupled extrusion of Na⁺ ions being replaced by at least two antiporter proteins for the uptake of protons (Guffanti et al., 1980; Guffanti, 1983; Kitada et al., 1989; Krulwich and Guffanti, 1989b; Ivey et al., 1992) (Figure 1.8, A). This secondary (ΔΨ-dependent) Na⁺ / H⁺ antiporter is thought
to maintain an acidic cytoplasmic pH relative to $\text{pH}_o$ and generate a $\Delta p\text{Na}^+$ (trans-membrane chemical gradient of sodium). It is not known whether there is a direct relationship between the number of $\text{Na}^+ / \text{H}^+$ antiporter systems, and the alkali-tolerance of the organism.

Following $\text{Na}^+$ extrusion linked to $\text{H}^+$ uptake, $\text{Na}^+$ must then re-enter the cell. This is thought to take place via a number of hypothetical and characterised routes, including $\text{Na}^+ / \text{solute symport mechanisms}$ (Koyama et al., 1976; Kitada and Horikoshi, 1977) (Figure 1.8, B) and flagella-mediated motility (Hirota et al., 1981; Hirota and Imae, 1983; Sugiyama et al., 1985; Muramoto et al., 1994) (Figure 1.8, C).

The regulation of pH in alkaliphiles has also been found to depend on novel cell wall components and adaptations, including the maintenance of a highly negatively charged cell membrane (Aono and Horikoshi, 1983; Aono, 1989). It was proposed that the acidic nature of the cell membrane in alkaliphiles served to exclude hydroxyl ions from the cytoplasm (Krulwich, 1995).

With the characterisation of specific membrane associated proteins, the method by which alkaliphilic organisms maintain their intracellular pH has become clearer. However, the regulation of these membrane processes remains to be elucidated. Transcription or translation of certain respiratory-chain proteins is known to be activated under conditions of low $\Delta \bar{\mu}_\text{H}^+$. (Quirk et al., 1991; 1993). This suggests there may be some mechanism for monitoring of bulk $\Delta \bar{\mu}_\text{H}^+$ (Figure 1.8). Furthermore, it is probable that alkaliphilic organisms possess the ability to monitor $\text{pH}_o$ and also perhaps elevated $\text{Na}^+$ levels (Krulwich, 1995). It therefore appears that the utilisation of $\text{Na}^+$ coupled processes alleviates the problems arising from a very low $\Delta \bar{\mu}_\text{H}^+$ since $\Delta \text{pH}$ would not be a component of the chemiosmotic driving force under these circumstances (Krulwich and Guffanti, 1992).
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$\Delta \mu_{H^+}$-associated ATP synthesis

It has been found that alkaliphiles extrude protons similar to their neutrophilic counterparts at pH values of 10.5 and above (Krulwich and Guffanti, 1989b). However, due to the inverted $\Delta p$H and the resulting low $\Delta \mu_{H^+}$, ATP synthesis via the uptake of protons through the membrane-bound ATP synthase would seem to be unfavourable, if not impossible (Krulwich and Guffanti, 1983).

The ability to generate ATP via ATP synthase at high pH and low bulk $\Delta \mu_{H^+}$ has proved puzzling. Purified ATP synthase from both Bacillus firmus OF4 (Hicks and Krulwich, 1990) and Bacillus alcalophilus (Hoffman and Dimroth, 1991) have shown these proteins utilise $H^+$ ($F_1F_0$-type ATPase) but not $Na^+$ (V-type ATPase) translocation. The reason for this may not yet be clear, but it is possible that, although $Na^+$-coupled ATPase are kinetically competent at maximal $\Delta \mu_{H^+}$ (Kaim and Dimroth, 1994), these enzymes may be kinetically inadequate at low $\Delta \mu_{H^+}$.

It is therefore possible that some mechanism exists to 'feed' the ATPases with $H^+$ that have passed through the proton-translocating respiratory chain components. Numerous models have been proposed which detail a 'hand-off' pathway enabling a proton released by a respiratory component to reach an antiporter or ATPase without first entering the bulk environment (Figure 1.8, D, E and F), a summary of which can be found in Krulwich and Guffanti, 1989b and Krulwich, 1995.

The most probable mechanism involves the recycling of protons within the cell membrane by direct protein-protein transfer, first postulated by Williams (1961) (Figure 1.8, F). This hypothesis is supported by the finding that respiratory components are found in the membrane at very high concentrations (Lewis et al., 1980; Koyama et al., 1986; Quirk et al., 1991; Hicks et al., 1991), suggesting that they are in intimate contact with other
membrane proteins (Ivey et al., 1994). It has been hypothesised that a non-chemiosmotic gating system operates above pH 9.5, effectively preventing protons from entering the bulk, redirecting them towards the ATP synthase (Krulwich and Guffanti, 1992b; Krulwich, 1995). If this system were to operate in alkaliophiles, it would be necessary for at least some protons to enter the bulk, primarily to maintain a small $\Delta \mu_{\text{H}^+}$, which is required to energise H$^+$-associated uptake systems (Krulwich and Guffanti, 1992a).

The true nature of the H$^+$-recycling process has yet to be fully elucidated, but work is at present underway using mutant strains of *Bacillus firmus* OF4 (DA strains) to investigate further the membrane interactions of alkaliophilic organisms (Krulwich et al., 1996).

1.4.2 pH homeostasis in anaerobic alkaliophiles

Due to the relatively recent isolation of alkaliophilic obligate anaerobes, very little work has been carried out on the bioenergetics of these organisms. One study has focused on the ATPase isolated from the facultatively anaerobic alkaliophile *Amphibacillus xylanus* (Koyama, 1996), whilst Ni et al. (1994) have investigated the pH homeostasis of the alkaliophilic methanogen *Methanolobus taylorii* GS-16.

Koyama and co-workers have carried out extensive investigations into the energy production and growth of a facultatively anaerobic alkaliophile (Koyama et al., 1988; Koyama, 1989; Koyama, 1993; Koyama, 1996). Strain Ep01, later classified as *Amphibacillus xylanus* (Niimura et al., 1990), was found to grow optimally at pH 9.5 - 10.0, and had a pH range of 8.0 to 10.5. Early work revealed that, unlike its aerobic alkaliophilic counterparts, *A. xylanus* contained no cytochrome components or quinones in its cell wall. However, the pH of this organism was found to be regulated to around 1.0 to
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Figure 1.8 (Overleaf) Diagram showing the major interaction thought to take place across an alkaliphile cell membrane. Specific interactions include:

A. Na⁺ / H⁺ antiporter system for the maintenance of intracellular pH levels and the generation of ΔpNa⁺.

B. Na⁺ / solute symporter mechanisms for the maintenance of internal Na⁺ and solute levels.

C. Na⁺ uptake energising flagella motility.

D. Proton recycling by intimate membrane association preventing loss to the bulk.

E. Proton recycling by entrapment within an extracellular matrix, preventing loss to the bulk.

F. Proton recycling by intramembrane direct transfer between respiratory components and ATPases, mediated by a gating system dependent on ΔpH.
1.6 pH units below the pHo of 9.5. The presence of a Na⁺ / H⁺ antiporter was confirmed, but the system was found to be non-specific for Na⁺, operating sub-optimally in the presence of Li⁺, Ca⁺, Cs⁺, Rb⁺, K⁺ and Mg²⁺ (Koyama et al., 1988).

A further striking feature of *A. xylanus* was that, despite the extremely low level of ΔµH⁺, and the low levels of respiratory chain components, this organism exhibited similar growth rates to its aerobic counterparts. During attempts to elucidate the aberrant energy-coupling systems of this bacterium, NH₄⁺ was found to be critical for optimum growth at elevated pH (Koyama, 1989), enhancing the uptake of glucose, leucine and other amino acids up to three-fold. It was concluded that the this bacterium possessed a unique transport mechanism, specifically activated by NH₄⁺ (Koyama, 1989).

The stimulatory effects of NH₄⁺ were also demonstrated on ATPase activity of *A. xylanus*. It was found that ATPase activity increased markedly in the presence of both Na⁺ and NH₄⁺, with only a slight increase when Na⁺ and NH₄⁺ were applied individually. This was thought to be indicative of a NH₄⁺-stimulated Na⁺-dependent ATPase (Koyama, 1993). Further investigation revealed it to be a V-type enzyme, analogues of which are present mainly in eukaryotic cells (e.g. Vacuoles, clathrin-coated cells etc.) and Archaea. However, the V-type ATPase of *A. xylanus* differed from similar enzymes isolated from other organisms, and it was proposed that this may constitute a novel NH₄⁺-dependent V-type Na⁺-ATPase (Koyama, 1996), similar to that found in *Enterococcus hirae* (Kakinuma and Igarashi, 1990).

The methods of energy transduction in a newly isolated anaerobic, thermophilic alkaliphile, strain LBS3, have also been investigated (Prowe et al., 1996). The uptake of several amino acids, including leucine, were found to be Na⁺ dependent, although with this organism Li⁺ could not be substituted for Na⁺. Furthermore, the F-type ATPase activity of this strain was non-
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specifically stimulated by the addition of both Na\(^+\) and Li\(^+\) (Prowe et al., 1996). This method of energy transduction differs markedly from that found in aerobic alkaliphiles, which depend on H\(^+\)-linked systems. The method of cytoplasmic pH regulation under these conditions is not clear, but it may possibly be linked to K\(^+\) / H\(^+\) antiporter mechanisms, although further studies need to be carried out on this and similar organisms to elucidate these mechanisms.

Regulation of cytosolic pH in *Methanolobus taylorii* GS-16 has been found to be dependent on K\(^+\) / H\(^+\) antiporter mechanisms (Ni et al., 1994). The use of K\(^+\) over Na\(^+\) in this archaeobacterium is perhaps not surprising since both halophilic archaea (Csonka, 1989) and methanogens (e.g. *Methanobrevibacter arboriphilis*; Matheson, 1985) have been shown to accumulate K\(^+\) in response to salt stress. Indeed K\(^+\) has been found to be the most abundant cation inside the cells of methanogens (Lai et al., 1991).

It has been proposed that an electrochemical gradient of K\(^+\) (\(\Delta \mu_{K^+}\)) exists, mediated by either a K\(^+\)-ATPase, as found in *Streptococcus faecalis* (Kakinuma and Igarashi, 1988), or by a secondary K\(^+\) / H\(^+\) antiporter, similar to the Na\(^+\) / H\(^+\) antiporter systems described above (Ni et al., 1994).

It is impossible to draw conclusions from the limited data available on the bioenergetics of anaerobic alkaliphiles. However, from the studies already carried out, one may postulate that these organisms utilise Na\(^+\) cycling for primary and secondary energy transduction by the development of a transmembrane electrochemical gradient of sodium, \(\Delta \mu_{Na^+}\). The presence of Na\(^+\) / H\(^+\) antiporter systems cannot be ruled out, although a more likely candidate for pH homeostasis may be K\(^+\) / H\(^+\) antiporter systems. Under hypersaline conditions, the possibility of a K\(^+\) activated ATPase cannot be ignored. Generation of a \(\Delta \mu_K\) as opposed to a \(\Delta \mu_{Na^+}\) would prevent the
possible toxic effects of Na\(^+\) build-up, and would also concur with the accumulation of K\(^+\) by other halophilic organisms.
1.5 Alkaliphile diversity in soda lakes

1.5.1 Phototrophic Bacteria

Due to high ambient temperatures and high daily light intensities, combined with the almost unlimited supply of CO₂ from the carbonate charged waters, soda lakes are among the most productive natural environments in the world. The less saline lakes are usually dominated by vast blooms of cyanobacteria, resulting in gross photosynthetic rates of over 30 g O₂ m⁻² day⁻¹ (ca. 11 g C m⁻² day⁻¹) (Melack and Kilham, 1974), whilst the hypersaline lakes of Magadi and the Wadi Natrun support blooms of both cyanobacteria and alkaliphilic anoxygenic phototrophs belonging to the genus *Ectothiorhodospira*. (Imhoff *et al.*, 1978, 1979; Tindall, 1980). The primary productivity of these phototrophic bacteria drives all the biological processes in the soda lake environment.

**Aerobic phototrophs**

Studies into the cyanobacteria populations of the athalassic and brackish lakes have revealed a predominance of the filamentous species *Spirulina platensis*, *Spirulina maxima* and *Cyanospira* sp. (Melack and Kilham, 1974; Talling *et al.*, 1973; Tuite, 1981). The taxonomic position of the *Spirulina* genus has been the subject of some debate (Ciferri, 1983; Grant and Tindall, 1986), with numerous members being misclassified into the genus *Microcoleus* or *Arthrospira* (Rippka *et al.*, 1981). However, *S. platensis* and *S. maxima* are distinct from other members of the *Spirulina* genus since they are obligate alkaliphiles which are inhibited by high concentrations of divalent cations. The genus *Anabaenopsis* has also been recently reclassified as *Cyanospira* (Florenzano *et al.*, 1985), showing the uncertain taxonomic affiliation of a number of these cyanobacteria species.
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Vast populations of lesser flamingos found on a number of the soda lakes are supported solely by blooms of filamentous cyanobacteria. It has been calculated that the population at Lake Nakuru, which may exceed 1 million birds, can consume 182 tonnes cyanobacteria filaments per day (Brown, 1959). This is possible due to the large numbers of *Spirulina platensis*, which may exceed 14,000 filaments ml⁻¹ (Melack and Kilham, 1974).

Unicellular species belonging to either *Chroococcus* sp., *Synechococcus* sp. or *Synechocystis* sp. have also been found, and in some cases these may be the dominant primary producers (Melack and Kilham, 1974; Grant *et al*., 1990). Under hypersaline conditions, such as those found at Lake Magadi, Kenya, unicellular cyanobacteria appear to predominate over filamentous forms (Dubinin *et al*., 1996).

Diatoms belonging to the genera *Nitzchia* and *Navicula* have also been shown to predominate under certain conditions, although this only seems to occur in brackish or freshwater lakes (Hecky and Kilham, 1973).

Anaerobic phototrophs

Blooms of anoxygenic phototrophic bacteria were first recorded by Isachenko (1951) and Jannasch (1957), who classified the organisms as *Chromatium* sp. and *Thiospirillum* sp. Following this early work, a number of other anaerobic phototrophs were isolated from soda lake samples, belonging to the genera *Chromatium*, *Rhodopseudomonas*, *Lamprocystis*, *Thiocapsa* and *Ectothiorhodospira* (Tew, 1966, 1980; Imhoff *et al*., 1978, 1979; Grant *et al*., 1979; Tindall, 1980; Irgens, 1983; Tindall and Trüper, 1984).

However, of the organisms isolated only those belonging to the genus *Ectothiorhodospira* were found to be obligate alkaliphiles, with the others exhibiting varying degrees of alkali-tolerance (Grant and Tindall, 1986). Organisms belonging to the genus *Ectothiorhodospira* were first classified as
members of the Chromatiaceae family, or the Thiorhodaceae as they were originally called (van Niel, 1931). However, the unusual characteristics of these organisms, including growth at alkaline pH (van Niel, 1931; Chesnokov and Shaposhnikov, 1936) and the ability to oxidise sulphide to sulphate, depositing extracellular elemental sulphur as an intermediate (van Niel, 1931; Chesnokov and Shaposhnikov, 1936; Pelsh, 1937), led to the proposal of a new family called the Ectothiorhodaceae (Pelsh, 1937). Following extensive debate on the classification of these organisms (Trüper, 1968; Pfennig, 1977; Tindall, 1980), a new family was created, based on Pelsh's Ectothiorhodaceae, called *Ectothiorhodospiraceae* (Imhoff, 1984). A detailed account of the classification of this genus can be found in Tindall (1980), Stackebrandt et al. (1984) and Grant and Tindall (1986).

More recently, a re-evaluation of the Ectothiorhodospiraceae was proposed by Imhoff and Suling (1996). Utilising 16S rRNA analysis, the genus was separated into two major groups, one retaining the name *Ectothiorhodospira*, whilst the second was designated *Halorhodospira*. Differentiation of the two genera was based both on molecular analysis, including the presence of rDNA signature sequences for each genus, and on physiological properties. *Halorhodospira* species were found to be obligate phototrophs with a minimum concentration for growth of 10% total salts. Members of the genus *Ectothiorhodospira* were able to grow under chemoautotrophic conditions in the dark, and had growth optima well below 10% total salts (Imhoff and Suling, 1996).

Organisms re-classified as *Halorhodospira* included *Halorhodospira halophila* (*Ectothiorhodospira halophila*; Raymond and Sistrom, 1969), *Halorhodospira halochloris* (*Ectothiorhodospira halochloris*; Imhoff and Trüper, 1977) and *Halorhodospira abdelmalekii* (*Ectothiorhodospira*
abdelmalekii, Imhoff and Trüper, 1981). Following this re-classification, the alkaliphilic phenotype was present across both genera.

The broad salt tolerance of certain Ectothiorhodospiraceae is exemplified by the striations found beneath the trona crust at Lake Magadi (Grant and Tindall, 1986; personal observation). Underlying the thick trona crust, which may be coloured light pink due to the presence archaeal halophiles, lies a green layer containing cyanobacteria (Tindall, 1980). Beneath this are two layers containing members of the Ectothiorhodospiraceae, an upper purple-red layer, followed by a further green layer (Imhoff and Trüper, 1977, 1981), which may also house members of the Chlorobiaceae family (Tindall, 1980). The final layer beneath the trona crust of Lake Magadi consists of a thick, black, gelatinous liquor, possibly supporting extensive populations of sulphate-reducing bacteria (Jones et al., 1994) (see Page 48).

1.5.2 Aerobic Chemo-organotrophic Bacteria

Organisms with the ability to grow in highly alkaline conditions were first reported in the 1920's (Meek and Lipman, 1922; Downie and Cruickshank, 1928). However, these early isolates merely showed an extended alkali-tolerance as opposed to being true alkaliphiles. The first validly published obligate alkaliphiles were Bacillus alcalophilus, (Vedder, 1934) and Bacillus pasteurii (Gibson, 1934).

Despite the productivity of soda lake environments, research into non-phototrophic, aerobic, organotrophic bacteria has only recently taken place. This has, however, led to the rapid characterisation of a number of novel alkaliphilic isolates. Isolation of these bacteria has been carried out using a variety of nutrient-rich media (Duckworth et al., 1996), based on standard alkaliphile media (Grant and Tindall, 1980).
Introduction

Viable counts of aerobic organotrophs from the thalassic and brackish lakes (e.g. Bogoria, Elmenteita, Nakuru etc.) revealed populations in the order of $10^5 - 10^6$ cfu ml$^{-1}$ (Grant et al., 1990; Mwatha, 1991), whereas total counts gave populations of $10^7 - 10^8$ bacterial ml$^{-1}$ (Kilham, 1981). Despite widely fluctuating numbers of cyanobacteria during the dry and wet seasons due to varying conductivity and alkalinity, secondary producer populations remain relatively stable (Grant et al., 1990). It was found that the dominant bacterial populations were dependent on a number of variables, including conductivity, alkalinity, phosphate and nitrogen levels (Mwatha, 1991).

The isolation of novel, alkaliophilic bacteria has resulted from initial interest in alkali-stable extracellular enzymes. Enzymes already isolated from alkaliophilic organisms include proteases (Horikoshi, 1971a; Aunstrup et al., 1972), $\alpha$-amylases (Horikoshi, 1971b; Boyer and Ingle, 1972), isoamylase (Ara et al., 1993), pectinases (Horikoshi, 1972; Kelly and Fogarty, 1978), $\beta$-$1,3$-glucanase (Horikoshi and Atsukawa, 1973a), xylanases (Horikoshi and Atsukawa, 1973b; Ikura and Horikoshi, 1977), cellulases (Horikoshi et al., 1984; Fukumori et al., 1985), $\beta$-mannanase and $\beta$-mannosidase (Akino et al., 1987), $\beta$-lactamase (Sunaga et al., 1976), lipases (Watanabe et al., 1977; Horikoshi and Akiba, 1982), catalase (Kurono and Horikoshi, 1973), cyclomaltodextrin glucanotransferases (Nakamura and Horikoshi, 1976a, b) and pullulanase (Nakamura et al., 1975). The biotechnological applications of alkali-stable enzymes have been reviewed in detail (Horikoshi and Akiba, 1982; Krulwich and Guffanti, 1989a; Grant and Horikoshi, 1992), with great emphasis placed on the use of enzymes in biological washing powders (Grant et al., 1990; Horikoshi, 1996).

Numerous aerobic soda lake organotrophs have been isolated, and these have been subjected to extensive chemotaxonomic and phylogenetic analysis.
Introduction

(Jones et al., 1994; Duckworth et al., 1996). The majority of isolates were found to be obligate alkaliphiles, exhibiting no growth below pH 8.0. These organisms were categorised into cell wall groups based on KOH sensitivity (Fluharty and Packard, 1967; Gregersen, 1978), the aminopeptidase reaction (Cerny, 1976) and quinone analysis (Collins and Jones, 1981).

Phylogenetic analysis using 16S rRNA gene sequencing showed that all isolates were members of the γ3 subdivision of the Proteobacteria as defined by Woese et al. (1985), with the majority clustering within the Halomonas / Deleya group. Although these isolates clustered with previously described organisms, they were found to be distinct both phylogenetically and phenotypically (Duckworth et al., 1996).

Phylogenetic analysis of the Gram-positive isolates revealed a dominant association with the low mol % G+C division organisms, most notably with Bacillus sp. (Duckworth et al., 1996). These isolates fell into 3 groups, the first contained the alkaliphilic species Bacillus alcalophilus along with the recently renamed Bacillus clausii and Bacillus halodurans (Nielsen et al., 1995). A second diverse group contained the recently named alkaliphiles Bacillus agaradhaerens and Bacillus clarkii (Nielsen et al., 1995). One further isolate was found to associate most closely with the non-spore forming Lactobacillus and Listeria genera (Duckworth et al., 1996).

It was postulated that the two main groups of soda lake isolates within the low mol % G+C Gram-positives may occupy different niches within the alkaline environment. Those associated with Bacillus alcalophilus appeared to tolerate regions of fluctuating alkalinity and salinity, such as littoral muds. The second group seemed to predominate in lake waters or sediments, which exhibit more stable conditions (Duckworth et al., 1996).
A small number of isolates fell within the high mol % G+C division of the Gram-positive bacteria. One group of isolates were closely related to *Arthrobacter / Terrabacter* sub-group, whilst two further isolates clustered with *Dietzia maris* (formerly *Rhodococcus maris*; Rainey *et al.*, 1995).

### 1.5.3 Anaerobic Bacteria

The anaerobic, alkaline environment has received very little attention compared to that of the aerobic habitat. To date only five alkaliphilic obligate anaerobes have been described in the literature, namely *Clostridium paradoxum* (Li *et al.*, 1993), *Clostridium thermoalcaliphilum* (Li *et al.*, 1994), *Haloanaerobium alcaliphilum* (Tsai *et al.*, 1995), *Spirochaeta asiatica* (Zhilina *et al.*, 1996a) and *Natroniella acetigena* (Zhilina *et al.*, 1996b).

Souza *et al.*, (1974) first reported the isolation of a mesophilic sporeforming anaerobic alkaliphile capable of growth between pH 8.0 and 11.3, with an optimum of pH 9.5. Unfortunately further characterisation of this organism was not carried out. Although there have been reports of alkali-tolerant strict anaerobes isolated from aerobic surface sediments (Shiba *et al.*, 1989), no alkaliphilic strict anaerobes were described prior to *Clostridium paradoxum* (Li *et al.*, 1993).

The alkaliphilic thermophilic anaerobe *Clostridium paradoxum* was isolated from anaerobic digestors in Athens and Atlanta, Georgia, USA. This organism had a pH optimum of 9.8 - 10.3 and an optimal temperature of 50 - 58 °C for growth, although growth occurred up to pH 11.0 and above 60 °C (Li *et al.*, 1993). Unusually, this strain of *Clostridium* appeared to maintain motility once cells had sporulated, facilitated by two to six peritrichous flagella. Substrates utilised in the presence of yeast extract included glucose, fructose, sucrose, maltose and pyruvate, resulting in the end-products acetate, CO₂ and H₂, along with one further unidentified product (Li *et al.*, 1993).
Clostridium thermoalcaliphilum was also isolated from an anaerobic digester in Atlanta, Georgia, USA (Li et al., 1994). Phylogenetic analysis using 16S rRNA sequence data showed that this organism exhibited a 2% evolutionary distance from Clostridium paradoxum. However, %G+C content, temperature range for growth, asporegenesis, cell wall composition and envelope structure, showed that this organism did not belong to the species Clostridium paradoxum. Clostridium thermoalcaliphilum was found to be a motile (two to twelve peritrichous flagella), non-sporeforming rod with an optimum pH of 9.5 - 10.0 and a temperature optimum of 48 - 51 °C. The organism was capable of growth from pH 7.0 to pH 11.0, and from 27 °C to 57.5 °C. Yeast extract was required for growth, which occurred on glucose, fructose, sucrose, maltose, cellobiose and casamino acids. The main end-products of fermentation were found to be acetate, H₃, isovalerate and lactate, along with one further unidentified product similar to that found with Clostridium paradoxum (Li et al., 1994).

Following the publication of these first two strictly anaerobic alkaliphiles, a further isolate was described by Tsai et al. (1995). This organism was isolated from a sample taken 7 m beneath the salt crust of Great Salt Lake, Utah, USA. 16S rRNA sequence analysis showed it to be a new species of the genus Haloanaerobium, subsequently named Haloanaerobium alcaliphilum. The organism showed an optimal NaCl concentration of 10%, although growth was observed between 2.5 and 25% NaCl. The optimum temperature was 37 °C, with growth occurring between 25 and 50 °C, and the optimum pH for growth was found to be pH 6.7 - 7.0. Substrates utilised in the presence of yeast extract included fructose, glucose, maltose, mannose, sucrose, pyruvate, glycine betaine and N-acetylglucosamine. Fermentation end-products were shown to be acetate, butyrate, lactate, H₃ and CO₂.
Although *Haloanaerobium alcaliphilum* had a reported pH range for growth of 5.8 to 10.0, it seems that the species name may be somewhat inappropriate. The term *alcaliphilum* (*alcali*, from Arabic *al*, end, and *qaliy*, soda ash; *philum*, loving) suggests the organism 'loves' or prefers alkaline media. However, it is apparent that this is not the case, with the organism only tolerating media above neutrality. Based on the definitions of Krulwich and Guffanti (1989a), this organism is alkali-tolerant, since it has an optimum pH in the range of 7.0 - 9.0 (in this case pH 6.7 - 7.0). Although the boundaries of the alkali-tolerant and alkaliphilic phenotype should be regarded as somewhat flexible, this example is obviously not a true alkaliphile. Perhaps, therefore, a more appropriate name for this Great Salt Lake isolate would have been 'Haloanaerobium alcalitolerans'.

A strictly anaerobic alkaliphile, named *Spirochaeta asiatica* (Zhilina *et al*., 1996a), was isolated from Lake Khatyn in the Ulug-Khem Valley, Tuva, Russia. Members of the genus *Spirochaeta* have been isolated from a range of extreme environments, including hot springs (Patel *et al*., 1985; Aksenova *et al*., 1990), low-temperature lakes (Franzmann and Rohde, 1992) and hypersaline environments (Greenberg and Canale-Parola, 1976). However, *Spirochaeta asiatica*, together with the aero-tolerant isolates *Spirochaeta alkalica* and *Spirochaeta africana* isolated from Lake Magadi, Kenya, are the first alkaliphilic spirochetes to be described. *Spirochaeta asiatica* was found to have an optimal pH of 8.4 - 9.4, and showed limited growth within the range of pH 7.9 - 9.7. A minimum concentration of 2% NaCl was required, whilst no growth occurred above 8% NaCl. The optimum temperature was found to be 33 - 37 °C, although temperatures within the range of 20 - 43 °C were tolerated. This organism was able to utilise glucose, maltose, glycogen, mannose, trehalose, cellobiose, saccharose, starch, galactose, pectin and
xylane as growth substrates. End-products of glucose fermentation were found to include acetate, ethanol and lactate, although $\text{H}_2$ was not detected.

*Natroniella acetigena*, an extremely haloalkaliphilic, homoacetic bacterium, was isolated from beneath the trona deposits of Lake Magadi, Kenya. This organism grew in alkaline medium at pH 8.1 to 10.7, with an optimum pH of 9.7 to 10.0. The optimum salinity was found to be 12% NaCl, although it could survive in medium ranging from 10% to 20% NaCl. Being a homoacetic bacterium, the major product of fermentation was found to be acetate, with only small amounts of propionate produced when grown on propanol. Further substrates suitable for growth included lactate, pyruvate, glutamate, and ethanol. An obligate requirement for carbonate and NaCl was found, and growth was enhanced in the presence of yeast extract, suggesting the need for growth factors. Phylogenetic analysis of 16S rDNA sequence data placed *Natroniella acetigena* within the order Haloanaerobiales, in the group Halobacteroidaceae (Zhilina *et al.*, 1996b).

The limited nutritional characteristics of this organism are consistent with the habitat from which it was isolated. It is probable that this isolate would utilise the products of the primary anaerobes, the spirochetes, described above. The asporogenensis of this isolate together with the apparent rapid lysis in pure culture may indicate a propensity for development in a mixed community (Zhilina *et al.*, 1996b).

Although only a small number of organisms have been isolated in pure culture, anaerobic enrichments have successfully been obtained from alkaline environments (Wenk and Bachofen, 1995; Zhilina and Zavarzin, 1994).

Analysis of mixed alkaliphilic communities have exemplified the diversity of the alkaliphilic phenotype within anaerobic environments. Zhilina and Zavarzin (1994) described an alkaliphilic anaerobic community at pH 10.0, showing positive enrichments for bacteriolytic and cellulolytic bacteria, and
pure culture representatives of saccharolytic, proteolytic, acetogenic, methanogenic and sulphate-reducing bacteria. A trophic network for the decomposition of organic matter in alkaline sediments was postulated, showing interactions between the various functional groups of organisms (Zhilina et al., 1996a).

Although a number of organisms were presented as pure culture representatives of the main trophic groups, only the spirochetes and one haloalkaliphilic isolate have been further characterised (Zhilina et al., 1996a; Zhilina et al., 1996b). This suggests that either characterisation is currently progressing, or that these isolates could not be maintained in pure culture.

Wenk and Bachofen (1995) also presented evidence of positive enrichments for fermentative, sulphate-reducing and methanogenic bacteria, although no pure culture isolates were obtained. Growth was tested under a range of oligotrophic and eutrophic conditions, showing that eutrophication led to a shift in pH optima from 9.0 - 10.0 to 10.5 - 12.0.

These studies show that alkaliphiles are not restricted to one genus of anaerobic bacteria. They also reveal a great deal of nutritional diversity, enabling the organisms to utilise a number of substrates depending on the availability within the natural habitat. Of course, the organisms isolated to date are a result of the enrichment and isolation procedure and may not be representative of the dominant naturally occurring populations. However, the investigations into the growth of mixed cultures on a wide variety of substrates may give an insight into the nutritional diversity of the organisms present in alkaline sediments.

1.5.4 Sulphate-reducing bacteria

The dissimilatory sulphate-reducing bacteria (SRB) are unique in their ability to utilise inorganic sulphate as a terminal electron acceptor. Due to the
relatively large amounts of sulphate required for this process, massive quantities of hydrogen sulfide are released into the immediate environment as an end product. Because of the deleterious consequences that result from the growth of SRB, their physiology, biochemistry and ecology have been much studied.

The first SRB were isolated from a canal in Delft, The Netherlands by W.M. Beyerinck over 100 years ago (Beyerinck, 1895). This original isolate was named *Spirillum desulfuricans* based on its morphological and physiological characteristics, but it is certain he in fact isolated the first *Desulfovibrio* species (Kluyver and van Niel, 1936; Postgate and Campbell, 1966). Difficulty in obtaining pure cultures of SRB and maintaining them under laboratory conditions has led to a number of contradictory reports to be published (see Stackebrandt *et al.*, 1995). Extensive research into these salient micro-organisms has uncovered many new genera, including Gram-positives (*Desulfotomaculum*: Campbell and Postgate, 1965; Widdel and Hansen, 1992), Gram-negatives (*Desulfovibrio, Desulfomicrobium, Desulfobulbus, Desulfobacter, Desulfobacterium, Desulfoarcina, Desulfomonile, Desulfonema, Desulfobotulus, Desulfoarculus, Desulfohalobium, Thermodesulfobacterium*: Widdel and Hansen, 1992) and Archaea (*Archaeoglobus*: Stetter, 1988; Burggraf *et al.*, 1990).

Originally thought to be obligate anaerobes, these organisms have been isolated from highly aerated superficial waters and lake sediments (Hata *et al.*, 1964; Novozhilova and Berezina, 1968). These early findings suggested that SRB possessed protective mechanisms against oxygen exposure. Hardy and Hamilton (1981) confirmed this hypothesis by showing that a superoxide dismutase-containing strain of *Desulfovibrio vulgaris* was able to survive in both oxygenated and deoxygenated seawater. Superoxide (O$_2^-$), the toxic free radical of O$_2$, is produced as a result of the oxidation of flavoproteins (and
Introduction

possibly as a result of enzyme-catalysed oxidations and oxygenations). Superoxide dismutase prevents the possibly lethal accumulation of superoxide under aerobic and microaerophilic conditions, via the following reaction:

\[ \text{Superoxide dismutase} \quad 2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \]

To prevent the accumulation of H\(_2\)O\(_2\) most organisms, including SRB (Hatchikian, 1970), contain the enzyme catalase, which decomposes hydrogen peroxide to oxygen and water:

\[ \text{catalase} \quad 2H_2O_2 \rightarrow 2H_2O + O_2 \]

However, at that time it was concluded that its presence was as a defence mechanism to protect the strict anaerobes from oxygen contamination.

More recently it has been shown that some SRB are capable of microaerophilic growth, utilising a number of organic and inorganic growth substrates (Dilling and Cypionka, 1990; Abdollahi and Wimpenny, 1990). The isolation of SRB from the oxic zones of microbial mats (Canfield and Des Marais, 1991) and from within the oxic / anoxic interface of deep waters and sediments (Battersby et al., 1985; Hastings and Emerson, 1988) has further confirmed the aerotolerant nature of these organisms.

