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ROMANO MWIRICHIA KACHIURU

DOCTOR OF PHILOSOPHY
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Romano Mwirichia Kachiuru

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

ROMANO MWIRICHIA KACHIURU

Signature …… Date……...

This thesis has been submitted for examination with our approval as university supervisors

Signature……. Date……

Prof. Hamadi Iddi Boga,
JKUAT, Kenya

Signature……. Date……

Prof. Anne Wangari Muigai
JKUAT, Kenya

Signature ..... Date 07.05.2009

Prof. Dr. Erko Stackebrandt,
DSMZ, Germany

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LIST OF ABBREVIATIONS

ATPase	Adenosine Triphosphatase
BLAST	Basic Local Alignment Search Tool
Ca^{2+}	Calcium ion
CFU	Colony-Forming Units
Cl^-	Chlorine ion
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate ion
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen u.Zellkulturen
EDTA	Ethylenediaminetetraacetic acid
G+C	Guanine + Cytosine
HCO_3^-	Bicarbonate ion
H_2O	Water
H_2O_2	Hydrogen Peroxide
HPLC	High Performance Liquid Chromatography
KOH	Potassium Hydroxide
Mg^{2+}	Magnesium ion
Na_2CO_3	Sodium Carbonate
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic acid
SDS	Sodium Dodecyl Sulphate
SSU rRNA	Small Subunit Ribosomal Ribonucleic acid
tRNA	Transfer Ribonucleic acid
TLC	Thin layer Chromatography
TCA	Tri Carboxylic Acid
UV	Ultra Violet

ABSTRACT

The major goal of microbial ecology is to understand microbial diversity in natural habitats their interaction with one another and with their habitat. The soda lakes are highly productive environments and the soda lakes of the East African Rift valley have been shown to support a dense and diverse population of aerobic, organotrophic, halophilic, alkaliphilic and alkalitolerant representatives of major bacterial and archaeal phyla.

The isolation and characterization of organisms belonging to widespread but previously uncultivated groups of organisms can provide insights into the roles and functions of these organisms in their natural settings and assist in the formulation of hypotheses about metabolic interactions between microorganisms and their natural environment. Several studies have been carried out to document the microbial diversity of the Kenyan soda lakes by other researchers. However no comprehensive study has been done in Lake Elmenteita. The aim of this study was to assess the microbial diversity of Lake Elmenteita using both culture independent and culture dependent techniques. The application of both techniques was expected to provide new insights into the microbial diversity of the Lake as well as possible roles played by each group within the soda lake environment.

Application of molecular tools to study microbial ecology has widened our approximation of diversity in the environments. Clone Libraries were constructed from PCR amplicons from total environmental DNA. Primers specific for Bacteria and Archaea respectively were used. Partial sequences were generated for both the

clones and the isolates. The relatedness of the Lake Elmenteita bacterial rRNA sequences to known rRNA gene sequences was determined by BLAST analysis and by alignment to the sequences on the ARB database (Release, 1994).

Clones possessed a higher similarity to other environmental clones than to cultured microorganisms. A total of 655 clone sequences were sequenced. Of these 525 (80.15%) sequences were related to uncultured members of the Domain Bacteria. This indicates that a large proportion of deep phylogenetic groups are represented in the clone libraries. Sixteen percent of the clones had similarity values below 90% to both cultured and uncultured microorganisms. Forty three percent of the clones had similarity values between 90-95% as compared to 34.35% that had values between 96-98%. Only a mere 6.87% had values between 99-100%. However a number of factors including relatively low cell numbers of large organisms and a variable number of rRNA operons among organisms, as well as extraction and PCR bias, may lead to under-representation of phylotypes relative to their *in situ* abundance.

Cultured isolates are still very important in developing our understanding of bacterial physiology, genetics, and ecology. Isolation was done using both nutrient rich and nutrient poor media. A polyphasic approach was employed in the identification of the various strains. The majority of the isolates (36.75%) belonged to the genus *Halomonas* while 31.35% belonged to the Genus *Bacillus*. More than half of the isolates (59.45%) belonged to the *Gammaproteobacteria*. An overlap between the clone library and the isolates was observed in the Order *Bacillales* and

the *Actinobacteria* only. In this study novel isolates related to *Marinospirillum, Idiomarina, Streptomyces, Nocardia, Marinilactibacillus, Amphibacillus* and *Vibrio* were recovered. A polyphasic approach to characterization showed they represented novel taxa.

The study showed that the application of both culture dependent and culture independent methods gives a better picture of diversity in the environment. It can be concluded the soda lakes harbour novel uncultured groups of microorganisms and most of them are of biotechnological potential. Future work should focus on Archaeal diversity as well as the uncultured groups of bacteria.

CHAPTER ONE

1.0 General Introduction

The major goal of microbial ecology is to understand microbial diversity in natural habitats, their interaction with one another and with their habitat. Therefore, knowledge of both micro-organisms and habitats is essential. It is known that there are extreme environments on earth which were previously thought to prevent the existence of life (Horikoshi, 1991). These extreme environments are home to diverse groups of organisms that are specifically adapted to these particular conditions and are usually referred to as alkaliphiles, halophiles, thermophiles and acidophiles, reflecting the particular type of extreme environment which they inhabit (Horikoshi, 1991). Ecosystems with moderate to high pH referred to as alkaline environments are characterized by high concentrations of sodium carbonate (Na_2CO_3) and are depleted of magnesium ions. The presence of carbonate provides buffering capacity to the lakes (Grant *et al.,* 1990).

1.1 Distribution of soda lakes

In Africa most of these lakes are found within the East African Rift Valley (Table 1). These lakes have been investigated since the early 1930s (Jenkin, 1932; Tindall, 1988; Grant *et al.,* 1990; Jones *et al.,* 1994; Jones *et al.,* 1998). The salinities of these lakes range from approximately 5% total salts (w/v), in the case of more northerly lakes namely Lakes Bogoria, Nakuru, Elmenteita, and Sonachi. The salinity may reach saturation (30% or greater) in the southern lakes namely Lakes Magadi, Little Magadi,

and Natron. It is proposed that there are roughly equal proportions of Sodium carbonate and sodium chloride as the major salts (Grant, 1992). The pH values range from 9 to above 11.5 in the most concentrated lakes (Duckworth *et al.,* 1996).

Table 1.0 Soda Lakes in Africa (Table adapted from Baumgardt, 2003).

Africa	
Libya	Lake Fezzan
Egypt	Wadi Natrun
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, Lake Sonachi
Tanzania	Lake Natron, Lake Embagi, Lake Magadi, Lake Manyara, Lake Balangida, Basotu, Crate lakes, Lake Kusare, Lake Tulusia, El Kekhooito, Momela lakes, Lake Lekandiro, Lake Reshitani, Lake Garya, Lake Ndutu, Lake Ruckwa North
Uganda Lake	Lake Katwe, Lake Mahega, Lake Kikorongo, Lake Nyamunuka, Lake Munyanayange, Lake Murumuli, Lake Nunyampaka
Chad	Lakes Bodu, Rombou, Djikare, Momboio, Yoan

1.2 Formation of soda lakes

The formation of alkalinity in the soda lake environment requires a combination of geographical, topographical and climatic conditions. Such conditions are found in arid and semi-arid zones of tropical or subtropical areas in continental interiors or rain-shadow zones (Grant *et al.,* 1990). Tindall, (1988) proposed that climatic, geological, and topographical factors may be responsible for the formation of Soda Lakes. In this case climatic factors control the amount of water entering the system as rainfall or surface runoff and the amount leaving by evaporation, favouring the formation of a saline lake. Geochemical influences determine which ions enter the system. Topography allows the concentration of the salts in a shallow depression forming a closed drainage leading to formation of bodies of water (Baumgarte, 2003).

A vital condition necessary for the formation of a soda lake is that significant amounts of calcium and magnesium ions must be absent. Under those conditions groundwaters containing bicarbonate are produced where the molar concentrations of bicarbonate /carbonate ions greatly exceed those of calcium and magnesium ion. Through evaporative concentration, such waters rapidly achieve saturation with respect to alkaline earth cations which precipitate as insoluble carbonates, leaving sodium, chlorine and bicarbonate /carbonate as the major ions in solution (Grant *et al.,* 1986; Grant and Horikoshi, 1989; Grant, 1992; Jones *et al.,* 1994). Alkalinity develops due to a shift in the CO_2/ HCO_3^-/CO_3^{2-}equilibrium as: $2HCO_3 \rightarrow CO_2^- + CO_2\uparrow 2H_2O$. Alkalinity evolves concomitant with the precipitation of other ions, especially sodium and chlorine ions leading to the development of alkaline and saline conditions. The

relative salinity of any lake is dependent on the local geologic and climatic conditions. In lakes of lower salinity, the concentration of CO_3^{2-} usually exceeds that of Cl^-, but in brines of higher salinity Cl^- exceeds CO_3^{2-} concentrations (Grant *et al.,* 1986; Grant and Horikoshi, 1989; Grant, 1992; Jones *et al.,* 1994).

It has also been proposed that geochemical factors directly influence the pH balance within the soda lakes. This occurs due to the leaching of the sodium-rich rocks by high CO^{2-}containing groundwater under condition of low Ca^{2+} and Mg^{2+} content (Eugster, 1970, Jones *et al.,* 1977). The presence of sodium carbonate creates a unique stable natural alkaline habitat, although the total salt content and the sodium carbonate fraction in the soda lakes vary over a broad range, depending on the local conditions (Sorokin and Kuenen, 2005). A stable, high-to-extremely high pH (usually around 9.5–10.5) in the water results from the sodium carbonate/bicarbonate system, which can rarely be found in any other natural ecosystems (Dimitry *et al.,* 2005). This could be the reason behind a stable development of obligately alkaliphilic microorganisms growing optimally at pH around 10.

1.3 Biogenesis

Besides evaporative concentration, development of alkalinity involving bacterial sulphate reduction has been proposed by Abd-el-Malek and Rizk (1963) for the lakes of the Wadi-el- Natrun depression in Egypt. Microbial ammonification and sulphate reduction in soils can locally increase the pH even to values above 10, but these are rarely stable (Jones *et al*., 1998). Carbon fixation as a consequence of photosynthetic

activity can displace the carbon dioxide/ bicarbonate/carbonate equilibrium that is the most common pH-buffering mechanism in freshwater systems. Photosynthesis thus tends to increase the environmental pH, counterbalancing the buffering effect of carbon dioxide, which globally leads to neutral or acid pH. This pH increase is particularly remarkable in hypereutrophic systems, as a consequence of a very high primary production. In turn, photosynthesis is favoured under more alkaline conditions, since alkaline systems act as a trap for atmospheric carbon dioxide (Imhoff *et al*., 1979).

1.4 Microbial diversity and productivity

The soda lakes environments are extremely productive because of high ambient temperatures, high light intensities and unlimited supplies of CO_2. Primary production rates of more than 10g cm^2 day have been recorded (Melack and Kilham, 1974). They are also regarded as naturally eutrophic reservoirs hence they feature considerable microbial diversity (Zavarzin *et al.,* 1999). Organic matter in the soda lakes is produced by cyanobacteria and anoxygenic phototrophic purple bacteria (Zavarsin *et al.,* 1999). Daily fluctuations in heating and cooling result in cycles of stratification and mixing. High insolation and adequate supply of nutrients usually support abundant phytoplankton (Vareschi, 1982). Supersaturation of dissolved oxygen in the upper waters during the day often results from the high rates of photosynthesis (Melack and Kilham 1974; Vareschi 1982).

Alkaliphilic communities contain representatives of major trophic groups. The cyanobacteria are responsible for high level of primary production and nitrogen fixation (Melack and Kilham, 1974; Dubinini *et al.,* 1995). The optimal growth regions of these alkaliphiles is defined by pH–alkalinity–mineralization coordinates (Zarvasin *et al.,* 1999). Kenyan soda lakes also maintain dense populations of non-phototrophic aerobic organotrophic bacteria that utilize products of photosynthesis as well as products of anaerobic degradation (Baumgarte, 2003). Viable counts of aerobic organotrophs from a range of diluted lakes indicate 10^5 - 10^6 cfu ml^{-1} (Grant *et al.,* 1990). It can be postulated that the alkaliphilic community is composed of diverse groups and all major functional trophic groups are represented. This diversity is responsible for the high productivity and is the driving force for the major biogeochemical cycles.

CHAPTER TWO

2.0 General Literature Review

2.1 Microbial diversity of soda lakes

The soda lakes of the East African Rift Valley have been shown to support a dense and diverse population of aerobic, organotrophic, halophilic, alkaliphilic and alkalitolerant representatives of major bacterial and archaeal phyla (reviewed by Duckworth *et al.,* 1996; Jones *et al.,* 1998; Grant *et al.,* 1999; Zavarzin *et al.,* 1999). Obligately alkaliphilic anaerobes, mainly of the clostridial line of descent, have been isolated (Grant *et al.,* 1999).

2.1.1 Phototrophic primary producers

Photosynthetic primary production appears to play an important role in the soda lake environment and, presumably, supports the rest of the microbial community (Jones *et al.,* 1998; Grant *et al.,* 1999). The less alkaline lakes are usually dominated by dense blooms of cyanobacteria while the hypersaline lakes occasionally support blooms of both cyanobacteria and alkaliphilic anoxygenic phototrophs belonging to the genera *Ectothiorhodospira* and *Halorhodospira* (Jones *et al.,* 1998; Grant *et al.,* 1999; Ballot *et al.,* 2004).

It has been proposed that these blooms may be due to the persistent and almost unicyanobacterial blooms of *Arthrospira fusiformis* (syn. *Spirulina fusiformis* Voronichin) (Jenkin 1929; Rich, 1931; Beadle, 1932; Jenkin 1936; Iltis 1968; Iltis,

1969; Compère, 1974; Beadle, 1981; Melack, 1996). Species of *Arthrospira* have been described from alkaline-saline water bodies as well as freshwater habitats (Vonshak and Tomaselli, 2000). The genus *Arthrospira* is non heterocysteous. Due to the incorrect unification of the genus *Arthrospira* and the genus *Spirulina* by Geitler (1932) the term "*Spirulina*" often is used instead of "*Arthrospira*". The division in two separate genera has been suggested by several authors (Desikachary, 1959; Rippka *et al.,* 1981; Anagnostidis and Komárek, 1988; Tomaselli, 1997). Beside *Arthrospira fusiformis* other filamentous cyanobacteria e.g. *Anabaenopsis arnoldii* Aptekarj and *Anabaenopsis elenkinii* V. Miller have been reported as abundant in alkaline lakes (Iltis, 1969; Vareschi, 1982; Melack, 1988; Kebede and Willén, 1998).

Cyanobacterial biomasses have been estimated to be in the range of 197 mg L^{-1} in Lake Elmenteita as compared to 96 mg L^{-1} in Lake Nakuru and of 347 mg L^{-1} in Lake Simbi (Ballot *et al.,* 2004). *Anabaenopsis arnoldii* is described as a common species in alkaline lakes and is already reported from Lake Nakuru and Lake Elmenteita in studies conducted in the seventies (Iltis, 1969; Vareschi, 1982; Melack, 1988). *Anabaenopsis abijatae* was first described from the Ethiopian alkaline Lake Abijata by Kebede and Willén (1996). *Anabaenopsis abijatae* and *Anabaena* sp. were described from the Kenyan soda lakes for the first time by Ballot *et al.,* (2004).

Several authors have described changes in the composition of the phytoplankton communities of Kenyan alkaline lakes (Tuite, 1981; Vareschi, 1982; Melack; 1988). A recent study done on Lake Elmenteita by Ballot *et al.,* (2004) showed that the

cyanobacterial community changed from one dominated by *A. abijatae* in June 2001 to one dominated by *A. fusiformis* and *A. arnoldii* in August 2001. However, in the sample from September 2002, *A. fusiformis* and *A. abijatae* were not present and only *A. arnoldii* was found. *Synechococcus* and *Synechocystis* species were present in all samples whereas *Spirulina subtilissima*, *Spirulina subsalsa* and *Pseudanabaena* species were present in some of the samples. *Arthrospira*, one of the dominant species in the alkaline lakes is regarded as non-toxic. However, several investigations have indicated a possible toxicity of *Arthrospira* (Gilroy *et al.,* 2000; Iwasa *et al.,* 2002).

2.1.2 Anoxygenic Phototrophic sulphur bacteria

Organic matter is produced also by anoxygenic phototrophic purple bacteria (Zavarzin *et al.,* 1999). Purple sulphur bacteria of the genera *Ectothiorhodospira* and *Halorhodospira* oxidize hydrogen sulphide with intermediate extra-cellular sulphur deposition (Baumgarte, 2003). The most thoroughly studied are alkaliphilic purple bacteria from Lake Wadi-el-Natrun, Egypt (Imhoff *et al.,* 1979) and from the lakes of the Kenyan Rift Valley (Tindall, 1980; 1988). *Thiorhodospira sibirica* and *Thioalkalicoccus limnaeus* which are strictly anaerobic obligately phototrophic purple sulphur were isolated from low saline soda lakes in the steppe of south-east Siberia (Bryantseva *et al.,* 1999a; 2000a). Under anoxic conditions, these bacteria use hydrogen sulphide and elemental sulphur as photosynthetic electron donors. *Rhodobaca bogoriensis* is an alkaliphilic *Alphaproteobacterium* isolated from Lake Bogoria in Kenya. The strain is capable of both phototrophic/chemotrophic growth

and is a representative of purple nonsulfur bacteria described from soda lake environments (Milford *et al.,* 2000). Two alkaliphilic heliobacteria *Heliorestis daurensis* and *H. baculata* that grow photo-heterotrophically (Bryantseva *et al.,* 1999a; 2000b) and an alkaliphilic Bacteriochlorophyll a-containing "aerobic phototroph" *Roseinatronobacter thiooxidans* (Sorokin *et al.,* 2000b) have been isolated from Siberian low-salt soda lakes.

2.1.3 Aerobic alkaliphiles

The soda lakes of the Rift Valley maintain dense populations of non-phototrophic, aerobic organotrophic bacteria that utilize products of photosynthesis as well as products of anaerobic destruction. A number of aerobic chemoorganotrophic, alkaliphilic isolates obtained from several soda lakes in East Africa have been studied in detail (Jones *et al.,* 1994; Duckworth *et al.,* 1996).

The majority of Gram-negatives isolated so far are members of the Gamma subdivision of the Proteobacteria which include many proteolytic organisms related to members of the genus *Halomonas* (Duckworth *et al.,* 2000), lipolytic isolates are affiliated to members of the genera *Pseudomonas sensu strictu* rRNA group1, *Stenotrophomonas* and few strains grouped with typical aquatic bacteria such as *Aeromonas, Vibrio* and *Alteromonas* (Duckworth *et al.,* 1996; Jones *et al.,* 1998). Studies on some lakes of the Kenyan Rift Valley for example Lakes Bogoria, Elmenteita, Nakuru Magadi and Crater Lake Sonachi reveal the presence of a diverse population of aerobic sulphur-oxidising bacteria of the genera *Thioalkalimicrobium*

and *Thioalkalivibrio* (Sorokin *et al.,* 2001). Within the genus *Halomonas* two species are recognized as haloalkaliphilic; *H. desiderata* and *H. magadiensis*. The ability to oxidize thiosulfate to tetrathionate seems widely distributed among the haloalkaliphilic *Halomonas* (Dimitry *et al.,* 2005). Several members of these groups are able to grow and concomitantly oxidize sulphide and polysulfide to elemental sulphur anaerobically under denitrifying conditions.