16S rRNA sequence analysis using several Desulfovibrio sp. and a number of aerobic bacteria has led to the rather surprising hypothesis that the Desulfovibrio sp. evolved from aerobic bacteria (Devereux et al., 1990). A full and detailed account on the effects of oxygen on the growth of SRB can be found in Le Gall and Xavier (1996).
Bioenergetics

SRB play a key role in the biological cycling of sulphur within natural environments. Although most bacteria, fungi and plants reduce the sulphate ion ($\text{SO}_4^{2-}$) to sulphide ($\text{S}^2$) prior to incorporation into amino acids (assimilatory sulphate reduction) (Postgate, 1984), the SRB are able to utilise the sulphate ion as an oxidant for the degradation of organic material (dissimilatory sulphate reduction) (Widdel and Hansen, 1992). For each mole of sulphate reduced, an equivalent mole of sulphide is produced (Berner, 1974).

\[ \text{2CH}_2\text{O} + \text{SO}_4^{2-} \rightarrow \text{H}_2\text{S} + 2\text{HCO}_3^- \]

Table 1.2 details the energy substrates that SRB have been shown to utilise. As can be seen, the SRB are a nutritionally diverse group of micro-organisms with the capability of utilising numerous organic compounds ranging from simple fatty acids to complex aromatic compounds. It has also been shown that, together with sulphate, numerous other ions may act as electron acceptors. These include thiosulphate ($\text{S}_2\text{O}_3^{2-}$), tetrathionate ($\text{S}_4\text{O}_6^{2-}$), sulphite ($\text{SO}_3^{2-}$) and elemental sulphur ($\text{S}^0$) (Postgate, 1951; Biebl and Pfennig, 1977; Cypionka, 1987). The production of large quantities of $\text{H}_2\text{S}$, which is a very strong reducing agent, inhibits the growth of certain aerobic organisms and may serve as an electron donor for the growth of some sulphur bacteria (Gibson, 1990). This is just one of many complex interactions that take place within natural environments.

Alkaliphilic SRB

Although dissimilatory sulphate reduction has been observed in relatively acidic environments (pH 2.5 to 4.5) (Tuttle et al., 1969; Herlihy and Mills, 1985; Spratt et al., 1987; Gyure et al., 1990), reports of sulphate reduction in
Table 1.2 Substrates which have been shown to be utilised by sulphate-reducing bacteria (based on Hansen, 1988, with data from Gibson, 1990 and Fauque, 1995).

<table>
<thead>
<tr>
<th>Type</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic</td>
<td>hydrogen, carbon monoxide</td>
</tr>
<tr>
<td><strong>Monocarboxylic acids</strong></td>
<td>formate, acetate, propionate, butyrate, isobutyrate,</td>
</tr>
<tr>
<td></td>
<td>2- and 3-methylbutyrate, pyruvate, lactate, fatty</td>
</tr>
<tr>
<td></td>
<td>acids up to C₁₈</td>
</tr>
<tr>
<td><strong>Dicarboxylic acids</strong></td>
<td>succinate, fumarate, malate, oxalate, maleinate,</td>
</tr>
<tr>
<td></td>
<td>glutarate, pimelate</td>
</tr>
<tr>
<td>Alcohols</td>
<td>methanol, ethanol, propanol, butanol, ethylene</td>
</tr>
<tr>
<td></td>
<td>glycol, glycerol, 1,2- and 1,3-propane-diol</td>
</tr>
<tr>
<td>Amino acids</td>
<td>glycine, serine, cystein, threonine, valine, leucine,</td>
</tr>
<tr>
<td></td>
<td>isoleucine, aspartate, glutamate, phenylalanine</td>
</tr>
<tr>
<td>Others</td>
<td>Choline, furfural, oxamate, fructose, benzoate,</td>
</tr>
<tr>
<td></td>
<td>stearate, 2-, 3- and 4-OH-benzoate, glucose,</td>
</tr>
<tr>
<td></td>
<td>aniline, cyclohexanecarboxylate, hippurate,</td>
</tr>
<tr>
<td></td>
<td>nicotinic acid, indole, anthranilate, quinoline,</td>
</tr>
<tr>
<td></td>
<td>phenol, p-cresol, catechol, roсорcinol,</td>
</tr>
<tr>
<td></td>
<td>hydroquinone, protocatechuate, phloroglucinol,</td>
</tr>
<tr>
<td></td>
<td>pyrogallol, 4-OH-phenylacetate, 3-phenylpropionate, 3-</td>
</tr>
<tr>
<td></td>
<td>aminobenzoate, dihydroxyacetate, acetone</td>
</tr>
</tbody>
</table>
Introduction

alkaline environments are not well documented. This lack of research is perhaps surprising considering the ecological and economic importance of SRB in industrial processes. In the oil industry, SRB have been implicated in corrosion of platform structures and pipelines (Hamilton, 1985), in the build-up of sulphide in enclosed working environments and in the souring (high sulphide content) of the produced oil and gas (Herbert, 1987). The importance of alkaliphilic sulphate reduction cannot be underestimated since alkalisation has been used in the control of SRB in industrial settings (Zobell, 1958). SRB have also been implicated in the corrosion and odour pollution of sewerage systems (Mori et al., 1992).

Reports on the isolation of alkaliphilic SRB have been limited and inconclusive. Zhilina and Zavarzin (1994) reported the isolation of numerous organisms from Lake Magadi, forming a mixed alkaliphilic community. One of the organisms isolated was presented as a H₂-utilising SRB, strain Z-7935. This isolate was described as a typical small, motile vibrio, but no further phenotypic or taxonomic work was carried out.

Although SRB have been isolated from acidic environments (< pH 5), they have been shown to be inhibited below pH 5.5 (Tuttle et al., 1969). It is possible this may be due to the toxic effects of H₂S, the dominant sulphide species under these conditions (Figure 1.9) (Hao et al., 1996). It has been proposed that any sulphide species (H₂S, HS⁻ or S²⁻) may inhibit SRB by combining with iron-containing compounds essential to the cell (e.g. ferredoxin and cytochrome), causing a cessation of the electron transport system (Okabe et al., 1992). The absence of H₂S from alkaline lake sediments is apparent at a number of sites where sulphate reduction appears to be taking place. Often thick, black mud may be observed, with no distinctive H₂S smell (personal observations).
Figure 1.9 Theoretical graph showing the sulphide speciation as a function of pH (Hao et al., 1996).
1.5.5 Archaea

Aerobic Archaea

Haloalkaliphilic Archaea, found to be confined to soda lake environments, have been isolated from Lake Magadi (Tindall et al., 1980); Wadi Natrûn, Egypt (Soliman and Trüper, 1982); Owen’s Lake, California (Morth and Tindall, 1985); and Chinese (Yang and Tang, 1989) and Russian (Zvyagintseva and Tarasov, 1987) soda lakes. Archaea from alkaline, hypersaline environments differ from normal halobacteria and halococci by having an alkaline pH optimum for growth, and a requirement for reduced concentrations of Mg\(^{2+}\) (< 1 mM) (Tindall et al., 1980; Grant and Larsen, 1989). These organisms have been classified into two new genera *Natronococcus* and *Natronobacterium* (Tindall et al., 1984).

Natronobacteria and natronococci are present in the lakes as secondary producers, with primary production being carried out by *Ectothiorhodospira* sp., or perhaps more rarely by cyanobacteria (Grant et al., 1990). Although the haloalkaliphilic archaea possess photoreactive retinal pigments, they themselves are incapable of photoautotrophic growth since they lack the light-mediated bacteriorhodopsin proton pump required for energy production found in other archaea (Bivin and Stoeckenius, 1986).

Jones et al. (1994) carried out numerical and chemotaxonomic analysis on twenty-five soda lake isolates which showed growth at 15% (w/v) NaCl or greater, and at least pH 10. Four clusters were obtained, two of which were Archaea containing sixteen isolates, and two were Bacteria containing nine isolates. Phylogenetic analysis on six of the Archaea revealed they were most closely related to the previously described *Natronococcus* and *Natronobacterium* genera (Duckworth et al., 1996). These findings confirmed the ubiquity of haloalkaliphilic archaea in hypersaline soda lakes.
Anaerobic Archaea

Although evidence is somewhat limited, methanogenesis almost certainly takes place within soda lakes (Oremland et al., 1982; Zhilina and Zavarzin, 1994). The majority of methanogens reported have an optimum pH in the range of 7.0 to 8.2, with *Methanococcus vannielii* having the highest pH optimum of 8.2 and a maximum pH for growth of 9.2 (Stadtman and Barker, 1951).

More recently methanogens have been isolated from soda lakes and deserts which exhibit alkali-tolerant and alkaliphilic properties. Mathrani et al. (1988) isolated *Methanohalophilus zhilinae* from Wadi Natrûn, Egypt, which had an optimum of pH 9.2 for growth, the highest for any methanogen described to date. This organism also had an obligate requirement for a minimum of 3.4% (w/v) NaCl. From the same location *Methanobacterium alcaliphilum* was isolated, which was also an obligate halophile and which had a pH optimum of around 8.3 (Worakit et al., 1986).

Further isolates with pH optima above neutrality include *Methanobacterium thermoalcaliphilum* (Blotevogel et al., 1985) isolated from the Wadi Natrûn, Egypt, with an optimum pH between 7.5 and 8.5; *Methanohalophilus oregonense* (Liu et al., 1990) isolated from an alkaline, saline aquifer near Alkali Lake, with an optimum pH for growth between 8.4 and 9.0; and *Methanobacterium arbophilicum* (Zeikus and Henning, 1975) an alkali-tolerant organism with a pH optimum between 7.5 and 8.0.

Two unidentified isolates NY-218 (Nakatsugawa, 1991); and Z-7936, Lake Magadi (Zhilina and Zavarzin, 1994) have been reported. Strain Z-7963 was enriched and isolated using a medium at pH 10.0, although an optimum pH for growth was not reported.
Although rates of methanogenesis at alkaline pH appear to be much lower than their neutrophilic counterparts (Wenk and Bachofen, 1995), it is apparent that a small number of methanogens have an alkali-tolerant or an alkaliophilic pH optimum for growth.
1.6 Phylogenetic inference from 16S rRNA sequence data

Recent years have seen a massive proliferation of methods for the analysis of DNA sequence data. Although this has given researchers a comprehensive arsenal of techniques to work with, the choice of which method is most relevant and applicable has become extremely complicated. This problem has been confounded by the practice of researchers selecting methods based on availability or historical inertia rather than on the relative advantages and disadvantages of each approach. It therefore seems appropriate to discuss recent advances in phylogenetic inference techniques, with specific reference to rRNA analysis.

1.6.1 Ribosomal RNA as a phylogenetic marker

Ribosomal RNA genes are of particular use in phylogenetic analyses not only because of their ubiquity in all non-viral organisms, but also because they allow meaningful comparisons between distantly related organisms (Woese, 1987). Although the possibility of obtaining evolutionary information from molecular sequences was first suggested in the mid-60's (Zuckerkandl and Pauling, 1965), it was not until more recently that molecular phylogenies were inferred for micro-organisms (Woese et al., 1992).

The use of rRNA to reconstruct an organism's past raises the question, can a single gene be representative of an entire organism. Although this can not be conclusively proven at the moment, analyses of the genes encoding for RNA polymerases, proton-translocating ATPases and elongation factor G indicate similar phylogenies to those found with rRNA (Pühler et al., 1989; Gogarten et al., 1989; Cammarano et al., 1992). Thus, until sufficient conflicting data are produced, it is reasonable to assume that present phylogenies are reliable.
16S rRNA sequence analysis led to the reclassification of the prokaryotes into two separate groups of organisms, the Archaea and the Bacteria. Together with the Eucarya, a ‘universal tree of life’ could be constructed, showing the relationship between these three major domains (Woese et al., 1990). rRNA molecules are useful in determining phylogenies since they comprise numerous conserved and variable regions. The conserved regions, although phylogenetically redundant, allow the use of universal amplification techniques which are independent of the host organism. Slightly variable sites allow the elucidation of the more deeply branching or distantly related groups, whereas sites with increased variability allow the separation of more closely related species. However, it should be noted that the most rapidly changing sequence positions may lead to errors in the resulting phylogenies, and so these positions should also be ignored in any phylogenetic analyses.

The propensity of base substitutions rather than insertions and deletions also makes small sub-unit rRNA molecules suitable candidates for phylogenetic inference. The low frequency of insertions and deletions simplifies the alignment procedure, and improves the robustness of the resulting phylogenies (Olsen and Woese, 1993).

Based on these findings, one can assume that the use of 16S rRNA gene sequences provides an accurate indication of an organisms ancestry and interrelationships with other organisms. Although phenotypic data must also be taken into account, contemporary molecular techniques allow the rapid identification of unknown isolates.

1.6.2 Selection of analytical method

Once sequence data have been obtained and confirmed from test organisms, subsequent analyses can be broken down into five distinct steps.
Introduction

1. Appropriate sequences for comparison must first be selected. This is usually straightforward since certain phenotypic characteristics reveal closely related species. When examining 16S rRNA data, sequences can be obtained from databases enabling rapid initial screening.

2. Sequences under investigation must then be aligned, based on regions of homology. This procedure may be straightforward if only pairwise differences exist, indicating substitutional or mutational events. However, the presence of insertions or deletions makes alignment of sequences increasingly difficult. It is of paramount importance that sequences are aligned correctly, since all subsequent phylogenetic analyses rely on this fact.

3. Once sequences have been aligned, a method of phylogenetic inference must then be selected. This decision is based on a number of factors, relating to the assumptions of the inference methods. Pertinent questions include: (a) Are a few broad assumptions preferable to many detailed assumptions about evolution? (b) What parameters of sequence evolution have been examined for the sequences of interest? (c) How variable are the rates of change within the sequences? (d) Are reconstruction of accurate branch lengths, or reconstruction of accurate branch order most important, or a combination of both of these?

4. Once the method of analysis has been selected and the appropriate software obtained, a strategy must be developed to obtain the optimal tree given the imposed criteria of the inference method. Any tree topology resulting from analysis will be a ‘best guess’ since it would be impossible to find this information computationally (Felsenstein, 1978). For example, with just 50 taxa there are over $2.8 \times 10^{74}$ distinct, bifurcating trees. Even if a computer existed than could analyse 1
trillion trees per second, it would still take $8.9 \times 10^{44}$ years to evaluate all possible trees for 50 taxa ($2 \times 10^{45}$ times the age of the earth).

5. The final objective in phylogenetic inference is to establish the confidence levels of the result. Ideally this would show which nodes of the tree are well supported by the data, and which are not.

**1.6.3 Obtaining 16S rRNA sequences**

Numerous databases exist which hold a variety of sequence information, including gene sequence data for a large number of proteins, along with 5S, 16S and 23S rRNA sequence data. The two main repositories for such data are the American database GenBank (Benson *et al.*, 1996) and the European database EBI (RodriguezTome *et al.*, 1994). These databases have a large number of sequences deposited, and are updated daily. Database searching is facilitated by proprietary software, available for use on-line, allowing complex querying and data extraction.

A number of electronic databases exist which hold specifically rRNA sequences obtained from GenBank and EBI. More recently these databases have developed alignment strategies, allowing pre-aligned sequence data to be downloaded. Previously all data was accessed non-interactively via email (electronic mail through the interconnected computer networks of the world). However, this method has been superseded by access via the world wide web. The two main repositories for 16S rRNA data are the Ribosomal Database Project (RDP) found at [http://rdpwww.life.uiuc.edu/](http://rdpwww.life.uiuc.edu/) (Maidak *et al.*, 1996) and the ribosomal database at the University of Antwerp found at [http://rrna.uia.ac.be/](http://rrna.uia.ac.be/) (Van de Peer *et al.*, 1996a). The RDP, together with sequence data, houses a number of additional programmes to aid sequence analysis. These include alignment of a user-supplied sequence to the most similar RDP sequences, comparison of a specific probe to a set of sequences,
retrieval of the most similar sequences to that supplied by the user and a programme to approximately place a user-supplied sequence within the RDP tree. The database at Antwerp has the added advantage of including secondary structure information within aligned sequences, assisting the alignment procedure. This web site also houses useful software for the manipulation and analysis of sequence data.

1.6.4 Alignment

The procedure of sequence alignment is complex, with many contributing factors having to be taken into account. The simplest taxa to align are those which are most closely related, exhibiting only a small number of substitutions and no insertions or deletions. As taxa become more divergent, so alignment becomes increasingly difficult.

The first step of alignment is a pairwise comparison between each of the taxa within the study group. This involves aligning conserved regions and inserting alignment gaps in variable regions. Usually matches are scored +1, mismatches are scored 0, and gaps are assigned a negative penalty, usually greater than the positive value for matches. Homologous features are assumed to be due to common descent rather than convergent or parallel evolution (Mindell, 1991). Gaps of more than one position are not usually weighted in direct proportion to the length of the gap, since it is most likely that adjacent nucleotides were inserted in a single event. Many pairwise approaches to sequence alignment have been developed, based mainly on the algorithm described by Needleman and Wunsch (1970).

Following pairwise comparison of all sequences in the data set, the optimal alignment is taken to be the one which is least costly. For example, in Figure 1.10 alignment (b) would be chosen over alignment (a) since it had a better
penalty score. Should two alignments have similar penalty scores, the one with fewer changes to the primary structure is selected.

Verification based on observed secondary structure must then be carried out to ensure the correct alignment has been obtained. This process has been simplified with the proliferation of pre-aligned sequence databases for 16S rRNA (Maidak et al., 1996; Van de Peer et al., 1996a). Utilising these pre-aligned databases and their associated software (De Rijk and De Wachter, 1993), primary and secondary alignment has been greatly simplified. Figure 1.11 shows a sample 16S rRNA alignment including secondary structure symbols, along with the corresponding structure produced. One further factor must be taken into account when aligning 16S rRNA sequences. Certain areas of the 16S gene are extremely variable whilst others are extremely conserved (Lane, 1985). When completing an alignment, base substitutions, insertions and deletions within highly conserved regions of the gene should be minimised.

1.6.5 Methods of phylogenetic inference

The choice of which phylogenetic inference method to use is dependent on which assumptions and models are acceptable. Usually it is best to chose a method specific for the molecule being examined, in this case the 16S rRNA molecule. Most inference models contain certain assumptions: characters evolve independently of each other, positional homology has been inferred correctly and generally nucleotide changes are neutral.

Sequence data used in phylogenetic analyses are a character-based information source, comprising of the bases A, C, G and T (or U for RNA) and additional gap characters. However, multiple mutational events can effectively conceal the phylogenetic history of a sequence, impairing character-based phylogenetic reconstruction (Figure 1.12).
Figure 1.10 Theoretical alignment of four DNA sequences. Alignment (a) requires no gaps, but has 15 substitutions at 13 positions. This sequence has 17 of 30 sequence positions conserved. Alignment (b) is favoured however since it requires 4 gaps and only 3 substitutions, and contains 26 of 30 conserved bases. Assuming gaps are scored as -2, alignment (a) has a score of +17 whereas alignment (b) has a score of +18.

Alignment (a)

Seq. a  AGCCAGGCTC CCGCGGTATA CCAGATCAGG
Seq. b  AGCCAGGCTC CCGCGGTATA CCAGATCAGG
Seq. c  AGCCAGGCTA CCGCGGTTTA CCAGATCAGG
Seq. d  AGCCCAAGCT ACGCGGTGTTT ACCAGACAGG

Alignment (b)

Seq. a  AGCC-AGGCC ACCGCGGTAT -ACCAGATCA GG
Seq. b  AGCC-AGGCC ACCGCGGTAT -ACCAGATCA GG
Seq. c  AGCC-AGGCT ACCGCGGTTT -ACCAGATCA GG
Seq. d  AGCCCAAGCT AC-GCGGTGT TACCAGA-CA GG

* * * * * * * * * *
**Figure 1.11** Sample 16S rRNA alignment showing two theoretical sequences. The following secondary structures are shown within the alignment: [ and ] denote the beginning and end of a stem structure; ^ denotes [[ (a new helix starting immediately after a previous one; ( and ) indicate a non-standard base pair; { and } indicate the beginning and end of an internal loop or bulge. Below is the secondary structures created from the primary sequence shown. Example taken from DCSE user manual.

<table>
<thead>
<tr>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
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</table>


--- 1 --- 2 --- 3 --- 3' --- 1' ---

1. **Organism 1**
   - GAT
   - GUU
   - CUG
   - A

2. **Organism 2**
   - UG
   - A
   - AU
   - GC
   - GA
   - AU
   - CG
   - U
   - A
   - CUG
   - A

---
Figure 1.12 Example of (a) convergent evolution and (b) reversal between species I and species II. The diagram shows that, although three mutational events have occurred since the common ancestor, no difference can be seen between the base composition of the two species. The evolution of these species has effectively been concealed.
There are therefore two methods of analysing phylogenetic data, namely character-based methods (analysing nucleotides themselves), or distance-based methods (analysing pairwise comparisons of whole sequences). Character-based methods, such as parsimony, rely on phylogenetically informative positions, ignoring variation unique to single taxa. Distance-based methods convert the sequence data into a matrix of pairwise comparisons (branch lengths separating each pair), attempting to correct for unobserved substitutional events occurring between divergent taxa (Figure 1.12). The branch lengths are taken to represent the product of time and rate of evolution, and a tree is chosen which best illustrates the pairwise distances.

**Character-based methods**

Character-based methods of sequence analysis treat each substitution separately rather than reducing all substitutions to one divergence value. There are three methods which determine phylogeny by the distribution of observed mutations, parsimony, maximum likelihood and method of invariants (or evolutionary parsimony). Character-based methods have the advantage that all sequence data is conserved, with none being lost during the production of a distance matrix. However, they have the disadvantage of requiring greatly increased computational time, and have greater difficulty in correcting for multiple substitution events.

**Maximum parsimony**

Maximum parsimony (Kluge and Farris, 1969) is the most popular method of character-based sequence analysis (Fitch, 1971). The basic product of this method is the most parsimonious tree that requires the fewest evolutionary steps to explain the original data. The method assumes that homoplasy (multiple substitution events leading to convergent evolution or reversals) do not interfere with phylogenetic inference. To reduce the effects of homoplasy,
greater weight can be assigned to conserved areas over rapidly mutating ones. For example, non-uniform weighting of stem and loop structures of rRNA reflecting the secondary structural constraints on stem structures which have to maintain base pairing. A detailed review of the weighting procedures used in parsimony analysis can be found in Swofford and Olsen (1990).

**Maximum likelihood**

The first ML method was developed by Cavalli-Sforza and Edwards (1967) to analyse gene frequency data. This was later followed by a method of constructing unrooted phylogenetic trees from nucleotide sequence data (Felsenstein, 1981). Saitou (1988) has recently proposed a stepwise tree-searching algorithm similar to that used in the distance matrix-based neighbour joining method.

The principle of maximum likelihood can be explained using the following example. Figure 1.13 shows an unrooted tree of five organisms (terminal nodes) A, B, C, D and E, with three internal nodes X, Y and Z. Considering just one nucleotide site $N_i$ (where $i = A, B, C, D$ or $E$), this nucleotide is known since it is contained within observed sequence data. However, a nucleotide at node $N_j$ (where $j = X, Y$ or $Z$) is unknown and can be any of the four bases. This gives a likelihood ($L$) for this site of

$$L = \sum_{N_Y} g_Y P_{YC} \left( \sum_{N_X} P_{YX} P_{XA} P_{XB} \right) \left( \sum_{N_Z} P_{YZ} P_{ZD} P_{ZE} \right)$$

where $g_Y$ is the probability that node Y has nucleotide $N_Y$, $P_{ij} = P(N_i, N_j, d_{ij}$ where $i = A, B, C, D$ or E and $j = X, Y$ or Z) is the probability of observing nucleotides $N_i$ and $N_j$ in sequences i and j respectively and $d_{ij}$ is the expected number of nucleotide substitutions between these two sequences. $P(N_i, N_j, d_{ij})$ is dependent on a pattern of nucleotide substitution. The simplest one-
parameter model which specifies random substitutions (Jukes and Cantor, 1969) would resolve as follows

\[
P(N_r, N_t, d_v) = \frac{1}{4} + \left(\frac{1}{4}\right) \exp\left(-\frac{4d_v}{3}\right) \quad \text{(if } N_r = N_t) \\
P(N_r, N_t, d_v) = \frac{1}{4} + \left(\frac{1}{4}\right) \exp\left(-\frac{4d_v}{3}\right) \quad \text{(if } N_r \neq N_t)
\]

If a two-parameter model is considered (Kimura, 1980), where transitions and transversions can occur at different rates, then these equations become increasingly complex and consequently time-consuming. Which ever method is used, the likelihood for each nucleotide is multiplied for all sites and computed for many combinations of branch lengths for a given tree topology. The branch length combination which shows the highest likelihood is then selected as the ML solution.

The maximum likelihood method of phylogenetic inference relies on models of molecular evolution to compensate for multiple hits, with the result being dependent on the model chosen and how well it reflects the evolution of the molecule of study. Therefore this method can be viewed as a cross-over between character methods and distance methods, since it considers each site individually, but applies a model of evolution to the result. Although increasingly complex models are developed, which account for unequal rates, site-specific rate variability and random distribution and / or clustering of variable sites, this method requires detailed information about the evolution of the molecule of study, which is not normally available. Extensive information on phylogenetic inference using the maximum likelihood method is available from Felsenstein (1981), Kishino and Hasegawa (1989), Swofford and Olsen (1990) and Saitou (1990).
Figure 1.13 Theoretical phylogenetic tree showing five nucleotide sequences (terminal nodes) with three internal nodes.
\textit{Evolutionary parsimony}

Evolutionary parsimony uses operator invariants, each of which reflects specific patterns of shared transversions corrected for homoplastic similarity (Lake, 1987). The calculations for operator invariants are based on the variable positions with two purines and two pyrimidines. Chi-squared or binomial tests are used to identify the correct topology for zero-value invariants, representing cases where multiple mutational events have cancelled each other out. Evolutionary parsimony assumes that the two types of transversions occur with equal frequency, although a recent modification proposes to correct for these inequalities by accounting for base compositional differences (Sidow and Wilson, 1990).

However, Jin and Nei (1990) showed that the evolutionary parsimony method of tree construction was not suitable under a number of conditions as it made some unrealistic assumptions. They compared the methods of maximum parsimony, evolutionary parsimony and neighbour joining for producing the correct tree from a set of data, and concluded that neighbour joining was more accurate than the other two methods.

\textbf{Distance matrix methods}

The simplest methods of phylogenetic inference involve dividing the total number of substitutions in a sequence by the number of base pairs examined, with no attempt to correct for evolutionary distance (uncorrected divergence) (Miyamoto \textit{et al.}, 1987).

\[ S = 1 - \frac{M}{L} \]

where \( S \) = dissimilarity, \( M \) = number of identical nucleotides between two sequences and \( L \) is the total number of nucleotides under investigation. When
two sequences are similar (<10 % dissimilarity), corrected and uncorrected divergence estimates give similar values. However, as sequences become increasingly distant, uncorrected divergence estimates become increasingly underestimated. Corrected approaches attempt to account for unobserved parallel substitutions and reversals in addition to observed differences, correcting for multiple substitution events by adopting some distribution of nucleotide change and by weighting substitutions (Table 1.3).

**Jukes and Cantor**

The most popular method for estimating the number of nucleotide substitutions between two taxa is that of Jukes and Cantor (1969). This one-parameter model assumes that all substitutions at a given position are independent, and that all positions are equally subject to change. Furthermore it assumes that there is no bias in the direction of change (i.e. substitutions occur randomly between A, G, C and T), and that there are no insertion or deletion events. Based on these assumptions, the number of nucleotide substitutions that have occurred since divergence of sequences A and B (evolutionary distance $d$) is:

$$d = -\frac{3}{4} \ln\left(1 - \frac{4}{3} S\right)$$

It should be noted that $d$ cannot be computed using this algorithm when $S > 0.75$.

**Kimura**

Kimura (1980) developed the one-parameter model to account for unequal transition and transversion rates (i.e. $A \rightarrow G$ and $C \rightarrow T$ occurring more frequently than other base substitutions).

$$d = -\frac{1}{2} \ln[(1 - 2P - Q)\sqrt{1 - 2Q}]$$
Table 1.3 Different methods, both corrected and uncorrected, for the estimation of divergence between differing taxa.

<table>
<thead>
<tr>
<th>Method</th>
<th>Comments</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Uncorrected approach</td>
<td>( p ) (difference)</td>
<td>Swofford and Olsen, 1990</td>
</tr>
<tr>
<td>Corrected approaches</td>
<td>One-parameter</td>
<td>Jukes and Cantor, 1969</td>
</tr>
<tr>
<td></td>
<td>All substitutions equal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Two-parameter</td>
<td>Kimura, 1980</td>
</tr>
<tr>
<td></td>
<td>Transitions and transversions treated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>differently</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Three-parameter</td>
<td>Kimura, 1981</td>
</tr>
<tr>
<td></td>
<td>Two classes of transversion, transitions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>treated equally</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Four-parameter</td>
<td>Tajima and Nei, 1984</td>
</tr>
<tr>
<td></td>
<td>Two classes of transversion, two classes of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>transition</td>
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</tr>
<tr>
<td></td>
<td>Six-parameter</td>
<td>Hasegawa et al., 1985</td>
</tr>
<tr>
<td></td>
<td>Four classes of transversion, two classes of</td>
<td></td>
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<tr>
<td></td>
<td>transition</td>
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where $P$ is the fraction of sequence positions differing by a transition and $Q$ is the fraction of sequence positions differing by a transversion.

**Jin and Nei**

All of these distance estimations assume that the rate of change among nucleotides is equal for all sites, an assumption that very rarely holds (Van de Peer et al., 1993). Jin and Nei (1990) showed that rates of substitution varied according to the gamma distribution, specified by the parameter $a$ which is the square of the inverse of the coefficient of variation of substitution rate (Nei, 1991). Applying this to the Jukes and Cantor one-parameter model, the following equations are derived:

for $a = 1$:

$$d = \frac{S}{1 - \frac{4}{3}S}$$

for $a = 2$:

$$d = \frac{3}{2} \left[ \frac{1}{\sqrt{1 - \frac{4}{3}S}} - 1 \right]$$

for $a = \frac{1}{2}$:

$$d = \frac{3}{8} \left[ \frac{1}{\left(1 - \frac{4}{3}S\right)^2} - 1 \right]$$

and for any other value of $a$ (Rzhetsky and Nei, 1994):

$$d = \frac{3}{4} a \left[ \left(1 - \frac{4}{3}f\right)^{-\frac{1}{a}} - 1 \right]$$
The correction could also be applied to Kimura’s two-parameter model, leading to the following equations:

for $a = 1$:

$$d = \frac{2P + Q}{2(1 - 2P - Q)} + \frac{Q}{2(1 - 2Q)}$$

for $a = 2$:

$$d = \frac{1}{\sqrt{1 - 2P - Q}} + \frac{1}{2\sqrt{1 - 2Q}} - \frac{3}{2}$$

for $a = \frac{1}{2}$

$$d = \frac{1}{4(1 - 2P - Q)^2} + \frac{1}{8(1 - 2Q)^2} - \frac{3}{8}$$

and for any other value of $a$ (Nei, 1991):

$$d = \frac{a}{2} \left[ (1 - 2P - Q)^{\frac{1}{a}} + \frac{1}{2} (1 - 2Q)^{\frac{1}{a}} - \frac{3}{2} \right]$$

Increasingly complex models attempt to incorporate numerous parameters of substitution into their calculations. This has led to the development of complex models involving three, four, six and twelve parameters (Lanave et al., 1984, Saccone et al., 1990).

Van de Peer

A new algorithm has been developed specifically for 16S rRNA which takes into account unequal rates of nucleotide substitution (Van de Peer et al., 1996b, c). Van de Peer and co-workers (1993) estimated that, although previous methods of distance calculation assumed equal rates of substitution with all nucleotide positions, rates of change within 16S rRNA varied by more than a factor of 1000. In an attempt to compute evolutionary distance ($d$), the following method was developed. Firstly, pairwise evolutionary
distances $d$ are computed according to the equation of Jukes and Cantor (1969).

These pairwise distances are then classified into distance intervals depending on the value of $d$ (e.g. distances from 0 to 0.005, distances from 0.005 to 0.010, etc.). The fraction of sequence pairs found within a distance interval grouping, and characterised by a nucleotide change, is then computed for every nucleotide position. This fraction can then be plotted against the mean of the distance interval (Van de Peer et al., 1993), resulting in a curve obeying the equation:

$$p_i = \frac{3}{4} \left[ 1 - \exp \left( -\frac{4}{3} v_i d \right) \right]$$

computed by non-linear regression. This equation expresses the probability that nucleotide $i$ ($P_i$) contains a different nucleotide in two sequences as a function of the evolutionary distance $d$ separating them. The slope of the curve at the origin yields the specific nucleotide substitution rate $v_i$ for the position under consideration.

This method is still sub-optimal since it is derived from a evolutionary distance value $d$ based on a distance matrix (i.e. it still assumes equal rates of substitution). Therefore following estimation of $v_i$ values for each position, all alignment positions with similar variability values are grouped together, producing a variability spectrum. Once the shape of the spectrum is determined, the fraction of observed substitutions ($S$) between two sequences can be derived as a function of the evolutionary distance separating them ($d$):

$$S = \frac{3}{4} \left[ 1 - \exp \left( -\frac{4}{3} p \ln \left( 1 + \frac{d}{p} \right) \right) \right]$$
where parameter $p$ is dependent on the shape of the substitution rate spectrum. The inverse of this equation is then used to convert the dissimilarity values into evolutionary distances, giving a more accurate result than the method of Jukes and Cantor (1969):

$$d = p \left[ \left(1 - \frac{4}{3}S\right)^{-\frac{3}{4p}} - 1 \right]$$

This equation can then be used to convert pairwise dissimilarity values ($S$) into evolutionary distance ($d$). The relative substitution rates for every alignment position are then calculated again as described above, and a new spectrum of evolutionary rates ($v_i$) is produced. This iterative process is repeated until the nucleotide substitution rates no longer change (generally no more than three iterations) (Van de Peer et al., 1996b) (Figure 1.14).

1.6.6 Construction of phylogenetic trees from distance matrix data

Methods for the representation of divergence data as a phylogenetic tree can be broken down into two groups, either single-tree methods or multiple-tree methods.

Single-tree methods

Two methods exist for data analysis using single-tree methods. The first assumes that data are ultrametric (rely on clock-like mutation rates e.g., constant rate of evolution hypothesis resulting in identical distances from the root to each terminal node), although this property is often not satisfied by sequence data. Two algorithms are available for analysis by this method, namely UPGMA (unweighted pair group method algorithm) and WPGMA (weighted pair group method algorithm). However, these methods have been largely replaced by others which do not assume the data are ultrametric.
Figure 1.14 Schematic showing the iterative process for the calculation of substitution rates for each nucleotide within the test molecule.
The most popular algorithm for the analysis of sequence data involves a heuristic approach for estimating minimum evolution phylogeny, named the neighbour joining method (Saitou and Nei, 1987; Studier and Keppler, 1988). This method does not depend on ultrametric data, but does depend on data being additive, meaning the evolutionary distance between any two taxa is equal to the sum of the branches that join them. This method constructs a modified matrix in which the separation between each pair of nodes is adjusted on the basis of their average divergence from all other nodes. This has the effect of normalising the divergence of each node towards an average clock rate. The least distant pairs of taxa as defined by the modified matrix are then combined into a new one. This new node is than added to the tree, removing the replaced taxa and their branches. The process of two terminal nodes being replaced by one new one is continued, reducing the size of the tree, until only two nodes remain separated by a single branch. A worked example of the neighbour joining method can be found in Swofford and Olsen (1990).

This method of tree construction is not only very effective in producing correct tree topology (Saitou and Nei, 1987; Saitou and Imanishi, 1989; Nei, 1991), but it is also a fast and non-computationally intensive technique.

**Multiple-tree methods**

The second type of tree analysis, unlike the single tree method which produces one definitive result, produces a result based on estimations of tree topology, selecting the best alternative. Multiple-tree methods, or least squares methods are they are more commonly known, rely on a defined criterion of optimality, defined as objective quantity, measuring the conformity of the original distance matrix data \(d_{ij}\) to the observed data from the tree \(\delta_{ij}\). The quantity \(E\) is then minimised for each pair of taxa \((i,j)\):
There are four main approaches to the multiple-tree method of analysis. The Fitch and Margoliash (1967) method selects for the tree which maximises fit of observed pairwise data against tree-derived (patristic) distances, measured by the percent standard deviation. The distance Wagner method (Farris, 1972) prevents patristic distances from being shorter than observed pairwise distances (disallows negative branch lengths). The best tree topology is chosen on the basis of minimum tree length, where tree length is the sum of all the constituent branch lengths. The neighbourliness method (Fitch, 1981) compares four-taxon clusters based on paired taxa distances. For example, the four taxa A, B, C and D are clustered based on distance \(d\) such that \(d(A, B) + d(C, D) < d(A, C) + d(B, D)\) and \(d(A, B) + d(C, D) < d(A, D) + d(B, C)\). If the data contain more than four taxa, comparisons are computed for all possible subclusters. The final method, called minimum evolution, is extremely complex, based on the single-tree method of neighbour joining. Branch lengths are optimised using the method of Fitch and Margoliash, and the tree with the minimal overall length is selected.

**1.6.7 Evaluating the robustness of inferred phylogeny**

Once a phylogenetic tree has been produced, it is necessary to assess the reliability of the original data and the robustness of the resulting tree. The testing of phylogenetic reliability is based on comparing the support for one tree to that of another, assuming data are randomly distributed. Methods in assessing confidence levels can be broken down into two groups, those utilising analytical techniques and those utilising resampling techniques.
Analytical techniques

The earliest approaches for the testing of parsimony procedures involved the Wilcoxon rank-sum test to compare the number of unique changes favouring one topology over another (Felsenstein, 1988). Further tests include confidence limits without clock (Felsenstein, 1983), confidence limits with clock (Felsenstein, 1985a) and Williams / Goodman confidence limits (Williams and Goodman, 1989), which all compare support for the best tree against that expected for a worst-case situation, looking for a statistically significant difference within imposed limits.

Evolutionary parsimony uses chi-square or binomial tests to determine which phylogenetic invariants deviate significantly from zero and which do not. Tree topology is well supported if invariants deviate significantly from zero (Lake, 1987).

Maximum likelihood methods may be tested in one of two ways. The logarithm of the ratio of the maximum likelihood scores for the best tree to unresolved phylogeny may be treated as a chi-square statistic, with one degree of freedom. Alternatively a heuristic approach may be implemented, comparing two trees by mean and variance of their likelihood scores (Kishino and Hasegawa, 1989).

Analysis of distance-based trees may be carried out by determining the significance of internal branch lengths. A branch is only considered significant if its length plus or minus two standard errors exceeds zero (Hasegawa et al., 1985).

Resampling techniques

This method of confidence testing involves the random sampling of original data, producing a tree from the new matrix of distances. The method involves multiple resampling (usually ≥ 100) of the original data, with the frequency of
replication of a group taken as a measure of its statistical reliability. The most commonly used method of resampling is bootstrapping (Felsenstein, 1985b).

Bootstrapping involves the creation of a new data set, the same size as the original, by sampling the available characters by replacement, leading to a data set where some characters are omitted, and others are duplicated. This is equivalent to the variation one would obtain from collecting new data sets.

The second resampling technique, called jack-knifing, randomly drops one or more characters, thus creating smaller data sets by resampling without replacement (Penny and Hendy, 1986). This method may also be adapted, leading to the dropping of taxa as opposed to characters (Lanyon, 1985).

In both techniques of resampling, phylogenies are reconstructed from the new data sets, and the frequency with which a node reappears among different permutations is taken as a measure of reliability.
1.7 Aims

Investigations into the microbiology of soda lakes have led to the isolation of numerous alkaliphilic organisms. With an increasing number of isolates being described, so a greater insight into the interactions taking place within these environments develops. However, the majority of this work has been carried out aerobically, with the anaerobic organisms being all but ignored.

The present project was therefore undertaken to investigate the anaerobes found within the sediments of five soda lakes. The main aims were to develop suitable culturing techniques for the enrichment and isolation of alkaliphilic obligate anaerobes, and to utilise these techniques in the analysis of the sediment samples. Characterisation of the resulting enrichments and pure culture isolates would then provide a useful insight into the anaerobic interactions occurring within the soda lakes.
2 Materials and Methods

2.1 On-site Measurements

2.1.1 Temperature

Temperature measurements were taken using a Pentacourt Jenway portable temperature probe (model PHM9). This instrument was calibrated prior to the sampling trip.

2.1.2 Conductivity

The Conductivity of the lake sediments and water was measured using a Hanna Instruments portable conductivity meter (model HI 8663). This was calibrated using a standard solution (HI 7030, Hanna Instruments) of 12.88 mS cm\(^{-1}\) at 25 °C.

On site the probe was programmed to compensate for \textit{in situ} temperature. However, due to the limitations of the instrument this was not possible for temperatures above 50 °C, and as such under these conditions the probe failed to give accurate readings.

2.1.3 Dissolved Oxygen

Dissolved oxygen was measured using a Hanna Instruments portable dissolved oxygen meter (model HI 8543). This was calibrated to zero O\(_2\) using a solution of sodium metabisulphite, and saturated O\(_2\) using either air or oxygen saturated water. The probe was also calibrated to compensate for elevation above sea level.
On site the probe was capable of automatic *in situ* temperature compensation, although no compensation could be made for salinity. Unfortunately, under field conditions the probe soon failed, producing erratic and unreliable values.

### 2.1.4 Redox Potential

Measurements of the redox potential at each sampling location were attempted using a Hanna Instruments portable redox potential value tester (model ORP). This instrument was calibrated using a standard solution of +200 - +250 mV at 20 °C, which was supplied by the manufacturer. As with the oxygen meter, this meter failed to perform with any degree of confidence under field conditions.


2.2 Sampling Procedure

Samples were collected by either removing the upper surface of the sediment using a sterile tongue depressor revealing the anoxic substrata beneath, or by removing core samples using a 1.5 m perspex coring rod.

At each site a 100 ml Duran bottle was filled with approximately 80 ml of sediment sample and then topped up with lake water. A spatula tip (ca. 10 mg) of sodium dithionite was then added to scavenge residual oxygen. The bottle was then sealed with a butyl rubber stopper in the following manner. The stopper was placed in the neck of the bottle together with a 21G needle attached to a 1 ml syringe, such that the needle was trapped between the stopper and the neck of the bottle. The syringe contained 1 ml of anaerobic reagent (sterile 3 g l⁻¹ cysteine-HCl, 3 g l⁻¹ Na₂S·9H₂O in oxygen free deionised water). The stopper was then forced down and, once it could be pushed in no further, then anaerobic reagent was injected. The needle was then removed whilst the stopper was held in place. To secure the stopper, a Duran cap with its centre removed was then screwed on to the bottle. The drilled hole in the cap also allowed injection into and removal of sample during storage.

Samples were stored at ambient temperature until return to Leicester, whereupon they were transferred to 4 °C for long-term storage.
2.3 Enrichment and Cultivation

Various media were developed to enrich and maintain the anaerobic organisms found within the sediment samples.

All media were prepared under similar conditions. The organic component of each medium was sterilised separately from the Na₂CO₃ and NaCl component. Immediately following sterilisation at 121 °C, 1.05 kg cm⁻² for 15 minutes, the bottles were either sealed with butyl rubber stoppers and cooled under a continuous flow of anaerobic gas (10% H₂, 90% N₂ (v/v)), or placed inside an anaerobic cabinet (Don Whitley Scientific, Shipley, England) with an atmosphere of 10% H₂, 90% N₂ (v/v). For medium containing agar, the latter method was found to be more reliable since a continuous flow of gas tended to cool the surface of the liquid too quickly, causing the agar to solidify prematurely.

Vitamins mixture and vitamin B₁₂ solution (Table 2.4) were stored aerobically at 4 °C, whilst thiamine solution (Table 2.4) was prepared fresh when required. Trace elements solution (Table 2.5) was stored aerobically at room temperature. For use in anaerobic media, appropriate volumes of these stock solutions were removed and placed inside an anaerobic chamber for 24 h. Reductant solutions (Table 2.6, Table 2.7) were prepared fresh under anaerobic conditions as previously described.

All manipulations of sediment samples and cultures were carried out under strictly anaerobic conditions within an anaerobic chamber. An atmosphere of 10% H₂, 90% N₂ (v/v) was used since it was found that the standard anaerobic gas mix of 10% H₂, 10% CO₂, 80% N₂ (v/v) caused the alkaline plates to drop in pH from 10.5 to 8.5 due to adsorption of CO₂.
**Materials and Methods**

**Table 2.4** Vitamin mixtures supplemented to media prepared without yeast extract. Thiamine solution prepared fresh when required and filter sterilised prior to use. Other vitamin solutions filter sterilised and stored aerobically in the dark at 4°C. Application = 1 ml l\(^{-1}\) for each solution (Widdel and Bak, 1992).

<table>
<thead>
<tr>
<th>Vitamin Solution</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate buffer</td>
<td>(10 mM; pH 7.1)</td>
</tr>
<tr>
<td>4-aminobenzoic acid</td>
<td></td>
</tr>
<tr>
<td>D(+) Biotin</td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td></td>
</tr>
<tr>
<td>Ca-D(+) pantothenate</td>
<td></td>
</tr>
<tr>
<td>Pyroxdine di-HCl</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thiamine Solution</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>10 ml</td>
</tr>
<tr>
<td>Thiamine chloride di-HCl</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamin B(_{12}) Solution</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>5 mg</td>
</tr>
</tbody>
</table>
Table 2.5 Trace elements mixture used as a supplement to media prepared without yeast extract. Once prepared this solution was autoclaved at 121 °C, 1.05 kg cm$^{-2}$ for 15 minutes, then stored aerobically at room temperature. Application = 1 ml l$^{-1}$ (Widdel and Bak, 1992).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>987 ml</td>
</tr>
<tr>
<td>HCl (25%=7.7M)</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>2100 mg</td>
</tr>
<tr>
<td>HBO$_3$</td>
<td>30 mg</td>
</tr>
<tr>
<td>MnCl$_2$·4H$_2$O</td>
<td>100 mg</td>
</tr>
<tr>
<td>CoCl$_2$·6H$_2$O</td>
<td>190 mg</td>
</tr>
<tr>
<td>NiCl$_2$·6H$_2$O</td>
<td>24 mg</td>
</tr>
<tr>
<td>CuCl$_2$·2H$_2$O</td>
<td>2 mg</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>144 mg</td>
</tr>
<tr>
<td>NaMoO$_4$·2H$_2$O</td>
<td>36 mg</td>
</tr>
</tbody>
</table>
Table 2.6 Reductant solution supplemented to all anaerobic media. This solution was prepared freshly when required and autoclaved at 121 °C, 1.05 kg cm² for 15 minutes. Following sterilisation, this solution was handled under strictly anaerobic conditions. Application = 10 ml l⁻¹ (Widdel and Bak, 1992).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>10 ml</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.1 g</td>
</tr>
</tbody>
</table>

Table 2.7 Dithionite solution used as a supplement to further reduce the media. Prepared freshly when required using oxygen-free water, and filter sterilised prior to use. Application = 1 ml l⁻¹ (Widdel and Bak, 1992).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>10 ml</td>
</tr>
<tr>
<td>Sodium dithionite</td>
<td>0.02 g</td>
</tr>
</tbody>
</table>
2.3.1 Sulphate-reducing bacteria (SRB)

All enrichments were carried out using liquid medium in 50 or 100 ml Wheaton serum bottles sealed with butyl rubber stoppers and crimped caps. Each bottle was filled to approximately 60% volume with medium, leaving a 40% headspace of anaerobic gas. Growth was found to be improved when a headspace of gas was included as opposed to the bottles being completely filled with medium.

Enrichment

An adapted SRB medium was developed to selectively enrich for alkaliphilic sulphate-reducing bacteria. The medium was based on Widdel and Bak (1992) Medium C with the following adaptions (Table 2.8).

The Mg$^{2+}$ concentration was lowered from 2 g l$^{-1}$ to 0.5 g l$^{-1}$ to emulate the reduced availability of divalent cations in the soda lake environment. Similarly the concentration of NaCl was increased, from 2.5 g l$^{-1}$ to 20 g l$^{-1}$ (2% w/v), reflecting the salinity of the alkaline lakes.

FeSO$_4$ was included in the medium to indicate sulphate reduction by the formation of a black FeS precipitate. Varying FeSO$_4$ concentrations were tested to find the optimal amount to prevent false positives. It was found that, although 2 ml l$^{-1}$ FeSO$_4$ solution (FeSO$_4$·2H$_2$O) was enough to show sulphate reduction under chemically induced conditions, 6 ml l$^{-1}$ FeSO$_4$ was needed to visualise biological sulphate reduction.

At the elevated pH, it was found that the FeSO$_4$ reacted with yeast extract, producing a black colour. To prevent this false positive, yeast extract was omitted from the medium, which was instead supplemented with trace element and vitamin solutions. NH$_4$Cl was replaced with NaNO$_3$ since at the
**Materials and Methods**

Table 2.8 Sulphate-reducing bacteria medium. Organic component, salt component (containing carbonate) and iron sulphate component were all prepared separately. All solutions were autoclaved at 121 °C, 1.05 kg cm\(^{-2}\) for 15 minutes, and then handled under strictly anaerobic conditions. Reductant solutions added as outlined above.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Widdel and Bak (1992) (g l(^{-1}))</th>
<th>Modified Recipe (g l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium lactate (50%)</td>
<td>12.0 (9.5 ml)</td>
<td>1-2% (v/v)</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>CaCl(_2)·2H(_2)O</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>NH(_4)Cl</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>NaNO(_3)</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td>KH(_2)PO(_4)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO(_4)·7H(_2)O</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.5</td>
<td>20.0</td>
</tr>
<tr>
<td>Na(_2)CO(_3)</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>FeSO(_4) Solution</td>
<td>10 ml</td>
<td>6 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 ml</td>
<td></td>
</tr>
<tr>
<td>FeSO(_4)·7H(_2)O</td>
<td>0.5 g</td>
<td></td>
</tr>
</tbody>
</table>
elevated pH the ammonium would be chemically converted to ammonia, possibly leading to nitrogen limiting conditions.

**Cultivation**

The defined medium used for the enrichment of SRB was found not to be suitable for rapid growth to acceptable cell densities. However, once positive enrichments had been confirmed, there was no need to have an efficient sulphate reduction indicator. Therefore a medium was developed (Table 2.9), based on the standard medium for *Desulfovibrio desulfuricans* (D.S.M., Germany) which allowed more rapid growth to higher cell densities.

Betaine was included in this medium as a possible compatible solute. Trisodium citrate was included when agar was used to prevent precipitation of iron sulphate.

### 2.3.2 Chemo-organotrophic bacteria

**Enrichment**

Organisms with the ability to ferment large carbon compounds were isolated using a minimal medium (MM) supplemented with 1% (w/v) carbon source (Duckworth *et al.*, 1996) (Table 2.10). Four substrates were investigated as growth substrates: starch, birchwood xylan, guar gum (galactomannan) and carboxymethyl cellulose. Due to difficulties in the preparation of media containing gum-based carbon sources, the amount of substrate was subsequently reduced to 0.5%. This reduction in substrate concentration did not appear to affect the growth of the isolates.

Samples were first enriched in liquid media, and then transferred onto solid media.
Table 2.9 Adapted *Desulfovibrio desulfuricans* medium. Organic component prepared separately from salt (plus carbonate) and iron sulphate components. All components sterilised by autoclaving at 121 °C, 1.05 kg cm$^2$ for 15 minutes. Where required, agar was added to a final concentration of 2% (w/v), along with sodium citrate to increase the solubility of the iron sulphate, prior to autoclaving. Reductant solutions added as outlined above.

<table>
<thead>
<tr>
<th>Component</th>
<th>g l$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_2HPO_4$</td>
<td>0.5</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>1.6</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>2.0</td>
</tr>
<tr>
<td>CaCl$_2$.$2$H$_2$O</td>
<td>0.06</td>
</tr>
<tr>
<td>MgSO$_4$.$7$H$_2$O</td>
<td>0.5</td>
</tr>
<tr>
<td>Betaine</td>
<td>1.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.0</td>
</tr>
<tr>
<td>Na lactate (70%)</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>20</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>10</td>
</tr>
<tr>
<td>FeSO$_4$ solution</td>
<td>6 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>2% (w/v)</td>
</tr>
<tr>
<td>Tri-sodium citrate</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Materials and Methods

Table 2.10 Minimal medium (MM) (Duckworth et al., 1996). Organic component prepared separately from salt and carbonate component. Both sterilised by autoclaving at 121 °C, 1.05 kg cm$^{-2}$ for 15 minutes. Where required, agar was added to a final concentration of 2% (w/v) prior to autoclaving. Reductant solutions added as outlined above. The carbon source was initially included at 1% w/v. Due to difficulties in the media preparation, this concentration was dropped to 0.5% and then to 0.1% (w/v).

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>0.5 g l$^{-1}$</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>10 g l$^{-1}$</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>1 g l$^{-1}$</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.2 g l$^{-1}$</td>
</tr>
<tr>
<td>Carbon source</td>
<td>1%, 0.5% [0.1%] (w/v)</td>
</tr>
<tr>
<td>NaCl</td>
<td>20 g l$^{-1}$</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>10 g l$^{-1}$</td>
</tr>
<tr>
<td>Agar</td>
<td>2% (w/v)</td>
</tr>
</tbody>
</table>
Materials and Methods

Cultivation

MM was used for the cultivation of these organisms, in both liquid and solid forms. However, the substrate concentration was reduced from 1% to 0.1% (w/v) to allow easier manipulation of the gum based compounds (Table 2.10).

Chemo-organotrophic bacteria (low salt)

Obligately anaerobic chemo-organotrophic bacteria were isolated by serially diluting the low salt lake samples and spreading on solid medium inside an anaerobic chamber. Grant’s Alkaliphile Medium (GAM) was used for the enrichment of these organisms (Grant et al., 1979) (Table 2.11). Resulting colonies were repeatedly subcultured both aerobically and anaerobically.

Haloalkaliphilic bacteria

High salt samples were initially serially diluted and spread on to one of two media. Grant’s Haloalkaliphile Medium (GHAM) (Duckworth et al., 1996) (Table 2.12) was used, along with an adapted version of Oren’s Hypersaline Medium (Oren, 1987) (Table 2.13). This method resulted in very little growth, so a small amount of each sediment sample was spread directly onto the surface of solid medium. Resulting colonies were picked out from within the sediment lumps and subcultured aerobically and anaerobically.
Materials and Methods

Table 2.11 Grant’s alkaliphile medium (GAM) (Grant et al., 1979). Organic component prepared separately from the salt and carbonate component, and autoclaved at 121 °C, 1.05 kg cm\(^{-2}\) for 15 minutes. When required, agar was added to a final concentration of 2% (w/v) prior to autoclaving. Reductant solutions were added as detailed above.

<table>
<thead>
<tr>
<th>Component</th>
<th>g l(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10</td>
</tr>
<tr>
<td>Peptone</td>
<td>5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5</td>
</tr>
<tr>
<td>K(_2)HPO(_4)</td>
<td>1</td>
</tr>
<tr>
<td>MgSO(_4) \cdot 7\text{H}_2\text{O}</td>
<td>0.2</td>
</tr>
<tr>
<td>NaCl</td>
<td>40</td>
</tr>
<tr>
<td>Na(_2)CO(_3)</td>
<td>10</td>
</tr>
<tr>
<td>Agar</td>
<td>2% (w/v)</td>
</tr>
</tbody>
</table>
Table 2.12 Grant’s haloalkaliphile medium (GHAM) (Duckworth et al., 1996). Organic component prepared separately from the salt and carbonate component, and autoclaved at 121 °C, 1.05 kg cm\(^{-2}\) for 15 minutes. When required, agar was added to a final concentration of 2% (w/v) prior to autoclaving. Reductant solutions were added as detailed above.

<table>
<thead>
<tr>
<th>Component</th>
<th>g l(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>7.5</td>
</tr>
<tr>
<td>Tri-sodium citrate</td>
<td>3.0</td>
</tr>
<tr>
<td>KCl</td>
<td>2.0</td>
</tr>
<tr>
<td>MgSO(_4)_7H(_2)O</td>
<td>1.0</td>
</tr>
<tr>
<td>MnCl(_2)_4H(_2)O</td>
<td>1 ml of 0.36g/l</td>
</tr>
<tr>
<td>FeSO(_4)_7H(_2)O</td>
<td>0.1 ml of 50g/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>200</td>
</tr>
<tr>
<td>Na(_2)CO(_3)</td>
<td>10</td>
</tr>
<tr>
<td>Agar</td>
<td>2% (w/v)</td>
</tr>
</tbody>
</table>
Table 2.13 Oren's hypersaline medium (Oren, 1987). Organic component prepared separately from the salt and carbonate component, and autoclaved at 121 °C, 1.05 kg cm⁻² for 15 minutes. When required, agar was added to a final concentration of 2% (w/v) prior to autoclaving. Reductant solutions were added as detailed above.

<table>
<thead>
<tr>
<th></th>
<th>Oren (1987)</th>
<th>Adapted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g l⁻¹</td>
<td>g l⁻¹</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>KCl</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>20.3</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>7.35</td>
<td>0.06</td>
</tr>
<tr>
<td>NaCl</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>-</td>
<td>18.5</td>
</tr>
<tr>
<td>Agar</td>
<td>2% (w/v)</td>
<td>2% (w/v)</td>
</tr>
</tbody>
</table>

Materials and Methods
2.4 $S^{2-}$ production by SRB

A simple visible test for the production of $S^{2-}$ was employed to test positive enrichments.

**Materials**

- **H$_2$S Reagent:** CuSO$_4$ 0.02g (5 mM final concentration)
- HCl 1 ml, 4N (50 mM final concentration)
- H$_2$O 79 ml

**Method**

a. 0.2 ml of enrichment culture was added to 1 ml $S^{2-}$ test reagent via a syringe

b. When injecting the culture into the test solution, the tip of the syringe needle was kept beneath the level of the solution to prevent the escape of volatile $S^{2-}$.

c. The mixture was then shaken, and the intensity of the resulting brown colour indicated the amount of $S^{2-}$ present. A positive SRB control and a negative control of uninoculated medium was included in the test

2.5 Viable Counts

Due to the limited growth of haloalkaliphilic organisms, viable counts were only carried out on the low-salt lake samples using GAM. Serial dilutions were prepared using (w/v) 4% NaCl, 1% Na$_2$CO$_3$, 0.02% MgSO$_4$·7H$_2$O to a dilution of $1\times10^{-6}$. 100 µl of each dilution was spread on a GAM agar plate and incubated at 37°C.
2.6 Morphology

2.6.1 Colony Morphology

The morphology of each isolate was recorded following 2 to 7 days incubation at 37 °C. Morphological characteristics noted were colony colour, size (diameter in mm), form (regular, irregular, filamentous), elevation (flat, low convex, domed, umbonate) and margin (entire, undulate, lobate, erose, filamentous).

2.6.2 Cell Morphology

Microscopic analysis was carried out on wet mounts of each isolate. Gram stains were attempted for each organism, but these proved to be unsuccessful, probably due to the alkaliphily of the isolates. Cellular shape (rod, cocci), size (length and width in μm), motility and spatial organisation were examined using phase contrast microscopy at ×1000 magnification.

2.7 Physiology

2.7.1 Salt range for growth

The salt range for growth of fermentative bacteria was tested using GAM agar plates with varying salt concentrations at pH 10.5. Salt concentrations tested were (w/v) 0%, 4%, 8%, 12% and 16%. Haloalkaliphilic isolates were tested using GHAM agar plates with (w/v) 0%, 4%, 8%, 12%, 16%, 20%, 24% and 30% NaCl at pH 10.5.

2.7.2 pH range for growth

The pH range for growth of fermentative bacteria was tested using adapted GAM or GHAM agar plates. To facilitate buffering at lower pH, the level of
Na₂CO₃ was reduced to 1 g l⁻¹, and 4.64 g l⁻¹ Tris(hydroxymethyl)-
aminomethane (TRIS) was added to the salts component of the medium. The
pH of this was then adjusted using HCl to the desired level. The pH levels
tested for both groups of organisms were 7.5, 8.5, 9.5 and 10.5.

2.7.3 Antibiotic sensitivity

Sensitivity to the antibiotics vancomycin and colicin was tested by placing
antibiotic disks (30 μg) onto freshly inoculated agar plates. Sensitivity was
found to be reduced at both high pH and high salt concentrations, therefore
these tests were carried out at pH 8.5, 4% NaCl (w/v) for fermentative
organisms, and pH 9.5, 16% NaCl (w/v) for haloalkaliphilic isolates.

2.7.4 Substrate utilisation

The ability to utilise a range of different growth substrates was tested using
the Biolog identification system (Biolog Inc., Hayward, California, USA).
Each 96 well Biolog plate houses 95 separate compounds designed to
determine the fermentative capabilities of test organisms. Each well contains
all the required nutrients to support growth, along with an indicator to show
when the substrate has been broken down.

Materials

Pre-warmed, anaerobic Biolog plates

Multi-channel pipette

Pre-warmed, anaerobic salts solution (4% or 16% w/v NaCl; 1% or 1.85%
Na₂CO₃, pH 10.5)
Method

a. 20 ml liquid cultures were incubated for 3 to 7 days (depending on growth rate) until a turbid culture was obtained.

b. These were then spun at 2500 rpm, 30 °C for 20 minutes to pellet the cells, and then immediately returned to the anaerobic cabinet.

c. The supernatant was poured off, and 10 ml anaerobic salts solution was added, and cell pellets were resuspended.

d. Samples were then centrifuged again, and immediately returned to the cabinet once more. The supernatant was again discarded, and 15 - 20 ml fresh salts solution was added.

e. Each pre-warmed, anaerobic Biolog plate was rapidly inoculated outside the cabinet (150 µl per well) using a multi-channel pipette, and then immediately returned anaerobic environment

f. Biolog plates were then incubated anaerobically at 30 °C, and examined every 24 hours for 7 days.
2.8 Metabolic end product analysis

End products of metabolism were analysed using isothermal gas chromatography (GC). Organic acid standards were included in the analysis to allow the identification of a small number of metabolites. All GC analyses were carried out in the Department of Chemistry, University of Leicester.

2.8.1 Sample preparation

Samples were prepared using a standard protocol, with minor modification to account for the alkalinity and buffering capacity of the media.

Materials

Rotary evaporator
Separation tubes
Storage vials
NaOH
50% HCl solution
basified water (10 ml H₂O, 2 drops NaOH)
Chromatography grade di-ethyl ether

Method

a. 15 ml cultures were centrifuged at 2500 rpm for 20 minutes, and the supernatant was retained. Control samples of uninoculated medium were included throughout.

b. Each supernatant was then filtered, firstly through a 0.45 μm pore size cellulose acetate filter, then through a 0.2 μm pore size filter.
**Materials and Methods**

Filtered supernatants could then be stored at -20 °C until needed.

c. Samples were dried using a rotary evaporator at 45 °C under vacuum. Care was taken not to over-dry the samples, as this would make resuspension difficult.

d. Dried residues were then resuspended in 5 ml basified water.

e. Basified samples were then extracted twice by shaking with 2 ml di-ethyl ether. After each extraction, the upper organic phase was removed and discarded.

f. Each sample was then acidified by adding 6 - 12 drops HCl solution. Due to the buffering capacity of the samples, the pH was tested during acidification using pH indicator strips. HCl was added until a pH <4 was obtained.

g. Once acidified, samples were extracted by shaking twice with di-ethyl ether, firstly with 300 µl, then with 250 µl. The upper, organic phases were carefully removed and pooled.

h. Extracts were stored at -20 °C ready for GC analysis in small storage vials.

**2.8.2 Gas Chromatography (GC)**

**Materials**

- Pye Unicam PU4500 capillary chromatograph
- HP-FFAP (crosslinked FFAP) chromatography column
- Hewlett Packard integrator
- Organic acid standards (25 mM)
  - Acetic acid
iso-Butyric acid
Butyric acid
iso-Valeric acid
Valeric acid
Caproic acid

Method

a. GC analysis was carried out iso-thermally under the following conditions.

i. Injector temperature 250 °C
ii. Detector temperature 300 °C
iii. Column temperature 150 °C
iv. Carrier gas Helium (1 kg cm²)
v. Hydrogen 1 kg cm²
vi. Air 0.5 kg cm²

b. An attenuation was chosen which gave good peak resolution whilst maintaining a low background noise level.

c. Once the GC had equilibrated to the correct temperatures, standard solutions were run to ensure the correct operation of the machine, and to determine the retention times of the known compounds.

d. Samples were run by applying 10 μl each extract to the column using a Hamilton syringe. The average length of run was approximately 15 minutes, after which time no further compounds came off the column.
Materials and Methods

e. Periodically, standards were re-run to reveal any changes in retention times during the analysis.

2.9 Long term storage of bacterial isolates

The method used for the long term storage of isolated organisms was that of Jones et al. (1991). During the beading protocol, manipulations were carried out under strictly anaerobic conditions using anaerobic media. To reduce the ingress of oxygen during storage, vials were sealed using layers of Parafilm.

2.9.1 Materials

Acid-washed 2 mm glass embroidery beads (Creative Beadcraft Ltd)
1.75 ml capacity screw-cap glass vials
Liquid growth medium with 15% (v/v) glycerol
-70 °C freezer

2.9.2 Method

a. Approximately 20 - 30 beads were placed in each vial, which were then sterilised by autoclaving.

b. 1 ml of beading medium was then aseptically added to each vial, which were then agitated to remove air pockets from within the beads.

c. Surface growth from solid media was removed using a sterile loop, and vigorously mixed with the beading medium. Following this, excess medium was removed using a sterile pipette to facilitate easy withdrawal of beads once frozen.

d. Vials were then placed in a -70 °C freezer for long-term storage.
2.10 Denaturing gradient gel electrophoresis (DGGE)

DGGE was used to resolve mixed PCR amplification products following touchdown PCR of SRB enrichments. This work was carried out at the Department of Environmental Microbiology, University of Warwick.

2.10.1 Preparation

Materials

Bio-Rad D-Gene denaturing gel electrophoresis system (Bio-Rad, California, USA)

50× TAE buffer

Tris base 242 g
Acetic acid 57.1 ml
0.5 M EDTA (pH 8) 100 ml
Distilled H₂O to 1000 ml

0% Denaturing solution (5% acrylamide)

40% Acrylamide / Bis-acrylamide (37.5 : 1) solution 12.5 ml
50× TAE 2.0 ml
Distilled H₂O to 100 ml

100% Denaturing solution (5% acrylamide)

40% Acrylamide / Bis-acrylamide (37.5 : 1) solution 12.5 ml
50× TAE 2.0 ml
Formamide 40 ml
Urea 42 g
Distilled H₂O to 100 ml

Loading buffer

  Bromophenol blue  0.05g
  Xylene cyanol  0.05g
  1× TAE  10 ml

10% Ammonium persulphate (APS)

TEMED

0.5× TAE running buffer

**Materials and Methods**

**Method - perpendicular gels**

a. Perpendicular gels had a total volume of 9 ml. For a gradient of 0% - 100% denaturant, 4.5 ml each denaturing solution was used.

b. 40 μl APS and 4 μl TEMED were added to each light (0%) and heavy (100%) solution, and briefly vortexed.

c. Bearing in mind that perpendicular gels were bottom filled, each solution was taken into a syringe and placed on the appropriate side of the gradient delivery system.

d. The gradient was poured by slowly rotating the cam wheel of the delivery system in one continuous motion.

e. Following pouring, gels were left to polymerise for approximately 1 hour on a level surface.

**Method - parallel gels**

a. Parallel gels had a total volume of 26 ml. Based on information from the perpendicular gels, a gradient of 20% - 50% denaturant was used.
Materials and Methods

b. 20% denaturing solution was prepared by mixing 2.6 ml 100% denaturing solution with 10.4 ml 0% denaturing solution.

c. 50% denaturing solution was prepared by mixing 6.5 ml of both 100% and 0% denaturing solutions.

d. 115 μl APS and 11.5 μl TEMED were added to each solution and mixed.

e. Parallel gels were top filled, so each solution was taken up into a syringe and attached to the appropriate side of the gradient delivery system.

f. The gradient was poured by slowly rotating the cam wheel of the gradient maker in one continuous motion.

g. The top of the gel was then immediately filled with 0% denaturing solution, and a comb was inserted. The gel was then allowed to crosslink for at least 1 hour.

2.10.2 Running conditions

The electrophoresis kit was assembled as per the manufacturers instructions. The running buffer (0.5× TAE) and gels were allowed to equilibrate to 60 °C before samples were loaded.

Perpendicular gels

For perpendicular gels, the sample had to be loaded across the entire width of the gel. Therefore a large sample volume was required for this procedure.