An alkaliphilic aerobic heterotrophic bacterium *Alkalilimnicola halodurans* was isolated from sediments of Lake Natron and the strain is affiliated to members of the family *Ectothiorhodospiraceae* (Yakimov *et al.,* 2001). A few alkaliphilic methane-oxidising bacteria of the *Methylococcaceae* have been isolated from several moderately saline soda lakes. The methanotroph *Methylobacter alcaliphilus* was isolated from soda lakes in Tuva, Central Asia (Khmelenina *et al.,* 1997) while strains of methanotrophic bacteria isolated from sediments of south-eastern Transbaikal soda lakes were identified as *Methylomicrobium buryantense* (Kaluzhnaya *et al.,* 2001). The most interesting representative of this group might be *Methylomicrobium* sp. AMO1, isolated from a mixed sample of sediments from five Kenyan soda lakes (Sorokin *et al.,* 2000a). This alkaliphilic methanotroph is able to oxidize ammonia to nitrite at pH 10- 10.5 and is also capable of oxidising organic sulphur compounds at high pH (Baumgarte, 2003).

Gram-positive aerobic isolates from both the high G+C and low G+C divisions have been described from the soda lake environments. From the low G+C groups, isolates

described are especially *Bacillus alcalophilus* (rRNA group 6, according to Nielsen *et al.,* 1994; 1995) and *Bacillus clarkia* (rRNA group 7 according to Duckworth *et al.,* 1996). Within the high G+C divisions of the Gram-positive bacteria two alkaliphilic organotrophic bacteria have been isolated from Lake Oloiden, Kenya and are affiliated with members of the genus *Dietzia*, and were named *Dietzia natronolimnaea* (Duckworth *et al.,* 1998). Other Gram-positive high G+C isolates were loosely associated with known species of the genera *Arthrobacter* and *Terrabacter* (Duckworth *et al.,* 1996). Two high G+C bacteria, *Bogoriella caseilytica* (Groth *et al.,* 1997) and *Cellulomonas bogoriensis* (Brian *et al.,* 2005) have so far been described from Lake Bogoria in Kenya.

2.1.4 Anaerobic microorganisms

The microbial sulphur cycle seems to be one of the most active in the soda lakes with anaerobic phototrophic purple sulphur bacteria and sulphate reducing alkaliphiles as the main actors (Imhoff *et al.,* 1979). However, until recently, no attempts had been made to study the aerobic chemolithotrophic bacteria capable of oxidizing reduced inorganic compounds, such as methane, hydrogen, sulphide and ammonia produced by the haloalkaliphilic anaerobes in the soda lakes during degradation of organic compounds, although signs of their activity (Imhoff *et al.,* 1979, Cloern *et al.,* 1983; Joye *et al.,* 1999) and the presence of characteristic genes (Ward *et al.,* 2000, Giri *et al.,* 2004) had been noticed in several soda lakes.

Sulphate reduction is responsible not only for the final steps of organic matter degradation but also for generating alkaline conditions as a result of transformation of sulphate to sulphide (Zavarzin *et al.,* 1999). The first alkaliphilic sulphate reducing bacterium identified as *Desulfonatronovibrio hydrogenovorans* was isolated from mud in a drainage ditch at Lake Magadi (Zhilina *et al.,* 1997b). The study of secondary anaerobes and their biodiversity in soda lakes of Tuva in Central Asia also revealed the presence of *Desulfonatronovibrio hydrogenovorans* (Pikuta *et al.,* 1997) indicating that this organism may play the universal role of hydrogen sink in a sulfidogenic anaerobic alkaliphilic community (Zavarzin *et al.,* 1999). Another alkaliphilic sulphate reducer; *Desulfonatronum lacustre* Z-7951, was obtained from Lake Khadyn (Tuva), an oligotrophic alkaline lake with low mineralization (Pikuta, *et al.,* 1997; 1998).These three species belong to the class *Deltaproteobacteria*.

Several groups are involved in the anaerobic degradation pathway, *Natroniella*, *Tindallia*, and *Natronoincola,* which participate primarily in acetogenesis while the hydrogen sink in the community is provided by alkaliphilic hydrogenotrophic sulphate reducers *Desulfonatronovibrio* and *Desulfonatronum* (Zarvasin *et al.,* 1999). The anaerobic oxidation depends on groups such as the phototrophic members of the family *Ectothiorhodospiraceae* e.g. the genus *Thiorhodospira* and *Heliospira.* Other groups responsible for anaerobic oxidation comprises the H_2-utilizing and sulphur-oxidizing bacterium *Natronohydrogenobacter thiooxidans*, heterotrophic and obligately autotrophic sulphur-oxidizing bacteria, methane-oxidizing *Methylobacter*

alcaliphilus and alkaliphilic *Methylomicrobium* sp. able to oxidize methane and ammonia (Zarvasin *et al.,* 1999).

Hydrogen-utilizing, extremely alkaliphilic strains of methanogens were isolated from Lake Wadi-el-Natrun, Egypt (Boone *et al.,* 1986) and were also detected in slurry samples from Lake Magadi (Zhilina and Zavarzin, 1994) and in mixed samples from Tuva lakes (Zavarzin *et al.,* 1999). Hydrogen acetogenesis may provide an available hydrogen sink. A representative of the homoacetogenic bacteria (strain Z-7937) was isolated from Lake Magadi samples (Zhilina & Zavarzin, 1994). Another hydrogen sink can be provided by nitrate reduction. An anaerobic culture of a hydrogenotrophic denitrifier, morphologically similar to *Paracoccus*, was isolated from Lake Magadi, Kenya (Zavarzin *et al.,* 1999).

A different pathway of anaerobic degradation of organic matter involves organotrophic acetogenesis. The first obligately haloalkaliphilic acetogenic bacterium *Natroniella acetigena* was isolated from the bottom mud of Lake Magadi, Kenya (Zhilina *et al.,* 1996a). *Halonatronum saccharophilum*, a moderately haloalkaliphilic chemoorganotrophic representative of the order *Halanaerobiales* was isolated from the coastal lagoon mud of Lake Magadi (Zhilina *et al.,* 2001a).

Organisms fermenting amino acids, called acetogenic ammonifiers have been isolated from soda lakes. Two strains, *Natronincola histidinovorans* (Zhilina *et al.,* 1998) and the non-spore-forming bacterium *Tindallia magadiensis* (Kevbrin *et al.,* 1998). A

number of alkaliphilic saccharolytic clostridia strains have been isolated from Lakes Elmenteita, Bogoria and Magadi (Jones *et al.,* 1998). The isolates from Lakes Elmenteita and Bogoria were found to be associated with members of group XI of *Clostridia*, while the haloalkaliphilic strains isolated from Lake Magadi were related with members of the genus *Moorella* (Group VI of Clostridia). Saccharolytic anaerobes have been isolated from the slightly mineralized Lake Nizhee Beloe in the south-eastern Transbaikal region (Tourova *et al.,* 1999) and from Lake Magadi (Zhilina *et al*., 2001b).

Facultative anaerobes and related to members of the genus *Amphibacillus* or obligately anaerobic strains clustering within the order *Halanaerobiales* have been detected in the Soda lakes. In an anaerobic community saccharolytic spirochetes utilize sugars and a limited range of polysaccharides to produce acetate, lactate, ethanol and Hydrogen (Zavarzin *et al.,* 1999). Two haloalkaliphilic strains *Spirochaeta alkalica* and *S. africana* have been isolated from Lake Magadi, and an alkaliphilic species *S. asiatica* from Lake Khatyn, Central Asia (Zhilina *et al.,* 1996b).

Chemoorganotrophic populations may hydrolyze different polymers to produce sugars and amino acids. These may be used as substrates for the fermentation of simple compounds by anaerobic fermentors. Fatty acids produced by anaerobes may be consumed by other groups such as the acetogenic bacteria, including *Natroniella acetigena*, *Thermosyntropha lipolytica* (Svetlichnyi *et al.,* 1996), and *Tindallia magadiensis* (Zavarzin *et al.,* 1999). Organic material degraded by anaerobic digestion

produces substrates for methanogens such as *Methanosalus zhilinaeae* isolated from Lake Magadi (Zhilina and Zavarzin, 1994). The methane produced is oxidized by methane-oxidizers, methanotrophs, assigned to the *Methylobacter* genus, although a recently isolated methane oxidizer, AMO1, is most closely related to *Methylmicrobium pelagium* (Sorokin *et al.,* 2000).

The sulphur cycle in these lakes utilizes sulphur and sulphate presumably generated by *Ectothiorhodospira* and *Halorhodospiria* sp. (the link between the carbon and sulphur cycles), and also aerobic sulphur-oxidizers. Sulfuroxidizing bacteria belong to two groups, those similar to the non-lithotrophic *Halomonas deleya* and those assigned to the novel genus *Thioalkalovibrio* (Sorokin *et al.,* 2001). Sulphate-reducing bacteria then complete the cycle; *Desulphonatronovibrio hydrogenovorans* and *Desulphonatrum lacustre* (Zhilina *et al.,* 1997; Pikuta *et al.,* 1998) have been isolated from Siberian soda lakes.

Lithotrophic, nitrite-oxidising bacteria (*Nitrobacter alkalicus*) is a member of *Alphaproteobacteria* isolated from soda lakes located in the Kunkur steppe in Siberia and from Lake Nakuru and Crater Lake in Kenya (Sorokin *et al.,* 1998). These chemolitho-trophic nitrifying bacteria play an important role in biological nitrogen cycling by converting reduced inorganic nitrogen compounds to nitrate. The nitrogen cycle in these lakes involves the production of ammonia by fermentative anaerobes such as *Tindallia magadii* (Kevbrin *et al.,* 1998). Ammonia is utilized by

methanotrophs and nitrifiers, producing nitrate. Nitrate, in turn, is utilized by the chemoorganotrophs, creating a link between the nitrogen and carbon cycles.

2.1.5 Archaea

Halobacteria are the most halophilic organisms known and form the dominant microbial population when hypersaline waters approach saturation, frequently importing a red coloration to the brines because of C50 carotenoids (Rodríguez-Valera *et al.,* 1981). Saline soda lakes support blooms of halobacteria and harbour alkaliphilic representatives of the genera *Natronobacterium* and *Natronococcus, Natronomonas, Natrialba, Natronorubrum* and *Halorubrum*. Functionally, they have a specific trophic position and flourish on the organic matter concentration arising from evaporation of brine and the death of its microbial population (Zavarzin *et al.,* 1999).

Methanotrophic methanogens isolated from several soda lakes were found to be related with members of the *Methanosarcinaceae* within the Euryarchaeota. The first haloalkaliphilic strains of methanogens were isolated from Lake Wadi-el-Natrun, Egypt (Boone *et al.,* 1986). One of these isolates, the methylotrophic strain WeN5, was later described as *Methanohalophilus zhilinae* (Mathrani *et al.,* 1988). Another strain of this species Z-7936 was later isolated from Lake Magadi (Zhilina and Zavarzin, 1994; Kevbrin *et al.,* 1997). The methylotrophic methanogen *Methanohalophilus oregonense* was isolated from an anoxic aquifer near Alkali Lake,

a hypersaline, alkaline desert lake in south central Oregon in the United States (Liu *et al.,* 1990).

2.2 Molecular microbial ecology

The first molecules to be analyzed for purposes of elucidating the phylogeny of microorganisms using molecular techniques were cytochromes and ferredoxins (Zuckerkandl and Pauling, 1965; Ambler *et al.,* 1979). Carl Woese and co-workers demonstrated the usefulness of small subunit (SSU) ribosomal RNA (rRNA) as a universal phylogenetic marker (Woese *et al.,* 1990). Several other macromolecules have been examined for their potential as molecular markers. These include the beta subunit of ATPase, elongation factor Tu, chaperonin, various ribosomal proteins, RNA polymerases, and tRNAs (Vandamme *et al.,* 1996).

The SSU rRNA is currently the most powerful phylogenetic marker, in terms of information content, depth of taxonomic resolution, and database size and scope (Ludwig *et al.,* 1998; Rosselló-Mora and Amann, 2001). The 16S rRNA approach is widely used a standard technique in microbial taxonomy and as an integrated part of a polyphasic approach for new descriptions of bacterial species or higher taxa (Stackebrandt and Goebel, 1994; Ludwig *et al.,* 1998). The SSU rRNAs have been a choice in phylogeny because they ubiquitous in all life forms, they exhibit functional constancy, change slowly in sequence, and they are experimentally tractable. As the central component of the complex translation apparatus of protein synthesis, rRNAs

are thought among the most refractory of molecules to horizontal gene flow (Woese, 2000). The varying degree of sequence conservation allows the reconstruction of phylogenies.

2.3 Limitations of the 16S rRNA approach

It is important to realize that libraries of PCR-amplified 16S rRNA and 16S rRNA genes may not represent a complete or accurate picture of the bacterial community. Firstly, the species diversity is so great (Torsvik *et al.,* 1990; Curtis *et al.,* 2002; Gans *et al.,* 2005). Even all of the currently published sequences combined would seem to constitute an incomplete census of all of the 16S rRNA genes on earth (Schloss and Handelsman, 2004). In addition, there may be biases in the contributions of the various bacterial groups to libraries. The efficiencies of nucleic acid extraction may be different for different bacteria, the number of copies of 16S rRNA or 16S rRNA gene copy per cell varies, and there may be preferential amplification of some sequence types relative to others by PCR (Embley and Stackebrandt, 1997; von Wintzingerode *et al.,* 1997; Frostegård *et al.,* 1999). Some sequences may arise from contaminating DNA and may not represent bacteria actually present in the sample being studied (Tanner *et al.,* 1998).

The resolving power of the 16S rRNA has been recognized to be insufficient to guarantee correct delineation of bacterial species (Fox *et al.,* 1992; Martinez-Murzia *et al.,* 1992). Assigning physiologies and functions to the hosts of 16S rRNA gene sequences is complicated in many cases by the lack of characterized close relatives

(Dojka *et al.,* 2000; Hugenholtz *et al.,* 2001; Mummey and Stahl, 2003) and by the diversity of phenotypes among close relatives in some groups (Achenbach and Coates, 2000; Saito *et al.,* 1998). Some, but not all, of these biases may be overcome as metagenomic data sets accumulate (Liles *et al.,* 2000; Quaiser *et al.,* 2003; Tringe *et al.,* 2005). Given the low phylogenetic resolving power at the levels of close relatedness (above 97% 16S rRNA sequence similarity), it is highly recommended to support conclusions based on SSU rRNA sequence data analysis by alternative comparative data, such as genomic DNA reassociation studies (Stackebrandt and Goebel,1994).

2.4 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) analysis of 16S rRNA gene segments has been used to profile complex microbial communities (Muyzer *et al.,* 1993) and to infer the phylogenetic affiliation of the community members (Muyzer and de Waal, 1994, Muyzer *et al.,* 1995). In this method, the Polymerase Chain Reaction (PCR) is used to amplify a region of the 16S rRNA gene or a functional gene (Wawer and Muyzer**,** 1995), from mixed microbial populations. The forward PCR primer has a GC-rich sequence (GC clamp) on its 59 end that imparts melting stability to the PCR products in a denaturing gradient gel. The resulting products, essentially all the same size, are separated into discrete bands during electrophoresis through an acrylamide gel that contains an increasing linear gradient of denaturants (Ferris *et al.,* 1996**)**. Individual double-stranded DNA molecules denature along their length

adjacent to the GC clamp according to their melting characteristics (i.e., sequences). This partial denaturation causes their migration to essentially halt at unique positions, forming discrete bands in the gel. DGGE offers a rapid means of detecting predominant populations which are PCR amplifiable (Ferris *et al.,* 1996**)**. However, as with all methods used to study microorganisms in nature, DGGE is not without limitations. Limited sequence data do not permit a robust evaluation of phylogenetic relationships of the sequence types detected (Ward *et al.,* 1992).

2.5 Justification

Isolation and characterization of organisms belonging to widespread but previously uncultivated groups of organisms can provide insights into the roles and functions of these organisms in their natural settings. This could also assist in the formulation of hypotheses about metabolic interactions between microorganisms in a natural environment (Zinder and Salyers, 2001). Lake Elmenteita was selected in this study due to its ecological importance. Several studies have been carried out to document the microbial diversity of the Kenyan soda lakes with the best studied being Lake Magadi. There are no comprehensive microbial diversity studies done on the Lake Elmenteita. Lake Elmenteita also serves as refuge for flamingos when the climatic conditions in Lake Nakuru are not favourable.

Results from the studies done on the Kenyan soda lakes so far point to an underestimation of the microbial diversity as compared to other well studied lakes such as the Wadi el Natrun in Egypt. The aim of this study was to assess the microbial diversity of Lake Elmenteita using both culture dependent and culture independent techniques. The application the two techniques was expected to provide new insights into the microbial composition of the Lake as well as the roles played by each group within the soda lake environment. The study would also form a basis for future work especially on isolation of strains with a biotechnological potential.

2.6 Objectives

2.6.1 Broad objective.

- To characterize the prokaryotic microbial community in Lake Elmenteita using both culture dependent and culture independent techniques

2.6.2 Specific objectives

- To characterize the diversity of bacteria and archaea from Lake Elmenteita.
- To isolate and characterize phylotypes represented in the clone library
- To understand the possible role of the microorganisms in the Soda Lake Ecosystem

CHAPTER THREE

3.0 General materials and methods.

3.1 Study site

Lake Elmenteita is situated 0°27' S, 36°15' E on the floor of the Kenyan Rift Valley at 1776 m above sea level and has no direct outlet (Figure3.1). The region is characterized by a hot, dry and semi-arid climate with a mean annual rainfall of about 700 mm. Due to the high temperatures; there are very high evaporation rates during the drier seasons leading to a reduction in the total surface area. Both lake Nakuru and Elmenteita are believed to have been one large lake basin with a total surface area of around 800 km^2 (McCall, 1967). The present sizes of Lakes Nakuru and Elmenteita are only about 40 and 20 km^2 respectively, while the depths rarely exceed 1.0 m.