Samples were prepared by mixing 25 μl PCR product with 75 μl TE and 100 μl loading buffer, giving a total volume of 200 μl. This was loaded onto the gel using a Gilson pipette.
Perpendicular gels were run at a constant voltage of 200 V, and at a constant temperature of 60 °C for 90 minutes.

Parallel gels

Samples were prepared by mixing 20 µl PCR product with 20 µl loading buffer. These were applied to wells within the gel using a Gilson pipette.

Parallel gels were run at a constant voltage of 200V, and at a constant temperature of 60 °C for 200 minutes.

2.10.3 Staining procedure

The gel was carefully removed from the glass plate sandwich and placed in a staining tank containing 250 ml 0.5× TAE and 100 µl ethidium bromide (1 mg ml⁻¹). The staining tank was gently rocked for 10 - 15 minutes to stain the gel.

Following this, the gel was transferred to a tank containing 0.5× TAE, where it was destained for 15 minutes. Bands within the gels could then be visualised using a UV transilluminator.

2.10.4 DNA recovery

DNA was recovered from the DGGE gels by excising the bands using a razor blade, and transferring to a small volume of 10 mM Tris-HCl in a microfuge tube. Each gel slice was then fragmented and incubated at 4 °C for 24 hours. This incubation time allowed the small amount of DNA present to elute from the gel into the surrounding milieu.

Eluted samples were then amplified using the standard PCR protocol described below, except that 10 µl each sample was used per reaction. Following PCR, samples were sequenced in the normal way.
2.11 16S rRNA gene analysis

2.11.1 Whole cell DNA extraction

Fresh cultures of each organism were used for whole cell DNA extraction (Pitcher et al., 1989). These were incubated for 2 - 7 days, depending on growth rate, in 15 ml cultivation medium. Clean DNA was obtained using a modified rapid DNA extraction technique (Pitcher et al., 1989).

Chemical extraction

Materials

Lysis solution

- Guanidium thiocyanate 60 g
- EDTA (0.5 M, pH 8) 20 ml
- deionised water 20 ml

Heated to 65 °C with mixing, then cooled.

- Sacrosyl (10% v/v) 5 ml
- deionised water to 100 ml

Lysozyme solution  (50 mg ml⁻¹ in TE, pH 8)

Ammonium acetate  (7.5 M)

Chloroform / isoamylalcohol (24:1)

Propan-2-ol

Ethanol (70 % v/v)

RNase solution (10 mg ml⁻¹ RNase)
Materials and Methods

Method

a. Broths were harvested at 2500 rpm, room temperature for 20 minutes. The pellet was then resuspended in 1.5 ml salts solution and transferred to a 2 ml microfuge tube. Samples were then centrifuged again at 13,000 rpm for 2 minutes.

b. The resulting cell pellets were resuspended by vortexing briefly in 150 μl lysozyme solution, and incubated at 37 °C for 30 minutes.

c. 500 μl lysis solution was then added, and samples were briefly vortexed again then incubated on ice for 10 - 20 minutes.

d. Lysates were then mixed with 250 μl cold ammonium acetate and incubated on ice for a further 10 minutes.

e. Organic extraction was carried out by mixing the lysates with 500 μl chloroform / isoamylalcohol and centrifuging at 13,000 rpm for 10 minutes to separate the phases.

f. The upper, aqueous layer was removed, and DNA was precipitated by adding 0.54 volumes cold propan-2-ol. Complete precipitation of DNA was ensured by incubating at -20 °C for 20 minutes.

g. The precipitated DNA was retrieved by centrifuging at 13,000 rpm for 10 minutes. Following careful removal of the supernatant, the DNA pellet was washed briefly with 70% ethanol, and recentrifuged.

h. The resulting DNA pellet was dried under vacuum using a rotary vacuum dryer for 10 minutes.

i. The pellet was then resuspended in 50 - 100 μl Tris·HCl (10 mM, pH 8) with 2 μl RNase solution overnight at 4 °C. DNA was stored for short periods at 4 °C, or for longer at -20 °C.
Materials and Methods

Crude extraction

A rapid, crude method of DNA extraction was also utilised, although this did not work for all organisms.

A small number of colonies were scraped from the surface of an agar plate and suspended in 100 μl Tris·HCl (10 mM, pH 8). 10 μl of this solution was used directly in the PCR mix without prior treatment. It was found that the initial 8 minute, 98 °C heating step of the PCR reaction caused enough disruption of the cells to enable DNA amplification.

2.11.2 DNA quantitation

DNA extractions and PCR amplifications, along with appropriate negative controls, were checked using agarose gel electrophoresis. Each gel was prepared using 1% agarose in TAE (pH 7.5), and samples were prepared for loading by mixing 2 μl with 1 μl 6x loading buffer. All samples were run alongside a 1 kb size marker. Electrophoresis was carried out in TAE (pH 7.5) buffer supplemented with 0.5 μg ml⁻¹ ethidium bromide, at a constant voltage of 70 V. Following electrophoresis, gels were destained in TAE for 15 minutes, and then bands were visualised using an ultraviolet transilluminator.

DNA concentration was determined spectroscopically using a UV/Vis scanning spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Samples were diluted 1:50 in nanopure water, and absorption at 260 nm and 280 nm was determined. An absorption ratio (A₂₆₀ / A₂₈₀) of 1.8 for DNA and 2.0 for primers indicated high purity product. Quantity of DNA was calculated using the following equations.

Double-stranded DNA (μg ml⁻¹) = A₂₆₀ × 50 × dilution factor.

Single-stranded DNA (μg ml⁻¹) = A₂₆₀ × 33 × dilution factor.
2.11.3 Primer selection and development

Selection - universal primers

Universal primers were used for the amplification and sequencing of DNA extracted from organisms in pure culture. Each primer was designed to anneal to a conserved region of the 16S rRNA gene found in all bacteria. Primers FD1 and rP1 were used to amplify a 1500 kb region of the 16S gene. Following amplification, the fragment was fully sequenced using these primers in conjunction with 4 additional internal primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>position</th>
</tr>
</thead>
<tbody>
<tr>
<td>FD1</td>
<td>AGA GTT TGA TCC TGG CTC AG</td>
<td>8 - 27</td>
</tr>
<tr>
<td>rP1</td>
<td>ACG G(TCA)T ACC TTG TTA CGA CTT</td>
<td>1512 - 1492</td>
</tr>
<tr>
<td>FD2N</td>
<td>CAG C(AC)G CCG CGG TAA TAC</td>
<td>519 - 536</td>
</tr>
<tr>
<td>FD8</td>
<td>ATT AGA TAC CC(TG) GGT AGT CC</td>
<td>789 - 807</td>
</tr>
<tr>
<td>rD2</td>
<td>G(TA)A TTA CCG CGG C(GT)G CTG</td>
<td>536 - 519</td>
</tr>
<tr>
<td>rD8</td>
<td>GAC TAC C(AC)G GGT ATC TAA TC</td>
<td>807 - 789</td>
</tr>
</tbody>
</table>

Selection - SRB specific primer

A PCR primer specific for the amplification of SRB was designed for the selective amplification of DNA isolated from SRB enrichment cultures. This was used in conjunction with a second primer bearing a 3' 'GC-clamp' for use with denaturing gradient gel electrophoresis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>position</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRB247F</td>
<td>TGA AGA TGG GTC CGC GTA CC</td>
<td>247 - 267</td>
</tr>
</tbody>
</table>
Materials and Methods

SRB247GC  CGC  CCG  CCG  CGC  GCG  GCG  GGC
        GGG  GCG  GGG  GCA  CGG  GGG  GTG
        AAG  ATG  GGT  CCG  CGT  ACC

Primer synthesis

Primers were synthesised using an Applied Biosystems 380B DNA synthesiser, and were cleaned up using the following protocol.

a. Each primer solution was divided into two giving more manageable volumes.

b. To remove any solid support material which may be carried over with the primers, each aliquot was centrifuged at 13,000 rpm for 2 minutes.

c. Primer solutions were then mixed with 0.1 volumes 3M sodium acetate (pH 5.2), and precipitated by adding 2.5 volumes ice cold 100% ethanol. After gentle mixing, these were incubated at -20 °C for 20 minutes.

d. Precipitated DNA was then collected by centrifuging at 13,000 rpm for 20 minutes.

e. The resulting DNA pellet was washed with 70% (v/v) ethanol, and recentrifuged at 13,000 rpm for 10 minutes.

f. Primer pellets were then dried using a rotary vacuum desiccator, and resuspended in 500 μl nanopure water.

Primer concentration was determined spectrosopically as previously described, using the following equation.

Concentration (μg ml⁻¹) = A_{260} \times 33 \times \text{dilution factor}.
Alternatively the molarity of the primer could be calculated, giving a more useful figure for use in PCR amplifications and cycle sequencing. This concentration was ascertained by multiplying the sum of each base within the primer with its respective base value; A = 15.4, C = 7.3, G = 11.7 and T = 8.8. This value \( B \) was then used in the following equation.

\[
\text{Concentration (pmol } \mu\text{l}^{-1}) = \frac{A_{\text{sum}}}{B} \times 1000
\]

Generally, for PCR reactions, a primer concentration of 20 pmol \( \mu \text{l}^{-1} \) was used, whereas for cycle sequencing a primer concentration of 3.2 pmol \( \mu \text{l}^{-1} \) was used. All primer solutions were stored at -20 °C.

2.11.4 PCR amplification of 16S rDNA

Materials

- Perkin Elmer Cetus DNA thermal cycler
- 10× PCR buffer 670 mM Tris·HCl (pH 8.8)
- 20 mM MgCl₂
- Nucleotide mix 25 mM each dNTP
- Forward primer 20 pmol \( \mu \text{l}^{-1} \) FD1
- Reverse primer 20 pmol \( \mu \text{l}^{-1} \) rPl
- Template DNA 10 - 100 ng \( \mu \text{l}^{-1} \)
- Taq polymerase 2 U
- Sterile nanopure water

Method - standard PCR

a. For each reaction to be carried out, the following reagents were used.

10 µl 10× PCR buffer, 1 µl each primer and 0.5 µl dNTP mix. These
component volumes were multiplied by the number of reactions (including a negative control) +1, and were then mixed in a microfuge tube. *e.g.* For 10 reactions, 110 µl PCR buffer, 11 µl each primer and 5.5 µl dNTP mix were combined in a microfuge tube and then aliquoted into reaction tubes, 12.5 µl in each vessel.

b. An appropriate volume of DNA was added to each reaction tube, with sterile nanopure water used as the negative control.

c. The volume of each tube was then brought to 98 µl with sterile nanopure water, and each reaction mix was overlaid with 40 µl light mineral oil.

d. 1 - 2 drops of light mineral oil was added to each well on the heating block of the PCR machine to ensure an even surface contact.

e. Reaction vessels were then added to these wells, and the DNA was denatured at 98 °C for 5 - 8 minutes (hot-start PCR).

f. Following this denaturing step, the reaction tubes were cooled to 94 °C, and 2 µl *Taq* polymerase (1 U µl⁻¹) was added to each vessel.

g. The following PCR reaction was carried out for 25 cycles.

<table>
<thead>
<tr>
<th>Ramp to</th>
<th>94 °C</th>
<th>30 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>94 °C</td>
<td>60 s</td>
</tr>
<tr>
<td>Ramp to</td>
<td>55 °C</td>
<td>60 s</td>
</tr>
<tr>
<td>Hold</td>
<td>55 °C</td>
<td>60 s</td>
</tr>
<tr>
<td>Ramp to</td>
<td>72 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>Hold</td>
<td>72 °C</td>
<td>60 s</td>
</tr>
</tbody>
</table>
Materials and Methods

h. A final extension was carried out at 72 °C for 7 minutes, following which the temperature was reduced to 4 °C until the reaction was ended.

Method - ‘touchdown’ PCR

The method of dropdown PCR (Don et al., 1991) was used to amplify 16S rDNA from SRB enrichments. This method was selected because it increased the specificity of primer binding, and reduced the production of non-specific PCR products. Each round of drop-down PCR was followed by a second round of amplification using the standard protocol, but using the SRB forward primer with the GC-clamp attached.

a. Reaction vessels were prepared in the same way as before.

b. Template DNA was melted at 96 °C for 5 minutes. Following this, reaction vessels were cooled to 80 °C for the addition of Taq polymerase.

c. The maximum ramp rate of the PCR machine was used to change between each temperature. Cycles were carried out as follows:

<table>
<thead>
<tr>
<th>Ramp to</th>
<th>94 °C</th>
<th>1 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>94 °C</td>
<td>60 s</td>
</tr>
<tr>
<td>Ramp to</td>
<td>annealing temp</td>
<td>1 s</td>
</tr>
<tr>
<td>Hold</td>
<td>annealing temp</td>
<td>60 s</td>
</tr>
<tr>
<td>Ramp to</td>
<td>72 °C</td>
<td>1 s</td>
</tr>
<tr>
<td>Hold</td>
<td>72 °C</td>
<td>120 s</td>
</tr>
</tbody>
</table>

The initial annealing temperature was calculated at 10 °C above the known annealing temperature of the primer.
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d. Two cycles were carried out at this annealing temperature, following which the annealing temperature was dropped by 1 °C, and another two cycles performed.

e. This protocol of a 1 °C drop every two cycles was continued until the known annealing temperature of the primer was attained. At this point, ten cycles were performed at constant annealing temperature, followed by a final extension at 72 °C for 7 minutes.

f. Samples were then held at 4 °C until the reactions were recovered.

PCR product purification

Following PCR, reactions were cleaned up using Qiagen quickspin PCR purification columns (Qiagen Ltd., Dorking, England). The method used was adapted from the manufacturers protocol.

a. PCR products were mixed with 5 volumes PB buffer.

b. This mix was applied to the Qiagen columns placed in 2 ml collection tubes.

c. Columns were then spun at 13,000 rpm for 60 s, and the contents of the collection tubes discarded.

d. Each column was washed by the addition of 750 µl PE buffer, and centrifugation for 60 s.

e. To ensure the complete removal of all ethanol, collection tubes were drained and the columns were respun for a further 60 s.

f. The columns were then transferred to fresh, sterile 1.5 ml microfuge tubes, and the DNA was eluted from the column by applying 30 µl of
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10 mM Tris·HCl to the centre of the gel matrix. This was then centrifuged again for 60 s.

g. To ensure all DNA was recovered from the column, a further 20 µl Tris·HCl was added to the gel matrix, and the columns were spun for 120 s.

Samples were eluted with 10 mM Tris·HCl to prevent auto-catalysis of the DNA, which may occur if the acidic DNA is not buffered. The DNA concentration of each reaction was calculated spectroscopically as previously described, and samples were either stored at 4 °C for immediate use, or held at -20 °C for long term storage.

2.11.5 Taq dye terminator cycle sequencing

DNA samples obtained from PCR were sequenced using the Applied Biosystems Taq-FS dye terminator cycle sequencing kit, followed by automated DNA sequencing using an Applied Biosystems automated sequencing unit (Applied Biosystems, Warrington, England).

Materials

Perkin Elmer Cetus DNA thermal cycler (Perkin Elmer, Warrington, England)

Primer 3.2 pmol each reaction

Template DNA 0.1 - 0.2 µg each reaction

ABI Taq-FS dye terminator reaction premix

Sterile nanopure water
Method

a. Using the DNA concentration value for each sample, calculated following the PCR reaction, an appropriate volume of PCR reaction product was mixed with sterile nanopure water to a final volume of 7 μl.

b. 1 μl primer was added to 4 μl water, giving a final volume of 5 μl.

c. Each reaction vessel was then made up using the following volumes. 8 μl reaction premix, 1 volume (5 μl) primer mix and 1 volume (7 μl) template mix, giving a final reaction volume of 20 μl.

d. Each reaction was overlaid with 40 μl light mineral oil.

e. Tubes were then immediately transferred to a preheated (96 °C) thermal cycler, and cycling was started.

f. The following cycling protocol was used for all sequencing reactions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ramp to</td>
<td>96 °C</td>
<td>1 s</td>
</tr>
<tr>
<td>Hold</td>
<td>96 °C</td>
<td>28 s</td>
</tr>
<tr>
<td>Ramp to</td>
<td>54 °C</td>
<td>1 s</td>
</tr>
<tr>
<td>Hold</td>
<td>54 °C</td>
<td>13 s</td>
</tr>
<tr>
<td>Ramp to</td>
<td>60 °C</td>
<td>1 s</td>
</tr>
<tr>
<td>Hold</td>
<td>60 °C</td>
<td>240 s</td>
</tr>
</tbody>
</table>


g. Following the reaction, the samples were held at 4 °C.

Total time for the sequencing reaction was approximately 2 h 52 min, within the recommended reaction time of 2 h 47 min ± 5 minutes.
Sequencing product purification

a. reaction volumes were increased to 100 µl by the addition of 80 µl sterile nanopure water.

b. 100 µl sequencing grade chloroform was added with mixing to remove the mineral oil, and samples were centrifuged at 13,000 rpm for 60 s.

c. The upper aqueous layer was removed to a fresh tube, ensuring there was no carry over of the chloroform.

d. 0.1 volume 3M sodium acetate (pH 5.2) was added with mixing, and extension products were precipitated by adding 2.5 volumes 95% (v/v) ethanol. Samples were allowed to precipitate at -20 °C for 20 minutes, and then were centrifuged at 13,000 rpm for 20 minutes.

e. Following centrifugation, the supernatant was carefully removed using a Gilson pipette, taking care to avoid the area of the DNA pellet, which could not normally be seen.

f. The pellet was then washed with 70% (v/v) ethanol, which was removed without the need for centrifugation.

g. Pellets were then dried using a rotary vacuum desiccator.

2.11.6 Automated DNA sequencing

Cycle sequencing products were sent for analysis to the Centre for the Mechanisms of Human Toxicology, University of Leicester, where they were analysed using an Applied Biosystems 373 automated DNA sequencer.
2.12 Phylogenetic analysis

Sequence data obtained using automated DNA sequencing was used to classify organisms based on their phylogenetic relationship to known samples. The majority of this work was carried out on Macintosh and PC based computer systems.

2.12.1 Fragment assembly

The six sequencing products obtained for each isolate were assembled using the Applied Biosystems SeqEd programme on a Macintosh computer. Each fragment was loaded into the programme, and reverse complemented where necessary (reverse primers). Following the removal of unusable sequence from the beginning and end of the fragments, overlaps were found either by eye, or by using the programmes built in automated overlap utility.

This resulted in an entire sequence, the majority of which was covered by at least 2 sequencing products. These overlaps enabled the confirmation of ambiguous bases by comparing the chromatograms produced by the automated sequencer. The majority of the ambiguities could be resolved by comparing chromatograms, but those which could not were assigned an ambiguity base code (Table 2.14).

Once fragments had been assembled and ambiguities resolved, a consensus sequence was produced by the computer programme which could be used for subsequent phylogenetic analysis.
Table 2.14 Ambiguity codes used in sequence determination.

<table>
<thead>
<tr>
<th>Ambiguous bases</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>T / G</td>
<td>K</td>
</tr>
<tr>
<td>T / A</td>
<td>W</td>
</tr>
<tr>
<td>C / G</td>
<td>S</td>
</tr>
<tr>
<td>C / A</td>
<td>M</td>
</tr>
<tr>
<td>C / T</td>
<td>Y</td>
</tr>
<tr>
<td>A / G</td>
<td>R</td>
</tr>
<tr>
<td>G / T / A</td>
<td>D</td>
</tr>
<tr>
<td>C / G / A</td>
<td>V</td>
</tr>
<tr>
<td>T / A / C</td>
<td>H</td>
</tr>
<tr>
<td>C / G / T</td>
<td>B</td>
</tr>
<tr>
<td>G / A / C / T</td>
<td>N</td>
</tr>
</tbody>
</table>
2.12.2 Primary sequence analysis

Consensus sequences obtained from the SeqEd application were first analysed using the FASTA database query programme. This programme was available on-line using the World Wide Web (WWW), allowing access to GenBank and EMBL sequence databases which were updated daily. The FASTA programme carries out a very basic alignment of the test sequence to all other sequences in the database, returning a list of the most closely related organisms. This allowed preliminary identification of the test organism, which could then be used when selecting organisms for detailed phylogenetic analysis.

2.12.3 Sequence alignment

Using the information returned from the FASTA search, a number of pre-aligned sequences were downloaded, via the WWW, from the small-subunit rRNA database in Belgium (Van de Peer et al., 1996a). This source of sequence data was used because all sequences were pre-aligned, based on primary sequence along with secondary structure information, and also a number of custom computing tools were available to assist in the handling of the data.

The first of these tools, DCSE (Dedicated Comparative Sequence Editor) was used in the multiple alignment of the sequences (De Rijk and De Wachter, 1993). Pre-aligned sequences from the low G + C group of the Gram Positive bacteria were downloaded in DCSE file format. A secondary structure data file for this alignment was also downloaded.

Test sequences were added to this alignment by aligning them to their closest relative, based on the FASTA information. Preliminary alignment was carried out using the internal pairwise alignment option within the DCSE package. Following this, the alignment was checked and secondary structure elements
Materials and Methods

were corrected. Alignment data could be easily converted from DCSE format into other formats using the sister programme to DCSE, CONVERS (De Rijk and De Wachter, 1993).

One of the benefits of the DCSE package is that it acts as a database, allowing the extraction of a small number of organisms from the full alignment. It also allows easy editing of the alignment since bases can be coloured, and secondary structure elements can be highlighted and numbered.

Once alignments had been completed, closely related organisms were selected for output and subsequent phylogenetic analysis. Each group also contained a distantly related organism which would be used as an outgroup during the construction of phylogenetic trees.

2.12.4 Phylogenetic tree construction

All phylogenetic trees were constructed using the TREECON for Windows package (Van de Peer and De Wachter, 1994). This package brings together all the tools needed for distance calculation and tree construction, although there are some limitation in the choice of algorithms.

Sequence alignments produced using the DCSE package were input into the first element of the TREECON programme. This part of the application carried out distance estimation calculations using the substitution rate calibration of Van de Peer et al. (1996b, c). The topology of the tree for these distance calculations was inferred using the second application in the TREECON package. This was based on the neighbour-joining method for inferring tree topology. Due to the fact that this tree drawing package can only produce rooted trees, a distantly related outgroup included in the alignment was forced out using the third application of the TREECON package. Following this, a tree could be drawn using the drawing component of the application.
Results and Discussion: Sample Collection and Site Descriptions

During an expedition in December 1992 to the Kenya section of the East African Rift Valley, anaerobic samples were collected from five alkaline lakes. From these five lakes, a total of 16 samples were obtained; 11 from the northern, non-saline lakes, and 5 from the hypersaline Lake Magadi in the south. All samples were stored and manipulated under completely anaerobic conditions.

Samples and on site measurements for each location were obtained using the methods described Section 2.1 and Section 2.2. The lake sites and sample locations are presented in chronological order as visited during the expedition rather than physical (north - south) location within the rift valley.
3.1 Sample Locations

3.1.1 Lake Elmenteita

The water level of Lake Elmenteita was found to be relatively high during the time of the expedition (Figure 1.4). Large numbers of flamingos were present on the lake, along with a small number of storks. Initially there appeared to be little *Spirulina*, but it was noticed that as the sun warmed the water column, *Spirulina* could be seen rising to the surface forming green ribbons close to the shore. Physical measurements are presented in Table 3.1.

North-east Shore (9.30 am)

The shore of the lake consisted of brown/black gritty soil whilst the lake bed had a thin layer of silt above a stony subsurface. Also present on the lake bed were numerous silty mounds which may have been worm casts.

Sample **E1NE** taken from a silty mound 2 m from the edge of lake. At this location there was no detectable smell of sulphide (Figure 3.1 A).

South-east Bay (11.55 am)

The edge of this bay was swamp-like in appearance, with dense growth of rushes and reeds. Small pools were present near the lake edge which were claret red in colour, presumably containing phototrophic bacteria. The lake water, which was brown/green in colour, was quite shallow and teaming with larvae. Below the water column lay a 10 cm thick layer of soft mud overlaying a hard, stony layer. Bubbles of gas could be seen emanating from the sediment, and there was a powerful smell of sulphide.

Sample **E2SE1** taken using the coring rod from sediment at the waters edge (Figure 3.1 B).
Results and Discussion: Sample Collection and Site Descriptions

Sample E3SE2 was taken from the shallow water of red/brown pool which was bordered by rushes and reeds (Figure 3.1 C).

3.1.2 Lake Nakuru

The level of the water in this lake was found to be very low, exposing large areas of dried soda flats. The lake was inhabited by vast numbers of flamingos, some stork and a few smaller birds (Figure 1.3). Also present were a number of hippopotami. Physical measurements are shown in Table 3.2.

Hippo Point (8 am)

Samples were taken adjacent to an inflow (pH 9.5) which originated from a sewage treatment plant serving the town of Nakuru. A distinctive smell of sulphide was detected emanating from the thick heavy mud underlying a thin layer of soft surface sediment.

Sample N4HP was taken from the edge of the lake using a tongue depressor to dig through the soft surface sediment revealing the black mud underneath (Figure 3.2 A).

West Lagoon (9.30 am)

This lagoon, situated between Baboon Cliff and Pelican Point, consisted of extensive mud and soda flats. The lake was heavily populated by lesser flamingos which were seen to be feeding. The shore of the lake was thick with excrement and decaying matter, and had a fetid smell. The receding level of the lake could clearly be seen at this site.

Sample N5WL was taken from a ridge in the polluted sediment covered by shallow water using a tongue depressor (Figure 3.2 B).
--- Results and Discussion: Sample Collection and Site Descriptions

Table 3.1 Measurements taken from the sampling sites of Lake Elmenteita. T = temperature (°C); pH = pH reading (± 0.5 pH units); C = conductivity (mS cm⁻¹); O₂ = oxygen concentration (ppm); R = redox potential (mV). Values thought to be incorrect due to equipment failure are indicated with (?). No anaerobic samples were collected from the north-west shore of Lake Elmenteita.

<table>
<thead>
<tr>
<th>Sample Zone</th>
<th>T</th>
<th>pH</th>
<th>C</th>
<th>O₂</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE Bay: Soil</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NE Bay: Lake edge</td>
<td>23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NE Bay: 5 cm deep</td>
<td>20</td>
<td>10-10.5</td>
<td>26.1</td>
<td>5.5</td>
<td>-140</td>
</tr>
<tr>
<td>NE Bay: Sediment</td>
<td>19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-310</td>
</tr>
<tr>
<td>SE Bay: Edge water</td>
<td>30</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SE Bay: Edge sediment</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SE Bay: Water 1 m out</td>
<td>28</td>
<td>10</td>
<td>25</td>
<td>14 (?)</td>
<td>-90</td>
</tr>
<tr>
<td>SE Bay: Sediment 1 m out</td>
<td>23</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-470</td>
</tr>
<tr>
<td>SE Bay: Red pool water</td>
<td>32</td>
<td>10</td>
<td>16.8</td>
<td>18 (?)</td>
<td>-125</td>
</tr>
<tr>
<td>SE Bay: Red pool sediment</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-500</td>
</tr>
<tr>
<td>NW Bay: Water column</td>
<td>29</td>
<td>10.5-11</td>
<td>27.8</td>
<td>9</td>
<td>-65</td>
</tr>
<tr>
<td>NW Bay: Silt mound</td>
<td>28</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-190</td>
</tr>
</tbody>
</table>
Results and Discussion: Sample Collection and Site Descriptions

**Figure 3.1** Map showing Lake Elmenteita and the surrounding area. The major tributaries to the lake are also shown. Sampling locations are indicated as follows: A = E1NE, B = E2SE1, C = E3SE2.
Results and Discussion: Sample Collection and Site Descriptions

Table 3.2 Measurements taken from the sampling sites of Lake Nakuru. T = temperature (°C); pH = pH reading (± 0.5 pH units); C = conductivity (mS cm⁻¹); O₂ = oxygen concentration (ppm); R = redox potential (mV). No anaerobic samples were collected from the southern flats of Lake Nakuru.

<table>
<thead>
<tr>
<th>Sample Zone</th>
<th>T</th>
<th>pH</th>
<th>C</th>
<th>O₂</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippo Point</td>
<td>16</td>
<td>10.5</td>
<td>33.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>West Lagoon</td>
<td>20</td>
<td>10.5</td>
<td>51.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Southern Flats: Red pool</td>
<td>34</td>
<td>10.5</td>
<td>12.6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.2 Map showing the major tributaries of Lake Nakuru. Sample locations are indicated as follows: A = N4HP, B = N5WL.
3.1.3 Lake Bogoria

The water level of this lake was much lower than that observed in previous years (Grant and Jones, personal observations), with once submerged sedge beds now high and dry on the shore. Flamingo nests were observed 20 - 30 m from the present water level suggesting that the lake level was much higher during the flamingo mating season. A further 10 m from the lake were a number of more eroded nests, presumably from the previous mating season.

An extensive hot spring complex on its western edge continually fed the lake with hot, slightly alkaline spring water (Figure 1.2). The succession of microorganisms in the streams running from the hot springs could clearly be seen. The hottest areas (e.g. surrounding an active fumarole) were covered in an orange microbial mat, presumably *Chloroflexus* sp. This was replaced in the slightly cooler waters by a green mat of possibly *Synocystis* sp. Further down the hot streams, green ‘streamers’ could be seen, followed by a microbial mat which was dark brown and gelatinous. Microbial growth was isolated mainly to the cooler periphery of the streams as opposed to the more heated and faster flowing central area, producing the characteristic ‘V’ growth often found in hot spring areas. Numerous flamingos could be seen feeding around the hot outflow streams originating from the hot springs. Physical measurements recorded at the sampling locations are presented in Table 3.3.

**South-east Hot Springs (11.45 am)**

This site was slightly further south than the main spring complex, and was dominated by a large (8 m diameter), deep, gently boiling and gassing pool with a neutral pH. The pool was situated approximately 15 m from the edge of the lake, elevated by approximately 0.5 m. From the pool, an outflow meandered through some sedge beds to the lake. There was also evidence of subterranean seepage from the pool, since the whole area was very unstable.
under foot, and the coring rod could be easily pushed through the upper crust of the shore, down some 1.5 m in to the water filled sediment complex beneath.

Sample **B6HS** was taken close to the mouth of the pool outflow where the mud was thick and gelatinous, with a patchy brown and black colour and a strong sulphide (Figure 3.3 A).

**Fig Tree Camp (2 p.m.)**

This site consisted of a rocky shore bordered by a thickly wooded area. The lake water was a milky green/brown colour, and wave action had caused foam to collect around the waters edge. The sediment under the rocks was rough and gritty, and was not particularly black. Nearby a fast flowing stream entered the lake via a brown-coloured channel (Fe³⁺).

Sample **B7FT** - a core sample from partially submerged sediment which was found to be gritty and silty, but not very black (Figure 3.3 B).

**North Shore (4 p.m.)**

Extensive soda flats dominated this area, consisting of white soda crust overlying a thick gelatinous sulphurous mud. Flamingos could be seen feeding on the lake, and the shore line was a mixture of flamingo excrement and mud.

Sample **B8NS1** was lake mud taken from the shallow periphery of the lake (Figure 3.3 C).

Sample **B9NS2** was taken inland, 1 m from the shore line of the lake. The surface soda crust was removed and the thick gelatinous underlying mud was sampled (Figure 3.3 D).
### Results and Discussion: Sample Collection and Site Descriptions

Table 3.3 Measurements taken from the sampling sites of Lake Bogoria. T = temperature (°C); pH = pH reading (± 0.5 pH units); C = conductivity (mS cm⁻¹); O₂ = oxygen concentration (ppm); R = redox potential (mV).

<table>
<thead>
<tr>
<th>Sample Zone</th>
<th>T</th>
<th>pH</th>
<th>C</th>
<th>O₂</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE Hot Springs</td>
<td>52</td>
<td>10</td>
<td>13.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fig Tree Camp</td>
<td>34</td>
<td>11 - 11.5</td>
<td>72.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>North Shore</td>
<td>33 - 34</td>
<td>11</td>
<td>59.6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.3 Map of Lake Bogoria showing the major tributaries and the location of hot springs. Sampling locations are indicated as follows: A = B6HS, B = B7FT, C = B8NS1, D = B9NS2.
3.1.4 Lake Sonachi (Crater Lake)

The water of this lake, which lies within the crater of an extinct volcano, was found to be pea-green in colour. This was bordered by thick sedge beds extending into the lake itself (Figure 1.5). Surrounding the lake and sedge beds was dense, inaccessible forest and vegetation. On-site measurements are presented in Table 3.4.

North-east Shore (11.45 am)

The sampling area was covered by thick sedge beds and a few dead trees. Open water beyond the sedge beds had a depth of about 30 cm and a gritty sediment with a sulphide smell.

Sample C10LB was taken using the coring rod from lake bed. The core had a stony, gritty texture, and a strong sulphide smell (Figure 3.4).

Sample C11SB was a core sample taken from within the sedge beds. This core contained brown mud and detritus, with no detectable smell of sulphide (Figure 3.4).

3.1.5 Lake Magadi

Lake Magadi consists mainly of vast trona flats with very little surface water (Figure 1.6). The lake has a very high NaCl concentration which reaches saturation in the hot, arid environment. This evaporative concentration is exploited commercially, with both salt and soda harvested from the lake (Figure 1.7). Physical measurements recorded at each sampling location are presented in Table 3.5.
Results and Discussion: Sample Collection and Site Descriptions

Table 3.4 Measurements taken from the sampling sites of Lake Sonachi. T = temperature (°C); pH = pH reading (± 0.5 pH units); C = conductivity (mS cm⁻¹); O₂ = oxygen concentration (ppm); R = redox potential (mV).

<table>
<thead>
<tr>
<th>Sample Zone</th>
<th>T</th>
<th>pH</th>
<th>C</th>
<th>O₂</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Sonachi: Water</td>
<td>22</td>
<td>10.5</td>
<td>14.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lake Sonachi: Sedge bed</td>
<td>22</td>
<td>-</td>
<td>12.7</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Results and Discussion: Sample Collection and Site Descriptions

Figure 3.4 Map showing the location of lakes Naivasha and Little Naivasha (Oloiden), and Lake Sonachi (Crater Lake). The major tributaries to Lake Naivasha are also indicated.
Results and Discussion: Sample Collection and Site Descriptions

Salt Evaporation Ponds (4 p.m.)

The salt ponds were bright pink in colour due to the presence of large numbers of halophilic bacteria (Figure 1.7). Salt and soda precipitates covered the sediments of these evaporation ponds.

Sample **M12SP** was a core sample taken from evaporation pond P1. The core consisted of green/black mud with no apparent sulphide smell (Figure 3.5 A).

Main Causeway (7 am)

The first sample site was about 200 m west of pump station 17, beside a lift station. Running adjacent to the causeway was a channel cut into the trona bed which was filled with a slow moving dark brown liquor, flowing east to west. The trona surface was unstable and treacherous, and could be removed to reveal a thick black gelatinous mud which descended about 15 cm to a further hard surface, probably another trona layer. It was not clear whether this showed the various depositional events during the history of the lake, or whether this material had been dredged from the nearby channel. The edge of the channel was pared away to reveal indistinct layering of white trona overlying pink, then black and dark green layers.

Sample **M13CC** was taken using the coring rod from the sediment of the channel (Figure 3.5 B).

Sample **M14CH** was extracted from the sediment underlying the thick trona crust at the side of the channel by digging a hole through the trona surface. The mud was collected using a tongue depressor (Figure 3.5 C).

The second sample site on the main causeway was mid-way between the lift station and pump station number 17. The site was much the same as the previous one, with the exception that the channel was now much smaller,
Results and Discussion: Sample Collection and Site Descriptions

consisting of a shallow rivulet of black liquor over a bed of soft thick black mud.

Sample **M15CL** was a core sample taken from the rivulet at second site. The core was about 1 m in depth, and only the lowest regions of the core were retained (Figure 3.5 D).

**North-west Causeway (10 am)**

The eastern end of this causeway was characterised by numerous soda springs rising in the gravel of the foreshore and trickling down to the lake. A larger spring feeding a small stream was populated by fish, thought to be *Tilapia*. The shore consisted of a solid surface approximately 5 cm thick covered by orange and green algal mats overlying a liquid filled subterranean matrix into which the coring rod could be pushed 1.5 m.

Further along the causeway at a passing place, the bed of the lake was found to consist of a thick black mud covered by a green algal mat. Beyond the passing place, the lake bed was dry and covered with acres of pink trona crust.

Sample **M16NWC** was taken from the black mud at the side of the passing place, dredged up using a sampling cup (Figure 3.5 E).
Results and Discussion: Sample Collection and Site Descriptions

Table 3.5 Measurements taken from the sampling sites of Lake Magadi. T = temperature (°C); pH = pH reading (± 0.5 pH units); C = conductivity (mS cm⁻¹); O₂ = oxygen concentration (ppm); R = redox potential (mV).

<table>
<thead>
<tr>
<th>Sample Zone</th>
<th>T</th>
<th>pH</th>
<th>C</th>
<th>O₂</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt Ponds</td>
<td>33</td>
<td>10.5</td>
<td>33.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Western Causeway</td>
<td>32</td>
<td>12</td>
<td>114.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NW Causeway: water</td>
<td>34</td>
<td>11</td>
<td>112.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NW Causeway: Mud</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.5 Map showing Lake Magadi and Little Magadi. The approximate locations of fissure springs are indicated, along with the main rivers and streams flowing in the immediate area. Sampling locations are indicated as follows: A = M12SP, B = M13CC, C = M14CH, D = M15CL, E = M16NWC.
4 Results and Discussion: Sulphate-reducing Bacteria

4.1 Enrichment of sulphate-reducing bacteria

The sampling locations investigated during the present study could be classified into two broad categories, those composed of gelatinous mud (E2SE1, N4HP, N5WL, B6HS, B8NS1, B9NS2, M13CC, M14CH, M15CL, M16NWC), and those composed mainly of grit and silt (E1NE, E3SE2, B7FT, C10LB, C11SB, M12SP). Although the redox-potential meter failed under field conditions, initial readings indicated low redox potentials within the sediments (-310 mV to -470 mV), suitable for the growth of anaerobic bacteria. Conductivity measurements showed that the concentration of the lake waters varied greatly between locations. Conductivity readings of ca. 14, 26, 50, 75 and 110 mS cm⁻¹ were found for Lakes Sonachi, Elmenteita, Nakuru, Bogoria and Magadi respectively. These levels indicated a gradual increase in dissolved salts from 1988 to 1992 (Brian Jones, personal communication), concomitant with a decrease in the water level of each lake.

Although sulphate reduction appeared to be taking place at a number of the sites, as evidenced by black gelatinous mud, H₂S could only be detected at a small number of locations (E2SE1, N4HP, B6HS and C10LB). However, it has already been noted that the major sulphur species above pH 9.0 is S²⁻ (Hao et al., 1996), and so the absence of the distinctive H₂S smell was perhaps not surprising.

Various media were tested for suitability of supporting growth of SRB at alkaline pH. Postgate’s media B and C (Postgate, 1984) were initially used,
Results and Discussion: Sulphate-reducing Bacteria

However it was soon found that FeSO₄, which was included as both a sulphate donor and an indicator of sulphate reduction, formed a black precipitate in the absence of bacterial sulphate reduction. Varying the amount of FeSO₄ added to the medium (g l⁻¹: 0.5, 0.3, 0.25, 0.2, 0.15, 0.125, 0.1) had no effect on the formation of a black precipitate. At the lowest FeSO₄ concentrations tested (g l⁻¹: 0.075, 0.05, 0.025 and 0.01) where no black precipitate formed spontaneously, the reaction could not be induced by the addition of Na₂S, showing that at these levels, the FeSO₄ could not serve as an indicator of sulphate reduction.

It was found that omitting yeast extract prevented the spontaneous formation of a black precipitate. This suggested a constituent of the yeast extract was reacting with the FeSO₄ at elevated pH. To alleviate this problem, a defined medium was adapted for enrichment purposes which contained no yeast extract (Table 2.8, Widdel and Bak, 1992). This medium was supplemented with trace elements and vitamins to stimulate growth.

A further problem when raising the pH of the SRB medium was the development of a grey / white precipitate, forming a sediment in each bottle. It was concluded that this was probably calcium or magnesium carbonate, and would not be detrimental to the growth of the organisms. Furthermore the ammonium chloride, present as a nitrogen source, had to be replaced due to the chemical conversion of ammonium to ammonia at elevated pH. Therefore sodium nitrate was used in all SRB media to prevent possible nitrogen limitation.

Substrates used for the enrichment of SRB were lactate, acetate, butyrate, formate, fumarate and ethanol. Each of these substrates was also tested for growth at high salt (20% w/v) using samples obtained from Lake Magadi. Unfortunately, under hypersaline conditions, positive stable enrichments could not be maintained. Xylan, carboxymethyl cellulose, guar gum, trehalose
and betaine were also tested as possible substrates, but again stable enrichments could not be maintained. There was, however, significant gas production within these enrichment cultures, indicating the presence of fermentative organisms.

Each SRB enrichment was repeatedly subcultured to remove traces of the original sediment sample. The subculturing process could only be carried out every one to two months, since the formation of a black precipitate indicating sulphate reduction was sometimes very slow to appear (Figure 4.1). For the first one to two years of the study, subculturing led to the loss of many previously positive enrichments. Exhaustive attempts to re-subculture bottles which had lost sulphate-reducing activity were unsuccessful. Although the majority of enrichment cultures either showed no initial SRB activity, or lost SRB activity during subculturing, a small number of samples remained stable throughout the test period (Table 4.1). From a total of 161 enrichment cultures under varying conditions, 16 proved to show stable sulphate reduction following repeated subculturing (Table 4.2).

Contrary to expected findings, the sampling locations composed of gelatinous black mud were not the most successful sites for the enrichment of SRB. Positive enrichments were found across all sampling locations, and were independent of the site morphology. Unfortunately a relationship between the on-site redox potential and successful enrichments could not be determined due to the failure of equipment under field conditions.

Ethanol was found to be the most successful growth substrate, resulting in 7 positive enrichments from a total of 11 (excluding those carried out at 20% (w/v) NaCl). Although ethanol has been identified as a common substrate for SRB (Widdel and Hansen, 1992), it is curious that this compound performed so much better than the other substrates under the conditions tested. It is of
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Figure 4.1 Typical growth of SRB enrichment cultures. Enrichments showing active sulphate reduction turn black in colour due to the reduction of FeSO₄ to FeS. Enrichments showing no sulphate reduction do not change colour.
Table 4.1 Positive, stable enrichments of SRB on various substrates. $S^2$ production was measured using the method detailed above (Section 2.4) with *Desulfovibrio desulfuricans* as a positive control, and an uninoculated medium sample as a negative control. Level of $S^2$ production was determined by eye, depending on the colour change of the test reagent compared to the controls (- = no colour change; + = light brown, ++ = mid-brown, +++ = dark brown).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sample</th>
<th>$H_2S$ Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>N5WL</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B6HS</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B7FT</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>B8NS1</td>
<td>+++</td>
</tr>
<tr>
<td>Acetate</td>
<td>E3SE2</td>
<td>+</td>
</tr>
<tr>
<td>Butyrate</td>
<td>N4HP</td>
<td>++</td>
</tr>
<tr>
<td>Formate</td>
<td>E2SE1</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>N5WL</td>
<td>++</td>
</tr>
<tr>
<td>Fumarate</td>
<td>E1NE</td>
<td>++</td>
</tr>
<tr>
<td>Ethanol</td>
<td>E1NE</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>E2SE1</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>E3SE2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>N5WL</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>B7FT</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>B9NS2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C10LB</td>
<td>+++</td>
</tr>
</tbody>
</table>

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course possible that the hydrogen present in the gas phase stimulated the SRB within these enrichments. Hydrogen, together with lactate, are the only substrates so far identified that SRB are capable of utilising under alkaliphilic conditions (Zhilina and Zavarzin, 1994). The classical SRB substrate, lactate, also resulted in a number of positive enrichments, with 4 from 11 showing $S^2-$ production. The remaining substrates, acetate, butyrate, formate and fumarate, were less successful as SRB substrates, although all resulted in at least 1 stable positive enrichment (Table 4.1).

Although stable enrichments could not be maintained for the Lake Magadi samples, initial SRB activity was detected in all enrichments when grown on lactate. This was the only substrate which resulted in sulphate reduction under hypersaline, alkaline conditions (Table 4.2). This finding agrees with that of Zavarzin et al. (1996), who concluded that sulphidogenesis at higher salinities only occurs on lactate.

Enrichments containing complex carbon substrates also failed to yield stable SRB cultures. This was not surprising since SRB are generally unable to utilise fermentable substrates. The initial signs that sulphate reduction was taking place were probably due to the utilisation of metabolic end-products produced by fermentative bacteria. The voluminous gas production in these enrichments was further evidence for the existence of organotrophic organisms.

Samples from Lake Elmenteita showed the greatest number of positive enrichments, with 4 of the substrates (acetate, formate, fumarate and ethanol) resulting in 6 positives (Table 4.2). These 6 positives were spread evenly throughout the 3 sampling sites of Elmenteita. Samples from Lake Bogoria were most successful when enriched on lactate, with 3 of the 5 positive enrichments originating from this lake utilising this substrate. Lake Nakuru enrichments produced 4 positives, 3 of which originated from one sampling.
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location (N5WL). Enrichments from Lake Sonachi performed poorly, with only 1 enrichment showing stable sulphate reduction when grown on ethanol (Table 4.2).

Sampling location N5WL resulted in three positive enrichments on lactate, formate and ethanol. This was the most heavily polluted, and therefore probably the most organically rich site which was visited. It is possible that the organisms responsible for the sulphate reduction within these enrichments originated from the flamingoines that polluted the environment. SRB have been isolated from numerous animals, including termites (Trinkerl et al., 1990), sheep (Widdel and Pfennig, 1984) and humans (Gibson et al., 1991), although the occurrence of avian SRB has not been documented.

Although SRB enrichments in alkaline media have been obtained using lactate (Wenk and Bachofen, 1995; Zavarzin et al., 1996) and hydrogen (Zhilina and Zavarzin, 1994), these are the first indications that SRB capable of utilising a range of substrates are present in alkaline sediments. Zhilina and Zavarzin (1994) attempted to isolate SRB from Lake Magadi samples using lactate, formate and acetate, although positive enrichments were not obtained. During the present study, positive SRB enrichments were obtained on each of these substrates.

SRB may be sub-divided into three physiological-ecological groups, the hydrogen-lactate group, the acetate group and the fatty acid group (Widdel, 1988). The hydrogen-lactate (pyruvate) group contains predominantly species from the genus Desulfovibrio, which are generally able to utilise a range of substrates including ethanol, succinate and fumarate. These organisms tend to have short doubling times, resulting in the incomplete oxidation of substrates to acetate. With the present SRB enrichments, the most successful substrate was found to be ethanol. This substrate resulted in positive enrichments from all of the northern (brackish and marine) lakes visited.
Table 4.2 Positive SRB enrichment cultures obtained from each of the lakes visited. Figures indicate the number of successful, stable enrichments. + indicates positive enrichments were initially obtained, although SRB activity was subsequently lost. Lac = lactate, Ace = acetate, Form = formate, Fum = fumarate, Eth = ethanol, GG = guar gum, CMC = carboxymethyl cellulose, S = starch, X = xylan, Bet = betaine, Tre = trehalose.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Lac</th>
<th>Ace</th>
<th>But</th>
<th>Form</th>
<th>Fum</th>
<th>Eth</th>
<th>GG</th>
<th>CMC</th>
<th>S</th>
<th>X</th>
<th>Bet</th>
<th>Tre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elmenteita</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nakuru</td>
<td>1</td>
<td>+</td>
<td>1</td>
<td>1</td>
<td>+</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bogoria</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sonachi</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>Magadi</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>
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Growth on ethanol has been found with a number of sulphate-reducers, including *Desulfovibrio* spp. (Postgate and Campbell, 1966; Bak and Pfennig, 1987; Folkerts *et al.*, 1989), *Desulfomonas pigra* (Moore *et al.*, 1976), *Desulfobacter curvatus* (Widdel, 1987), *Desulfosarcina variabilis* (Widdel, 1980), *Desulfobacterium macestii* (Gogiova and Vainstein, 1989), *Desulfomicrobium apsthonum* (Rozanova *et al.*, 1988) and Gram-positive *Desulfotomaculum* spp. (Cord-Ruwisch and Garcia, 1985; Nazina *et al.*, 1989). Oxidation of ethanol by these organisms would normally result in the formation of acetate via the following reaction:

\[
2 \text{ ethanol} + \text{SO}_4^{2-} \rightarrow 2 \text{ acetate} + \text{HS}^- + \text{H}^+ + 4\text{H}_2\text{O} \quad \text{(Kremer *et al.*, 1988)}.
\]

Following this, the acetate produced would either be oxidised by the acetate group of SRB, or by methanogenic bacteria. During the present study, acetate was found to be a poor substrate for growth, with only one sample resulting in a positive enrichment. Acetate has also been found to be a poor growth substrate for SRB from other alkaline environments (Zhilina and Zavarzin, 1994; Zavarzin *et al.*, 1996). The acetate group of SRB tend to have a limited range of substrates which they can utilise, and also tend to have extended doubling times (Widdel, 1988), although they are capable of the complete oxidation of acetate (Widdel and Bak, 1992). Further SRB isolates are also capable of acetate utilisation, although this is not the preferred substrate and growth tends to be limited. It is unlikely that there are no SRB capable of utilising acetate within alkaline environments, since this substrate can be used by the majority of described species. It is therefore probable that the enrichment and isolation techniques used during the study, and indeed other investigations (Zhilina and Zavarzin, 1994; Zavarzin *et al.*, 1996), are incompatible with this group of micro-organisms.

The final group of SRB, the fatty acid group, can be sub-divided into the complete and incomplete oxidisers. Members of this group may also be
capable of lactate and ethanol utilisation, although for isolation purposes fatty acid substrates are required. During incomplete oxidation, fatty acids with odd numbers of carbon atoms result in the formation of acetate plus propionate, whereas fatty acids with even numbers of carbon atoms result in only acetate production. (Pfennig and Widdel, 1981).

Under anaerobic conditions, mineralisation is complex and generally requires the participation of a number of different families of micro-organisms (Vosjan, 1982). The positive enrichments obtained from the alkaline lake samples show that SRB are present and capable of sulphate reduction at elevated pH. It can therefore be assumed that they carry out a similar role to their neutrophilic counterparts. Using this information, a trophic network of degradation ending in complete oxidation of the organic intermediates may be postulated, involving alkaliphilic chemo-organotrophs, SRB, methanogens (Boone et al., 1986) and acetogenic bacteria (Zhilina and Zavarzin, 1996b) (Figure 4.2).

If SRB were absent from the trophic network, low molecular weight compounds would accumulate, inhibiting oxidative processes higher in the food chain (Bryant, 1976). Under conditions of sulphate limitation, methanogens are able to utilise the excess hydrogen and acetate. However, within environments such as alkaline lakes with their plentiful supply of sulphate, methanogenesis is generally inhibited due to the competition from SRB for mutual growth substrates (Oremland and Taylor, 1978; Robinson and Tiedje, 1984). Despite competition from SRB, populations of methanogens may still exist within sulphate-rich environments either by utilisation of substrates not used by SRB (e.g. methylamines), or due to sufficient electron donor concentrations which are able to support both groups of organisms (Boone et al., 1986; Gibson, 1990). A predominance of methylotrophic methanogens has been identified within alkaline environments, although
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hydrogen and acetate-utilising methanogens have not been observed (Zavarzin et al., 1996).

Syntrophic associations are commonplace within the anaerobic sediments of lake ecosystems (Figure 4.2). These develop as a result of a series of integrated microbial processes. For example, the sulphur atom may serve as an inter-species electron carrier, acting as both an electron acceptor for the dissimilation of organic matter by SRB, and as an electron donor for the growth of autotrophic bacteria. SRB may also grow in consortium with acetogenic bacteria (e.g. Syntrophomonas spp., Natroniella acetigena), where they play an essential role in the disposal of reducing equivalents (Boone and Bryant, 1980; McInerney et al., 1981; Zhilina et al., 1996b).
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Figure 4.2 The role of sulphate-reducing bacteria in the degradation of organic matter within the trophic network of an alkaline anaerobic community. (SRB = sulphate-reducing bacteria; MB = methanogenic bacteria; AB = acetogenic bacteria). Redrawn from Gibson (1990).

Carbohydrates, proteins, lipids, etc.

\[ \text{hydrolysis} \]

Low molecular weight products (e.g. sugars, long chain fatty acids, etc.)

\[ \text{Volatile fatty acids (acetate, propionate, butyrate)} \]

\[ \text{Fermentation intermediates (e.g. alcohols, lactate, pyruvate, etc.)} \]

\[ \text{SRB} \]

\[ \text{SO}_4^{2-} \]

\[ \text{S}^2- \]

\[ \text{Acetate} \]

\[ \text{CO}_2 \]

\[ \text{H}_2 \]

\[ \text{CH}_4 \]

\[ \text{MB (??)} \]

\[ \text{SRB (??)} \]

\[ \text{AB} \]

\[ \text{MB} \]

\[ \text{SRB} \]

\[ \text{SO}_4^{2-} \]

\[ \text{S}^2- \]
4.2 Attempted isolation of sulphate-reducing bacteria

Due to the slow growth of the SRB enrichment cultures, a second medium was developed in an attempt to stimulate more rapid growth of SRB appropriate for isolation in pure culture. Based on the medium described by the DSM for cultivation of *Desulfovibrio desulfuricans*, adaptations were made to make this suitable for the culturing of alkaliphiles. These adaptations included a reduction in the concentration of MgSO₄ and CaCO₃, a reduction in the amount of FeSO₄ and substitution of NH₄Cl for NaNO₃ (Table 2.9). Although this medium was very dark prior to inoculation, sulphate reduction could still be observed by a colour change from dark grey to black. A heavy precipitate also formed with this medium, although it was noticed that the bacteria appeared to associate with this forming a biofilm on the sides and bottom of the culture vessel (Figure 4.3). However, this complete medium was found to be unsuitable for the long term maintenance of SRB enrichments. After rapid growth over 7 - 10 days, cell numbers fell dramatically. The reason for this is not known, although the most likely explanation may be the build-up of toxic intermediates or end-products.

A diversity of cellular morphologies were seen using this growth medium, ranging from vibrio-shaped and straight rods to cocci. Also found within these cultures were some curious morphologies or associations. A number of the long, rod-shaped bacteria appeared to have spherical appendages attached. These appendages were found to be central, sub-terminal and terminal to the cell. Also seen within these SRB cultures were cells with a coiled morphology, similar to the *Spirochaeta* sp. described by Zhilina et al. (1996a) (Figure 4.4a). Large spherical bodies were also identified, corresponding to the spheroplasts observed within the *Spirochaeta* cultures (Figure 4.4b).
**Figure 4.3** Formation of a bacterial biofilm within SRB enrichment cultures containing yeast extract. Biofilm formation occurred on the sides of the glass culture vessel, and as a film on the surface of the undisturbed liquor (×400).
Figure 4.4 (a) *Spirochaeta*-like organisms found within SRB enrichment cultures in the presence of yeast extract. These organisms were not observed in defined media enrichments (×400). (b) Spherical bodies were also observed, which corresponded to those found with the *Spirochaeta* sp. (Zhilina et al., 1996a) (×1000).
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The complete medium, with the addition of agar and sodium citrate, was also used for anaerobic plate culturing. Sodium citrate was included at this stage to maintain the FeSO₄ in solution. Using this medium, numerous methods of plating were tested in an attempt to isolate single colonies of SRB.

Of all the methods tested, only direct plating of enrichment samples onto the surface of completely anaerobic and highly reduced agar plates had any degree of success. The plating of the enrichments, although generally unsuccessful, did lead to the development of limited number of large, black colonies after 3 to 5 days incubation at 30°C, together with numerous pin-point black colonies (Figure 4.5). Subculturing of the large colonies on both solid and liquid media was attempted using a variety of different methods. However, on subculturing growth either did not occur, or the resulted in colonies which were not black in colour. Subculturing into small volumes (1 ml) of liquid medium were also unsuccessful, even when the medium was supplemented with additional vitamins and trace elements. Extended incubation times saw an increase in the size of the pin-point black colonies. Unfortunately these took an inordinately long time to grow (> 6 months) and could not be successfully subcultured.

A further attempt to isolate single colonies involved the use of Wolfe bottles. These contained molten agar-based medium held at 60°C whilst all components were added. Bottles were then inoculated and the agar was allowed to set. Although some small, pin-point colonies were observed after extended incubation, these could not be subcultured into fresh media. Further attempts to isolate SRB included using deep semi-solid agar tubes, pour plates, bi-layer spread plates and bi-layer pour plates, together with microculture of any resulting black colonies in small volumes of liquid media.
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Figure 4.5 Development of black colonies on the surface of agar plates. Subculturing to obtain axenic cultures was unsuccessful, suggesting that the organisms survived only as members of syntrophic associations.
Unfortunately none of these methods were successful, and no SRB were obtained in pure culture.

To date, only one alkaliphilic SRB isolate has been reported, which originated from Lake Magadi and utilised hydrogen (Zhilina and Zavarzin, 1994). Unfortunately further characterisation of this isolate has not been carried out. Due to the difficulties involved in the isolation of SRB, and in particular isolation at elevated pH, alternatives to the standard microbiological procedures are required to investigate these potentially significant microorganisms.
4.3 Molecular analysis of SRB enrichments

4.3.1 SRB-specific PCR primer

Due to the problems encountered during attempted SRB isolation, alternative methods for the identification of the organisms present were implemented. Custom 16S rDNA PCR primers have been used for the selective amplification of sequence from numerous groups of organisms, the stringency of which can be altered to select for species, genera or phyla. Although an SRB-specific primer has been described previously (SRB385F, Amann et al., 1992), a FASTA search demonstrated this primer was not completely specific, also picking up *Frankia* spp. and members of the *Clostridium* assemblage (Table 4.3). Therefore a novel SRB-specific primer (SRB247F) was developed which greatly improved the specificity as determined by a FASTA search.

In order to locate regions of homology between the somewhat diverse SRB group, a homology alignment was obtained from the Ribosomal Database Project (Maidak et al., 1996). This homology alignment contained the bacteria *Escherichia coli*, *Clostridium fervidus*, *Pseudomonas aeruginosa* and *Bacillus subtilis*; the archaeon *Thermoproteus* sp.; and the SRB *Desulfovibrio desulfuricans*, *Desulfo bacter postgatei*, *Desulfobulbus propionicus*, *Desulfotomaculum orientus* and *Thermodesulfobacterium commune*. Using this alignment, a number of regions were selected as possible sites for the development of a SRB primer. Each site was checked for self-complementation, G+C content, and SRB specificity. Based on this information the following primer was chosen for the selective amplification of SRB 16S rDNA.
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<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>position</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRB-F</td>
<td>TGA AGA TGG GTC CGC GTA CC</td>
<td>247 - 267</td>
</tr>
</tbody>
</table>

FASTA searches carried out using this new primer sequence revealed a high specificity for SRB (Table 4.3). More importantly, non-SRB sequences which were also found to have a high overall homology with the primer, generally lacked 3' base pairing. Since 3' base pairing is required for extension during PCR, these sequences would not be amplified with this primer.

4.3.2 DNA extraction

Various methods of DNA extraction were tested for efficacy of extraction from SRB enrichments. Due to the large amounts of particulate matter within the enrichments, and the tendency for the organisms to associate with this, numerous methods of DNA extraction proved unsuccessful.

Using a standard extraction protocol utilising lysozyme and SDS for cell disruption, followed by phenol-chloroform extraction, no DNA was obtained (Owen and Pitcher, 1985). Only when incubation times were extended and freeze-thaw cycles were carried out prior to lysozyme treatment was DNA extracted from the enrichments. Unfortunately the quality of DNA obtained using this procedure was very poor. During the extraction with phenol and chloroform, the contents of vessel turned claret red in colour, making it extremely difficult to see the interface between the organic and aqueous phases. Following precipitation, the DNA was dark brown in colour due to the co-precipitation of a constituent of the original enrichment.

In an attempt to clean the contaminated DNA, several commercial purification methods were assessed. The first of these was glassmilk purification, which utilised a particulate matrix to bind DNA, allowing the elution of contaminants (GlassMAX, Life Technologies, Paisley, Scotland).
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Table 4.3 Results of a FASTA search for SRB385F (Amann et al., 1992) and SRB247F. SRB are shown in bold type.

<table>
<thead>
<tr>
<th></th>
<th>SRB385F</th>
<th></th>
<th>SRB247F</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EMBL</td>
<td>Organism</td>
<td>EMBL</td>
<td>Organism</td>
</tr>
<tr>
<td>1.</td>
<td>fsrrdy1</td>
<td>Frankia sp.</td>
<td>dvrr168</td>
<td>D. variabilis</td>
</tr>
<tr>
<td>2.</td>
<td>fsrgop</td>
<td>Frankia sp.</td>
<td>dprr1611</td>
<td>D. propionicus</td>
</tr>
<tr>
<td>3.</td>
<td>fsrgda</td>
<td>Frankia sp.</td>
<td>amcs310</td>
<td>unknown</td>
</tr>
<tr>
<td>4.</td>
<td>amcs308</td>
<td>unknown</td>
<td>dvrr169</td>
<td>D. vacuolatum</td>
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<tr>
<td>5.</td>
<td>fsrgdb</td>
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<td>darr1610</td>
<td>D. autotrophicum</td>
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<tr>
<td>6.</td>
<td>dsrr16s</td>
<td>Desulfovibrio sp.</td>
<td>bbrr16sb</td>
<td>Bdellovibrio sp.</td>
</tr>
<tr>
<td>7.</td>
<td>fsrgdc</td>
<td>Frankia sp.</td>
<td>dnrr1677</td>
<td>D. niacini</td>
</tr>
<tr>
<td>8.</td>
<td>akrg16s</td>
<td>Acetogenium kivui</td>
<td>ec16smaa</td>
<td>E. cellulosolvens</td>
</tr>
<tr>
<td>9.</td>
<td>dsrr16sa</td>
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<td>pnribdnab</td>
<td>Porphyrobacter sp.</td>
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<tr>
<td>10.</td>
<td>dcrr1614</td>
<td>D. curvatus</td>
<td>darr16s</td>
<td>D. australicum</td>
</tr>
</tbody>
</table>
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Unfortunately, using this procedure, the DNA from the enrichment samples did not bind to the support, and was removed along with the first buffer. Following this, gel purification was attempted using low melting point (LMP) agarose. A commercial gel purification kit (JETsorb, GENOMED GmbH, Bad Oeynhausen, Germany) was used as per the manufacturers instructions. This protocol proved successful and resulted in clean DNA, albeit at a greatly reduced concentration. Although this procedure was successful in recovering clean DNA, it was not ideal due to the problems of separating the organic and aqueous phases during the initial extraction. PCR of the DNA following this clean-up regime was often unsuccessful due to: a) the reduced concentration of the resulting DNA; and b) the poor quality of the resulting DNA as a result of the prolonged manipulation.

One final method was investigated in an attempt to obtain clean DNA in as few steps as possible. The method used was based on that of Pitcher et al. (1989), with a few modifications. Due to the large pellet obtained following centrifugation of an SRB culture, the volumes of reagents and incubation times were increased. The large pellet was a result of extensive precipitation within the medium, along with the large culture volumes required to obtain ample DNA (500 ml compared to 10 ml required for E. coli).

The cell pellet obtained following centrifugation of an SRB enrichment was washed with an anaerobic physiological salts solution (pH 10.5) and divided equally between two 2 ml microfuge tubes. 180 µl lysozyme solution (50 mg ml\(^{-1}\) lysozyme in TE, pH 8) was added and the tubes were incubated at 30 °C for 30 - 60 minutes. 600 µl lysis solution (guanidium thiocyanate) was then added, and the tubes were incubated on ice for 20 - 30 minutes. Following the addition of 300 µl cold ammonium acetate (7.5 M) and a further incubation of 20 - 30 minutes, an organic extraction was carried out using 600 µl chloroform / isoamylalcohol (24:1). Both tubes were then centrifuged to
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separated the aqueous and organic layers, allowing the removal of the extracted DNA. Following precipitation and washing of the DNA, extracts from the two tubes were pooled for further analysis.

Following these minor modifications to the extraction protocol, clean DNA was obtained from all enrichments. Also, since this protocol omitted the phenol extraction step, no deep red colour was observed thus preventing contamination of the DNA. This method was subsequently used for all SRB DNA extractions.

4.3.3 Drop-down PCR

To increase the specificity and reduce mis-priming of the SRB forward primer, an amplification regime using drop-down PCR was implemented (Don et al., 1991). This involved a first round of PCR using the SRB forward primer and an annealing temperature 10°C above the calculated annealing temperature. During the PCR reaction, the annealing temperature was reduced 1°C every two cycles, as described in Section 2.11.4. On completion of this PCR regime, 1 µl from each reaction was used as the template DNA in a second round of PCR. This PCR reaction followed the standard protocol, with an annealing temperature of 55°C, using the SRB forward primer with an attached 30 bp GC-clamp. The additional GC-rich region was included to provided a high-melting point domain, improving the resolution of subsequent DGGE analysis.

Using the method of drop-down PCR, non-specific priming of both SRB and control DNA was almost completely eliminated. Only a few small products could be seen in some of the SRB samples (Figure 4.6). Misprimed products were successfully removed using a commercial gel purification kit (QIAGEN), although the resulting DNA concentration was significantly reduced.
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**Figure 4.6** Amplification of SRB 16S rRNA using SRB247F and SRB247GC. Samples containing misprimed products were cleaned using a commercial gel purification kit (QIAGEN). Lane 1 = Kb ladder; 2 = -'ve control; 3, 5 = *Desulfovibrio desulfuricans*; 4, 6 = *Desulfovibrio desulfuricans* GC-clamp; 7 = E1NE (fumarate); 8 = E1NE (fumarate) GC-clamp; 9 = N4HP (butyrate); 10 = N4HP (butyrate) GC-clamp; 11 = N5WL (formate); 12 N5WL (formate) GC-clamp; 13 = B7FT (lactate); 14 = B7FT (lactate) GC-clamp; 15 = B8NS1 (lactate); 16 = B8NS1 (lactate) GC-clamp; 17 = C10LB (lactate); 18 = C10LB (lactate) GC-clamp; 19 = -ve control; 20 = Kb ladder

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

1 Kb
4.3.4 Denaturing gradient gel electrophoresis (DGGE)

The likelihood of obtaining one amplification product from a mixed microbial community, even with selective primers, is slight. Sequencing of the amplification product obtained from an enrichment culture confirmed that there was more than one DNA sequence present. Therefore the products had to be separated to allow representative samples to be sequenced. The classical approach to this problem was to include a multiple cloning site attached to the amplification primer. This would allow the products to be cloned and then sequenced using *E. coli*. However, the inclusion of cloning site has been found to reduce the specificity of SRB primers (Paul Rochelle, personal communication). Therefore a novel method of PCR product analysis was selected, namely denaturing gradient gel electrophoresis (DGGE).

DGGE was first reported by Fischer and Lerman (1979) as a procedure for the length-independent separation of DNA restriction fragments. They then further developed the procedure for the separation of single base pair transversions and transitions in *Eco RI* digests of *E. coli* (Fischer and Lerman, 1983). This involved the use of a formamide and urea gradient coupled to a running temperature of 60°C. During electrophoresis, the partially denatured DNA molecules exhibited varying mobility, allowing separation based on their nucleotide sequence. Following this early work, the procedure was modified by the inclusion of a GC-rich DNA sequence to improve the resolution of DGGE (Myers *et al.*, 1985a,b,c). The attachment of a GC-clamp altered the melting profile of the 5' end, preventing strand dissociation. Theoretical calculations indicated that about 95% of all possible single base substitutions would be separable when attached to a GC-clamp.

More recently DGGE has been used for the analysis of mixed microbial populations and environmental samples (Muyzer *et al.*, 1993; Wawer and Muyzer, 1995). Using this method, up to 10 distinguishable bands were
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obtained when analysing an uncharacterised aerobic bacterial biofilm. The representative organisms were then be identified by hybridisation with species-specific probes. The genetic diversity of *Desulfovibrio* spp. in environmental samples has been investigated by DGGE, using PCR-amplified [NiFe] hydrogenase gene fragments. Analysis of microbial mat samples revealed up to 5 distinguishable bands, compared to 2 bands from an experimental bioreactor. These findings suggested diversity in the natural system is far greater than in experimental bioreactors.