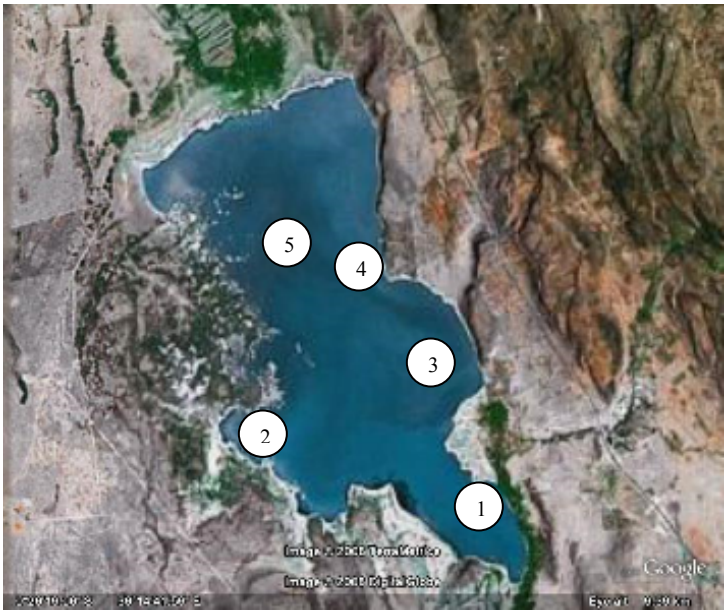

Figure 3.1 Satellite map of Lake Elmenteita (Source…Google maps, www.google.maps.com). The sampling points are indicated as 1, 2, 3, 4 and 5 on the map

3.2 Collection of samples.

Water and wet sediment samples were collected from each sampling point. The Water samples were collected in sterile bottles, capped on site, labelled and preserved in cooled boxes for transportation back to the laboratory. Dry mud and wet sediment samples were collected in sterile tubes, labelled and preserved in dry ice. Back in the laboratory the water samples were filtered through a sandwich of a Nucleopore filters (GF/F; Whatmann) and a glass fibre filter of 0.22µm (GF/F; Whatmann) using a vacuum pump. The filter papers were properly folded in an aluminium foil, labelled and preserved in dry ice. The samples filter papers, dry mud and wet sediment samples were shipped via courier service to DSMZ in Braunschweig, Germany under dry ice for immediate analysis.

3.3 DNA Extraction Protocol

The sediment and filter papers from **3.2** above were thawn from - 80^{o}C and 200 mg from each of the wet and dry sediment sample weighed into separate sterile eppendorf® tubes. The filter sandwich was cut into small pieces with a sterile scalpel and transferred to a sterile two ml eppendorf® tube. DNA extraction protocol was by a modification of the method described by Russel and Sambrook (2002). To each tube was added 500ul of solution A (50 mM Tris pH 8.5, 50 mM EDTA pH 8.0, 25 % Sucrose solution) and mixed by gently inverting several times and centrifuged at 13,000 rpm for one Minute. This was to remove the salts and exopolysaccharides from the sediments samples. The supernatant was discarded and the sample re-suspended in

200 µl of solution A. To it was added 5 µl of Lysozyme (20 mg/ml), 5µl of RNAse A (20 mg/ml) and mixed gently. The samples were allowed to incubate at exactly 37 °C for one hour. To this mixture was added 600 µl of solution B (10 mM Tris pH 8.5, 5 mM EDTA pH 8.0 and 1% SDS) and mixed by inverting several times after which 10 µl of Proteinase K (20mg/ml) was added and mixed gently. The mixture was incubated at 50 °C for 30 minutes.

DNA was extracted by adding equal volumes of Phenol : Chloroform (means if the mixture obtained from the step above was 400 µl then 200 µl of phenol and 200 µl of chloroform were added), mixed by inverting several times and centrifuged for 15 minutes at 13,000 rpm. The aqueous phase was carefully pipetted out and this contained the crude DNA. This extraction step was repeated once. A second extraction was done with an equal volume of Chloroform: Isoamylalcohol (24:1) mixed by inverting several times and centrifuged for 15 minutes at 13,000 rpm. The aqueous phase was carefully pipetted out and transferred to a new tube.

A second round of extraction was done to remove all the phenol. DNA was precipitated overnight at -80°C by addition of an equal volume of Isopropanol and 0.1 volumes 3M NaCl. The DNA samples were then thawed and centrifuged at 4°C for 30 minutes to pellet the DNA. The pellets were washed twice with 70 % Ethanol and air dried on the bench at room temperature for 20 minutes. The pellets were redissolved in 100 µl of TE buffer pre-warmed at 55°C Removal of humic substances from the DNA

was done using the Caesium Chloride method (Smalla *et al.,* 1993). Presence of DNA was checked on 1% Agarose gel. The DNA was aliquoted in 10 µl. Some samples were stored at -20^{o} C and for long storage at -80^{o} C.

3.4 PCR amplification

Almost full-length 16S rDNA bacterial genes were amplified using the primers bac8f (5' AG (A/G) GTTTGATCCTGGCTCAG-3') and bac1492r (5'-CGGCTACCTTGTTACGACTT-3'). These primers for PCR amplification of bacteria were based on the fD1 and rP1 primers described by Weisburg *et al.,* (1991) and modified by Sørensen *et al.,* (2005). A gradient PCR was initially done on an eppendorf Thermocycler with the annealing temperature ranging from 52.1 – 63.5 $^{\circ}$C to determine the optimum annealing temperature. PCR cycling consisted of a three minute initial pre-incubation step at 94°C followed by either 28 cycles of a denaturation step at 93°C for one minute, a one minute annealing step at 58°C, and a one minute elongation step at 72°C and a final extension step at 72°C for five minutes.

The PCR mix consisted of 5 ml of 10 X PCR buffer [100 mM Tris-HCl (pH 9), primers at a concentration of 0.5 mM, each deoxynucleoside triphosphate at a concentration of 200 mM, 2.0 mM $MgCl_2$, 20 ng of bovine serum albumin (BSA), 0.5 µl of template DNA, 2.5 U of Taq DNA polymerase (Roche). The volume was adjusted to a final volume of 50 ml with sterile MQ water. The presence of PCR products and their concentration were determined by analysing 5 ml of product on 2%

agarose gels after staining with Ethidium bromide (3 µl/100 ml) and comparison with a molecular weight marker (Smartladder; Eurogentec).

3.5 Microbial community profiling via DGGE

The DNA extracted from the water samples was amplified for DGGE analysis using the PCR procedure described by Muyzer *et al.,* (1993). The PCR primers were F357GC (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCT ACGGGAGGCAGCAG-3'), which contains a GC-rich `clamp' and is specific for most Bacteria, and R518 (5'-ATTACCGCGGCTGCTGG-3') which is specific for most Bacteria, Archaea and Eukarya. PCR cycling was done on a Biozym Maxicycler PTC-100. Cycling conditions were as follows: After a pre-incubation at 94 °C for five minutes a total of 20 cycles of 94 °C for one minute, 65 °C for one minute and 72°C for one minute. This was followed by five cycles of: 94 °C for one minute, 55°C for one minute, 72°C for one minute and a final incubation at 72°C for 10 min. In the first 20 cycles, annealing temperature was decreased by 0.5°C, stepwise every one cycle, from 65°C in the first cycle to 55°C in the twentieth. This `touch-down' procedure decreases non-specific amplification (Muyzer *et al.,* 1993).

The PCR products were checked on 1% agarose for the correct amplicons size of about 200bp. The PCR products were separated by DGGE on a Bio-Rad Dcode system (Muyzer *et al.,* 1993). Two stock solutions were prepared, representing 0 and 100% denaturing agent, respectively. The 0% solution consisted of 10% (wt/vol)

acrylamide-bisacrylamide (37.5:1) in 0.5X Tris-acetic acid-EDTA buffer (TAE), and the 100% solution consisted of 10% (wt/vol) acrylamide-bisacrylamide, 420 g of urea/litre, and 400 ml of formamide /litre in 0.5X TAE. The DGGE gels had a linear denaturing gradient between 35% and 70%. To each well was loaded 20µl of PCR products, and the gels were run for 18 hours at 70 V and 60°C. The gels were stained on a shaker for 30 min in 250 ml TAE buffer containing 25µl Ethidium Bromide, visualized and photographed on Gel documentation equipment (Kodak EDAS 290, Biometra Transilluminator).

3.6 Clone library construction

The PCR products were purified with QIAquick® spin columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The PCR products were eluted using 50 µl of TE Buffer (pH 8.0). One µl of the cleaned PCR products was ligated onto pGEM-T Easy vector system II (Promega) and transfected through heat shock to *E. coli* JM109 High Efficiency Competent cells (Promega). The cells were plated on LB/Ampicillin/X-Gal/IPTG media and incubated overnight at 37°C. Transformants showing the Lac2 phenotype (white colonies) were picked with sterile toothpicks to LBA (Ausubel, 1995) containing 100 mg of ampicillin per ml. Plasmid DNA was extracted from the clones by resuspending a loopful of cell material in 100 µl of sterile MQ water and heating to 98°C for 10 minutes. The cell lysates were centrifuged at 13,200 rpm for three minutes to sediment the cell debris leaving the DNA in the aqueous phase. 80 µl of the supernatant (containing the clone DNA) was carefully

pipetted to a sterile Eppendorf tube. The presence of correct inserts was determined by performing a PCR using the primers M13F (5'-GTA AAACGACGGCCAG-3') and M13R (5'-AGGAAACAGCTATGAC-3'). These primers flank the cloning site on the vector. The PCR products were checked on 1% agarose gel. Further screening was done via ARDRA to select for a few representative clones for sequencing. A double digestion was done using the restriction enzymes *Eco*NI and *Stu*I for bacteria and *Cvi*II for the Archaeal clones. The restriction enzymes were from New England Biolabs [NEB], (Beverly, Mass.). The Restriction fragments were then separated on 2% agarose gel at 37°C for two hours and 100V. Representative profiles were picked and the PCR amplicons purified with QIAquick® spin columns (Qiagen, Hilden, Germany).

3.7 Phylogenetic analysis

Representative clones were selected for sequencing and partial sequences were generated using the Sequencing primer 518r. These primers target one of the conserved regions. The reads were manually edited and the Sequence data was BLAST (www.ncbi.nlm.nih.gov/**BLAST**/) analyzed against the GenBank 16S rRNA database. The sequences were aligned, and checked for chimeric structures using the CLUSTAL W program (Higgins and Sharp, 1988) and Mallard program (Ashelford *et al.,* 2006).

Phylogenetic relationship of the partial sequences was determined using neighbour-joining (Felsenstein, 1993) and maximum-likelihood analyses (Olsen *et al.,* 1994). The analyses were conducted in MEGA4 (Tamura *et al.,* 2007). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004). The resultant tree topologies were evaluated in bootstrap analyses of the Neighbour joining method based on 1000 re samplings (Felsenstein, 1985).

CHAPTER FOUR

4.0 Diversity of Bacteria in Lake Elmenteita

4.1 Introduction

The soda lakes environments are extremely productive because of high ambient temperatures, high light intensities and unlimited supplies of CO_2. They are also regarded as naturally eutrophic reservoirs hence they feature considerable microbial diversity (Zavarsin *et al.,* 1999). The soda lakes of the East African Rift Valley have been shown to support a dense and diverse population of aerobic, organotrophic, halophilic, alkaliphilic and alkalitolerant representatives of major bacterial and archaeal phyla (Duckworth *et al.,* 1996; Jones *et al.,* 1998; Grant *et al.,* 1999; Zavarzin *et al.,* 1999). Obligately alkaliphilic anaerobes, mainly of the clostridial line of descent, have been isolated (Grant *et al.,* 1999). Several studies have been carried out to document the microbial diversity of the Kenyan soda lakes and the best studied is Lake Magadi. Results from the studies done on the Kenyan soda lakes so far point to an underestimation of the microbial diversity as compared to other well studied lakes such as the Wadi al Natrun in Egypt. There are no comprehensive microbial diversity studies done on Lake Elmenteita. The aim of this study was to assess the microbial diversity of Lake Elmenteita using a culture independent approach.

4.2 Materials and methods

The study site, sample collection, DNA extraction and PCR amplification are described in section 3.1 to 3.4. Microbial community profiling using DGGE and clone library construction was done as described in 3.5 and 3.6. Representative clones were

selected for sequencing and partial sequences were generated using the Sequencing primer 518r. This primer targets one of the conserved regions. The reads were manually edited and the Sequence data was BLAST (www.ncbi.nlm.nih.gov/**BLAST**/) analyzed against the GenBank 16S rRNA database. The sequences were aligned, and checked for chimeric structures by using the using the CLUSTAL W program (Higgins and Sharp, 1988) and Mallard program (Ashelford *et al.,* 2006). The partial sequences were automatically aligned and analysed as described in section 3.5

4.3 Results

In this study, the Phenol: chloroform method gave DNA of high molecular weight that could be amplified even without purification (Figure 4.1).

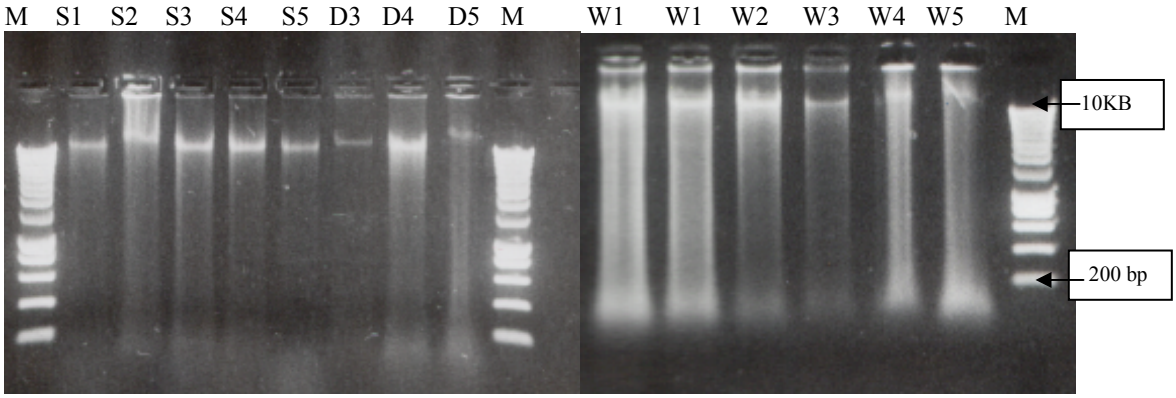

Figure 4.1: DNA yields from the various samples using the Phenol: chloroform extraction method (S- Sediment, D – dry mud and W – water).

The DNA fragment length was estimated by comparison to a molecular weight marker (Smartladder; Eurogentec). The ladder has a molecular weight ranging from 200 bp to 10,000bp and is used for easy quantification as well as size determination of DNA.

Figure 4.2 below shows PCR products from amplification of the DNA extracted without further clean-up. Though the DNA was brownish in colour PCR amplification using 16S rRNA gene universal primers was successful. It was realized that purification of the DNA using columns DNA clean-up columns (GeneClean® Turbo kit, Promega) led to loss of most of the DNA. This may be due to the retention of the Nucleic Acids in the purification columns.

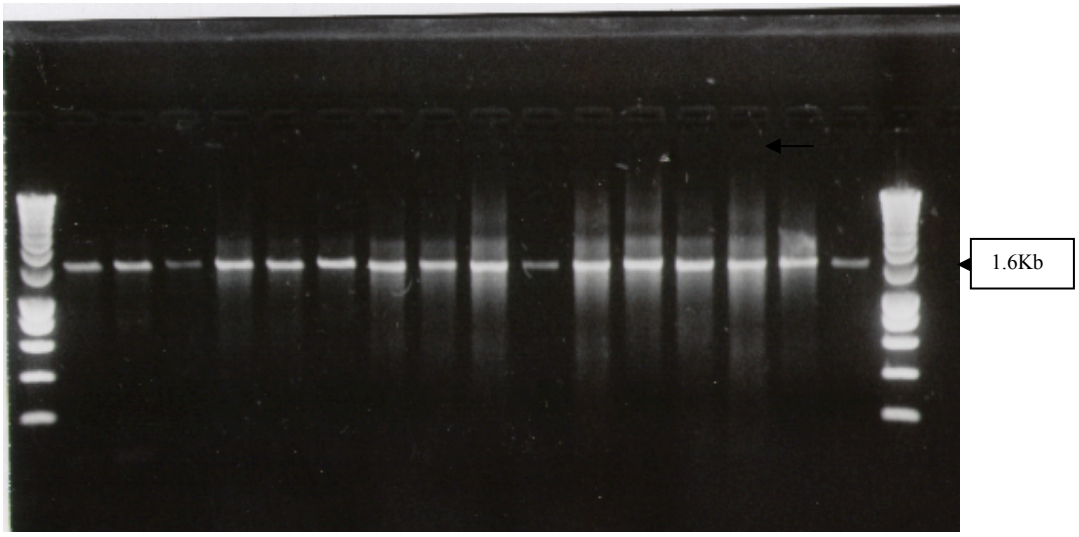

Figure 4.2: Amplified PCR products from Figure 4.1. The arrow indicates a size of 1.6kb

Figure 4.3 below shows the electrophoretic profiles of the different samples on a Denaturing Gradient Gel for bacteria. The profiles show that there is great diversity in all the samples though some groups are common in all the samples. Due to the heterogeneity cutting out some of the bands would definitely capture only the dominant groups so it was preferable to clone the 16S rRNA amplicons directly.

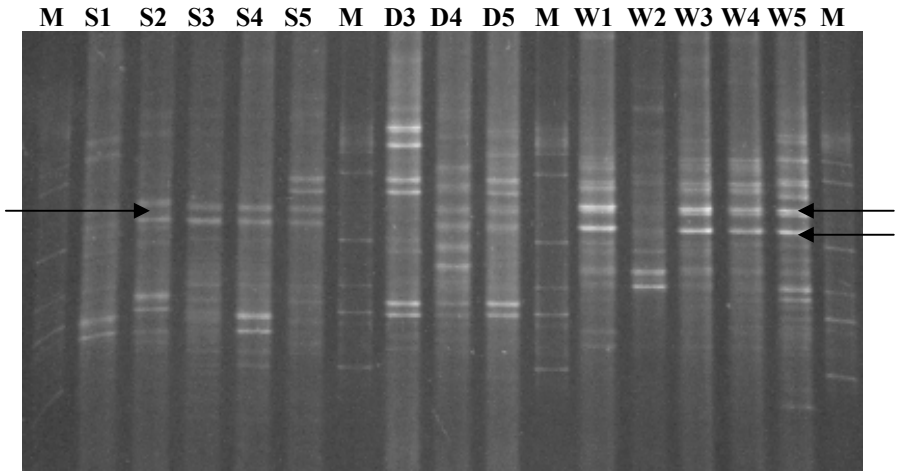

Figure 4.3: DGGE Agarose Gel for Bacteria. The arrows indicate Bands common in several samples. M= marker, S = sediment, D=dry mud and W= Water. The dry mud and water samples have a more diverse profile indicating they may be richer in diversity than sediment samples.

4.3.1 Clone library Results

The clones picked were screened for the right sized inserts through PCR amplification using the M13 primers (Figure 4.4 below). The right insert size expected was approximately 1.6 kb reflecting the size of the 16S rRNA genes.

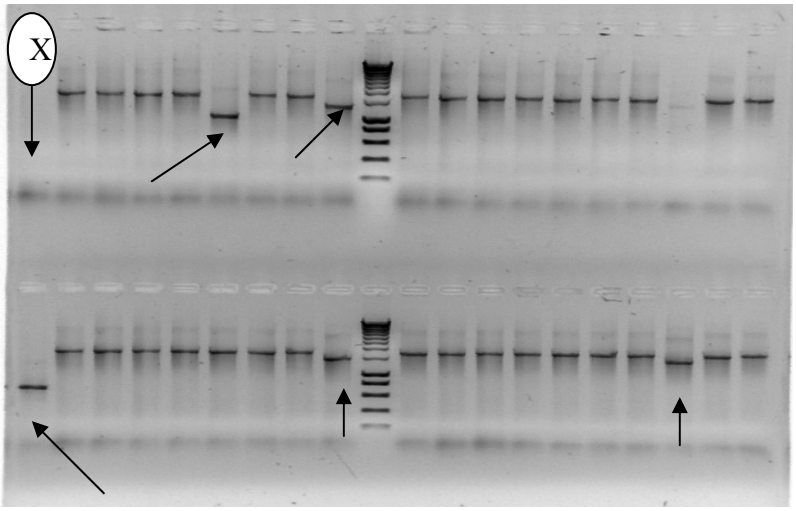

Figure 4.4: Size screening for the right sized insert of 16S rRNA clones. **The arrows indicate plasmids with incorrect insert.** The box marked X shows a plasmid with no insert. All the other bands had the desired Insert of 1.6 kb.