The present study attempted to analyse mixed microbial populations by the selective amplification of SRB 16S rRNA. Amplification using a primer with a GC-clamp attached was found to be inefficient. Therefore two rounds of PCR were carried out, the first of which used a primer without the GC-clamp attached. This also allowed the reamplification of low-yield samples.

DGGE was carried out at 60°C, to partially melt the DNA double helix. At this temperature only the low melting point (low G+C) domains disassociated. As the DNA migrated through the gel, the increasing formamide concentration had caused increasingly stable domains within each sequence to melt. Eventually each sample reached a critical concentration of denaturant, where the two DNA strands had separated so much the molecule could no longer migrate through the gel, with the distance travelled by the molecule being dependent solely on the base composition. The GC-clamp included during the PCR reaction served to hold the two strands of DNA together should they become completely melted.

Running polyacrylamide gels at 60°C presented a whole host of problems, due mainly to the incompatibility of available equipment. The problems were further compounded by the need to continually circulate the cathodic and anodic buffers to prevent acidification at the anode. Despite numerous
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tries to adapt Bio-Rad Protean II and Pharmacia Multiphor equipment to carry out this procedure, the method proved entirely unsuccessful.

Using purpose built DGGE apparatus at the University of Warwick (D-Gene Electrophoresis System, Bio-Rad), the potential of this protocol for the analysis of mixed cultures was exhibited. Initially, a control sample of Desulfovibrio desulfuricans DNA was run on a perpendicular gel with a gradient of 0 - 100% (Figure 4.7). This allowed the determination of the optimal gradient for parallel gels. Based on these findings, a denaturing concentration of 20 - 65% was chosen for the parallel gels. SRB enrichment samples were run on a gel with this denaturant concentration. Although conditions were not optimal for running SRB samples, definite separation and banding could be seen with the majority of the samples (Figure 4.8).

Further optimisation of the technique was required to improve the resolution of these samples, which was impossible due to time constraints at the University of Warwick. If further work had been possible, a shorter gradient of 30 - 50% combined with a run time of 4 hours would probably have been successful. Although not fully optimised, the potential of this method for the isolation of individual amplification products from mixed cultures is apparent.

A further factor influencing the success of this technique was the size of the amplification products. Fragments over 1 kb require extended electrophoresis time, which may be incompatible with subsequent sequence analysis. Therefore future investigations should employ shorter amplification products of around 500 bp.
Figure 4.7 Photograph showing *Desulfovibrio desulfuricans* 16S rRNA amplified using SRB247F followed by SRB247GC. A denaturant gradient of 0 - 100% was used (left to right), and the sample was run perpendicular to this (top to bottom). Electrophoresis was carried out at a constant voltage of 200 V for 1.5 hours.
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Figure 4.8 Photograph of SRB enrichment samples run on a parallel gradient gel of 20 - 65% denaturant. Electrophoresis was carried out at 170V for a total of 2 hours. Although the duration of the run was too short, banding of the samples is still apparent. Lanes 1, 9 = *Desulfovibrio desulfuricans* (no dropdown PCR); 2, 10 = *Desulfovibrio desulfuricans* (dropdown PCR); 3, 11 = E1NE (fumarate); 4, 12 = N4HP (butyrate); 5, 13 = N5WL (formate); 6, 14 = B7FT (lactate); 7, 15 = B8NS1 (lactate); 8, 16 = C10LB (ethanol).
4.3.5 DNA sequencing from DGGE gels

Due to the poor quality of the DGGE banding (tight bands near the top of the gel), excision for subsequent amplification was difficult. Despite this, a number of bands were successfully excised and the DNA was eluted. This eluent was used in a further round of PCR, using an internal forward primer (FD8) and the universal reverse primer (rP1), resulting in positive amplification for these samples. Elution of DNA from the polyacrylamide gel appeared to be inefficient, resulting in low PCR product yield. This problem may be overcome by using a gel with reversible crosslinking.

Automated sequencing of the resulting PCR products was relatively unsuccessful, with only one sample producing useful sequence data. The results from the automated sequencer showed that the DNA was of poor quality, probably due to the manipulations carried out following electrophoresis.

A FASTA search using the limited useful sequence data revealed the isolated DNA belonged to a *Clostridium* sp., although further identification could not be carried out based on the data available (Table 4.4).

Despite the numerous difficulties involved, DGGE has the potential to be a rapid and robust technique for the screening of mixed microbial populations. Optimisation of conditions is essential to the success of this technique. The conditions suitable for the resolution of one sample type (e.g. microbial mat) may not be suitable for the resolution of another (e.g. SRB enrichment). However, once suitable running conditions are obtained, samples can be amplified, separated using DGGE and sequenced within 2 - 3 days. Alternatively, hybridisation analysis can be carried out using genus or species specific probes.
Table 4.4 FASTA search using the limited sequence data available following DGGE analysis.

<table>
<thead>
<tr>
<th>EMBL</th>
<th>Organism</th>
<th>% identity in 339 bp overlap</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. m23930</td>
<td>Clostridium pasteurianum</td>
<td>90.3</td>
</tr>
<tr>
<td>2. l37588</td>
<td>Clostridium botulinum BP2</td>
<td>90.3</td>
</tr>
<tr>
<td>3. x73844</td>
<td>Clostridium botulinum type A</td>
<td>90.0</td>
</tr>
<tr>
<td>4. l08062</td>
<td>Clostridium tyrobutyricum</td>
<td>90.0</td>
</tr>
<tr>
<td>5. l37593</td>
<td>Clostridium botulinum type F</td>
<td>90.0</td>
</tr>
<tr>
<td>6. l37585</td>
<td>Clostridium botulinum type A</td>
<td>90.0</td>
</tr>
<tr>
<td>7. x68189</td>
<td>Clostridium sporogenes</td>
<td>90.0</td>
</tr>
<tr>
<td>8. x68172</td>
<td>Clostridium botulinum type F</td>
<td>90.0</td>
</tr>
<tr>
<td>9. l37587</td>
<td>Clostridium sporogenes</td>
<td>90.0</td>
</tr>
<tr>
<td>10. x68186</td>
<td>Clostridium botulinum type B</td>
<td>90.0</td>
</tr>
</tbody>
</table>
Results and Discussion: Sulphate-reducing Bacteria

Utilisation of this method will inevitably lead to the discovery of novel sequence data belonging to organisms present within enrichments but which cannot be isolated using present methods. Furthermore, direct extraction of DNA from sediment samples would almost certainly reveal a diversity of new sequence data from viable but non-culturable organisms. The data obtained from such analyses would give further insight into the component members of the trophic network within environments, and could also be used to produce DNA probes for comparisons with other habitats. Eventually, the composition of a complex microbial community could be determined without the need to enrich or culture any of the constituent members. However, this technique should not be used instead of, but rather in concert with existing microbiological methods, assisting in the determination of nutritional requirements of the component organisms and aiding their isolation.
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Anaerobic micro-organisms exist in closely associated communities, probably with intrinsically linked degradative processes. Isolation of axenic cultures from such communities may therefore be extremely difficult. Prior to the inception of present study, no alkaliphilic, obligate anaerobes had been described in detail. Subsequently a small number of novel anaerobic alkaliphiles have been described, comprising of Clostridium spp., Haloanaerobium sp., Spirochaeta sp. and Natroniella sp. Only two of these organisms have been isolated from soda lakes, Natroniella acetigena from Lake Magadi, Kenya (Zhilina et al., 1996b), and Spirochaeta asiatica from Lake Khatyn, Tuva, Russia (Zhilina and Zavarzin, 1996a).

During the alkaliphile isolation procedure, a number of factors were soon noted as being important for successful culturing. The most obvious of these was the use of reductants to maintain a low redox potential (Widdel and Bak, 1992). Despite being toxic at elevated concentrations, sodium thioglycollate in concert with ascorbic acid was found to reduce the media to a satisfactory level (ca. -300 to -350 mV). However, preparation of the media still resulted in small amounts of contaminating oxygen, as determined by the pink colourisation of resazurin included in each recipe. It was found that sodium dithionite removed this oxygen contamination and promoted the growth of the anaerobic alkaliphiles. Numerous elaborate techniques have been developed for the preparation of anaerobic media (see Widdel and Bak, 1992), however a more simplified approach was developed for the isolation of the present isolates. Although the method of preparation did not allow for the production
of large volumes of media, it proved to be a very reliable method for obtaining oxygen free media.

5.1 Isolation of chemo-organotrophic bacteria

Two media were used for the isolation of chemo-organotrophic Bacteria, a complete medium with glucose (GAM; Grant et al., 1979) and a minimal medium used in conjunction with complex carbon substrates (MM). Isolation was carried out under strictly anaerobic conditions using media prepared in the absence of oxygen and reduced using a combination of reductants. Throughout the isolation process aerobic subcultures were performed to ensure the anaerobicity of the resulting organisms.

5.1.1 Enrichment using novel carbon substrates

Complex carbon substrates were used during the enrichment process in an attempt to isolate organisms with interesting enzymatic properties. The substrates used in this study were guar gum (galactomannan; mannose:galactose - 2:1), birchwood xylan, carboxymethyl cellulose and starch, to look for mannanase, xylanase, cellulase and amylase enzymes respectively. Enrichments were carried out in liquid media within anaerobically sealed bottles. Growth with each substrate was initially determined by the amount of gas produced and by microscopic examination of wet mounts.

Guar gum exhibited the best growth, with 11 of the 16 samples producing gas as a result of fermentation, including all but one of the non-saline lakes (Table 5.1). Starch and CMC were found to be poorer substrates for growth. Xylan supported good growth for a number of the samples, including the majority of the samples from Lake Magadi which were cultured in media containing 20% (w/v) NaCl.
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Table 5.1 Growth of enrichment cultures on the complex carbon substrates guar gum, birchwood xylan, carboxymethyl cellulose (CMC), and starch. During liquid enrichment, + → + + + indicates increasing amounts of gas produced whilst - indicates no gas produced. On solid media, + → + + + indicates increasing colony diversity.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Guar Gum</th>
<th>Xylan</th>
<th>CMC</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>liquid</td>
<td>solid</td>
<td>liquid</td>
<td>solid</td>
</tr>
<tr>
<td>E1NE</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>E2SE1</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>E3SE2</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>N4HP</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>N5WL</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>B6HS</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>B7FT</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>B8NS1</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>B9NS2</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>C10LB</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C11SB</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M12SP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M13CC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M14CH</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M15CL</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M16NWC</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

180
Isolation of the organisms enriched in liquid media was attempted using the same medium recipe with the addition of 2% (w/v) agar. Initial spread plates showed a great diversity of colony types within a number of the enrichments. The majority of samples enriched on xylan showed good growth, although C10LB and C11SB were somewhat limited and N4HP showed no growth at all (Table 5.1). All guar gum-enriched samples led to growth on solid media, although a number of the colonies grew within the agar substrate rather than as surface colonies, hindering subsequent manipulations. The growth of starch utilisers was much more restricted, with only a small number of colonies appearing on each plate. On testing for starch hydrolysis using iodine, a few of the colonies showed areas of clearing revealing amylase activity. Growth on CMC was the most limited of the substrates tested, with only a small number of pin-point colonies appearing after extended incubation (Table 5.1).

From these spread plates, individual colonies were picked off and subcultured in an attempt to obtain pure cultures. This process resulted in 21 xylan isolates, 19 guar gum isolates, 24 starch isolates and 22 CMC isolates (Table 5.2). Although a number of these had similar colony characteristics, they were not assumed to be the same organism and were treated independently. During the process of subculturing to ensure axenic cultures, a number of isolates could not be maintained whilst other samples were found to house more than one colony type. This resulted in a total of 25 isolates from xylan, 25 from guar gum, 24 from starch and 20 from CMC (Table 5.2). Once each of the isolates had been confirmed as clean, they were each subcultured aerobically to identify facultative anaerobes.

Aerobic subculturing revealed only 14 of the 94 isolates were unable to grow in the presence of O₂ on the substrates tested. Of the 14 isolates, 7 were grown on xylan, 2 on guar gum, 3 on starch and 2 on CMC (Table 5.3). However,
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Table 5.2 Isolation of organisms using complex carbon substrates. ‘Initial isolates’ shows the number of isolates obtained from enrichment cultures; ‘Lost during subculture’ indicates the number of isolates which failed to grow when subcultured; ‘Mixed cultures’ shows the number of plates containing mixed cultures, together with the total number of isolates obtained from those plates in brackets; ‘Total’ shows the total number of axenic cultures; ‘Total anaerobic’ indicates the number of organisms which appeared to only grow anaerobically.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Initial isolates</th>
<th>Lost during subculture</th>
<th>Mixed cultures</th>
<th>Total</th>
<th>Total anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guar Gum</td>
<td>19</td>
<td>1</td>
<td>7 (14)</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>Xylan</td>
<td>21</td>
<td>1</td>
<td>4 (9)</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>CMC</td>
<td>22</td>
<td>3</td>
<td>1 (2)</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Starch</td>
<td>24</td>
<td>3</td>
<td>3 (6)</td>
<td>24</td>
<td>3</td>
</tr>
</tbody>
</table>
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when these apparent obligate anaerobes were tested using GAM (glucose as the substrate) both aerobically and anaerobically, all showed varying degrees of aerobic growth. This finding was unexpected, since aerobic growth was not detected using the complex substrates. When these isolates were again checked for aerobic growth on the complex substrates, it was found that very slow growth occurred, with visible signs only appearing after 2 - 4 weeks incubation compared to 24 - 48 hours anaerobically. It seemed that, although these isolates were facultative anaerobes, they were only capable of efficient breakdown of the substrates (extracellular enzyme production) under anaerobic conditions.

The reason why these organisms seemed unable to utilise the carbon substrates under aerobic conditions is not known. It is possible that enzyme production was only induced under anaerobic conditions, although the advantage of this is not clear. Another possibility may be that the enzymes lose activity under oxidising conditions. From a biotechnological point of view, this loss of activity may be linked to a pH drop of around 2 units when plates are removed from the CO₂-free environment of the anaerobic cabinet to an aerobic environment. If this were the case, then it would suggest the enzymes operate optimally at high pH.

The majority of the organisms isolated on these substrates originated from Lake Sonachi (Table 5.3). This lake was encircled by thick vegetation, and had high rates of primary production as evidenced by the thick suspension of cyanobacteria. The actual sampling site was close to a number of dead trees, which may account for the large number of organisms capable of xylan degradation.
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Table 5.3 Organisms isolated on complex carbon substrates which appeared to show only anaerobic growth. Figures indicate the number of isolates from each lake location.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Guar Gum</th>
<th>Xylan</th>
<th>CMC</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elmenteita</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Nakuru</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bogoria</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonachi</td>
<td></td>
<td>5</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Magadi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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Many species of *Clostridium* produce exoenzymes, resulting in an ability to grow on a wide range of substrates (Hippe *et al*., 1992). Generally, work on the isolation of enzymes has been carried out using thermophilic isolates, to obtain thermotolerant enzymes. Enzymes isolated from thermophiles include α-amylase, β-amylase and pullulanase from *C. thermosulfurogenes* and *C. thermohydrosulfuricum* (Antranikian *et al*., 1987a, b; Hyun and Zeikus, 1985) and cellulases from *C. thermocellum* (Lamed and Bayer, 1988), along with pectinase from *C. felsineum* (Vozniakovskaya *et al*., 1974) and xylanase from *C. xylanolyticum*, both of which are mesophilic.

The fact that the organisms isolated here are all facultative anaerobes takes them outwith the scope of this project. However, one reason for using the substrates described above was to detect the presence of alkalophilic enzymes. A number of the isolates obtained during the period of study have been commercially screened for the presence of such enzymes, and some of these have shown commercial potential (Brian Jones, personal communication).

5.1.2 Direct isolation on agar media

Alkaliphilic anaerobes were obtained by carrying out serial dilutions using GAM medium with agar. Each sediment sample was shaken vigorously to mix the sediment with the overlying lake water. Aliquots were then removed and serially diluted down to $1 \times 10^4$ as described in Section 2.5. From these serial dilutions the number of viable organisms capable of anaerobic growth was calculated for each sample. Viable counts ranged from $1.85 \times 10^3$ (N4HP, B6HS) to $2.5 \times 10^6$ (E3SE2) bacteria ml$^{-1}$ (Figure 5.1).
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**Figure 5.1** Viable counts from sediment samples obtained from the non-saline lakes. Serial dilutions were performed to $1 \times 10^4$ and plated on GAM under strictly anaerobic conditions.
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Site E3SE2 housed the largest number of culturable bacteria, followed by site N5WL. The sample from Lake Elmenteita was not from the lake itself, but from a red/brown pool close by. This pool was bordered by thick beds of rushes, and there was evidence of animal activity close by. Gas bubbling through the water indicated microbial activity, and there was a strong sulphide smell from the sediment. The Lake Nakuru sample was heavily polluted by the vast flamingo population present on the lake. Therefore, from this site large numbers of bacteria were expected, although with such high levels of pollution toxic compounds could conceivably inhibit microbial activity. The low numbers found with B6HS may be due in part to the fluctuating temperatures in this area. This sample was obtained from the mixing zone between a hot spring outflow and the lake water itself. This would lead to fluctuating pH, conductivity and temperature, restricting the establishment of stable microbial populations. Incubation at a higher temperature may have revealed a larger viable population.

From these dilution plates, individual colonies were subcultured in an attempt to obtain pure cultures. This initial isolation work was carried out inside a cabinet with an atmosphere of N₂CO₂H₂. However, due to the adsorption of CO₂ into the agar, the pH of the plates within the cabinet dropped from ca. pH 10.5 to ca. pH 8.5 over a period of a few hours. During work within this atmosphere 36 organisms were isolated in pure culture, 31 of which survived after three rounds of subculture. Subsequently manipulations were carried out in a cabinet containing an atmosphere of H₂N₂ which did not affect the pH of the media. During the change between these two incubation atmospheres, growth was examined to test for any adverse reaction by the isolates.

Of the 31 stable isolates obtained, 4 failed to grow (both in N₂CO₂H₂ and H₂N₂) following the transfer to the new atmosphere. Of the 27 isolates which remained, 14 grew more vigorously in H₂N₂, 5 grew better in N₂CO₂H₂ and the
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remaining 8 grew equally well in both atmosphere (Table 5.4). Aerobic subculturing of these isolates revealed 14 were facultative anaerobes, exhibiting good aerobic growth. For those isolates which grew only anaerobically, 7 were found to grow better in the H$_2$N$_2$ atmosphere, suggesting a preponderance for alkaliphilic conditions (Table 5.4).

During the course of the study, a number of these obligate anaerobes could not be maintained in culture, despite exhaustive efforts to do so. It seems apparent that most of the organisms present in the alkaline sediments survive in association with other organisms, and cannot survive when grown in pure culture. From the original 36 isolates, only 6 obligate anaerobes could be maintained in stable pure culture. These isolates were designated E2SE1-B, E2SE1-C, B7FT-A, B8NS1-A, B8NS1-C and B8NS1-C-FT. Interestingly, 5 of the 6 isolates which resulted in stable laboratory cultures belonged to the group of isolates which grew better in an atmosphere of H$_2$N$_2$.

In an attempt to increase the number of obligate anaerobes isolated, liquid SRB enrichments were spread directly on GAM and the resulting colonies were cleaned through subculturing. This isolation procedure resulted in a total of 25 isolates: 16 from the ethanol enrichments; 5 from the formate enrichments; 2 from the lactate enrichments and 2 from the butyrate enrichments (Table 5.5). Despite best efforts only 9 of these 25 isolates could be maintained in stable laboratory culture. Of the 9 organisms that could be maintained, all were found to be facultative anaerobes (Table 5.5).

Although none of the anaerobic bacteria obtained from SRB enrichment cultures could be maintained in pure culture, one isolate was fully sequenced before it was lost. This isolate was obtained from a lactate enrichment of N5WL, the heavily polluted sampling site at Lake Nakuru. Phylogenetic data for this isolate are presented in Section 5.3.5.
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Table 5.4 Numbers of isolates obtained from serial dilution plates. The data are divided into those organisms which grew better in an atmosphere of \( \text{N}_2\text{CO}_2\text{H}_2 \), those which grew better in \( \text{H}_2\text{N}_2 \), and those which grew equally well in both atmospheres. The data are then further subdivided into numbers of facultative anaerobes and numbers of obligate anaerobes.

<table>
<thead>
<tr>
<th>Site</th>
<th>Better growth in ( \text{N}_2\text{CO}_2\text{H}_2 )</th>
<th>Better growth in ( \text{H}_2\text{N}_2 )</th>
<th>Equal growth in both</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>facultative</td>
<td>obligate</td>
<td>facultative</td>
</tr>
<tr>
<td>E1NE</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>E2SE1</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>E3SE2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N4HP</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N5WL</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6HS</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>B7FT</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B8NS1</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B9NS2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10LB</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>C11SB</td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>3</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

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**Results and Discussion: Chemo-organotrophic Bacteria**

**Table 5.5** Isolation of chemo-organotrophic alkaliphiles from SRB enrichment cultures using GAM. Figures shown indicate the number of organisms isolated from SRB enrichment cultures with a particular substrate: E = ethanol, F = formate, L = lactate and B = butyrate.

<table>
<thead>
<tr>
<th>Sample site</th>
<th>Number of Isolates</th>
<th>Obligate Anaerobes</th>
<th>Anaerobes failed on subculture</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1NE</td>
<td>3E, 1F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2SE1</td>
<td>2E</td>
<td>1E</td>
<td>1</td>
</tr>
<tr>
<td>E3SE2</td>
<td>3E, 2F</td>
<td>1E</td>
<td>1</td>
</tr>
<tr>
<td>N4HP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N5WL</td>
<td>2E, 2F, 2L, 2B</td>
<td>1F, 1L, 1B</td>
<td>3</td>
</tr>
<tr>
<td>B6HS</td>
<td>3E</td>
<td>2E</td>
<td>2</td>
</tr>
<tr>
<td>B7FT</td>
<td>2E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B8NS1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B9NS2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10LB</td>
<td>1E</td>
<td>1E</td>
<td>1</td>
</tr>
<tr>
<td>C11SB</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results and Discussion: Chemo-organotrophic Bacteria

5.2 Phenotypic analysis of chemo-organotrophic bacteria

Following the initial enrichment and isolation procedures, six alkaliphilic obligate anaerobes were obtained. These isolates originated from Lake Elmenteita and Lake Bogoria. All attempts to maintain the organisms isolated from Lake Sonachi and Lake Nakuru were wholly unsuccessful.

The two isolates from Lake Elmenteita both originated from the same sampling site at the south-east bay. The sample from this location was composed of a core extracted from the waters edge, where the temperature was 25 °C and the pH was 10.0. The conductivity at this location was found to be 25 mS cm\(^{-1}\). The Lake Bogoria isolates were obtained from two different sampling sites, at Fig Tree Camp and the north shore. B7FT-A was isolated from a core sample taken from beneath the water column, where the water temperature was 34 °C. pH measurements of 11.0 - 11.5 and a conductivity of 72.3 mS cm\(^{-1}\) indicated more saline and alkaline conditions compared to Lake Elmenteita. The remaining three Lake Bogoria isolates originated from the north shore. pH and temperature measurements agreed with those found at Fig Tree Camp, although the conductivity at this location was slightly lower, at 59.6 mS cm\(^{-1}\).

5.2.1 Colony morphology

When the organisms were first isolated, doubling times were long, and growth was relatively poor. However, once stable cultures had been obtained, and the media used had been refined, growth occurred much more rapidly. Visible growth could be seen after only 24 hours incubation anaerobically at 37°C, although fully formed colonies did not appear until 48 - 72 hours incubation. Colony morphology was recorded after 48 hours growth (Table 5.6). The
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growth rate of isolate E2SE1-B was consistently lower than that found with the other isolates. Despite this, the culture was maintained throughout the course of the study.

5.2.2 Cell morphology

Microscopic analysis of wet mounts showed all organisms had a rod-shaped cellular morphology. The Lake Bogoria isolates (B7FT-A, B8NS1-A, B8NS1-C and B8NS1-C-FT) and one of the Lake Elmenteita isolates (E2SE1-B) were also found to be motile. Indeed, the only isolate which appeared to be non-motile was isolate E2SE1-C. Two isolates, B7FT-A and B8NS1-A, produced spores, although this occurred relatively infrequently, and only in old cultures (Table 5.7). Although spores were only observed in two of the six isolates, all isolates may possibly sporulate under the correct conditions. Conditions which stimulate sporulation in one strain do not necessarily stimulate spore formation in another. With some strains, the initiation of spore formation is extremely difficult (Cato and Salmon, 1976; Moore et al., 1976). Although CO₂ has been found to stimulate sporulation and favour growth of clostridia (Smith and Sullivan, 1989), this gas was excluded during the growth of the present isolates due to its effect on medium pH.

Gram stains carried out using samples from both liquid and solid media were ubiquitously Gram negative. The difficulty in Gram staining alkaliphiles has been reported previously (Jones et al., 1994), and similar problems were encountered with the present isolates. During the Gram stain procedure, the cells seemed to lyse or become disrupted, leading to an inferior mount with extensive background staining. Due to the difficulties in using the classical Gram-stain procedure, alternative methods for determining the Gram type were employed.
Table 5.6 Colony morphology of obligate anaerobes maintained in stable culture. Data presented represents colony morphology after 48 hours growth anaerobically at 37°C.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colour</th>
<th>Size (mm)</th>
<th>Form</th>
<th>Elevation</th>
<th>Margin</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2SE1-B</td>
<td>white</td>
<td>&lt;1</td>
<td>regular</td>
<td>flat</td>
<td>entire</td>
<td>very limited growth on solid media</td>
</tr>
<tr>
<td>E2SE1-C</td>
<td>light brown</td>
<td>2 - 3</td>
<td>regular</td>
<td>domed</td>
<td>entire</td>
<td>extremely mucoid</td>
</tr>
<tr>
<td>B7FT-A</td>
<td>cream / white</td>
<td>6</td>
<td>regular</td>
<td>low convex</td>
<td>entire</td>
<td></td>
</tr>
<tr>
<td>B8NS1-A</td>
<td>milky / white</td>
<td>4</td>
<td>regular</td>
<td>domed</td>
<td>entire</td>
<td>mucoid growth</td>
</tr>
<tr>
<td>B8NS1-C</td>
<td>white</td>
<td>8 - 10</td>
<td>regular</td>
<td>flat</td>
<td>translucent, undulate</td>
<td>‘watery’ appearance</td>
</tr>
<tr>
<td>B8NS1-C-FT</td>
<td>white</td>
<td>8 - 10</td>
<td>regular</td>
<td>flat</td>
<td>translucent, undulate</td>
<td>‘watery’ appearance</td>
</tr>
</tbody>
</table>
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Table 5.7 Microscopic analysis of wet mounts of each isolate. The presence of spores was often difficult to determine as they were only apparent in very old cultures. Measurements of cell size are approximations of the mean, since cellular size varied in each given sample.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Motile</th>
<th>Spores</th>
<th>Cellular morphology (length × width in µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2SE1-B</td>
<td>+</td>
<td>-</td>
<td>short rods with rounded ends, some in pairs (1 × 0.5)</td>
</tr>
<tr>
<td>E2SE1-C</td>
<td>-</td>
<td>-</td>
<td>long and thin rods, curved or slightly spiralled (10 × 0.4)</td>
</tr>
<tr>
<td>B7FT-A</td>
<td>+</td>
<td>+</td>
<td>rods with rounded ends, many in pairs (4 × 0.5)</td>
</tr>
<tr>
<td>B8NS1-A</td>
<td>+</td>
<td>+</td>
<td>rods with rounded ends (3 × 0.4)</td>
</tr>
<tr>
<td>B8NS1-C</td>
<td>+</td>
<td>-</td>
<td>shorter rods, some in pairs, some curved (2.5 × 0.4)</td>
</tr>
<tr>
<td>B8NS1-C-FT</td>
<td>+</td>
<td>-</td>
<td>rods, some curved, with rounded ends (3 × 0.4)</td>
</tr>
</tbody>
</table>
5.2.3 Gram reaction of cells

Due to the difficulties in determining the Gram reaction of these organisms, alternative methods were investigated. Common laboratory methods for the identification of the Gram reaction of organisms include the potassium hydroxide test (Gregersen, 1978), the L-alanine aminopeptidase test (Cerny, 1976) and the vancomycin sensitivity test (Halebian et al., 1981). A recent evaluation of these methods for the identification of anaerobic bacteria found the vancomycin susceptibility was the most useful diagnostic method (Bamarouf et al., 1996).

Based on these finding, the vancomycin susceptibility test was used to identify the Gram reaction of the present organisms. This test was used in conjunction with the colicin susceptibility test which had the inverse effect of vancomycin. At the normal growth pH of 10.5, all organisms were universally resistant to both antibiotics. This phenomenon has also been noted under conditions of high salt (Jörg Kunte, personal communication), and was probably due to the inactivation of the antibiotic at elevated pH. Subsequent sensitivity tests were therefore carried out at pH 8.5. Although growth at this pH was slower, antibiotic sensitivity could be correctly determined.

All of the organisms under consideration were found to be vancomycin sensitive and colicin resistant (Table 5.8). Although the degree of vancomycin susceptibility varied among the isolates, the results were definitive. This indicated that, although the organisms stained Gram-negative using the standard laboratory Gram-stain technique, all the organisms were in fact Gram-positive.

5.2.4 pH range for growth

Each isolate was tested for growth at various pH levels, ranging from pH 7.5 to pH 10.5. Below pH 10.5, the standard carbonate buffering system was
substituted for a Tris-based buffering system, although low levels of carbonate were still included as carbonate is sometimes required by alkaliphiles (Zhilina et al., 1996b). Plates were incubated anaerobically at 37°C, and growth was determined after 2 - 7 days.

Most of the organisms, with the exception of E2SE1-C, showed a restricted pH range for growth in the alkaline region, with no growth occurring below pH 8.5 (Table 5.8). Isolate E2SE1-C exhibited a slightly increased pH tolerance, with noticeable growth occurring at pH 7.5. For the pH values tested, the best growth for all isolates was at pH 10.5, indicating a pH optimum of above pH 9.5. Values above pH 10.5 were not tested due to difficulties in buffering batch media at these high levels (Grant and Horikoshi, 1992).

### 5.2.5 Salt range for growth

Isolates were tested for the ability to grow on media with varying salt concentrations, from 0% to 20% (w/v) NaCl. Determination of the salt levels tolerated by each isolate was carried out at pH 10.5.

Salt concentrations tolerated were generally quite broad, with the exception of E2SE1-C which could only survive a salt concentration between 0% and 4%. B7FT-A, B8NS1-C and B8NS1-C-FT showed the greatest salt tolerance, with growth occurring up to 12%, although this was somewhat limited (Table 5.8).

These findings concur with the conductivity readings obtained on site. The Elmenteita isolates generally tolerate only low salt concentrations, whereas the Bogoria isolates have a greater degree of salt tolerance. On site measurements indicated that Lake Bogoria was more saline than Lake Elmenteita.
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Table 5.8 Phenotypic characteristics of each isolate. Gram-type was determined by antibiotic sensitivity tests. pH range was tested using media with 4% (w/v) NaCl. Tolerated salt range was determined with media poised at pH 10.5. Values shown are % (w/v) NaCl. Colicin and vancomycin antibiotic sensitivity tests were carried out with media at pH 8.5 and 4% (w/v) NaCl. R = resistant, S = Sensitive.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gram</th>
<th>pH range</th>
<th>Salt range</th>
<th>Colicin</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2SE1-B</td>
<td>+</td>
<td>8.5-10.5</td>
<td>0-8</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>E2SE1-C</td>
<td>+</td>
<td>7.5-10.5</td>
<td>0-4</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>B7FT-A</td>
<td>+</td>
<td>8.5-10.5</td>
<td>0-8</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>B8NS1-A</td>
<td>+</td>
<td>8.5-10.5</td>
<td>0-8</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>B8NS1-C</td>
<td>+</td>
<td>8.5-10.5</td>
<td>0-12</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>B8NS1-C-FT</td>
<td>+</td>
<td>8.5-10.5</td>
<td>0-12</td>
<td>R</td>
<td>S</td>
</tr>
</tbody>
</table>
5.2.6 Biolog analysis

Biolog analysis was carried out as described in Section 2.7.4. Although the recommended incubation time for organisms prior to inoculation was 4 - 18 hours, an incubation period of 24 - 48 hours was used for the present isolates since these were generally more slow growing. Following inoculation, Biolog plates were incubated anaerobically at 30°C, and read every 24 hours for 7 days. Following repeated testing, an optimum incubation time of 48 hours was used for the present isolates, much longer than the recommended incubation time of 4 - 16 hours. However, this extended incubation time improved the reproducibility of the diagnostic procedure (Figure 5.2). An uninoculated test plate containing physiological salts solution showed only biological activity resulted in positive wells.

Despite extensive efforts, isolate E2SE1-B proved incompatible with the Biolog plate identification system, with all wells, including the negative control, turning positive after only 1 hour incubation. This is known to occur with particular isolates with membrane proteins which interact with the redox dye. Oligotrophic bacteria are also known to produce universally positive results when used with this system (Biolog Reference manual, Biolog Inc., California, USA).

Of the 95 substrates included as possible growth substrates, 8 were utilised by all isolates (glucose, D-fructose, arabinose, lactulose, piscose, turanose, glucose-6-phosphate and glucuronamide). Isolates E2SE1-C, B8NS1-A, B8NS1-C and B8NS1-C-FT could also utilise mannose, cellobiose, melibiose and glucuronic acid. Isolate B7FT-A showed a positive reaction with the broadest range of substrates, which including aspartic acid, urocanic acid, p-hydroxy phenylacetic acid, serine, histidine, threonine, thymidine, uridine, phenylalanine, alaninamide and glycerol (Figure 5.3).
Figure 5.2 Typical Biolog result following 48 hours incubation anaerobically at 30 °C. Each well contained a different growth substrate, along with other nutrients required for growth. Utilisation of a substrate was indicated by a colour change from clear to pink / purple.
Figure 5.3 Results of Biolog analysis for pure culture isolates. Only substrates utilised by one or more of the organisms are shown. Plates were read after 48 hours anaerobic incubation at 30°C. + indicates a positive reaction.