4.3.2 ARDRA screening

Figure 4.5 below show the Restriction Pattern for Bacterial Clones S2- 001 to S2 – 046. The ARDRA patterns indicate that there is very high bacterial diversity.

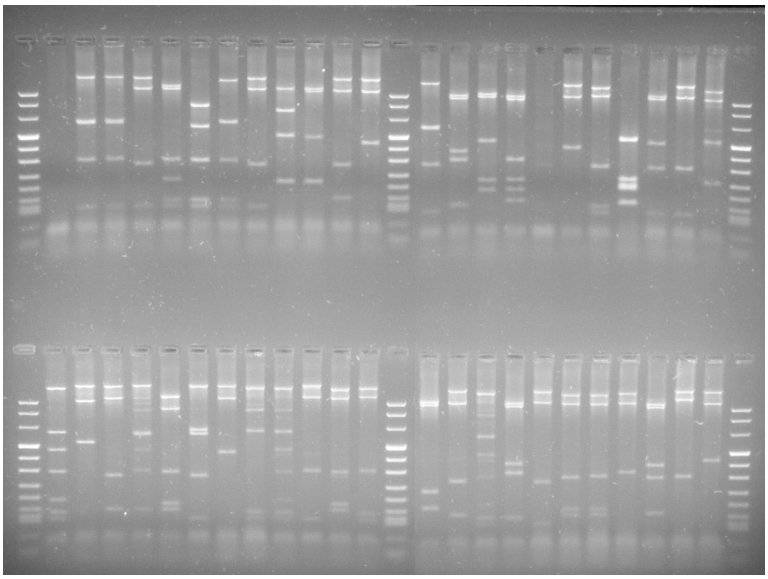

Figure 4.5: ARDRA pattern for selected Bacterial clones S2- 001 – S2 – 046. Due to the large number of gels, only representative ARDRA profiles and those that were unique were selected for sequencing.

4.3.3 Clone Library Analysis

The affiliation of the Lake Elmenteita bacterial rRNA sequences to known rRNA gene sequences was determined by BLAST analysis and by alignment to the sequences on the ARB database (Release, 1994). Figure 4.6 below shows the distribution of the clones in different phylotypes whereas Table 4.1 shows their distribution per sample. The two dominant groups were the Phylum Cyanobacteria and the Phylum Firmicutes. The Firmicutes group was the second in terms of numbers but the most diverse in terms of the Genera represented.

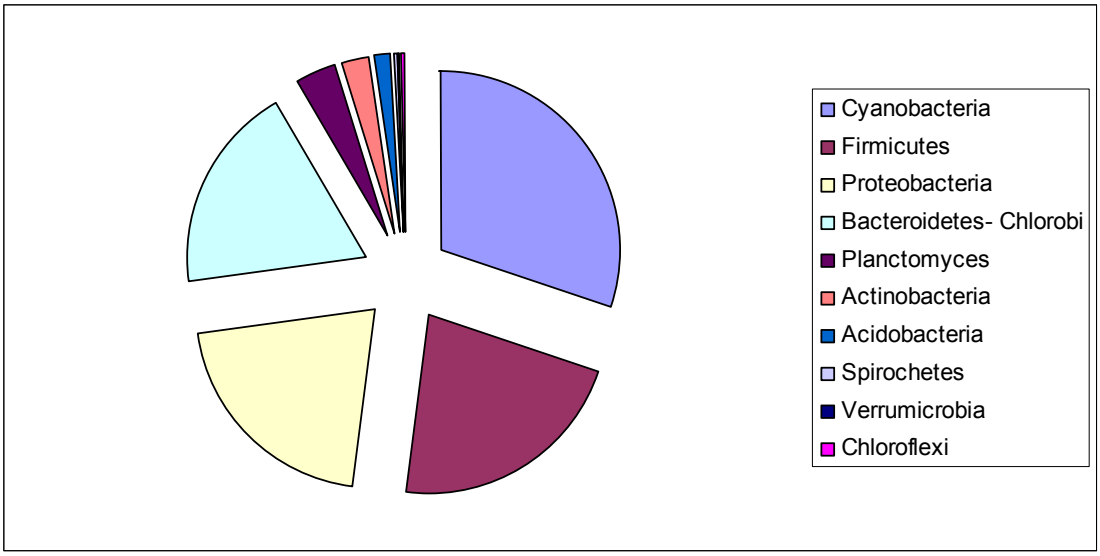

Figure 4.6 Summary of phyla represented in the clone library for Bacteria

Table 4.1: Summary of the clones in the Domain Bacteria

Phyla/Class	Order	Dry mud	Sediment	water	Total
Cyanobacteria	*Chroococcales*	3	9	81	93
Cyanobacteria	*Nostocales*		5	13	18
Cyanobacteria	*Oscillatoriales*	7	21	20	48
Cyanobacteria	*Stigonematales*	1	-	-	1
Firmicutes	*Bacillales*	3	4	-	7
Firmicutes	*Clostridiales*	17	64	4	85
Firmicutes	*Dethiobacter*	1	7	-	8
Firmicutes	*Thermoactinomycete*	-	3	-	3
Firmicutes	*Thermoanaerobacteria*	-	3	-	3
Firmicutes	*Lactobacillales*	15		-	15
Alphaproteobacteria	*Rhizobiales*	1	3	-	4
Alphaproteobacteria	*Rhodobacterales*	4	13	18	35
Alphaproteobacteria	*Spingomonadales*	-	1	4	5
Betaproteobacteria	*Burkholderiales*		-	36	36
Betaproteobacteria	*Methylophillales*	-	-	3	3
Gammaproteobacteria	*Chromatiales*	1	3	4	8
Gammaproteobacteria	*Methylococcales*	-	4	-	4
Gammaproteobacteria	*Thiotrichales*	1	4	2	7
Gammaproteobacteria	*Xanthomonadales*	-	3	-	3
Deltaproteobacteria	*Bdellovibrionales*	1	1	1	3
Deltaproteobacteria	*Desulfovibrionales*	-	2	-	2
Deltaproteobacteria	*Desulfuromonadales*	-	1	-	1
Deltaproteobacteria	*Myxococcales*	-	1	-	1
Bacteroidetes	*Bacteroidales*	1	10	1	12
Bacteroidetes	*Flavobacteriales*	1	10	15	26
Bacteroidetes	*Sphingobacteriales*	10	37	17	64
Chlorobi	*Chlorobiales*	1	18	-	19
Planctomyces	*Planctomycetales*	-	10	11	21
Acidobacteria	*Acidobacateriales*	-	2	-	2
Acidobacteria	*Solibacteriales*	7	-	-	7
Actinobacteria	*Actinomycetales*	1	6	16	23
Chloroflexi	*Chloroflexales*	-	4	-	4
Deinococcus	*Deinococcales*	-	1	-	1
Aquificae	*Aquificales*	1	6	-	7
Candidate Div. BD	*Gemmatimonadetes*	-	-	1	1
Spirochetes	*Spirochete*	1	-	-	1
Fusobacteria	*Verrucomicrobiales*	-	1	-	1

A total of 655 clone sequences were sequenced. Clones possessed a higher similarity to other environmental clones than to cultured microorganisms. Of these 525 (80.15%) sequences were related to uncultured members of the domain bacteria. Sixteen percent of the clones had similarity values below 90% to both cultured and uncultured microorganisms, forty three percent of the clones had similarity values between 90-95% as compared to 34.35% that had similarity values between 96-98% to cultured and uncultured microorganisms. Only a mere 6.87% had similarity values between 99-100% to cultured and uncultured microorganisms.

4.3.4 Predominance of different Groups

4.3.4. 1 Cyanobacteria

The Cyanobacteria is one of the most important groups of microorganisms within the soda lake environment. The table below summarizes the Cyanobacterial clones detected in this study and their phylogenetic affiliation.

Table 4.2 Cyanobacterial groups represented in the clone library

Order	Genus	No. of Clones
Chroococcales	*Cyanobium*	2
	Gloeothece	1
	Microcystis	1
	Synechococcus	86
	Synechocystis	3
Nostocales	*Anabaena*	3
	Nodularia	2
	Nostoc	6
Oscillatoriales	*Calothrix*	7
	Arthrospira	3
	Leptolyngbya	15
	Oscillatoria	6
	Spirulina	19
	Trichodesmium	5
Stigonematales	*Stigonema*	1

Figure 4.7a below shows the phylogenetic relatedness of the representative clones from the phylum Cyanobacteria clones to the closest relatives on ARB database and also BLAST analysis. Clones from this study and their accession are indicated in blue font.

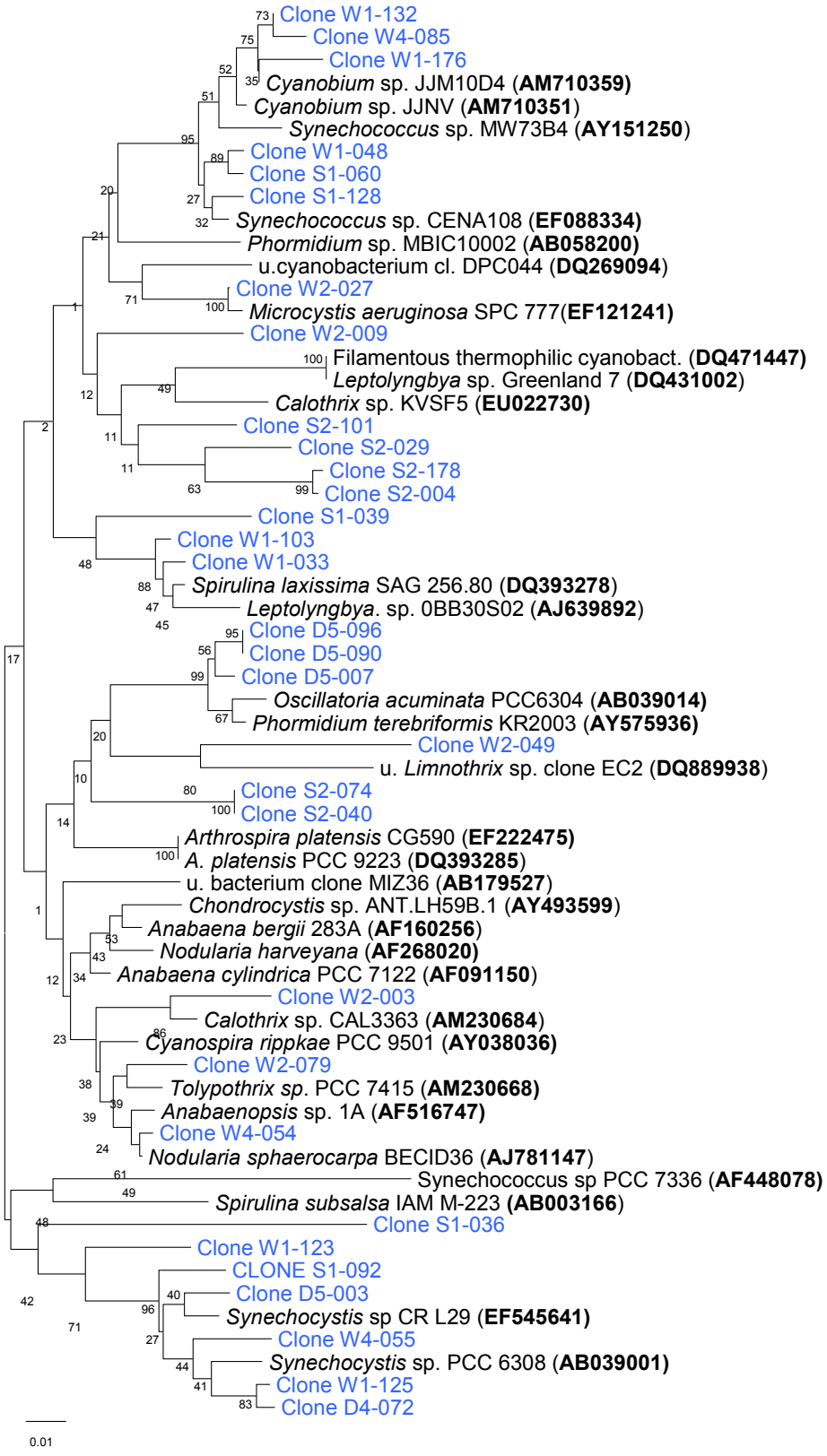

Figure 4.7a Evolutionary relationships of Taxa in Cyanobacteria clones detected in lake Elmenteita.

A total of 160 clones (Table 4.2) from Lake Elmenteita were aligned on ARB database. Two clones (S2-004 and S2-094) were related to a filamentous thermophilic cyanobacterium of the genus *Leptolyngbya* (similarity values of 93 and 94% respectively) from a thermal spring in Jordan. Close to this were 12 clones with similarity values of 91-93% distantly related to a *Leptolyngbya* sp (DQ431002) isolated from microbial mats from Arctic hot springs (Greenland). There were a total of 17 clones related to *Spirulina* species. 15 clones were related (94-98%) to *Spirulina laxissima* strain SAG 256.80 and the remaining two related (99%) to *Spirulina subsalsa* and were both from the hotspring water samples. All the *Spirulina* clones were exclusively from the water.

4.3.4.2 The Phylum Firmicutes

The Phylum consists of three classes; clostridia, bacilli and mollicutes and the members are Gram-positive bacteria with low G+C content. Within the order *Clostridiales*, clones were identified affiliated to uncultured members of *Johnsonella*, *Ruminococcus* and *Acetobacterium*. A clade of 19 clones was not affiliated to any cultured clostridia. Two other clades were formed in the Genera *Tindallia* and *Alkaliphilus* respectively. Clone S2-021 and S2-052 were related to *Clostridium bifermentans* while clone S1-109 and S1-001 were closely related to *Clostridium mesophilum*. Clone S1-031 was closely related to *Alkaliphilus transvaalensis*. Within the *Tindalli*a group, clone D5-086 was closely related to *Clostridium elmenteitii* (Jones *et al.,* 1998). Figure 4.7b below shows the phylogenetic relatedness of representative clones to the closest relatives on ARB database and also BLAST analysis. Clones from this study are shown in blue font.

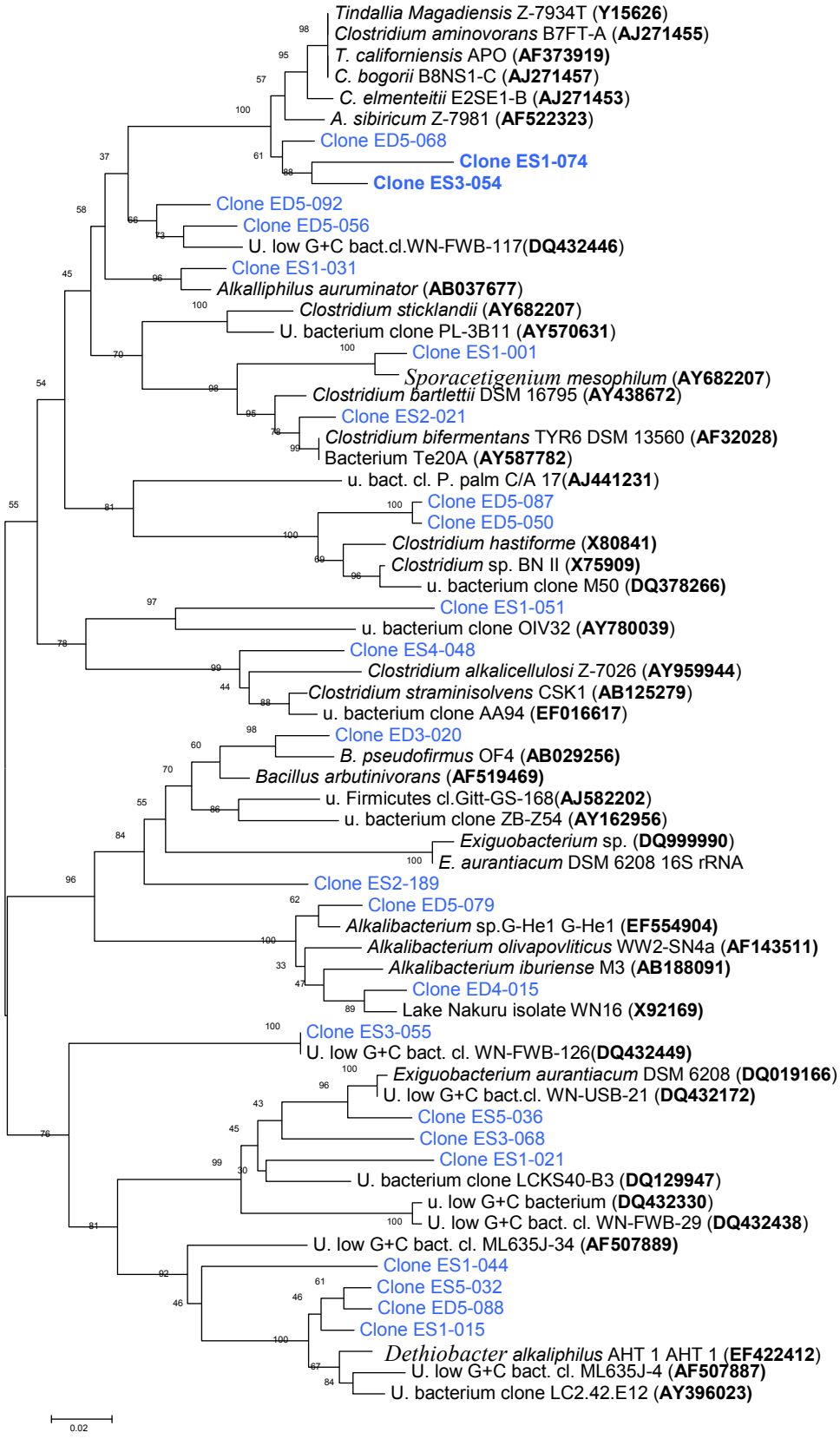

Figure 4.7b Evolutionary relationships of Taxa in the Phylum Firmicutes clones from Lake Elmenteita

The Bacilli class was less diverse. Clones related to *Alkalibacteria* were related to *Alkalibacterium iburiense* and *Alkalibacterium olivapovliticus*. Four clones related to Bacilli group (similarity values 88-98%) were all from hotspring sediment samples and one clone (D3-020) was from a dry mud sample. This may indicate that there could be a preference to the warm temperatures by the *Bacillus* group. Ten clones, all from the dry mud samples were similar (94-98%) to a Firmicutes bacterium isolate WN16 from Lake Nakuru and *Exiguobacterium* was represented by two clones.