<table>
<thead>
<tr>
<th></th>
<th>α-D-glucose</th>
<th>D-fructose</th>
<th>D-galactose</th>
<th>maltose</th>
<th>D-mannose</th>
<th>L-arabinose</th>
<th>cellobiose</th>
<th>gentiobiose</th>
<th>lactose</th>
<th>D-melibiose</th>
<th>L-rhamnose</th>
<th>L-glucose-6-phosphate</th>
<th>D-galacturonic acid</th>
<th>succinic acid</th>
<th>mono-methyl-succinate</th>
<th>L-aspartic acid</th>
<th>urocanic acid</th>
<th>D-glucuronic acid</th>
<th>p-hydroxy phenylacetic acid</th>
<th>L-serine</th>
<th>L-histidine</th>
<th>L-threonine</th>
<th>thymidine</th>
<th>uridine</th>
<th>L-phenylalanine</th>
<th>glucuronamide</th>
<th>N-acetyl-D-glucosamine</th>
<th>N-acetyl-D-galactosamine</th>
<th>tween 80</th>
<th>glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2SE1-B</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>E2SE1-C</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
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<td>+</td>
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<td>+</td>
<td>++</td>
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<tr>
<td>B7FT-A</td>
<td>+</td>
<td>++</td>
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<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B8NS1-A</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>++</td>
<td>+</td>
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<tr>
<td>B8NS1-C</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>B8NS1-C-FT</td>
<td>++</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
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It should be noted that the Biolog identification system was not specifically designed for the identification of anaerobic organisms, although its use under these conditions is documented (Biolog Reference manual, Biolog Inc., California, USA). Furthermore, this particular identification system was not designed for analysis at elevated pH, although it has been used under such circumstances (Jones et al., 1994). One further identification system, ATB (API-bioMérieux), was tested using the present isolates under anaerobic alkaline conditions. Unfortunately this system proved incompatible with the anaerobic, alkaline environment, and so could not be used.

Each isolate showed a relatively broad range of substrate utilisation, especially in the utilisation of different sugars. B7FT-A was significantly different from the other isolates in its ability to utilise a number of amino acids. Many clostridia which are capable of carbohydrate fermentation are unable to utilise single amino acids. Similarly, clostridia capable of amino acid fermentation often are unable to utilise carbohydrates (e.g. Clostridium tetani and Clostridium histolyticum) (Hippe et al., 1992). B7FT-A appears to have a limited substrate specificity for carbohydrates, but a broad specificity for amino acids.

5.2.7 Fermentation products

Gas chromatography was carried out to determine the major end products of fermentation. For the purposes of this analysis, all organisms were grown in liquid culture at pH 10.5 and 4% NaCl. Due to the highly alkaline nature of the growth media, care was taken to ensure complete acidification of the extracts. This resulted in the need for 2 - 3 times more 50% HCl to acidify the extracts when compared to neutral media.

The following standards were used during analysis: acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate and caproate (Figure 5.4). Each
sample was run for 15 minutes, after which time no further compounds were found. A negative control of uninoculated medium was also analysed, which resulted in no visible peaks.

The isolates from Lake Bogoria all produced acetate and propionate as their major end products. B7FT-A and B8NS1-A produced greater amounts of propionate, whereas B8NS1-C and B8NS1-C-FT produced mainly acetate (Figure 5.5). Isolate E2SE1-C was the only organism found to produce butyrate as an end product. This is a common end product for the clostridia, resulting in the distinctive 'butyric acid' Clostridium smell. Acetate was also produced as an end product by this isolate, which is again a common product produced by the clostridia. Although the amounts of product produced by E2SE1-B are very low, due to the slow growth of this organism in liquid culture, acetate appeared to be produced as almost the sole end product. Valeric and caproic acid were not detected in any of the samples. Iso-butyric and iso-valeric acids were produced by all isolates, although generally in much reduced quantities (Figure 5.5).
Results and Discussion: Chemo-organotrophic Bacteria

Figure 5.4 Representative gas chromatography trace showing 25 mM standards (top trace) and an alkaliphile extract (bottom trace). 3.7 = acetate, 4.5 = propionate, 4.9 = iso-butyrate, 5.8 = butyrate, 6.5 = iso-valerate, 11.6 = caproate. A control of uninoculated medium was also examined, which resulted in no visible peaks.
Figure 5.5 End products of fermentation represented as amount of product per ml of culture (mmol ml$^{-1}$).
5.3 Phylogenetic analysis of isolates

5.3.1 Sequence alignment

Before the raw 16S rRNA data could be used to construct phylogenies, the unknown sequences had to first be aligned to published sequence data. Correct alignment based on the secondary structure of the 16S rRNA molecule is vital for all subsequent phylogenetic analyses. For the purposes of the present study, data were obtained from the small subunit RNA database at the University of Antwerp, Belgium, via the world wide web (http://rRNA.uia.ac.be/) (Van de Peer et al., 1996a).

A total of 171 sequences were downloaded, all belonging to the low G+C group of the Gram-positive bacteria. Sequences were selected as representatives of the clusters found within the Clostridium phylum of the low G+C group of the Gram-positive Bacteria, as proposed by Collins et al. (1994). Bacillus alcalophilus was also included, as an outgroup to root the subsequent phylogenetic trees. Each of the downloaded sequences also contained secondary structure data, which assisted in the alignment of the unknown organisms.

The present isolates were aligned one at a time to the database isolates using the DCSE computer package (De Rijk and De Wachter, 1993). Utilising the data from a FASTA search, each novel isolate was initially aligned automatically to its closest described relative, using the built-in pairwise alignment option of the DCSE package. This initial crude alignment was then checked by eye and refined where necessary. Following this, secondary structure characters were copied from the known sequence to the novel sequence. Finally, the secondary structure data were checked by eye to ensure
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correct base pairing in the 16S helices. The complete alignment of the novel sequences and their closest relatives is presented in Appendix II.

5.3.2 Substitution rate calibration

Use of the nucleotide substitution rate calibration method of Van de Peer et al. (1996b) firstly required the calculation of the $p$ value. For this calculation, a large data set of 171 described species together with the 10 sequences obtained during the course of the present study were used to produce an accurate substitution rate spectrum.

For the purposes of distance estimation using substitution rate calibration, certain nucleotide sites had to be ignored. These sites included those which were absolutely conserved across all species, and those which were deleted in more than 75% of the sequences. From an initial data set of 2107 nucleotides covering bases 8 to 1512 of the 16S rRNA gene ($E. coli$ numbering, including alignment gaps), 501 positions were found to be absolutely conserved, and 611 were found to be unreliable (present in $< 25\%$ of the sequences). Therefore the remaining 995 significant bases were used for the computation of the $p$ value and for subsequent phylogenetic inference.

Evolutionary distance was first approximated using the equation of Jukes and Cantor (1969). However, as stated in the introduction, evolutionary distance calculations are inaccurate if individual nucleotides within the sequence show considerable differences in evolutionary rate, as found with 16S rRNA (Olsen, 1987). Jin and Nei (1990) proposed that substitution rates formed a gamma distribution over the sequence positions, a finding later confirmed by De Rijk et al. (1995). However, calculations based on a gamma distribution of substitution rates still did not take account of the substitution rates of individual nucleotide positions. Therefore the Jukes and Cantor evolutionary distance was used in a calculation of the relative variability of each nucleotide
position. Following this, alignment positions were grouped together into sets of similar variability (Figure 5.6). The shape of this variability spectrum was then used to derive a more exact expression for the dissimilarity between two sequences based on the evolutionary distance separating them. Repeated iterations using the recalculated distance matrix data were carried out to determine the $p$ value, with each iteration plotted as a function of dissimilarity ($f$) against distance ($d$) (Figure 5.7). It was found that four iterations were sufficient for changes to become imperceptible. From this analysis, the value of $p$ for this specific combination of data was found to be 0.32. Calculation of the $p$ value only had to be performed once. The reason for this was subsequent analyses only utilised data which was included in the original $p$ value calculation. Evolutionary distances were calculated using the following equation, included as part of the TreeCon package:

$$d = p \left[ \left(1 - \frac{4}{3}S \right)^\frac{3}{4p} - 1 \right]$$

Following substitution rate calibration, a subset of the sequence data was obtained, consisting only of the positions used in the previous analysis (i.e. not including completely conserved sequence positions of those which were deleted in > 75% of the sequences). From this subset of data, individual sequences were selected for the construction of phylogenetic trees.

### 5.3.3 Phylogenetic analysis of the genus *Clostridium*

Preliminary phylogenetic analyses utilising the on-line FASTA database querying programme of the European Bioinformatics Institute was performed. This revealed that all the present isolates fell within the *Clostridium* spectrum. In order to obtain an overall picture of the relationships between the novel and described isolates, a phylogenetic tree containing
Figure 5.6 Distribution of relative substitution rates, estimated for all alignment positions that were not completely conserved and that were contained in at least 25% of the aligned sequences. Substitution rates were measured relative to the average rate of the entire molecule.
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Figure 5.7 Graphical representation of dissimilarity ($f$) against evolutionary distance ($d$) based on the iterative process of the substitution rate calibration equation. $JC$ indicates the curve obtained from a substitution rate spectrum based on the Jukes and Cantor equation. 1, 2 and 3 indicate curves obtained following the recalculation of distances based on relative substitution rates. Evolutionary distance can be estimated by calculating the dissimilarity between two sequences, and using this value to determine distance.
Results and Discussion: Chemo-organotrophic Bacteria

representatives of all the clostridial clusters described by Collins et al. (1994) was constructed (Figure 5.8). The present isolates fell into three groups: the haloalkaliphiles which grouped between clusters VII and VIII (see Section 6.3); the low-salt isolates which fell within clostridial cluster XI; and one isolate which could not be maintained in pure culture, which was most closely related to cluster XV. Using this information, subsections of the clostridial tree were examined in more detail to show exact phylogenetic relationships.

5.3.4 Cluster XI isolates

Isolates E2SE1-B, E2SE1-C, B7FT-A, B8NS1-A, B8NS1-C and B8NS1-C-FT all fell with cluster XI of the genus Clostridium as described by Collins et al. (1994). Using this information, the following organisms were selected for inclusion in a phylogenetic tree: Peptostreptococcus anaerobius, Clostridium bifermentans, C. difficile, C. felsineum, C. formicoaceticae, C. ghoni, C. glycolinum, C. halophilum, C. irregularis, C. litorale, C. lituseburense, C. mangenotii, C. mayombeii, C. paradoxum, C. sordellii, C. sticklandii, C. thermoalcalophilum, C. villosus, Eubacterium tenue and Bacillus alcalophilus.

Using the $p$ value obtained from the analysis described above, the evolutionary distances between the present isolates and these previously described organisms were calculated using the substitution rate calibration method (Van de Peer et al., 1996b). Bootstrap analysis was carried out to place confidence intervals on the phylogenies, with data being resampled 1000 times.

Following distance estimation calibrated for relative substitution rates, a phylogenetic tree was constructed from the resulting distance matrix using the neighbour-joining of Saitou and Nei (1987) (Figure 5.9). The present isolates
Figure 5.8 Phylogenetic reconstruction of the *Clostridium* spectrum. The tree was constructed using the neighbour joining method, with bootstrapping of 100 trees. The areas coloured green represent the locations of the present novel alkaliphiles. Cluster designations correspond to those proposed by Collins *et al.* (1994). Scale bar indicated 0.1 substitutions per sequence position.
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were found to compose a distinct group within cluster XI, separate from the previously described alkaliphiles *C. paradoxum* and *C. thermoalcaliphilum*. This was perhaps not surprising since these cluster XI alkaliphiles were isolated from a man-made alkaline environment. The bootstrap data show that the present alkaliphiles form a robust group, although confidence in the tree topology within this group is somewhat lower at some of the later branches.

In order to further elucidate the relationships between the present isolates and previously described representatives of the *Clostridium* genus, percentage sequence similarity was calculated. These data were calculated using the BestFit computer programme which is part of the Wisconsin GCG package. BestFit makes an optimal alignment of the best segment of similarity between two sequences. These alignments are found by inserting gaps to maximise the number of base matches, using the algorithm of Smith and Waterman (1981). Using this alignment, the percentage similarity can be calculated.

The phylogenetic inference and sequence similarity data show the two Lake Elmenteita isolates are distinct from the Lake Bogoria isolates. Combining these data with the phenotypic analyses, it appears that these organisms represent two novel species of *Clostridium*. The two organisms have different colony and cellular morphology, and differ in their tolerances to neutral and saline media. Furthermore, Biolog analysis revealed differences between these two organisms, with E2SE1-B being incompatible with this method of identification. Finally, isolate E2SE1-C produced butyrate as a major end product of fermentation whereas E2SE1-B only produced significant quantities of acetate. All of these factors indicate that these isolates are distinct from one another. The phylogenetic data shows that they are also unrelated to any described species, although they fall within the genus *Clostridium*.  

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organisms can be obtained by summing the lengths of the branches connecting them along the horizontal axis. Bootstrap values are shown as percentages of 1000 trees. Scale bar represents 0.05 substitutions per nucleotide position.
Table 5.9 Percentage sequence similarity between the present alkaliphiles and selected described species. Similarity figures were calculated using the BestFit programme of the Wisconsin GCG computer package. *C. felsineum* was the most closely related organism as determined by a FASTA search. *C. paradoxum* and *C. thermoalcaliphilium* were included as previously described alkaliphiles.

<table>
<thead>
<tr>
<th></th>
<th>C. felsineum</th>
<th>E2SE1-B</th>
<th>E2SE1-C</th>
<th>B7FT-A</th>
<th>B8NS1-A</th>
<th>B8NS1-C</th>
<th>B8NS1-C-FT</th>
<th>C. paradoxum</th>
<th>C. thermoalc</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. felsineum</td>
<td>-</td>
<td>90.9</td>
<td>88.4</td>
<td>90.5</td>
<td>90.3</td>
<td>90.5</td>
<td>90.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E2SE1-B</td>
<td>90.9</td>
<td>-</td>
<td>92.0</td>
<td>96.9</td>
<td>96.7</td>
<td>97.0</td>
<td>97.0</td>
<td>86.8</td>
<td>-</td>
</tr>
<tr>
<td>E2SE1-C</td>
<td>88.4</td>
<td>92.0</td>
<td>-</td>
<td>91.9</td>
<td>92.0</td>
<td>92.1</td>
<td>92.0</td>
<td>85.8</td>
<td>-</td>
</tr>
<tr>
<td>B7FT-A</td>
<td>90.5</td>
<td>96.9</td>
<td>91.9</td>
<td>-</td>
<td>99.5</td>
<td>99.9</td>
<td>99.9</td>
<td>87.0</td>
<td>-</td>
</tr>
<tr>
<td>B8NS1-A</td>
<td>90.3</td>
<td>96.7</td>
<td>92.0</td>
<td>99.5</td>
<td>-</td>
<td>99.5</td>
<td>99.5</td>
<td>87.0</td>
<td>-</td>
</tr>
<tr>
<td>B8NS1-C</td>
<td>90.5</td>
<td>97.0</td>
<td>92.1</td>
<td>99.9</td>
<td>99.5</td>
<td>-</td>
<td>100</td>
<td>87.1</td>
<td>-</td>
</tr>
<tr>
<td>B8NS1-C-FT</td>
<td>90.5</td>
<td>97.0</td>
<td>92.0</td>
<td>99.9</td>
<td>99.5</td>
<td>100</td>
<td>-</td>
<td>87.0</td>
<td>-</td>
</tr>
<tr>
<td><em>C. paradoxum</em></td>
<td>-</td>
<td>86.8</td>
<td>85.8</td>
<td>87.0</td>
<td>87.0</td>
<td>87.1</td>
<td>87.0</td>
<td>-</td>
<td>98.3</td>
</tr>
<tr>
<td><em>C. thermoalc</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>98.3</td>
</tr>
</tbody>
</table>
Results and Discussion: Chemo-organotrophic Bacteria

The data supporting the Lake Bogoria isolates is somewhat less conclusive, with sequence similarity figures for all the isolates over 99%. Despite this fact, isolates B7FT-A and B8NS1-A appear to be distinct from B8NS1-C and B8NS1-C-FT. Colony and cellular morphological data revealed B7FT-A and B8NS1-A had similar growth characteristics. Furthermore, fermentation end-product analysis also revealed a degree of similarity between these two organisms. However, B7FT-A not only had a greater salt tolerance, but more significantly it utilised amino acid substrates in preference to carbohydrates. B8NS1-A appeared to be distinct from B8NS1-C and B8NS1-C-FT based on the morphological data, together with the substrate utilisation profile and fermentation end-product data. The phylogenetic data also shows this isolate to be somewhat unrelated to B7FT-A, despite the high sequence similarity values. Based on these findings, the two isolates appear to be new species within the Clostridium genus.

The differences between B8NS1-C and B8NS1-C-FT are less pronounced. They are both morphologically similar, and have identical tolerances to both salinity and pH. They also produce the same end-products of fermentation in roughly equal quantities. The only differences found between these two isolates was during the Biolog analysis. However, as noted previously, the Biolog identification system was not designed specifically for use with anaerobic bacteria. Bearing this in mind, the Biolog results are noticeably similar to each other. Finally, the phylogenetic data place these organisms extremely close together. All of these factors indicate that B8NS1-C and B8NS1-C-FT are in fact the same species.

Although the Lake Bogoria isolates are closely related, the data suggest the six novel alkaliphile isolates represent five new species within the Clostridium genus, two from Lake Elmenteita and three from Lake Bogoria. Numerous procedures have been described, which would further clarify the
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relationships between these isolates. The most powerful of these would be DNA-DNA hybridisation analysis, which has already been used to elucidate relationships within the clostridia (Johnson, 1973; Johnson and Francis, 1975) would further clarify the relationships between the Bogoria isolates. Further identification procedures which have been used in the identification of clostridia include whole cell protein pattern analysis by polyacrylamide gel electrophoresis (Cato et al., 1982), cell wall composition analysis (Weiss et al., 1981) and cellular fatty acid analysis (O'Leary and Wilkinson, 1988).

5.3.5 Isolate N5WL-A-lac

Organism N5WL-A-lac was isolated from an early SRB enrichment culture which had lactate present as the growth substrate. Although this isolate could not be maintained in pure culture, successful DNA extractions were obtained enabling 16S rRNA sequence analysis.

A preliminary FASTA search showed that this isolate was most closely related to Eubacterium limosum, found within cluster XV of the Clostridium phylum. Based on this information the following members of this cluster, together with members of closely related clusters, were selected for phylogenetic analysis: Clostridium barkeri (Eubacterium barkeri; Collins et al., 1994), C. innocuum, C. ramosum, C. rectum, C. spiroforme, Eubacterium alactolyticum, E. biforme, E. limosum, Lactobacillus catenaforme, L. vitulinus, Streptococcus pleomorphus and Bacillus alcalophilus.

The phylogenetic tree presented in Figure 5.10 was constructed as previously described, using bootstrap analysis of 1000 trees. Based on this information, isolate N5WL-A-lac appeared to fall within cluster XV of the Clostridium group. This cluster contains members of the Eubacterium genus, including the recently renamed Eubacterium barkeri (Collins et al., 1994).
Organisms of the *Eubacterium* genus are similar to those of the *Clostridium* genus except that they are universally non-sporeforming. To date they have only been isolated from mesophilic environments, and growth at elevated pH has not been documented. The failure of the present isolate to grow in laboratory culture is perhaps not surprising given the fastidiousness of a number of *Eubacterium* sp. Many species require supplements to complex media, including vitamins, volatile fatty acids and / or special substrates such as cellobiose or maltose (Andreesen, 1992). Even if all the necessary nutrients are present, the growth of some species is still poor (Hill *et al.*, 1987). It is possible that N5WL-A-lac required the presence of one or more fatty acids found within the SRB enrichment culture from which it was isolated. Whether this requirement was for lactate or perhaps for a metabolic end-product of lactate oxidation is impossible to ascertain. Supplementing the complex alkaliphile medium with an SRB enrichment extract would have undoubtedly assisted in the maintenance of this isolate.

Although N5WL-A-lac was found to be most closely related to members of the genus *Eubacterium*, sequence similarity levels suggest that this organism may belong to a new genus. Percentage similarity figures when compared to *Eubacterium limosum* show only 85% sequence similarity. This value is comparable to that obtained between N5WL-A-lac and E2SE1-B, which have a sequence similarity value of 84%, and which are only distantly related.

Although this organism could not be maintained in pure culture, it does indicate that novel bacteria are present which are perhaps incompatible with present culturing techniques. Using the data of the present study, further investigations can be targeted to isolate specific genera. Furthermore, the sequence data produced can be used to design DNA probes to look for similar organisms in the natural environment. The present study has revealed the presence of a diversity of novel alkaliphilic anaerobic organisms, many of
Results and Discussion: Chemo-organotrophic Bacteria

which cannot be cultured using contemporary methods. However, the number of isolates from anaerobic alkaline habitats will surely rise as a greater understanding of these fascinating ecological niches develops.
Figure 5.10 Evolutionary tree showing the relationship between the isolate N5WL-A-lac and previously described isolates. Distance values were calculated using the substitution rate calibration method (Van de Peer et al., 1996b), and the tree was constructed using the neighbour joining method (Saitou and Nei, 1987), based on bootstrap analysis of 1000 trees. Scale bar represents 0.05 substitutions per nucleotide position.
6 Results and Discussion: Haloalkaliphilic Bacteria

Lake Magadi is a classical site of soda and salt deposition, with an active anaerobic microbial community (Tindall, 1988; Jones et al., 1994; Zhilina and Zavarzin, 1994). It is therefore an ideal location for investigations into novel haloalkaliphilic anaerobes.

Anaerobic haloalkaliphiles are a group of micro-organisms which have received very little scientific investigation. Carbohydrate-fermenting halophilic anaerobes comprise nine genera, represented by 14 species, within the domain Bacteria (Ollivier et al., 1994). The alkaliphile phenotype has only been identified in two described species, *Haloanaerobium alcaliphilum* (Tsai et al., 1995) and *Natroniella acetigena* (Zhilina et al., 1996b). However, of these two organisms only *N. acetigena* was found to be truly alkaliphilic, exhibiting a pH optimum of 9.7 to 10.0. *H. alcaliphilum* had a reported pH optimum of 6.7 - 7.0, and as such cannot be termed a true alkaliphile (Krulwich and Guffanti, 1989a).

The limited number of haloalkaliphilic anaerobes isolated indicate a need for further investigation. With the identification of new species, a fuller understanding of the processes involved in nutrient cycling within these extreme environments will undoubtedly prevail.

6.1 Isolation of haloalkaliphilic bacteria

Two media were initially used for the isolation of halophilic alkaliphiles, a standard haloalkaliphile medium with yeast extract and casamino acids
Results and Discussion: Haloalkaliphilic Bacteria

(GHAM, Duckworth et al., 1996), and an adapted anaerobic hypersaline medium with glucose as the carbon source (Oren, 1987). Isolations were carried out under strictly anaerobic conditions using media prepared in the absence of oxygen and reduced using a combination of reductants. Throughout the isolation process aerobic subcultures were performed to ensure the anaerobicity of the resulting organisms.

Initially isolation was attempted by performing serial dilutions using the sediment samples, and plating these on solid media at 37 °C. However, using this method, single colonies could not be obtained. Subsequently, large lumps of sediment were spread directly onto the surface of the agar without previous dilution.

Primary growth was superior on the adapted medium of Oren where glucose was the growth substrate. However, once pure cultures had been obtained, the growth of all the isolates was more robust on GHAM medium. From these primary spread plates, 17 isolates were obtained from the GHAM plates and 20 isolates were obtained from the adapted Oren plates (Table 6.1). Aerobic subculturing revealed 6 of the GHAM isolates and 7 of the Oren isolates were unable to grow in the presence of oxygen. Despite repeated efforts, only 3 of these isolates could be maintained in stable laboratory culture, 2 from GHAM (G-M14CH-4, G-M16NWC-4) and 1 from Oren (O-M12SP-2) (Table 6.1). These organisms were subsequently cultivated using GHAM medium, and were found to growth equally well using both liquid and solid media.

Due to the nature of the primary isolation of these organisms, viable counts were impossible to perform.

The diversity of isolates initially obtained from the Lake Magadi sediment samples again show that a plethora of micro-organisms exist within these environments. A number of haloalkaliphiles belonging to the Bacteria have
already been isolated (Jones et al., 1994), although it is not known whether these organisms are capable of anaerobic growth. The varied colony morphology of a number of the isolates that could not be maintained in laboratory culture suggests the presence of a number of distinct species. This, together with the isolation of a number of facultatively anaerobic haloalkaliphiles, shows that the hypersaline, alkaline, anaerobic environment of Lake Magadi houses an active and diverse population of micro-organisms.
Results and Discussion: Haloalkaliphilic Bacteria

Table 6.1 Isolation of haloalkaliphiles using two separate media. Numbers shown represent the total number of isolates within each category obtained from each sampling location. GHAM = Grant’s haloalkaliphile medium (Duckworth et al., 1996). Oren = Adapted hypersaline medium (Oren, 1987). Facultative = organisms found to be facultative anaerobes; Anaerobic = organisms found to be obligate anaerobes; Stable = organisms were maintained in stable laboratory culture.

<table>
<thead>
<tr>
<th>Sample</th>
<th>GHAM</th>
<th>Oren</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Facultative</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>M12SP</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M13CC</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M14CH</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>M15CL</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>M16NWC</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>6</td>
</tr>
</tbody>
</table>
6.2 Phenotypic analysis of haloalkaliphilic bacteria

Following the initial isolation procedure, three organisms could be maintained in stable laboratory culture, namely O-M12SP-2, G-M14CH-4 and G-M16NWC-4. Isolate O-M12SP-2 originated from a core sample taken from a primary evaporation pond at Lake Magadi, Kenya. These primary evaporation ponds were employed by the Magadi Soda Company for the initial precipitation of carbonates from the Lake. Further ponds were used for the subsequent precipitation of NaCl. G-M14CH-4 was isolated from the trona beds bordering the main causeway across the lake, 200 m west of pump station 17. The thick trona crust was removed using a machete, and the underlying gelatinous mud was collected with a sterile tongue depressor. The final isolate, G-M16NWC-4, originated from a sample taken beside the north-west causeway of Lake Magadi. This sample was collected using a sampling cup attached to a 2 m long pole, from the eastern side of the causeway at a passing-place between the mainland and 'Big Island'. At this location an algal mat community had colonised the surface of the trona.

All of the samples taken from Lake Magadi had a conductivity of \( \text{ca.} \ 110 \, \text{mS cm}^{-1} \) and a pH of 10.5 to 12. Generally the temperature varied from 30 to 35 °C depending on the time of the day.

6.2.1 Colony morphology

Visible growth could be seen only after 4 - 5 days incubation anaerobically at 37 °C, although fully formed colonies do not appear until 5 - 7 days incubation. Growth improved with the addition of \( \text{Na}_2\text{S} \), which served to maintain a highly reduced environment. Colony morphology was recorded after 7 days growth (Table 6.2).
6.2.2 Cell morphology

Microscopic analysis of wet mounts revealed all organisms had a rod-shaped cellular morphology. Isolate O-M12SP-2 consisted of short rods, some slightly curved, with no visible spores or motility. G-M14CH-4 consisted of extremely long and distended rods, each with many twists and curves. This isolate also did not produce spores, although the cells showed limited motility. Isolate G-M16NWC-4 was similar in morphology to O-M12SP-2 although curvature of the cells was not observed. This isolate was non-motile and did not appear to sporulate. It should be noted that although spores were not observed for these isolates, the possibility of sporulation under certain circumstances cannot be ruled out. Often sporulation of anaerobic bacteria under laboratory conditions is extremely difficult (Hippe et al., 1992) (Table 6.3).

6.2.3 Gram reaction of cells

As mentioned previously, alternative methods of determining the Gram reaction were employed. The vancomycin susceptibility test, together with the colicin susceptibility test were used for this purpose. In order for these tests to operate correctly, each organism was grown on a medium poised at pH 8.5 with 16% (w/v) NaCl. All of the organisms under consideration were found to be vancomycin sensitive and colicin resistant (Table 6.4), indicating that they were in fact Gram-positive.
Table 6.2 Colony morphology of obligate anaerobes maintained in stable culture. Data presented represents colony morphology after 48 hours growth anaerobically at 37 °C.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colour</th>
<th>Size (mm)</th>
<th>Form</th>
<th>Elevation</th>
<th>Margin</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-M12SP-2</td>
<td>pink/white</td>
<td>2 - 3</td>
<td>regular</td>
<td>low dome</td>
<td>entire</td>
<td>early growth pinpoint, white</td>
</tr>
<tr>
<td>G-M14CH-4</td>
<td>beige/white</td>
<td>3 - 5</td>
<td>regular</td>
<td>umbonate</td>
<td>entire</td>
<td>early growth pinpoint, white</td>
</tr>
<tr>
<td>G-M16NWC-4</td>
<td>pink/white</td>
<td>3 - 4</td>
<td>regular</td>
<td>low convex</td>
<td>entire</td>
<td>early growth pinpoint, white</td>
</tr>
</tbody>
</table>
Results and Discussion: Haloalkaliphilic Bacteria

Table 6.3 Microscopic analysis of wet mounts of each isolate. The presence of spores was often difficult to determine as they were only apparent in very old cultures. Measurements of cell size are approximations of the mean, since cellular size varied in each given sample.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Motile</th>
<th>Spores</th>
<th>Cellular morphology (length $\times$ width in $\mu$m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-M12SP-2</td>
<td>-</td>
<td>-</td>
<td>rods, some curved, individual. 0.6 $\times$ 8.0</td>
</tr>
<tr>
<td>G-M14CH-4</td>
<td>+/-</td>
<td>-</td>
<td>very long, distended rods, curved, individual. 0.6 $\times$ &gt;10.0</td>
</tr>
<tr>
<td>G-M16NWC-4</td>
<td>-</td>
<td>-</td>
<td>rods, short with rounded ends, individual. 0.8 $\times$ 4.0</td>
</tr>
</tbody>
</table>
6.2.4 pH range for growth

Each isolate was tested for growth at various pH levels, ranging from pH 7.5 to pH 10.5. Below pH 10.5, the carbonate buffering system was substituted for a Tris-based buffering system, although small levels of carbonate were still present. The salt concentration used for these tests was 16% (w/v) NaCl. Plates were incubated anaerobically at 37°C, and growth was determined after 7 days.

These haloalkaliphilic organisms were found to have a restricted pH range for growth, with only G-M16NWC-4 showing any growth below pH 9.5. Growth occurred most rapidly on medium poised at pH 10.5, indicating a pH optimum of above pH 9.5. Values above pH 10.5 were not tested due to difficulties in buffering batch media at these high levels (Grant and Horikoshi, 1992).

The restricted pH range of these organisms reflects the habitat from which they were isolated. Lake Magadi has alkaline ‘hot-spots’, which may be up to pH 12.0. Towards the periphery of the lake, volcanic springs form less alkaline and less concentrated lagoons (e.g. ‘Fish-Spring Lagoon’, pH 10.5). Each of the present organisms was isolated from highly alkaline locations, which may account for their requirement for alkaline conditions.

6.2.5 Salt range for growth

Lake Magadi is a hypersaline soda lake, and as such one would expect the organisms which inhabit this location to require NaCl for growth. Also, carrying out the isolation procedure at 20% NaCl would select for halophilic organisms. Isolates which have a lower salt optimum would tend not to grow under such hypersaline conditions.
Results and Discussion: Haloalkaliphilic Bacteria

Isolates were tested for the ability to grow on media with varying salt concentrations, from 0% to 30% (w/v) NaCl. Determination of the salt levels tolerated by each isolate was carried out at pH 10.5.

All of the isolates showed an obligate requirement for elevated levels of NaCl. O-M12SP-2 exhibited a limited salt tolerance range, with no growth occurring below 16% or above 24% (w/v) NaCl. This finding is consistent with the location from which the organism was sampled. The primary evaporation ponds of solar salterns tend to be more dilute than the later ponds. Therefore the more limited salt tolerance of this isolate is not surprising. The other two isolates exhibited a broader salt tolerance, growing from 12% to 26% (w/v) NaCl. Both of the sampling locations from which these isolates originated were found within the main body of the lake. Previous geological analyses have shown the upper beds of the lake to have a NaCl concentration in the range of 270 g l⁻¹ (Baker, 1958).

6.2.6 Biolog analysis

Extensive testing of the Biolog identification system revealed that, although these isolates were much slower growing than their non-halophilic counterparts, an incubation time of 48 hours was suitable for reproducible results. If incubation was extended beyond this time, all wells gradually transposed to give a positive result, including the negative control. Therefore an incubation time of 48 hours was used for analysis. Inoculation of a Biolog plate with sterile physiological salts solution resulted in no positive wells, showing only biological action resulted in a colour change.

Each of these haloalkaliphilic isolates showed broadly similar results, with 17 of the 95 possible growth substrates utilised by all strains (α-D-glucose, D-
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Table 6.4 Phenotypic characteristics of each isolate. Gram-type was determined by antibiotic sensitivity tests. pH range was tested using media with 16% (w/v) NaCl. Tolerated salt range was determined with media poised at pH 10.5. Values shown are % (w/v) NaCl. Colicin and vancomycin antibiotic sensitivity tests were carried out with media at pH 8.5 and 16% (w/v) NaCl. R = resistant, S = Sensitive.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gram</th>
<th>pH range</th>
<th>Salt range</th>
<th>Colicin</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-M12SP-2</td>
<td>+</td>
<td>9.5-10.5</td>
<td>16-24</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>G-M14CH-4</td>
<td>+</td>
<td>9.5-10.5</td>
<td>12-26</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>G-M16NWC-4</td>
<td>+</td>
<td>8.5-10.5</td>
<td>12-26</td>
<td>R</td>
<td>S</td>
</tr>
</tbody>
</table>
fructose, L-fructose, D-galactose, maltose, D-mannose, \(\alpha\)-D-lactose, L-arabinose, cellobiose, gentibiose, D-mellibiose, lactulose, D-piscose, turanose, L-rhamnose, glucose-6-phosphate, D-glucuronic acid and glucuronamide). Isolate G-M16NWC-4, in addition to these substrates, also showed a positive reaction for urocanic acid, L-serine, D-alanine, L-alanine, L-histidine, L-leucine, L-phenylalanine and alaninamide.

Based on these findings, O-M12SP-2 and G-M14CH-4 are largely limited to fermentable carbohydrates substrates for growth. Isolate G-M16NWC-4 is also capable of carbohydrate fermentation, but in addition to this it is able to utilise a limited range of amino acids.

6.2.7 Fermentation products

Gas chromatography was carried out on extracts from liquid cultures of each of the present isolates. A medium containing 20% NaCl at pH 10.5 was used for culturing prior to extraction. As noted previously, complete acidification of the highly alkaline extracts was essential to the success of this procedure.