4.3.4.3 The class *Betaproteobacteria*

A total of 38 clones were affiliated to members of the class *Betaproteobacteria*. All the clones in this group were from the water samples. Two clones aligned to uncultured members of the *Macromonas* group. These two clones were from the sampling site 2 which was a hotspring while the closest relative in ARB was from groundwater in a deep gold mine of South Africa (Lin *et al,* 2006). Seventeen clones could be affiliated to uncultured members of the *Hydrogenophaga* group and Clone W1-010 was closely related to *Malikia granosa* (Spring *et al.,* 2006). Clone W2-021 was found to be affiliated to *Aquabacrerium citratiphilum* (Kalmbach *et al.,* 1999). There were nine sequences related to Alcaligenes *group.* Two clones were closely related to uncultured members of the genus *Lautropia*. The family *Methylophillaceae* was represented by six clones all affiliated to uncultured members of the genus. Figure 4.7c shows the phylogenetic relatedness representative clones in this class to the closest relatives on ARB database and also BLAST analysis.

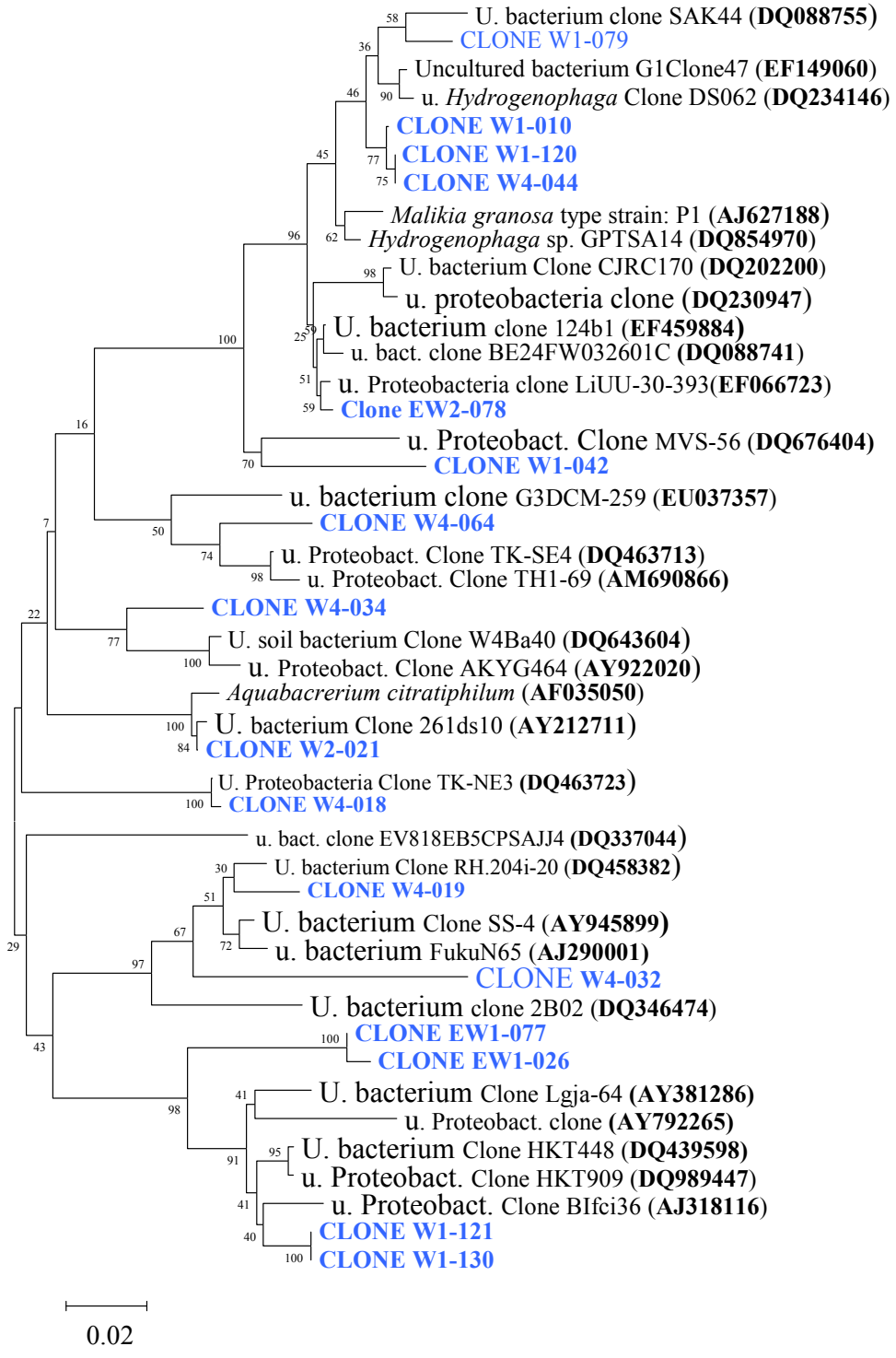

Figure 4.7c Evolutionary relationships of Taxa in the class *Betaproteobacteria.* Clones from Lake Elmenteita are indicated in blue font.

4.3.4.4 The class *Gammaproteobacteria*

A total of 20 clones representing various groups were aligned on ARB. The order *Xanthomonadales* was represented by three clones affiliated to uncultured Gamma Proteobacteria. These three clones were all from the hotspring sediment samples and the closet relative was a clone from the great Artesian Basin in Australia. Seven clones affiliated to the genus *Nitrosococcus* were also encountered and they were affiliated to uncultured bacteria. Six of these clones were from the hotspring water and one from the dry mud samples. Since there are no close relatives isolated, the clones could represent novel thermophilic bacteria. Clone S1-111 was affiliated to *Thioalkalivibrio.* Four clones affiliated to the family *Ectothiorhodospiraceae* were identified. Clone D5-037 was closely related to *Ectothiorhodospira shaposhnikovii* while clone W4-003 and W2-059 were related to *Ectothiorhodosinus mongolicum*. The later was isolated from a Mongolian soda lake. Three clones all related to *Thiothrix nivea* (Teske *et al.,* 1995) were encountered and the three were from the hotspring samples. The genus comprises of filamentous sulphide oxidizing bacteria. Figure 4.7d shows the phylogenetic relatedness representative clones to the closest relatives on ARB database and also BLAST analysis. Clones from this study are shown in blue font.

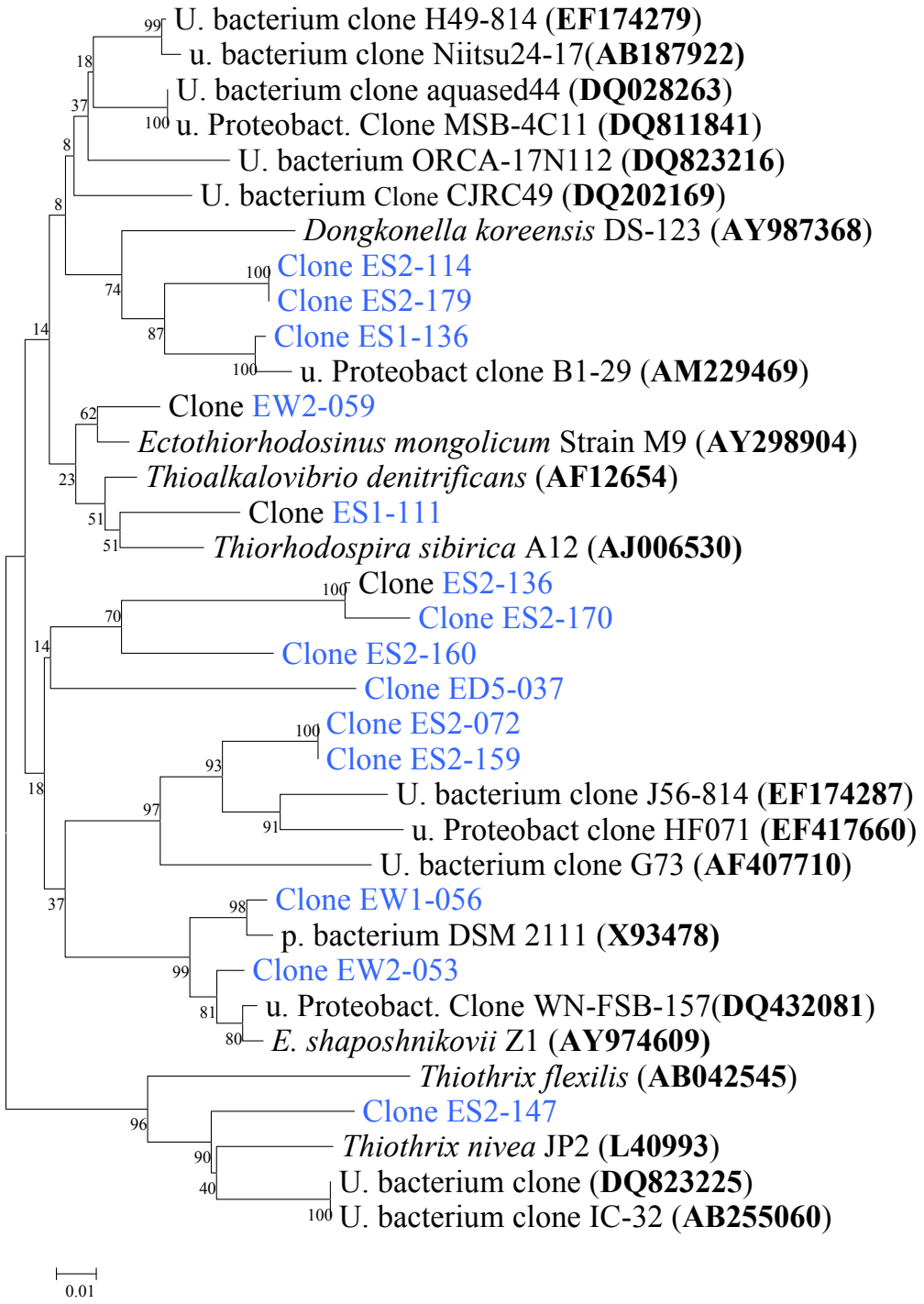

Figure 4.7d Evolutionary relationships of Taxa in the class *Gammaproteobacteria*. Clones from Lake Elmenteita are indicated in blue font.

4.3.4.5 The class *Alphaproteobacteria*

The most common representatives of this group in the soda lakes are the genera *Rhodobaca* and *Rhodobacter*. In this study, a total of 17 clones were related to *Rhodobaca bogoriensis*. Ten clones were related to *Rhodobacter*, six of them aligning to uncultured members of this genus. Clone S1-022 had 98% similarity to *Mesorhizobium* sp whereas Clone S1-09 was 98% similar to uncultured member of the genus *Rhodoblastus* which comprises of acidophilic bacteria. One clone (D5-027) from the dry mud samples was affiliated to the *Methylobacterium* group. The genus *Methylobacterium* is a group of strictly aerobic, facultatively methylotrophic, Gram-negative, rod-shaped bacteria that are able to grow on C1 compounds as the sole carbon and energy sources (Green, 1992). Two clones (W4-036 and W2-036) were affiliated to the *Erythrobacter* group. The sphingomonads and the *Sandracinobacter* were represented by one clone each. A single clone (S2-108) was related to *Rubrimonas cliftonensis*. Figure 4.7e below shows the phylogenetic relatedness representative clones from the class *Alphaproteobacteria* to the closest relatives on ARB database and also BLAST analysis.

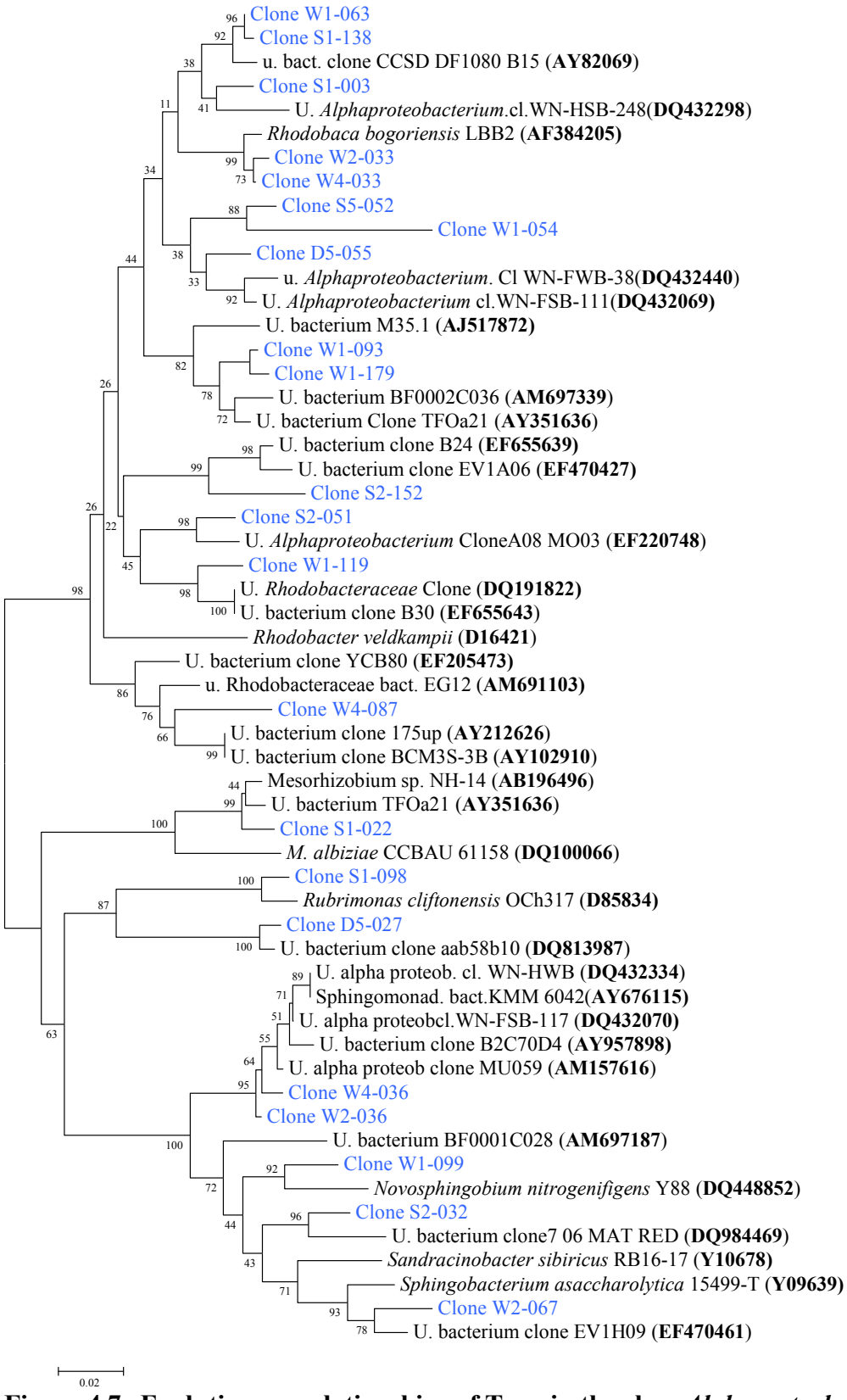

Figure 4.7e Evolutionary relationships of Taxa in the class *Alphaproteobacteria*. Clones from Lake Elmenteita are indicated in blue font.

4.3.4.6 The class *Deltaproteobacteria*

A single clone S2-056 was closely affiliated to *Myxobacterium/Polyangium*. This clone was from the hotspring samples. Other groups represented include the *Bacteriovorax* (W4-075), *Desulfobulbus* (D5-036) and *Desulfosarcina* (S1-119, S4-078 and S2-085). Clone S1-134 and clone S5-011 were closely affiliated to *Desulfonatronum cooperativum* (Zhilina *et al.,* 2005) and *D. thiodismutans* (Pikuta *et al.,* 2003). These two belong to the Order *Desulfovibrionales* and the latter is a sulphate reducing bacteria isolated from Mono Lake California.

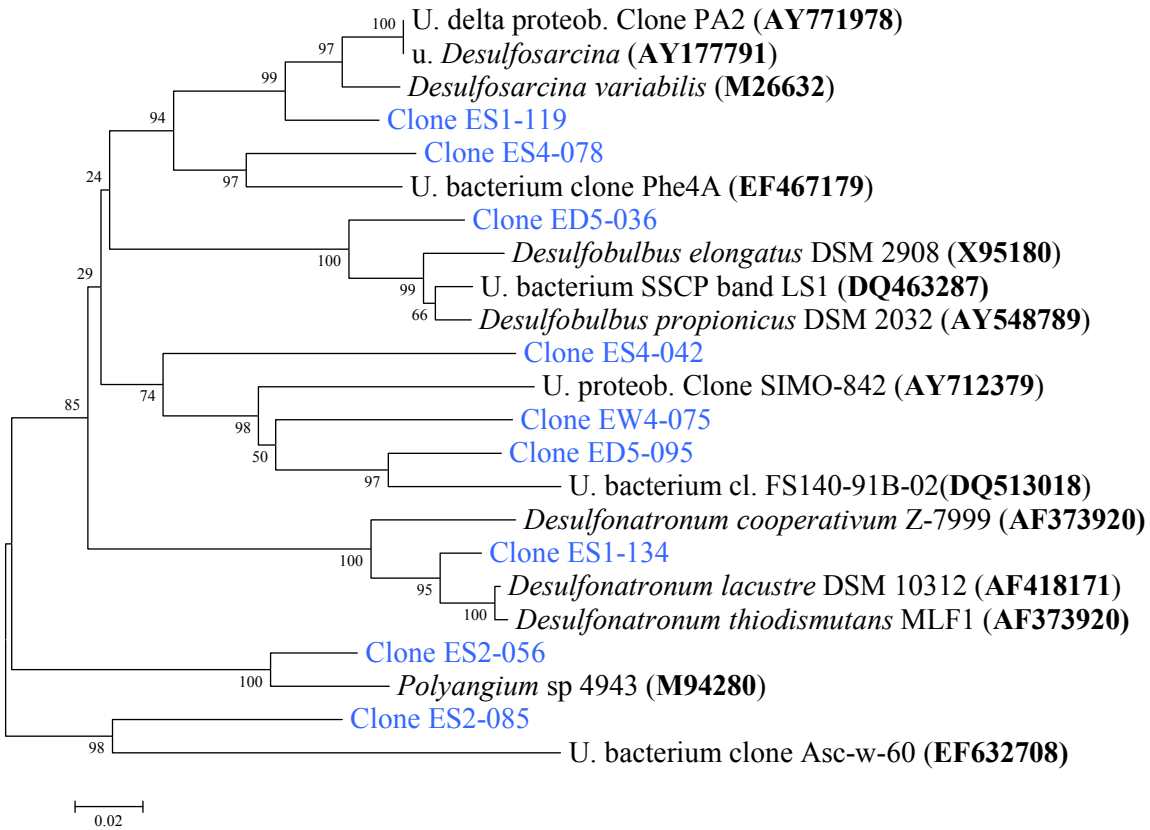

Figure 4.7f Evolutionary relationships of Taxa in the class *Deltaproteobacteria.* Clones from Lake Elmenteita are indicated in blue font.

4.3.4.7 Bacteroidetes – Chlorobi Group

A total of 102 clones belonging to the Bacteroidetes – Chlorobi were aligned to the ARB database. This group was diverse in terms of groups represented as shown on table 4.3 below. However most of the clones were affiliated to uncultured bacteria. Figure 4.7g shows the phylogenetic relatedness representative clones to the closest relatives on ARB database and also BLAST analysis. The *Chitinophaga-Flexibacter* clone sequences were from sediment samples from sampling site S1. All the clones in the family *Saprospiraceae* aligned to the haliscomenobacter and all of them were from the hotspring sediment samples. Three clones from the hot spring were affiliated to *Sphingobacteriaceae*. Eight clones from the *Saprospiraceae*, four from *Lewinella* and seven from Chlorobi form distinct clades not related to other members of their respective groups.

4.3.4.8 Planctomyces

Within the Planctomyces, a total of 18 clones were aligned on ARB database. Figure 4.7h shows the phylogenetic relatedness representative clones to the closest relatives on ARB database and also BLAST analysis. Five of the clones align to the *Pirellula* group, eight to the *Blastopirellula* and one clone formed a deep branch within the *Rhodopirellula*. Clone W-053 was affiliated to *Planctomycete* str. 449 (Griepenburg *et al.,* 1999). Two clones were not affiliated to any cultured planctomycete.

Table 4.3 Clones in the Bacteroidetes – Chlorobi Group

Taxon	Clones	Closest relative in BLAST search
Sphingobacteriaceae	7	uncultured bacteria
Flavobacteriaceae_1	3	*Bacteroidetes* bacterium T4-KAD-str1
Chitinophaga-Flexibacter	13	uncultured bacteria
Saprospiraceae	8	Uncultured bacteria
	19	Bacteroidetes bacterium clone Skagenf36
Lewinella	5	*Lewinella nigricans*
Flexibacter-Flexis	5	Uncultured bacteria
	2	CFB group bacterium clone SM1C08
Cyclobacteria	8	Uncultured Hongiella sp
Crenothricaceae	2	Uncultured bacteria
Chlorobi	10	Uncultured bacteria
Verrucomicrobia	1	Uncultured bacteria
Uncultured Bacteria	31	

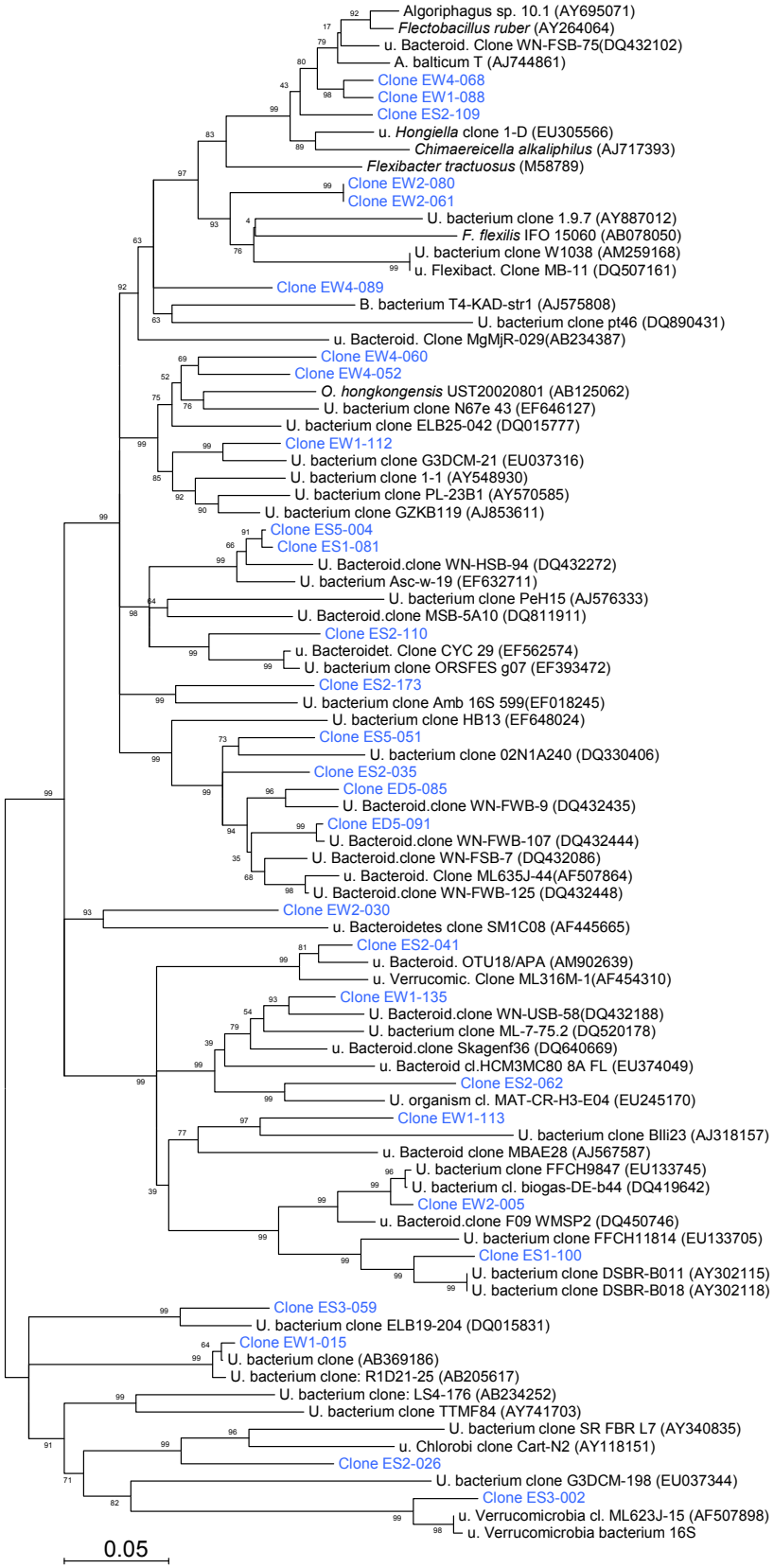

Figure 4.7g Evolutionary relationships of Taxa in the Bacteroidetes group. Clones from Lake Elmenteita are indicated in blue font.

4.3.4.9 Other groups

Only two clones belonging to the Spirochetes were detected. *Spirochaeta halophila*, a facultative anaerobe, was detected in the clone libraries of Wadi Al Natrun, Egypt (Mesbah *et al.,* 2006). Thirteen clones were affiliated to uncultured members of the family *Microbacteriaceae* in the Actinobacteria. One clone was affiliated to uncultured *Gemmatimonadete*. Seven clones belonged to the Acidobacteria group 4 and all the seven were from the Hotspring sediment samples. The sequences in this group are all from acidophilic bacteria. Three clones were affiliated to Chloroflexi group. "*Candidatus Chlorothrix halophilus*" is an obligately anaerobic sulphide-dependent phototrophic green nonsulfur bacterium that was cultured from a hypersaline microbial mat (Klappenbach and Pierson, 2004).

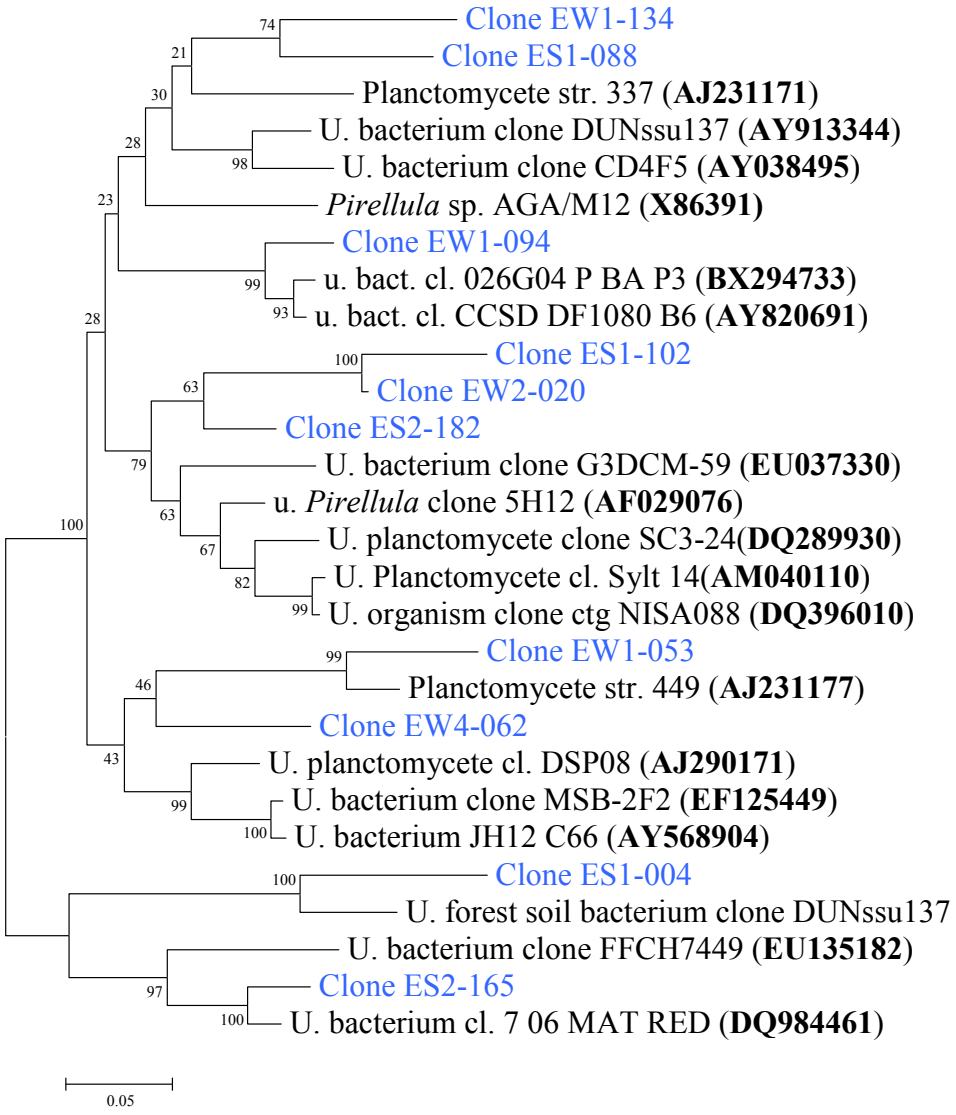

Figure 4.7h Evolutionary relationships of Taxa in the Planctomycetes group. Clones from Lake Elmenteita are indicated in blue font.

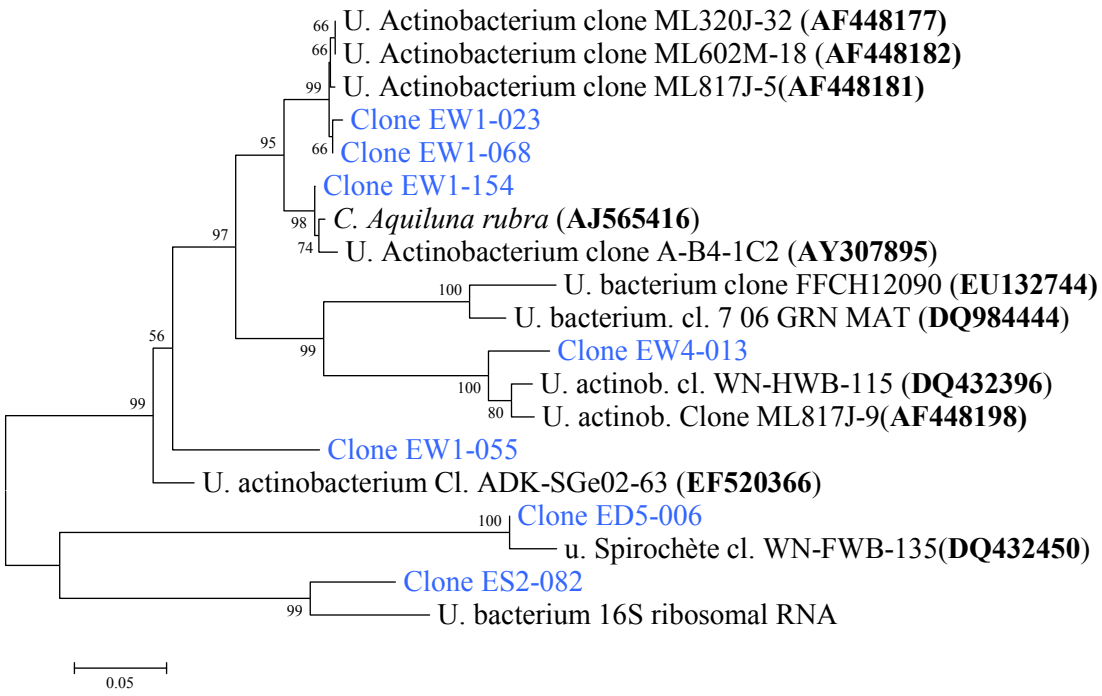

Figure 4.7i Evolutionary relationships of Taxa in the Actinobacteria, Spirochete, Acidobacteria and Chloroflexi groups. Clones from Lake Elmenteita are indicated in blue font.

4.4 Discussion

Numerous DNA extraction methods have been developed and evaluated, but all methods yield crude extracts that are contaminated (Wilson, 1997). Humic acids are the most widely reported contaminants in soil and sediment nucleic acid extracts (Wilson, 1997). Although the DNA extracted in this study was brownish in colour amplification was possible without further purification. From the DGGE profile it was not possible to cut out bands from the gel without losing much of the diversity.

A comparison of the profiles with previous attempts by Rees *et al.,* (2004) reveals that there is much higher diversity within Lake Elmenteita. This is confirmed by the fact that of the 655 clones sequences selected for analysis, 525 (80.15%) were related to uncultured members of the Domain Bacteria. More than 90% of the clones had similarity values below 80% to both cultured and uncultured microorganisms. This indicates that a large proportion of deep branching novel phylogenetic groups are represented in the clone libraries.

Interesting is the occurrence of the Betaproteobacteria which have not been previously reported from the East African soda lakes or the other soda lakes such as Wadi al-Natrun in Egypt. Lake Elmenteita has not been extensively studied as compared to Lake Magadi. The presence of groups commonly found in the hypersaline Lakes Magadi and Natron points to an evolutionary adaptation to varying salt concentration and alkalinity.

The results presented here point to a complex microbial community which drives the various geochemical cycles within the soda lake environment. The four dominant Phyla in the clone libraries are the Cyanobacteria, Firmicutes, Proteobacteria and the Bacteroidetes. This concurs with earlier reports that the less alkaline lakes such as Lake Elmenteita are usually dominated by dense blooms of cyanobacteria while the hypersaline lakes occasionally support blooms of both cyanobacteria and alkaliphilic Anoxygenic phototrophs belonging to the genera *Ectothiorhodospira* and *Halorhodospira* (Jones *et al.,* 1998; Grant *et al.,* 1999; Ballot *et al.,* 2004).

The four orders of Cyanobacteria were represented with clones in the genus *Synechococcus* being the most common (Table 4.3). Cyanobacteria groups reported so far from Lake Elmenteita include *Arthrospira, Arthrospira fusiformis, A. arnoldii, Synechococcus* sp., *Synechocystis* sp., *Spirulina subtilissima*, *Spirulina subsalsa* and *Pseudanabaena* sp. (Iltis, 1969; Vareschi, 1982; Melack, 1988; Jones *et al.,* 1998; Kebede and Willén, 1998; Grant *et al.,* 1999 Ballot *et al.,* 2004).

Organic matter is produced also by anoxygenic phototrophic purple bacteria (Zavarzin *et al.,* 1999). Purple sulphur bacteria of the genera *Ectothiorhodospira* and *Halorhodospira* oxidize hydrogen sulphide with intermediate extra-cellular sulphur deposition (Baumgarte, 2003). The family *Ectothiorhodospiraceae* represents a group of haloalkaliphilic purple sulphur bacteria that has been shown to be separated from, but related to species of the *Chromatiaceae*, according to their ribosomal RNA

oligonucleotide catalogues (Stackebrandt *et al.,* 1984) and 16S rDNA sequence similarities (Imhoff and Süling, 1996; Imhoff *et al.,* 1998). One of the features that distinguish the family *Chromatiaceae* from *Ectothiorhodospiraceae* is that they accumulate intracellular elemental sulphur while all strains of the family *Ectothiorhodospiraceae* produce external globules of elemental sulphur during photosynthetic sulphide oxidation (Imhoff, 1984).

The Phylum Chlorobi consists of green sulphur bacteria are anoxygenic photosynthetic bacteria. Some are unicellular or produce a network of cells. Carbon is fixed via a reductive TCA cycle rather than the Calvin cycle. Nineteen clones were retrieved from the mud samples. The Phylum Chloroflexi consists of green gliding bacteria and the members are metabolically versatile. Four clones were retrieved from the sediment samples. The members grow as heterotrophs or photosynthetically. The filamentous green nonsulfur bacteria are among the most abundant organisms in both oxic and anoxic layers of microbial mats from a variety of environments (Jonkers, *et al.,* 2003; Nübel *et al.,* 2001; 2002; Pierson *et al.,* 1994; Ward *et al.,*1997). They include both photo and chemotrophs, but the overall physiological capability and ecological role of the group is poorly understood. Therefore the occurrence clones from both *Chlorobi* and *Chloroflexi* contributes to primary production in the Lake.

Rhodobaca bogoriensis is an alkaliphilic Alphaproteobacterium isolated from Lake Bogoria in Kenya capable of both phototrophic and chemotrophic growth and is a

representative of purple nonsulfur bacteria described from soda lake environments (Milford *et al.,* 2000). The occurrence of 35 clones belonging to the order *Rhodobacterales* confirms that bacteria in the genera *Rhodobaca* and *Rhodobacter* play an important role in the soda lake environment.

A number of aerobic chemoorganotrophic, alkaliphilic isolates obtained from several soda lakes in East Africa have been studied in detail (Duckworth *et al.,* 1996; Jones *et al.,* 1994). These populations of non-phototrophic, aerobic organotrophic bacteria utilize products of photosynthesis as well as products of anaerobic destruction. A few alkaliphilic methane-oxidising bacteria of the *Methylococcaceae* have been isolated from several moderately saline soda lakes. In this study four clones affiliated to the *Methylococcales* were retrieved from the wet sediments. The methanotroph *Methylobacter alcaliphilus* was isolated from soda lakes in Tuva, Central Asia (Khmelenina *et al.,* 1997) while strains of methanotrophic bacteria isolated from sediments of south-eastern Transbaikal soda lakes were identified as *Methylomicrobium buryantense* (Kaluzhnaya *et al.,* 2001). The most interesting representative of this group might be *Methylomicrobium* sp. AMO1, isolated from a mixed sample of sediments from five Kenyan soda lakes (Sorokin *et al.,* 2000a). This alkaliphilic methanotroph is able to oxidize ammonia to nitrite at pH 10- 10.5 and is also capable of oxidising organic sulphur compounds at high pH (Baumgarte, 2003).