All haloalkaliphilic isolates produced \emph{iso}-valeric acid as their major end product of fermentation (Figure 6.2). Secondary products for O-M12SP-2 and G-M14CH-4 were acetic and \emph{iso}-butyric acids in roughly equal proportions, whereas G-M16NWC-4 produced increased levels of acetic acid over \emph{iso}-butyric acid. All of the isolates also produced limited quantities of propanoic acid. Valeric and caproic acids were not produced by these isolates under the conditions tested (Figure 6.2).

The only previously described haloanaerobic isolate found to produce \emph{iso}-valerate and \emph{iso}-butyrate was \emph{Sporohalobacter lortetii} (Oren \emph{et al}., 1987), which was originally classified as \emph{Clostridium lortetii} (Oren, 1983). This
Figure 6.1 Results of Biolog analysis for pure culture isolates. Only substrates utilised by one or more of the organisms are shown. Plates were read after 48 hours anaerobic incubation at 30 °C. + indicates a positive reaction.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>O-M12SP-2</th>
<th>G-M14CH-4</th>
<th>G-M16NWC-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-D-glucose</td>
<td>+ + + + + + + + + + + + + + +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-fructose</td>
<td>+ + + + + + + + + + + + + + +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-fructose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-galactose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maltose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-mannose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-arabinose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellubiose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gentiobiose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-melibiose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactulose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-psicose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>turanose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-rhamnose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose-6-phosphate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-D-lactose</td>
<td>+ + + + + + + + + + + + + + +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-glucuronic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-hydroxy phenylactic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-serine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-alanine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-alanine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-leucine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-phenylalanine gluturonamide alanamide</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results and Discussion: Haloalkaliphilic Bacteria

Figure 6.2 End products of fermentation represented as amount of product per ml of culture (mmol ml⁻¹).

![Diagram showing end products of fermentation for different isolates with acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate, and caproate categories.]
organism was reclassified on the basis of its Gram-negative cell wall. *Haloanaerobacter chitinovorans* was also found to produce iso-butyrate, together with acetate (Liaw and Mah, 1992).

The production of acetate has been found to be ubiquitous within the halophilic anaerobes. The alkaliphile *Natroniella acetigena* was found to produce acetate as the sole end-product of fermentation, although when grown on propanol, low levels of propionate were produced (Zhilina et al., 1996b). The alkaline-tolerant *Haloanaerobium alcaliphilum* was also found to produce acetate, together with lactate, butyrate, CO₂ and H₂ (Tsai et al., 1995).

These data suggest the present isolates are physiologically distinct from previously described halophilic anaerobes. They also appear to be distinct from the anaerobic *Clostridium* group, which produce mainly butyrate and/or propionate (Hippe et al., 1992).

### 6.3 Phylogenetic analysis of haloalkaliphiles

Phylogenetic analysis of the haloalkaliphilic isolates was carried out using the substitution rate calibration method, as described in Section 5.3.2. The sequence data from these organisms, together with the data from related known isolates, were included in the initial derivation of the *p* value. Therefore this value could be used for distance estimation of these organisms, using a sub-set of the original sequence data.

A FASTA search revealed that the present isolates were most closely related to *Clostridium thermoautotrophicum* and *C. thermoacetemicum*. In order to obtain an overall picture of the phylogeny of these isolates, a tree was constructed containing representatives of the major clostridial clusters.
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(Collins et al., 1994) (Figure 5.8). This revealed that the haloalkaliphiles fell between clusters VII and VIII of the Clostridium group.

Based on this information the following related species, were selected for further phylogenetic analysis: *Acetogenium kivui*, *Clostridium thermoamylyoliticum*, *C. thermocopriae*, *C. thermosaccharolyticum*, *Desulfotomaculum australicum*, *Pectinatus frisigenesis*, *P. cerevisiiphilus*, *Quinella ovalis*, *Selenomonas ruminantium*, *Se. sputigena*, *Sporomusa paucivorans*, *Sp. termitida*, *Syntrophomonas wolfei*, *Syntrophospora bryantii*, *Thermoanaerobacter brockii*, *T. ethanolicus*, *T. finii*, *T. lactoethylicum*, *T. saccharolyticum*, *T. thermohydrosulfuricus*, *T. thermosulfurigenes*, *T. xylanolyticum*, *Thermoanaerobacter acetoethylicus* and *Bacillus alcalophilus*.

Figure 6.3 shows that detailed phylogenetic analysis placed the present isolates between cluster VIII and cluster IX of the Clostridium group, although they do not seem closely related to either cluster. This area of the Clostridium phylum contains a heterogeneous collection of organisms, some of which are quite distantly related (Cato and Stackebrandt, 1989). Hence some of the bootstrap values for certain clusters appear quite low. However, the values for the haloalkaliphilic isolates show that this group is well defined and phylogenetically robust.

The use of the substitution rate calibration method for distance estimation helps to eliminate the shortening of branch lengths between more distantly related or rapidly evolving species (Van de Peer et al., 1996b). Therefore this is an ideal method when analysing areas where relationships between the representative organisms are somewhat distant.

According to the evidence from the phylogenetic analysis, the present haloalkaliphilic isolates constitute a novel cluster within the Clostridium
Figure 6.3 Phylogenetic tree showing the relationship between the present haloalkaliphilic isolates and other previously described organisms. Evolutionary distances were calculated using the substitution rate calibration method (Van de Peer et al., 1996b), and the tree topology was inferred using the neighbour-joining method (Saitou and Nei, 1987) based on bootstrap analysis of 1000 trees.
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phylum. BesFit analysis revealed 13 - 14% sequence divergence between these isolates and their closest relative *C. thermoaceticum* (Table 6.5). This level of sequence difference would suggest the presence of a new genus. Similarity differences within this group of haloalkaliphiles range from 94 - 98% (Table 6.5), suggesting the isolates represent three distinct species within the new genus.

*C. thermoaceticum* and *C. thermoautotrophicum* are both thermophilic, homoacetogenic organisms which ferment a range of carbohydrates producing acetate as their major end product (McBee, 1948; Wiegel *et al.*, 1981). Although alkaline-tolerance has not been documented with these organisms, acid-tolerant mutants have been described (Reed *et al.*, 1987; Brumm *et al.*, 1988). The present isolates appear to have a different mode of growth, with butyrate produced as the main end-product of fermentation.

The closely related members of cluster VIII both degrade fatty acids in syntrophic associations with methanogenic bacteria (McInerney *et al.*, 1979). Biolog analysis of the haloalkaliphiles showed they were unable to degrade fatty acids, indicating that although they are phylogenetically close to *Syntrophomonas wolfei* and *Syntrophospora bryantii*, they are phenotypically distinct, with a different mode of growth.

Cluster IX comprises a heterogeneous collection of spore-forming and non-sporeforming organisms, many of which are Gram-negative (Collins *et al.*, 1994). Each of the organisms within this cluster are phenotypically and phylogenetically distinct from the haloalkaliphilic isolates. *Selenomonas* sp. are anaerobic, Gram-negative rods which are generally obligately saccharolytic (Hespell *et al.*, 1992). The genus *Sporomusa* consists of anaerobic, Gram-negative, spore-forming rods which ferment a range of
Results and Discussion: Haloalkaliphilic Bacteria

Table 6.5 BestFit analysis showing the percentage sequence similarity between the present isolates and their closest relative, *C. thermoaceticum*.

<table>
<thead>
<tr>
<th></th>
<th>O-M12SP-2</th>
<th>G-M14CH-4</th>
<th>G-M16NWC-4</th>
<th><em>C. thermoaceticum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>O-M12SP-2</td>
<td>-</td>
<td>94.3</td>
<td>98.1</td>
<td>86.3</td>
</tr>
<tr>
<td>G-M14CH-4</td>
<td>94.3</td>
<td>-</td>
<td>94.0</td>
<td>87.4</td>
</tr>
<tr>
<td>G-M16NWC-4</td>
<td>98.1</td>
<td>94.0</td>
<td>-</td>
<td>85.6</td>
</tr>
<tr>
<td><em>C. thermoaceticum</em></td>
<td>86.3</td>
<td>87.4</td>
<td>85.6</td>
<td>-</td>
</tr>
</tbody>
</table>
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sugars. Pectinatus sp. together with Megasphaera sp. are strict anaerobes which contaminate unpasteurised, pre-packaged beer products. Due to recent changes in beer technology leading to a reduction in oxygen levels, the occurrence of these organisms has increased dramatically over the past few years. A notable feature of Pectinatus sp. is acid-tolerant growth down to pH 4.0 (Haikara et al., 1992).

The evidence for a new genus is compelling. The salt and pH tolerance of O-M12SP-2, G-M14CH-4 and G-M16NWC-4 separate them from genotypically related genera. The only phenotypically related organism, Natroniella acetigena, is phylogenetically distinct from the present organisms. Within the new genus, the evidence for three new species is also strong. Although O-M12SP-2 and G-M16NWC-4 are more closely related than G-M14CH-4, phenotypic data show the two organisms have distinct modes of growth. O-M12SP-2 is limited mainly to carbohydrate fermentations, whereas G-M16NWC-4 has the ability to utilise a number of amino acids addition to a range of carbohydrates.

It is of particular interest that the present isolates show greatest similarity to C. thermoaceticum and C. thermoautotrophicum, which are both homoacetogenic micro-organisms. This is significant because the only other alkaliphilic, obligate anaerobe isolates to date is Natroniella acetigena (Zhilina et al., 1996b). This organism was isolated from the sediments of Lake Magadi, and is a homoacetogenic haloalkaliphile. It is curious that the present isolates appear most closely related to these homoacetogenic organisms, and that the only other described isolate from this location is also homoacetogenic. The phylogenetic data show that the present isolates are unrelated to Natroniella acetigena, and gas chromatography analysis indicates they are not homoacetogenic.
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This link between the Lake Magadi organisms appears to be more than coincidence. As stated previously, many of the organisms isolated from alkaline environments produce acetate as an end product. However, to date utilisation of this substrate seems limited (Zhilina and Zavarzin, 1994; Wenk and Bachofen, 1995). It may be possible that, in vivo, the present haloalkaliphiles also produce acetate as their major end product. The question remains: which organisms utilise the acetate produced by the secondary anaerobes? Further work on haloalkaliphilic methanogens and sulphate-reducers is required to obtain an overall picture of the cycling within these extreme environments.
Conclusions

The anaerobic alkaline environment has been shown to house numerous organisms with diverse metabolic properties (Boone et al., 1986; Tindall, 1988; Zhilina and Zavarzin, 1994). The present study has confirmed these findings, and has indicated the presence of a greater range of novel organisms than previously thought. As the number of isolated anaerobic alkaliphiles grows, investigations into the bioenergetics and genetics of these salient micro-organisms are required to understand the processes occurring within these extreme environments.

The present study has indicated the presence of sulphate-reducing bacteria which utilise substrates not previously found (Zhilina and Zavarzin, 1994; Wenk and Bachofen, 1995). The presence of these organisms is not surprising, since they play a key role in the biological sulphur cycle (Widdel and Hansen, 1992). Despite the implication of SRB in the development of alkaline conditions (Abd-el-Malek and Rizk, 1963c), SRB isolates from alkaline environments have not been described.

The evidence for alkaline SRB activity relies solely on enrichment cultures (Wenk and Bachofen, 1995), except for one isolate obtained from Lake Magadi which has not been characterised (Zhilina and Zavarzin, 1994). This lack of data on alkaliphilic sulphate-reducers is perhaps a result of the difficulty in obtaining pure cultures of these organisms. Despite the lack of axenic cultures, an insight into the role of the SRB within alkaline environments can be obtained using the enrichment culture data.

The most pertinent question arising from this, and previous studies, involved the oxidation of acetate. Present data suggest an absence, or at least limited
numbers of SRB capable of acetate oxidation. However, during the
development of the knowledge base concerning neutrophilic SRB, similar
questions arose. Early metabolic data suggested the SRB had a restricted
metabolic potential, capable of incomplete oxidation of only a restricted range
of organic acids and alcohols. However, this conflicted with field data, which
suggested that less than a third of the organic matter within marine sediments
would be degraded if anaerobic acetate oxidation did not occur (Jørgensen
and Fenchel, 1974). Furthermore, Jørgensen (1977, 1982) showed that the
degradation of over half the organic matter within marine sediments was
directly attributable to SRB activity, suggesting previously undetected strains
of sulphate-reducers capable of oxidising lower fatty acids, particularly
acetate, were present. It was not until the isolation of *Desulfotomaculum
acetooxidans* that the complete oxidation of acetate to CO₂ was confirmed
(Widdel and Pfennig, 1977). It is therefore entirely possible that SRB capable
of acetate oxidation are present, and have remained undetected. The
successful enrichment of one Lake Elmenteita enrichment using acetate gives
credence to this hypothesis, despite only limited S^2− production.

The discovery of alkaliphilic SRB enrichments capable of utilising a range of
substrates affords a greater insight into the interactions within these anaerobic
environments. Neutrophilic interactions are already well documented
(Laanbroek and Veldkamp, 1982; Gibson, 1990; Widdel and Hansen, 1992;
Fauque, 1995). It would therefore appear that similar interactions occur
within alkaline environments, involving organotrophic, acetogenic,
methanogenic and sulphate-reducing bacteria (Tindall and Trüper, 1984;
Boone *et al.*, 1986; Jones *et al.*, 1994; Zhilina and Zavarzin, 1994; Wenk and
Bachenhof, 1995; Duckworth *et al.*, 1996; Zhilina *et al.*, 1996). This
information can be used to postulate a complex network of microbial
interactions within the alkaline lake environment (Figure 7.1).
Figure 7.1 Complex interactions thought to take place within the alkaline lake environment.

Conclusions
Although a small number of alkaliphilic anaerobes have been described recently (Li et al., 1993; Li et al., 1994; Zhilina et al., 1996; Zhilina et al., 1996), the present isolates are the first Gram-positive, alkaliphilic, obligate anaerobes to be isolated from naturally occurring alkaline environments. During the isolation procedure, a number of the organisms which were obtained could not be maintained in pure culture. This diversity was also reflected in the viable count numbers obtained for the non-saline lakes. It is obvious there are a great number of organisms present, the growth of which are incompatible with current culturing techniques.

Pure culture isolates were obtained from both the hypersaline and marine / brackish sediment samples. These isolates represent the first members of the *Clostridium* spectrum to be isolated from alkaline lake sediments. The organisms were capable of utilising a range of fermentable carbohydrates, and two interesting isolates (B7FT-A and G-M14CH-4) also utilised a number of amino acids. Interestingly, the alkaliphilic *Clostridium* spp. previously described are only partially related to the present species, suggesting that the alkaline lakes house a distinct population of alkaliphiles, separate from those found within man-made environments. All of the present isolates were found to be related to the *Clostridium* spectrum, although the halophilic isolates are sufficiently distinct to compose a new genus within this spectrum. These obligate anaerobes add to the increasing number of alkaliphilic organisms which have been isolated from soda lakes (Grant et al., 1979; Tindall et al., 1980; Grant et al., 1990; Mwatha, 1991; Mwatha and Grant, 1993; Jones et al., 1994; Duckworth et al., 1996; Dubinin et al., 1996; Zhilina et al., 1996a; Zhilina et al., 1996b).

Although the number of described species is continually growing, there are still vast numbers of organisms which remain undetected due to the limitations of contemporary culturing techniques. Because of the difficulties
in obtaining pure culture isolates, a more direct approach to the analysis of environmental samples is required. The present study utilised denaturing gradient gel electrophoresis for the analysis of mixed PCR products following amplification with selective primers. Although conditions could not be optimised for this procedure within the current timescale, its potential as a powerful diagnostic technique was demonstrated. With only minor developments, this technique would soon become an essential tool for the rapid elucidation of mixed microbial populations.

Prior to the inception of the present study, a number of objectives were established. The main objective was to isolate novel, alkaliphilic, obligate anaerobes and compare these to described isolates. This objective has been accomplished, with the isolation of five new *Clostridium* species and three haloalkaliphilic species comprising a new genus. Furthermore, the presence of sulphate-reducing bacteria capable of utilising a range of substrates has been determined. Finally, the breakdown of complex carbon substrates by organisms producing a wide range of exo-enzymes has been demonstrated, with the unusual finding that enzyme production appeared to take place only under anaerobic conditions. However, a great deal of work still remains to examine the processes taking place within the alkaline lake environment. Only by *in vitro* examination of microbial activity will the nutrient cycling and population composition within the sediments be ascertained.

Although the aims of the current study have been fulfilled, one question still remains unanswered: Why study the microbiology of anaerobic, alkaline environments? There are a number of answers to this question, the most obvious of which is to further the scientific knowledge in the field of alkaline lake ecology. However, it has been stated previously that there is also a large industrial market for alkaline active enzymes (Horikoshi and Akiba, 1982; Horikoshi, 1996). The majority of alkaline stable industrial enzymes are
produced for the detergent industry, and it has been estimated that detergent enzymes now represent over 30% of the world-wide enzyme production (Horikoshi, 1996). The continued isolation of novel alkaliphiles will surely lead to the discovery of increasing numbers of alkaline active enzymes.

It has already been proposed that soda lakes are possible modern-day analogues of Proterozoic microbial communities (Zavarzin, 1993). Therefore these environments may have been the centres of biodiversity origin, perhaps representing the location of the origins of life itself. Although the fossil record suggests cellular life originated some 3.5 Gyr ago, recent molecular data has suggested a divergence date during the Proterozoic, some 2 billion years ago (Gyr) (Doolittle et al., 1996). This 1.5 Gyr discrepancy is the subject of much debate, and the true date has yet to be resolved (Morell, 1996; Mooers and Redfield, 1996).

Calculations of the branching order of the Eubacteria show that the Thermotogales are closest to the root of the ‘tree of life’ (Olsen and Woese, 1993; Van de Peer et al., 1994). This is consistent with the hypothesis that life originated in a environment of high temperatures and low oxygen tension (Pace, 1991). Recently an anaerobic thermophile was isolated from Lake Bogoria, Kenya, using medium poised at an alkaline pH. Phylogenetic analysis placed this organism within the Thermotogales group (Duckworth et al., 1996).

Although we appear to be slowly moving closer to discovering the origins of life on Earth (Mooers and Redfield, 1996), attention is also turning to alternative methods of investigation. It is generally accepted that, at one time, great rivers and oceans flowed over the surface of Mars. At the moment, the planet appears to be in an extended “Ice Age”, with extensive permafrost covering the sterile, carbon-free surface (Kieffer et al., 1992). Although the pH of the Mars surface has not been measured, it has been hypothesised that
alkaline rivers may have existed in the past (Sims and Mills, 1995; Mills and Sims, 1995). Therefore it is possible that subsurface samples, which have not yet been investigated, may house microbial life. With detailed microbiological analysis of martian rock samples planned (http://mgs-jpl.nasa.gov; http://www.jsc.nasa.gov/pao/flash/marslife/index.html), the techniques for the culturing of anaerobic alkaliophiles detailed in the present study may prove useful in martian biological analyses. Microbiological analyses of martian samples may then give further insight into the origins of life.
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1 Appendix I

1.1 Sample Summary

**E1NE**
Elmenteita - Northeast corner. Sample taken about 2m from edge of lake, from a silty mound. No detectable smell of sulphur.

**E2SE1**
Elmenteita - Southeast corner. Core sample taken at waters edge (12 cm deep). Sulphide smell. Silt with stony sediment underneath.

**E3SE2**

**N4HP**
Nakuru - Hippo Point. Mud flats - heavy mud with a soft surface and a sulphide smell.

**N5WL**
Nakuru - West Lagoon. Sediments heavily contaminated with flamingo excrement. Sample taken from a ridge in the sediment (wave action).

**B6HS**
Bogoria - Hot Springs. Sample taken from outlet of hot spring where hot water meets lake water. Thick gelatinous mud with a strong sulphide smell.

**B7FT**
Bogoria - Fig Tree Camp Site. Core sample of sediment - gritty and silty, but not very black. Dark green/black mats on sediment surface.
Appendix I

B8NS1  Bogoria - North Shore. Sample taken from lakeside sediment under a few centimetres of water.

B9NS2  Bogoria - North Shore. Sample taken from thick black gelatinous mud found underneath the soda crust a short distance from the lake. Strong sulphide smell.

C10LB  Crater Lake - Lake Bed. Lake bed sample - stony and gritty with a strong sulphide smell.

C11SB  Crater Lake - Sedge Bed. Core sample taken from within extensive sedge beds. No significant sulphide smell.

M12SP  Magadi - Salt Ponds. Core sample taken - green/black colour, but no sulphide smell.

M13CC  Magadi - Causeway Channel. Core sample of sediment in a channel running parallel with the causeway.

M14CH  Magadi - Causeway Hole. Hole dug beneath the trona crust, and mud scooped out. Green/black in colour, but no sulphide smell.

M15CL  Magadi - Causeway Liquor. Core sample taken from rivulet of black liquor next to the causeway.

M16NWC Magadi - Northwest Causeway. Core sample taken from side of causeway - black mud with an algal mat on its surface.
1 Appendix II

Multiple sequence alignment showing the present isolates together with their closest described relatives. *B. alcalophilus* is also included as an outgroup. Bases used in the computation of the distance matrix are indicated by a ‘*’. The locations of the PCR primers are also indicated. Secondary structure elements are included within the alignment as follows: [ and ] denote the beginning and end of a stem structure; ^ denotes ][ (a new helix starting immediately after a previous one; ( and ) indicate a non-standard base pair; { and } indicate the beginning and end of an internal loop or bulge.

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10  20  30  40  50
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1 E2SE1 B
2 E2SE1 C
3 B7FT A
4 B8NS1 A
5 B8NS1 C
6 B8NS1 C FT
7 C. felsineum
8 C. paradoxum
9 C. thermoalc.
10 N5WL A lac
11 E. limosum
12 OTM2 SP
13 GML4CH 4
14 GML6NW 4
15 C. thermosaceticum
16 B. alcalophilus
17 Hn2
Appendix II

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**P231**

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1. E2SE1 B
2. E2SE1 C
3. B77T A
4. B0NS1 A
5. B0NS1 C
6. B0NS1 C FT
7. C. felsineum
8. C. paradoxum
9. C. thermoalkalilus
10. N5WL A lac
11. E. limosum
12. CM12 SP
13. CM14CH 4
14. CM16NC 4
15. C. thermoaceticum
16. B. alcalophilus
17. Hn2
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**Appendix II**

1. E2SE1 B
2. E2SE1 C
3. B7FT A
4. BBN1 A
5. BBN1 C
6. BBN1 C FT
7. C. felsineum
8. C. paradoxum
9. C. thermoaceticum
10. N5WL A lac
11. E. limosum
12. OM12 SP
13. OM14CH 4
14. OM16WNC 4
15. C. thermoaceticum
16. B. alcalophilus
17. Hn2
1.1 Proposal for five new *Clostridium* species

i. **Description of *Clostridium alcalibutyricum***. *Clostridium alcalibutyricum* (al.ca.li.bu.ty’ri.cum. alcali from the Arabic al the end, quali soda ash; Gr. n. boutyron butter; butyricum related to butter, butyric; M.L. adj. alcalibutyricum producing butyric acid in alkaline media). Cells are curved or slightly spiralled long, thin rods, that are ca. 10 by 0.4 μm, are non-motile and occur singly, but not in pairs or chains. Gram positive, but standard Gram reaction variable. Spores are not formed under normal culture conditions. Colonies (2 - 3 mm diameter) are round with entire edges, domed, opaque and light brown. Growth on solid media is extremely mucoid. Non-halophilic. NaCl optimum for growth, ca. 2%; range of NaCl concentrations for growth, 0 - 4%. Alkaliphilic. pH range for growth, 7.5 - 10.5; optimum pH > 9.5. Colicin resistant. Vancomycin sensitive.

Strictly anaerobic. Chemoorganotrophic. Ferments glucose, fructose, mannose, arabinose, cellibiose, gentibiose, melibiose, lactulose, piscose, turanose, rhamnose, glucose-6-phosphate, galactouronic acid, succinic acid, mono-methyl-succinate, glucuronic acid, glucuronamide, N-acetyl-D-galactosamine and tween 80. End products of glucose fermentation are acetate and butyrate in roughly equal quantities. Small amount of propionate and iso-valerate are also produced.
Appendix III

Strain E2SE1-C was isolated from pool close to the lake at the south-east shore of Lake Elmenteita, Kenya.

ii. Description of *Clostridium aminovorans*. *Clostridium aminovorans* (am.i.no'vo.rans. M.L. n. *aminum* amine; L. adj. *vorans* devouring, digesting; M.L. adj. *aminovorans* amino acid digesting). Cells are rods with rounded ends, that are *ca.* 4 by 0.5 μm, are non-motile and occur singly, but more often in pairs. Chains rarely observed. Gram positive, but standard Gram reaction variable. Spores are formed in older culture. Colonies (6 mm diameter) are round with entire edges, low-convex, opaque and cream / white. Non-halophilic. NaCl optimum for growth, *ca.* 4%; range of NaCl concentrations for growth, 0 - 12%. Alkaliphilic. pH range for growth, 8.5 - 10.5; optimum pH > 9.5. Colicin resistant. Vancomycin sensitive.

Strictly anaerobic. Chemoorganotrophic. Ferments a range of amino acids and carbohydrates, including glucose, arabinose, lactulose, piscose, turanose, rhamnose, glucose-6-phosphate, galactouronic acid, aspartic acid, urocanic acid, hydroxy phenylacetic acid, serine, histidine, threonine, thymidine, uridine, phenylalanine, glucuronamide, alaninamide, N-acetyl-D-galactosamine and glycerol. Main end products of glucose fermentation are propionate and acetate. *iso*-butyrate and *iso*-valerate are produced in lesser quantities.

Strain B7FT-A was isolated from a core sample taken near 'Fig-Tree Camp' at the south shore of Lake Bogoria, Kenya.

iii. Description of *Clostridium alcaliphilum*. *Clostridium alcaliphilum* (al.ca.li.phil'um. M.L. *alcali* from the Arabic *al* the end, *quali* soda ash; Gr. adj. *philus* loving; M.L. adj. *alcaliphilum* liking alkaline media). Cells are rods with rounded ends, that are *ca.* 3 by 0.4 μm, are non-
motile and occur singly, but not in pairs or chains. Gram positive, but standard Gram reaction variable. Spores are formed in older cultures. Colonies (4 mm diameter) are round with entire edges, domed, opaque and milky white. Growth on solid media is mucilaginous. Non-halophilic. NaCl optimum for growth, ca. 4%; range of NaCl concentrations for growth, 0 - 8%. Alkaliphilic. pH range for growth, 8.5 - 10.5; optimum pH > 9.5. Colicin resistant. Vancomycin sensitive.

Strictly anaerobic. Chemoorganotrophic. Ferments glucose, fructose, mannose, arabinose, cellibiose, melibiose, lactulose, piscose, turanose, glucose-6-phosphate, urocanic acid, glucuronic acid, histidine, glucuronamide and N-acetyl-D-galactosamine. End products of glucose fermentation are propionate and acetate in roughly equal quantities. With lesser amounts of iso-valerate, and small quantities of iso-butyrate also produced.

Strain B8NS1-A was isolated from the north shore of Lake Bogoria, Kenya.

iv. Description of *Clostridium bogorii*. *Clostridium bogorii* (bo.go'ri.i. M.L. gen. n. bogorii of Bogoria; named for Lake Bogoria, a soda lake in Kenya). Cells are rods with rounded ends, some curved, that are ca. 2.5 - 3.0 by 0.4 μm, are non-motile and occur singly or in pairs, but not in chains. Gram positive, but standard Gram reaction variable. Spores are not formed under normal culture conditions. Colonies (8 - 10 mm diameter) are round with entire edges, flat, opaque and white. Growth on solid media appears ‘watery’. Non-halophilic. NaCl optimum for growth, ca. 4 range of NaCl concentrations for growth, 0 - 12 Alkaliphilic. pH range for growth, 8.5 - 10.5; optimum pH > 9.5. Colicin resistant. Vancomycin sensitive.
Strictly anaerobic. Chemoorganotrophic. Ferments a range of substrates, including glucose, fructose, maltose, mannose, arabinose, cellibiose, gentibiose, melibiose, lactulose, piscose, turanose, rhamnose, glucose-6-phosphate, galactouronic acid, glucuronic acid and glucuronamide. Main end product of glucose fermentation is acetate, with propionate and iso-valerate produced in lesser quantities.

Strain B8NS1-C was isolated from the north shore of Lake Bogoria, Kenya.

v. **Description of Clostridium elmenteitii.** *Clostridium elmenteitii* (el.men.tei’ti.i. M.L. gen. n. elmenteitii of Elmenteita; named for Lake Elmenteita, a soda lake in Kenya). Cells are straight rods with rounded ends, that are ca. 1 by 0.5 μm, are motile and occur singly or in pairs, but never in chains. Gram positive, but standard Gram reaction variable. Spores are not formed under normal culture conditions. Colonies (< 1 mm diameter) are round with entire edges, flat, opaque and white. Limited growth on solid media. Non-halophilic. NaCl optimum for growth, ca. 4%; range of NaCl concentrations for growth, 0 - 8%. Alkaliphilic. pH range for growth, 9.5 - 10.5; optimum pH > 9.5. Colicin resistant. Vancomycin sensitive.

Strictly anaerobic. Chemoorganotrophic. Ferments glucose to produce mainly acetate, with propionate produced in lesser quantities.

Strain E2SE1-B was isolated from the south-east shore of Lake Elmenteita, Kenya.
1.2 Proposal for new alkaliphile genus

Description of *Natronoanaerobium* gen. nov. *Natronoanaerobium* gen. nov. (Na.tro.no.an.a.e.ro’bi.um. Gr. n. *natrun* soda, salt; Gr. pref. *an* not; Gr. n. *aër* air; Gr. n. *bius* life; M.L. gen. n. *Natronoanaerobium* soda anaerobe). Gram-positive, haloanaerobic alkaliphiles. Spores not formed under normal culturing conditions. Ferment a range of carbohydrates and/or amino acids, producing *iso*-valerate as the major end-product of glucose fermentation. Most closely related to cluster VI of the *Clostridium* spectrum.

i. Description of *Natronoanaerobium salstagnum*.

*Natronoanaerobium salstagnum* (sal.stag’num. L. gen. n. *sal* salt; L. neut. n. *stagnum* standing water, pond; M.L. neut. n. *salstagnum* salt pond, from a salt evaporation pond). Cells are rods, some curved, that are *ca.* 8 by 0.6 µm, are non-motile and occur singly, but not in pairs or in chains. Gram positive, but standard Gram reaction variable. Spores are not formed under normal culture conditions. Colonies (<2 - 3 mm diameter) are round with entire edges, low dome, opaque and pink/white. Limited growth on solid media during short incubation times. Moderately halophilic. NaCl optimum for growth, *ca.* 20%; range of NaCl concentrations for growth, 16 - 24%. Alkaliphilic. pH range for growth, 9.5 - 10.5; optimum pH > 9.5. Colicin resistant. Vancomycin sensitive.

Strictly anaerobic. Chemoorganotrophic. Ferments glucose, fructose, galactose, maltose, mannose, arabinose, cellibiose, gentibiose, melibiose, lactulose, piscose, turanose, rhamnose, glucose-6-phosphate, glucuronic acid and glucuronamide. Main end-product of fermentation is *iso*-valerate, with acetate and *iso*-butyrate produced in lesser quantities.
Strain O-M12SP-2 was isolated from a primary evaporation pond at Lake Magadi, Kenya.

ii. Description of *Natronoanaerobium halophilum*.  
*Natronoanaerobium halophilum* (ha.lo.ph.i’um. Gr. n. halo, halos the sea, salt; Gr. adj. philus loving; M.L. adj. halophilum salt-loving). Cells are very long, distended rods that are curved. Cells are ca. > 10 by 0.6 μm, which show limited motility and occur singly, but not in pairs or chains. Gram positive, but standard Gram reaction variable. Spores are not formed under normal culture conditions. Colonies (3 - 5 mm diameter) are round with entire edges, umbonate, opaque and beige / white. Limited growth on solid media during short incubation times. Moderately halophilic. NaCl optimum for growth, ca. 20%; range of NaCl concentrations for growth, 12 - 26%. Alkaliphilic. pH range for growth, 9.5 - 10.5; optimum pH > 9.5. Colicin resistant. Vancomycin sensitive.

Strictly anaerobic. Chemoorganotrophic. Ferments glucose, fructose, galactose, maltose, mannose, arabinose, cellibiose, gentibiose, melibiose, lactulose, piscose, turanose, rhamnose, glucose-6-phosphate, glucuronic acid and glucuronamide. Main end-product of fermentation is *iso*-valerate, with acetate and *iso*-butyrate produced in lesser, but roughly equal quantities. Small amounts of propionate are also produced.

Strain G-M14CH-4 was isolated from a hole in the trona crust adjacent to the main causeway at Lake Magadi, Kenya.

iii. Description of *Natronoanaerobium aggerbacterium*.  
*Natronoanaerobium aggerbacterium* (ag.er.bac.te’ri.um. L. n. agger causeway; Gr. dim. n. bakterion a small rod; M.L. neut. n.
aggerbacterium causeway bacterium). Cells are short rods with rounded ends, that are ca. 4 by 0.8 μm, are non-motile and occur singly, but not in pairs or chains. Gram positive, but standard Gram reaction variable. Spores are not formed under normal culture conditions. Colonies (3 - 4 mm diameter) are round with entire edges, low-convex, opaque and pink / white. Limited growth on solid media during short incubation times. Moderately halophilic. NaCl optimum for growth, ca. 20%; range of NaCl concentrations for growth, 12 - 26%. Alkaliphilic. pH range for growth, 8.5 - 10.5; optimum pH > 9.5. Colicin resistant. Vancomycin sensitive.

Strictly anaerobic. Chemoorganotrophic. Ferments a range of carbohydrates and amino acids, including glucose, fructose, galactose, maltose, mannose, arabinose, cellubiose, gentibiose, melibiose, lactulose, piscose, turanose, rhamnose, glucose-6-phosphate, lactose, urocanic acid, glucuronic acid, hydroxy phenylacetic acid, serine, alanine, leucine, phenylalanine, glucuronamide and alaninamide. Main end-products of fermentation are iso-valerate and acetate, with iso-butyrate produced in lesser quantities, and small amounts of propionate produced.

Strain G-M16NWC-4 was isolated from the north-west causeway at Lake Magadi, Kenya.