The major groups within the phylum Firmicutes are the *Bacillus* sp and *clostridia*. The *Bacilli* are aerobic or facultatively spore forming whereas the Clostridia are anaerobic fermentors. Some groups are sulphate reducers. One group is an exception, the *Heliobacteria* which are photosynthetic due to a unique Bacteriochlorophyll Bchl g. Clones from the orders *Bacillales* (7 clones), *Clostridiales* (85 clones), *Dethiobacter* (8 clones), *Lactobacillales* (15 clones), *Thermoactinomycete* (3 clones) and *Thermoanaerobacter* (3 clones) were retrieved. From isolation studies, Gram-positive aerobic isolates of both the high G+C and low G+C divisions have been found. From the low G+C isolates represented are especially *Bacillus alcalophilus* (rRNA group 6, according to Nielsen *et al.,* 1994; 1995) and *Bacillus clarkia* group, (Duckworth *et al.,* 1996).

The occurrence of clones with low similarity values to cultured isolates indicated that there are more novel uncultured groups within the Actinobacteria in the soda lake environments. Within the high G+C divisions of the Gram-positive several isolates have been reported. These include *Dietzia natronolimnaea* isolated from Lake Oloiden, Kenya (Duckworth *et al.,* 1998). Other Gram-positive high G+C isolates are associated with known species of the genera *Arthrobacter* and *Terrabacter* (Duckworth *et al.,* 1996). Two other high G+C bacteria; *Bogoriella caseilytica* (Groth *et al.,* 1997) and *Cellulomonas bogoriensis* (Brian *et al.,* 2005) have so far been described from Lake Bogoria in Kenya.

The microbial sulphur cycle seems to be one of the most active in the soda lakes with anaerobic phototrophic purple sulphur bacteria and sulphate reducing alkaliphiles as the main actors (Imhoff *et al.,* 1979). Aerobic chemolithotrophic bacteria are capable of oxidizing reduced inorganic compounds, such as methane, hydrogen, sulphide and ammonia produced by the haloalkaliphilic anaerobes in the soda lakes during degradation of organic compounds (Imhoff *et al.,* 1979, Cloern *et al.,* 1983; Joye *et al.,* 1999). The presence of characteristic genes had been noticed in several soda lakes (Ward *et al.,* 2000, Giri *et al.,* 2004).

The sulphur cycle in these lakes utilizes sulphur and sulphate presumably generated by *Ectothiorhodospira* and *Halorhodospira* sp. (the link between the carbon and sulphur cycles), and also aerobic sulphur-oxidizers. Sulfuroxidizing bacteria belong to two groups, those similar to the non-lithotrophic *Halomonas deleya* and those assigned to the novel genus *Thioalkalovibrio* (Sorokin *et al.,* 2001). Sulphate-reducing bacteria then complete the cycle; *Desulphonatronovibrio hydrogenovorans* and *Desulphonatrum lacustre* (Zhilina *et al.,* 1997; Pikuta *et al.,* 1998) have been isolated from Siberian soda lakes.

Sulphate reduction is responsible not only for the final steps of organic matter degradation but also for generating alkaline conditions as a result of transformation of sulphate to sulphide (Zavarzin *et al.,* 1999). The first alkaliphilic sulphate reducing bacterium identified as *Desulfonatronovibrio hydrogenovorans*, a member of the delta

subclass of the Proteobacteria was isolated from mud in a drainage ditch at Lake Magadi (Zhilina *et al.,* 1997b). The study of secondary anaerobes and their biodiversity in soda lakes of Tuva (Central Asia) also revealed the presence of *Desulfonatronovibrio hydrogenovorans* (Pikuta *et al.,* 1997) indicating that this organism may play the universal role of hydrogen sink in a sulfidogenic anaerobic alkaliphilic community (Zavarzin *et al.,* 1999). Another alkaliphilic sulphate reducer of the delta subdivision of Proteobacteria, *Desulfonatronum lacustre* Z-7951, was obtained from Lake Khadyn (Tuva), an oligotrophic alkaline lake with low mineralization (Pikuta, *et al.,* 1997; 1998).

Hydrogen-utilizing, extremely alkaliphilic strains of methanogens were isolated from Lake Wadi-el-Natrun, Egypt (Boone *et al.,* 1986) and were also detected in slurry samples from Lake Magadi (Zhilina and Zavarzin, 1994) and in mixed samples from Tuva lakes (Zavarzin *et al.,* 1999) but have not been fully characterized. Hydrogen acetogenesis may provide an available hydrogen sink. A representative of the homoacetogenic bacteria (strain Z-7937) was isolated from Lake Magadi samples (Zhilina & Zavarzin, 1994). Another hydrogen sink can be provided by nitrate reduction. An anaerobic culture of a hydrogenotrophic denitrifier, morphologically similar to *Paracoccus*, was isolated from Lake Magadi, Kenya (Zavarzin *et al.,* 1999).

A different pathway of anaerobic degradation of organic matter involves organotrophic acetogenesis. The first obligately haloalkaliphilic acetogenic bacterium

Natroniella acetigena was isolated from the bottom mud of Lake Magadi, Kenya (Zhilina *et al.,* 1996a). *Halonatronum saccharophilum*, a moderately haloalkaliphilic chemoorganotrophic representative of the order *Halanaerobiales* was isolated from the coastal lagoon mud of Lake Magadi (Zhilina *et al.,* 2001a).

Organisms fermenting amino acids, called acetogenic ammonifiers have been isolated from soda lakes. Two strains, *Natronincola histidinovorans* (Zhilina *et al.,* 1998) and the non-spore-forming bacterium *Tindallia magadiensis* (Kevbrin *et al.,* 1998) were isolated from Lake Magadi, Kenya, and were found to be related with members of Group XI of the Clostridium taxon (Collins *et al.,* 1994) including *Clostridium felsineum*, *C. formicoaceticum,* and the more distant *C. halophilum*.

A number of alkaliphilic saccharolytic clostridia strains have been isolated from Lakes Elmenteita, Bogoria and Magadi (Jones *et al.,* 1998). The isolates from Lakes Elmenteita and Bogoria were found to be associated with members of group XI of Clostridia, while the haloalkaliphilic strains isolated from Lake Magadi were related with members of the genus *Moorella* (Group VI of Clostridia). Zhilina *et al*., (2001b) isolated saccharolytic anaerobes from Lake Magadi. Saccharolytic anaerobes have also been isolated from the slightly mineralized Lake Nizhee Beloe in the south-eastern Transbaikal region (Tourova *et al.,* 1999).

Lithotrophic, nitrite-oxidising bacteria e.g. *Nitrobacter alkalicus* have been isolated from soda lakes located in the Kunkur steppe in Siberia, Lake Nakuru and Crater Lake

in Kenya (Sorokin *et al.,* 1998). These chemolitho-trophic nitrifying bacteria play an important role in biological nitrogen cycling by converting reduced inorganic nitrogen compounds to nitrate. The nitrogen cycle in these lakes involves the production of ammonia by fermentative anaerobes such as *Tindallia magadii* (Kevbrin *et al.,* 1998). Ammonia is utilized by methanotrophs and nitrifiers, producing nitrate. Nitrate, in turn, is utilized by the chemoorganotrophs, creating a link between the nitrogen and carbon cycles. The genus *Thioalkalivibrio* comprises obligately alkaliphilic and obligately chemolithoautotrophic sulphur oxidising bacteria that use nitrate or oxygen as electron acceptors (Sorokin *et al.,* 2001).

Of the three main orders in Phylum Bacteroidetes, *Sphingobacteriales* and *Flavobacteriales* consist mainly of aerobic organisms while known strains of order *Bacteroidales* are all anaerobic (Kirchman, 2002; Reichenbach, 1991). However due to the shallow depths of Lake Elmenteita, it may be possible that mixing of the water tilts the distribution of the phylotypes. The *Cytophaga-Flavobacteria-Bacteroides* cluster belongs to a diverse bacterial division that has been labelled differently over the years. The name used in the most recent edition of the *Bergey's Manual of Systematic Bacteriology* is simply *Bacteroidetes*, encompassing the three new classes *Bacteroidetes*, *Flavobacteria* and *Sphingobacteria* (Ludwig and Klenk, 2001). *Cytophaga* - *Flavobacteria* are chemoorganotrophic and can degrade various biopolymers such as cellulose, chitin, and pectin (Reichenbach, 1992). Members of the *Bacteroidetes* can be found in many habitats, such as the human gut, polluted to

seemingly pristine river water and in extreme habitats like Antarctic marine waters (Weller *et al.,* 2000).

Other phyla occurring in the clone Library include members of the Phyla Fusobacteria, Acidobacteria and Spirochete. Only one clone (ES3-002) was from the *Verrucomicrobia*. These are unique in that some members have bacterial tubulin genes. Few have been isolated in pure culture. The Acidobacteria are common in soils and sediments but few have been isolated. The Phylum consists of aerobic genus Acidobacterium, the homoacetogenic bacteria *Holophaga* and the iron reducing bacteria of the genus Geothrix. In an anaerobic community saccharolytic spirochetes act as dissipotrophs utilizing sugars and a limited range of polysaccharides to produce acetate, lactate, ethanol and hydrogen (Zavarzin *et al.,* 1999). Two haloalkaliphilic strains *Spirochaeta alkalica* and *S. africana* have been isolated from Lake Magadi, and an alkaliphilic species *S. asiatica* from Lake Khatyn, Central Asia (Zhilina *et al.,* 1996b).

From the alignment of the various clones, it would be probable that the Planctomyces inhabiting Lake Elmenteita represent novel taxa which have not been isolated so far. These organisms probably contribute to the mineralization of organic material created by phototrophic primary producers in the crust.

In spite of the limitations of the molecular approach such as differential DNA extraction, primer selectivity, and variable rRNA gene copies (Wintzingerode *et al.,* 1997), the approach remains as an effective method to identify the most prominent microorganisms in a natural environment. From the data obtained in this study it can be concluded that the microbial communities within the soda lakes are complex and more studies are needed to understand them better. The results also indicate that the lake harbours diverse groups of microorganisms than estimated in previous studies. Novel approaches for enrichment and isolation would also deepen our understanding of the roles played by the different groups within the ecosystem.

CHAPTER FIVE

5.0 Diversity of Archaea in Lake Elmenteita

5.1 Introduction

Soda lakes are characterized by high salinity and alkalinity but are also highly productive environments (Jones *et al.,* 1998). Studies on the lakes of the East African Rift Valley in Kenya have shown that they are habitats for novel species of bacteria and archaea (Duckworth *et al.,* 1996). The ability to recover and analyze 16S rRNA genes directly from environmental DNA provides a means to investigate microbial populations in any environment without the need for cultivation (Olsen *et al*. 1986; Ward *et al*., 1990; Amann *et al*., 1995; Hugenholtz *et al*., 1998; Dojka *et al*., 2000). The application of this method to study marine bacteria and archaea has revealed large numbers of unknown microorganisms, which appear to be largely unaffiliated with previous isolates from the same environment (DeLong, 1992). Such methods have also been applied to a number of soil, thermal, and hypersaline environments resulting in the description of as yet uncultivated groups of both Bacteria and Archaea (Amann *et al.,* 1995; Benlloch *et al.,* 1995). The hypersaline soda lakes Natron, Little Lake Magadi and Lake Magadi have over the years yielded a number of Archaea, all of which were relatively closely associated with the extant taxa *Natronobacterium* and *Natronococcus* (Grant *et al.,* 1999). The species *Natronobacterium* and *Natronococcus* were originally isolated and described from this site (Tindall *et al.,* 1984). To date no detailed phylogenetic study had been done on Lake Elmenteita. The

objective of this study was to apply a culture independent approach to elucidate the archaeal diversity in Lake Elmenteita.

5.2 Materials and Methods

The study site, sample collection and DNA extraction are described in section 3.1 to 3.3 above. Almost full-length 16S rDNA archaeal genes were amplified using the primers arc8f (5'-TCCGGTTGATCCTGCC-3') and arc1492r (5'-GGCTACCTTGTTACGACTT-3'). PCR cycling consisted of a three minute initial pre-incubation step at 94° C followed by 35 cycles of a denaturation step at 93°C for one minute, a one minute annealing step at 58°C, and a one minute elongation step at 72°C and a final extension step at 72°C for five minutes. The PCR mix consisted of 5 ml of 10 X PCR buffer [100 mM Tris-HCl (pH 9), primers at a concentration of 0.5 mM, each deoxynucleoside triphosphate at a concentration of 200 mM, 2.0 mM $MgCl_2$, 20 ng of bovine serum albumin (BSA), 0.5 µl of template DNA, 2.5 U of Taq DNA polymerase (Roche).The volume was adjusted to a final volume of 50 ml with sterile MQ water. The presence of PCR products and their concentration were determined by analysing 5 ml of product on 2% agarose gels after staining with Ethidium bromide (3 µl/100ml) and comparison with a molecular weight marker (Smartladder; Eurogentec). Community DGGE analysis was done as described in 3.5 but with the forward primer being 341f –GC (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACG GG (A/G) GG CAG CAG-3'). Clone library construction and analysis was done as described in Section 3.6 but restriction digestion of the clone PCR was done using the

restriction enzyme *Cvi*II from New England Biolabs [NEB], (Beverly, Mass.). Partial sequences were generated using the primer 514r for Archaea. Phylogenetic analysis was done as described in Section 4.4 above.

5.3 Results

Numerous DNA extraction methods have been developed and evaluated, but all methods yield crude extracts that are contaminated (Wilson, 1997). In this study, the Phenol: chloroform method gave DNA of high molecular weight that could be amplified even without purification being necessary (Figure 5.1).

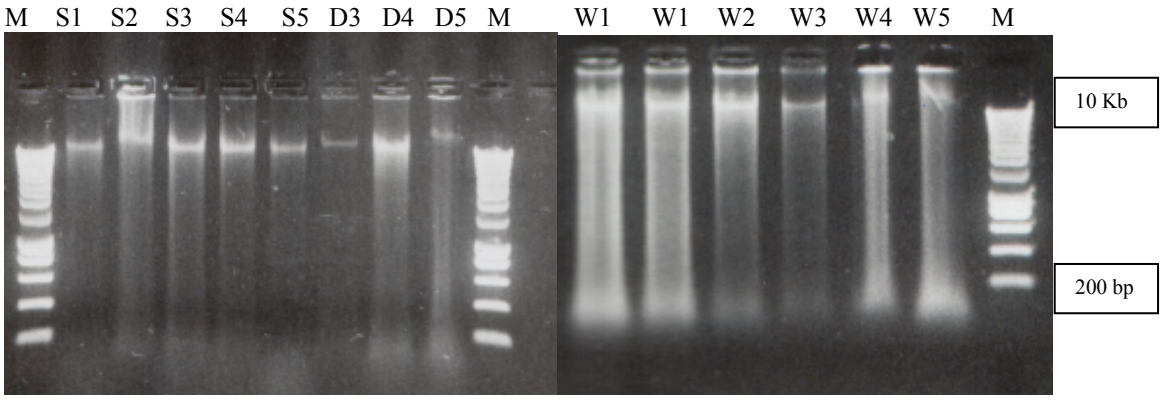

Figure 5.1: DNA yields from the various samples using the Phenol: chloroform extraction method (M- molecular weight marker, S - sediment, D – dry mud and W – water).

The DNA fragment size was estimated by comparison to a molecular weight marker (Smartladder; Eurogentec). The ladder has a molecular weight ranging from 200 bp to 10,000bp and is specially designed for easy quantification as well as size determination of DNA.

Figure 5.2 below shows PCR products from amplification of the DNA extracted without further clean-up. Though the DNA was brownish in colour PCR amplification using 16S rRNA gene universal primers was successful.

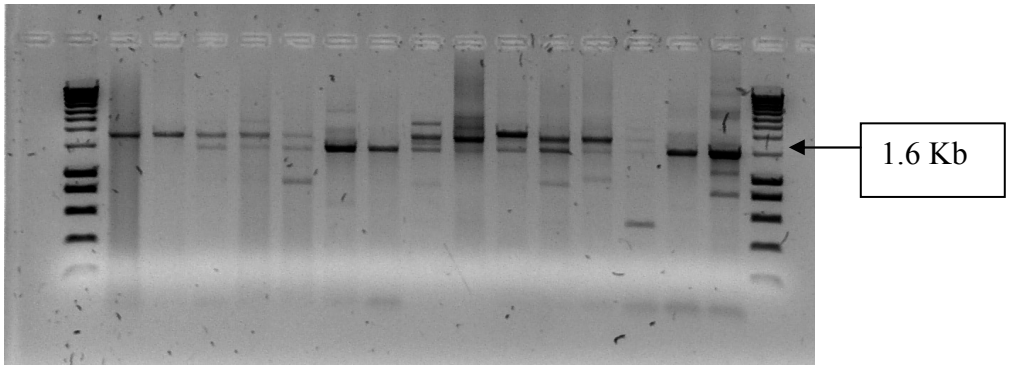

Figure 5.2: Amplified PCR products for Archaea

Figure 5.3 below shows the profiles of archaea in the different samples on a Denaturing gradient gel for bacteria.

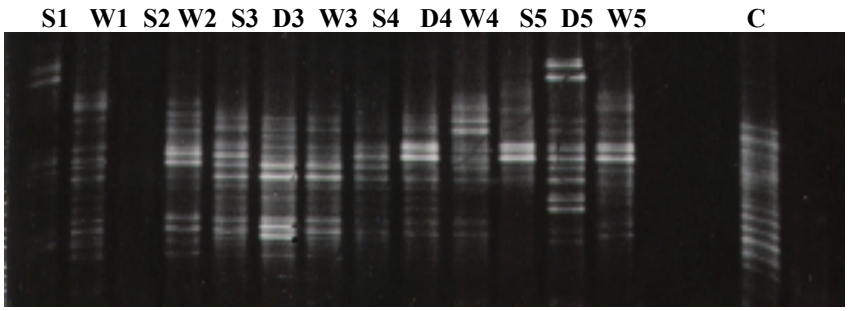

Figure 5.3**:** DGGE Agarose Gel for Archaea (Bottom). The samples were labelled as S- sediment, D- Dry mud and W – water. The water samples seem to have higher diversity than other samples.

Figure 5.4 shows clones having the correct insert size i.e. corresponding to the 16S gene after amplification using the M13 Primers

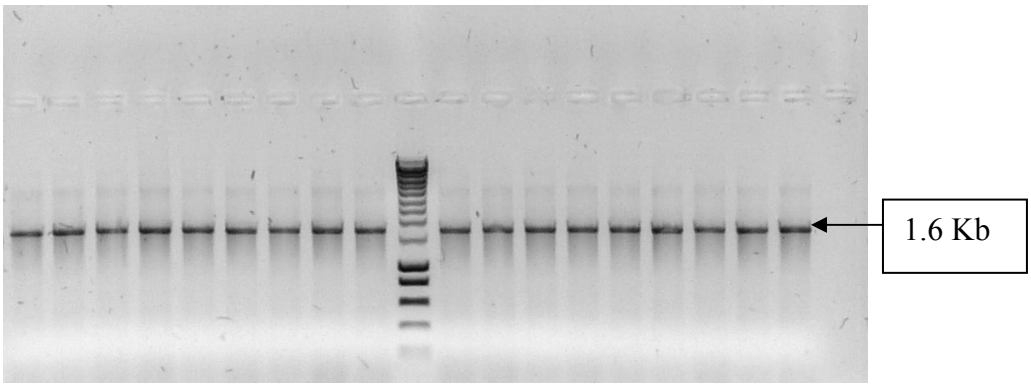

Figure 5.4: Size screening for the right sized insert of 16S rRNA clones.

Figure 5.5 shows and ARDRA pattern of selected clones. Due to the large number of clones, only representative clones that showed unique ARDRA profiles were selected for sequencing.

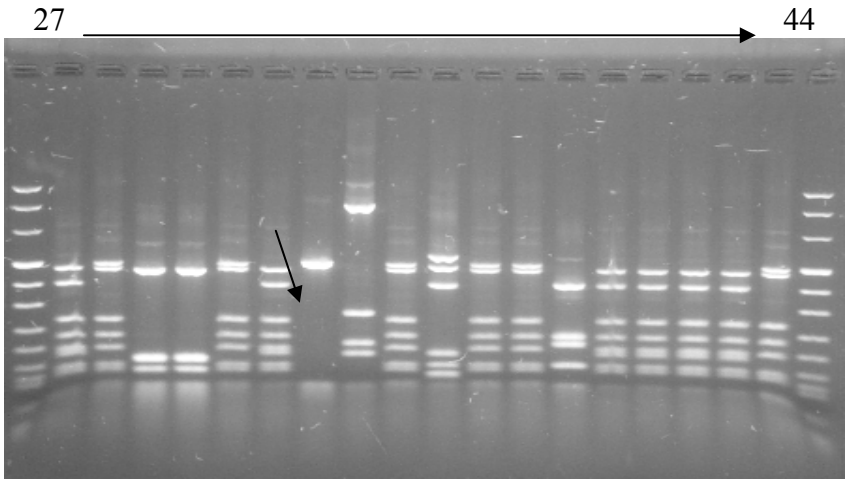

Figure 5.5: ARDRA pattern for selected Archaeal clones S2 – 027 to S2 – 044. The arrow in the Gel shows a Plasmid with no DNA insert.

5.3.1 Archaeal clone library results

A total of 1,399 clones were picked and analysed via ARDRA. Only 170 showed unique ARDRA patterns and these were selected for sequencing. After sequencing 149 clones gave good sequences. BLAST analysis showed that only 73 belonged to the domain Archaea and the rest (76) were affiliated to Eukaryotic groups. A total of 47 clone sequences could be properly aligned to the ARB database and all of them were affiliated to the Phylum *Euryarchaeota*. Only representative clones are shown in Figure 5.1 below. Three clones (ES5- 050, EW1- 017 and EW1- 087) were affiliated to *Methanocalculus*. Clone EW1-009 was related to *Methanospirillaceae*, a family whose members are spiral in shape. Within the *Methanomicrobiales* 2 clones (EW1 - 31 and EW4- 038) were affiliated to *Methanosaeta*; a genus consisting of obligate anaerobes which use acetate as only energy source. *Thermoplasmatales* were represented by 3 clones (EW1- 022, EW1-001 and ES1- 010) all related to uncultured bacteria.

Table 5.1: Summary of clones in the Order Euryarchaeota

Phylum	***Order***	**Dry mud**	**Sediment**	**Water**	**Total**
Euryarchaeota	*Halobacteriales*	20	9	13	**42**
	Methanomicrobiales		1	2	**3**
	Methanosarcinales			2	**2**
	No relatives			2	**2**

Figure 5.6 shows the phylogenetic relationship of selected clones in relation to the nearest neighbours as per BLAST analysis and alignment to the ARB database. Clones from Lake Elmenteita are indicated in a blue font

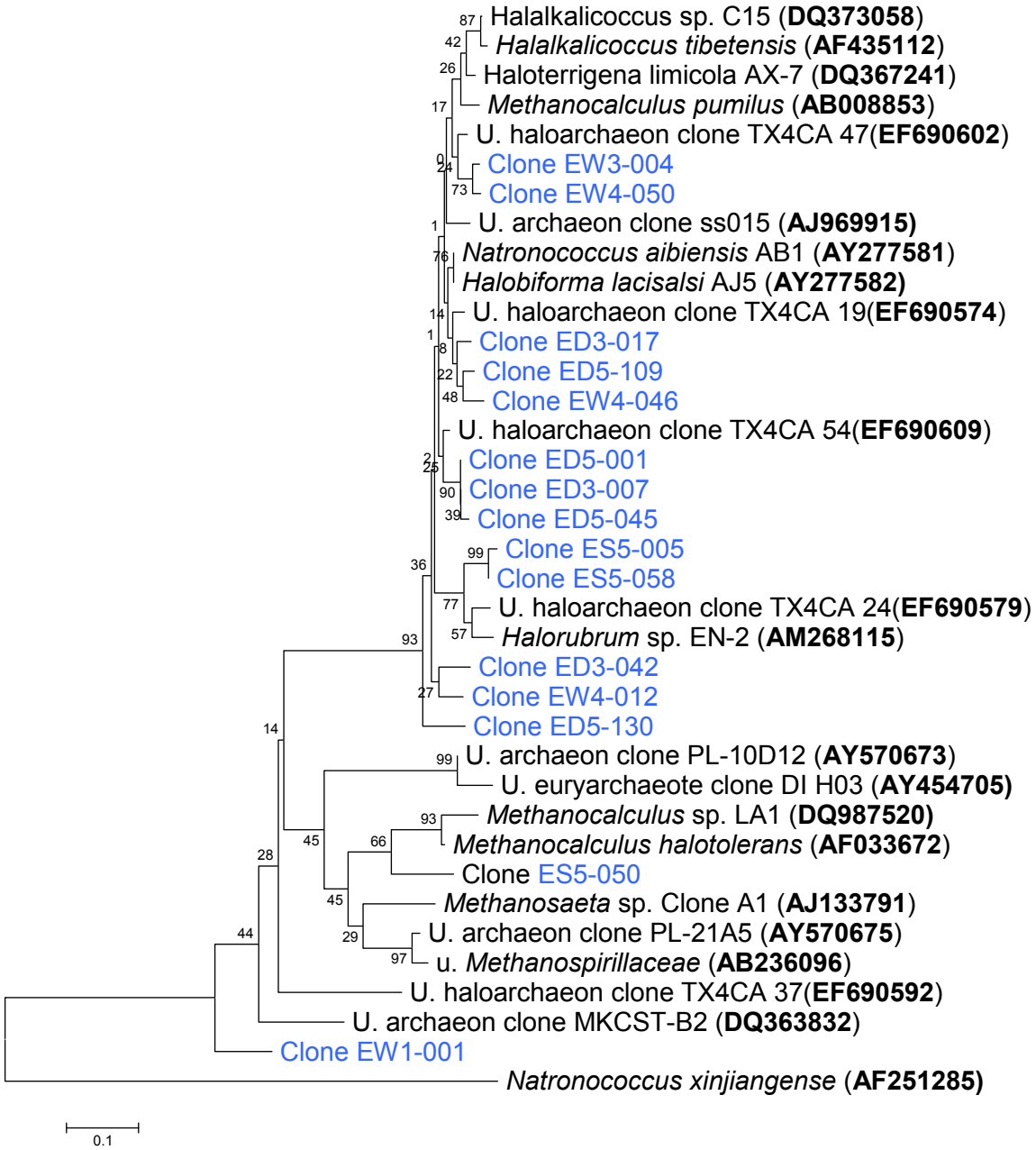

Figure 5.6: Phylotypes in the clone library for Archaea

The order *Halobacteriales* was more diverse. Clone W2-079 closely affiliated to *Halovivax asiaticus*. Clones W3-004, W4-050 and W4-046 belonged to the *Halobiforma* group whereas clones S5-005 and S5-058 relate to uncultured *Halorubrum*, D4-029 and W4-044 related to *Natronococcus amylolyticus*. Three

clones belonged to uncultured *Natronococcus*. BLAST analysis showed that clone S2-019 was related to *Halalkalicoccus tibetensis*. A total of 22 clones belonged to uncultured haloarchaea.

5.4 Discussion

According to small subunit ribosomal RNA sequence comparisons, all known Archaea belong to the Phyla Crenarchaeota, Euryarchaeota, Korarchaeota and Nanoarchaeota which grow attached to the surface of a specific archaeal host. In this study all the clones retrieved were from the phylum Euryarchaeota. This concurs with a study done on five Kenyan soda lakes (Rees *et al.,* 2004) and those Lake Wadi-el-Natrun, Egypt (Mesbah *et al.,* 2006). Within this phylum, members of the *Methanocalculus* group obtain their metabolic energy via reduction of CO_2 to methane whereas H_2 and formate are electron donors. Their distinguishing feature is tolerance to high salt concentration. *Methanosaeta* is a genus consisting of obligate anaerobes which use acetate as their only energy source. Members of the order *Halobacteriales* require high salt for growth and are chemo-organotrophic may be aerobic or facultatively anaerobic and are ubiquitous where salt concentration is high.

Members of the Genus *Natronococcus* are alkaliphilic and require a pH of at least 8.5 for growth. In a study by Rees *et al*., (2004) on Lake Elmenteita, of the fourteen archaeal-related amplicons retrieved, three were related to the genera *Halobacteria, Haloarcula*, and *Natronobacterium*. Many of the amplicons in the study were closely related to *Natronobacterium pharaonis,* with 98.05 – 99.53% identity. DNA from the

Halobacteriales has been extracted from the Dead Sea, solar salterns, Antarctic hypersaline lakes, alkaline African hypersaline lakes, and Solar Lake, Sinai (Benlloch *et al.,* 2001; Cytryn *et al.,* 2000; Moune´, *et al.,* 2003; Oren, 2002). Isolated strains of this group are aerobic halophiles growing at salinities up to NaCl precipitation, although some are capable of anaerobic growth either in the light using bacteriorhodopsin or in the dark by fermentation (Hartmann *et al.,* 1980; Oren and Trüper, 1990).

Saline soda lakes support blooms of halobacteria and harbour alkaliphilic representatives of the genera *Natronobacterium and Natronococcus, Natronomonas, Natrialba, Natronorubrum* and *Halorubrum*. Functionally, they have a specific trophic position and flourish on the organic matter concentration arising from evaporation of brine and the death of its microbial population (Zavarzin *et al.,* 1999).

Methanotrophic methanogens isolated from several soda lakes are related with members of the *Methanosarcinaceae* within the Euryarchaeota. Though only two clones from the *Methanosarcinales* were retrieved in this study, it indicates that members of this group are represented in Lake Elmenteita. The first haloalkaliphilic strains of methanogens were isolated from Lake Wadi-el-Natrun, Egypt (Boone *et al.,* 1986). One of these isolates, the methylotrophic strain WeN5, was later described as *Methanohalophilus zhilinae* (Mathrani *et al.,* 1988). Another strain of this species Z-7936 was later isolated from Lake Magadi (Zhilina & Zavarzin, 1994; Kevbrin *et al.,*

1997). The methylotrophic methanogen *Methanohalophilus oregonense* was isolated from an anoxic aquifer near Alkali Lake, a hypersaline, alkaline desert lake in south central Oregon in the United States (Liu *et al.,* 1990).

A number of factors including relatively low cell numbers of large organisms and a variable number of rRNA operons among organisms, as well as extraction and PCR bias, may lead to under-representation of phylotypes relative to their *in situ* abundance (Von Wintzingerode *et al.,* 1997). This study shows that microbial diversity in the Kenyan Soda lakes is much higher than that previously reported. The various groups occupy unique niches within the lake ecosystem and this is what drives the high levels of productivity. Novel approaches for enrichment and isolation would also deepen our understanding of the roles played by the different groups within the ecosystem.

CHAPTER SIX

6.0 Isolation and characterisation of bacteria from Lake Elmenteita

6.1 Introduction

Microbial isolates are still very important in developing our understanding of bacterial physiology, genetics, and ecology (Palleroni, 1997; Zinder and Salyers, 2001). Many of the isolates from the Kenyan soda lakes belong to the *Gammaproteobacteria*. A large number of aerobic organotrophic strains belonging to the halomonad lineage have previously been isolated from and around soda lakes in Kenya (Duckworth et al. 1996; Jones *et al.,* 1994). *Alkalimonas delamerensis* was isolated from Lake Elmenteita (Ma *et al.,* 2004a). Within the Cyanobacteria, *Anabaenopsis arnoldii* was described in the phytoplankton communities of Lakes Nakuru and Elmenteita (Vareschi, 1978; Melack, 1988). Recently *Arthrospira fusiformis* and *A. abijatae* were isolated from Lake Elmenteita (Ballot *et al.,* 2004). A number of alkaliphilic saccharolytic clostridia have been isolated from Lakes Elmenteita, Bogoria and Magadi (Jones *et al.,* 1998). Previous attempts at isolation have been based on the alkaline media described by Horikoshii (1991). However, as this medium is rich in organic carbon, heterotrophic microorganisms most probably outcompete slow growers. The objective of this study was to enrich for and isolate some of the groups represented in the clone library using both nutrient rich and nutrient poor media prepared using filter sterilized water from the lake.

6.2 Materials and Methods

6.2.1 Isolation Procedure

Media used for isolation was prepared with water collected from the Lake. The water was filtered through a 0.45 μm and then through a 0.22 μm membrane filters (Whatman). An enrichment strategy was established whereby 14 different liquid media were prepared with lake water, boiled to dissolve the compounds, dispensed into sterile Eppendorf tubes and sterilized for 15 minutes at 121^{o} C. This strategy was applied as due to the high level of dissolved carbonates in the soda lakes prolonged sterilization would have led to blackening of the media.

6.2.2 Media used for isolation

The various media used in enrichment and isolation are shown in Table 6.1. Strains obtained were purified on Dilute Tryptic Soy Broth supplemented with 15 g Bacto Agar (Difco), 3.5% NaCl and 1% $NaCO_3$. The strains were stocked in Tryptic soy Broth supplemented with 3.5% NaCl, 1% $NaCO_3$ and 20 % (v/v) glycerol.

6.2.3 Molecular Identification of the isolated microorganisms

DNA extraction was carried out using the SEQLAB Bacteria-DNA-Kit and PCR amplification of the 16S rRNA genes was carried at SeqLab (Göttingen, Germany). Partial gene sequences were obtained using the 530 reverse primer (5′-GKATTACCGCGGCKGCTG-3′). Sequence analysis was done as described in section 3.4.

Table 6.1 Media used in the isolation of bacteria

Media No.	Media Composition
1.	Trypticase soy Agar
2.	M1: 1% starch,0.4 % yeast extract,0.2 % peptone 1.5% agar
3.	Low medium 1: 0.2% starch, 0.08% Yeast extract, 0.01% peptone, 1.5% agar
4.	6 ml of 100% Glycerol, 1g Arginine, 1g K_2HPO_4, 0.5g of $MgSO_4.7H_2O$, 15g Agar
5.	6g Glucose, 2g Chitin, 14 g Agar
6.	2g Chitin,15g Agar and
7.	15g Agar and 100mg Cycloheximide
8.	7.5g Casamino acids (Difco), 10g Yeast extract (Difco), 3g Trisodium Citrate, 0.3g $MgSO_4.7H_2O$, 2 g Potassium Chloride, traces of Iron and manganese, 15g Agar
9.	3.75g Potassium Chloride, 0.267 g Ammonium chloride,0.174 g Potassium Hydrogen Phosphate, 3.7 g Magnesium Sulphate,0.5 g Calcium Chloride, 0.2 g Yeast extract,0.4 g Peptone,1.5% Agar
10.	5.0 g Peptone, 5.0 g Yeast Extract, 1.0 g K_2HPO_4, 0.2 g of $MgSO_4.7H_20$, 10 g Glucose and 15 g Agar
11.	5.0 g Peptone, 5.0 g Yeast Extract, 1.0 g K_2HPO_4, 0.2 g of $MgSO_4.7H_20$, 10 g Cellulose powder and 15 g Agar
12.	5.0 g Peptone, 5.0 g Yeast Extract, 1.0 g K_2HPO_4, 0.2 g of $MgSO_4.7H_20$, 10 g soluble Starch and 15 g Agar
13.	5.0 g Peptone, 5.0 g Yeast Extract, 1.0 g K_2HPO_4, 0.2 g of $MgSO_4.7H_20$, 10 ml Olive oil and 15g Agar
14.	5.0 g Peptone, 5.0 g Yeast Extract, 1.0 g K_2HPO_4, 0.2 g of $MgSO_4.7H_20$, 10 g Xylose and 15 g Agar

6.3 Results

A total of 221 isolates were selected on the basis of colony morphology and microscopy for partial sequencing. A total of 185 isolates gave readable sequences and the sequences were analysed using BLAST software (Altschul *et al.,* 1997). Table 6.2 gives a summary of the genera represented whereas Table 6.3 gives a summary of the almost complete sequenced isolates and their closest neighbours. The full list of the sequenced isolates in given in Appendix II

Table 6.2: Affiliation of the partial sequences from 185 isolates to different genera

Genus	**No of Isolates**
Halomonas	68
Bacillus	58
Marinospirillum	20
Idiomarina	15
Alkalibacterium	7
Vibrio	5
Enterococcus	2
Nocardiopsis	2
Alkalimonas	1
Streptomyces	1
Nitrincola	1
Halolactibacillus	1
Marinilactibacillus	1
Pseudomonas	1
Amphibacillus	1
TOTAL	**184**

Table 6.3 below gives a summary of the 34 representative isolates that were almost fully sequenced. The strains selected had low similarity values to nearest neighbours (BLAST analysis). The results show that a third (11 isolates) had similarity values below 97% to described isolates meaning they could represent novel genera or species.

Table 6.3 Almost Full length sequences for selected Isolates

Isolate No.	Nearest Neighbour in BLAST	%	Length in Bp
6	*Bacillus pseudofirmus*	98	1550
55	*Bacillus pseudalcaliphilus*	98	1361
127	*Bacillus pseudofirmus*	98	1551
124	*Bacillus sp*	99	1549
141	*Bacillus sp*	98	1552
163	*Bacillus pseudoalkaliphilus*	98	1606
172	*Bacillus sp*	98	1551
257	*Bacillus Pseudofirmus*	98	1584
272	*Bacillus Pseudofirmus*	98	1559
282	*Bacillus hemicellulolyticus*	98	1570
34	*Idiomarina Sp.*	96	1542
36	*Idiomarina Sp. JK 17*	99	1538
113	*Idiomarina JK 17*	97	1539
116	*Idiomarina JK 17*	99	1539
410	*Idiomarina JK16*	98	1425
292	*Marinospirillum alkaliphilum*	97	1519
408	*Marinospirillum alkaliphilum*	97	1521
463	*Marinospirillum alkaliphilum*	97	1522
466	*Marinospirillum alkaliphilum*	98	1523
468	*Marinospirillum alkaliphilum*	97	1538
38	*Nitrincola lacisaponensis*	97	1407
44	*Nitrincola lacisaponensis*	98	1350
48	*Nitrincola lacisaponensis*	98	1445
59	*Alkalibacterium indicireducens*	99	1606
119	*Alkalibacterium indicireducens*	99	1545
109	*Marinilactibacillus Piezzotolerans*	94	1544
273	*Marinilactibacillus Piezzotolerans*	94	1544
69	*Halomonas sp*	98	1498
70	*Streptomyces caelestis*	96	1564
90	*Lake Bogoria isolate*	98	1340
108	*Alkalimonas delamerensis*	97	1379
112	*Amphibacillus*	97	1571
327	*Vibrio metschnikovii*	98	1386
143	*Nocardiopsis exhalans*	99	1552

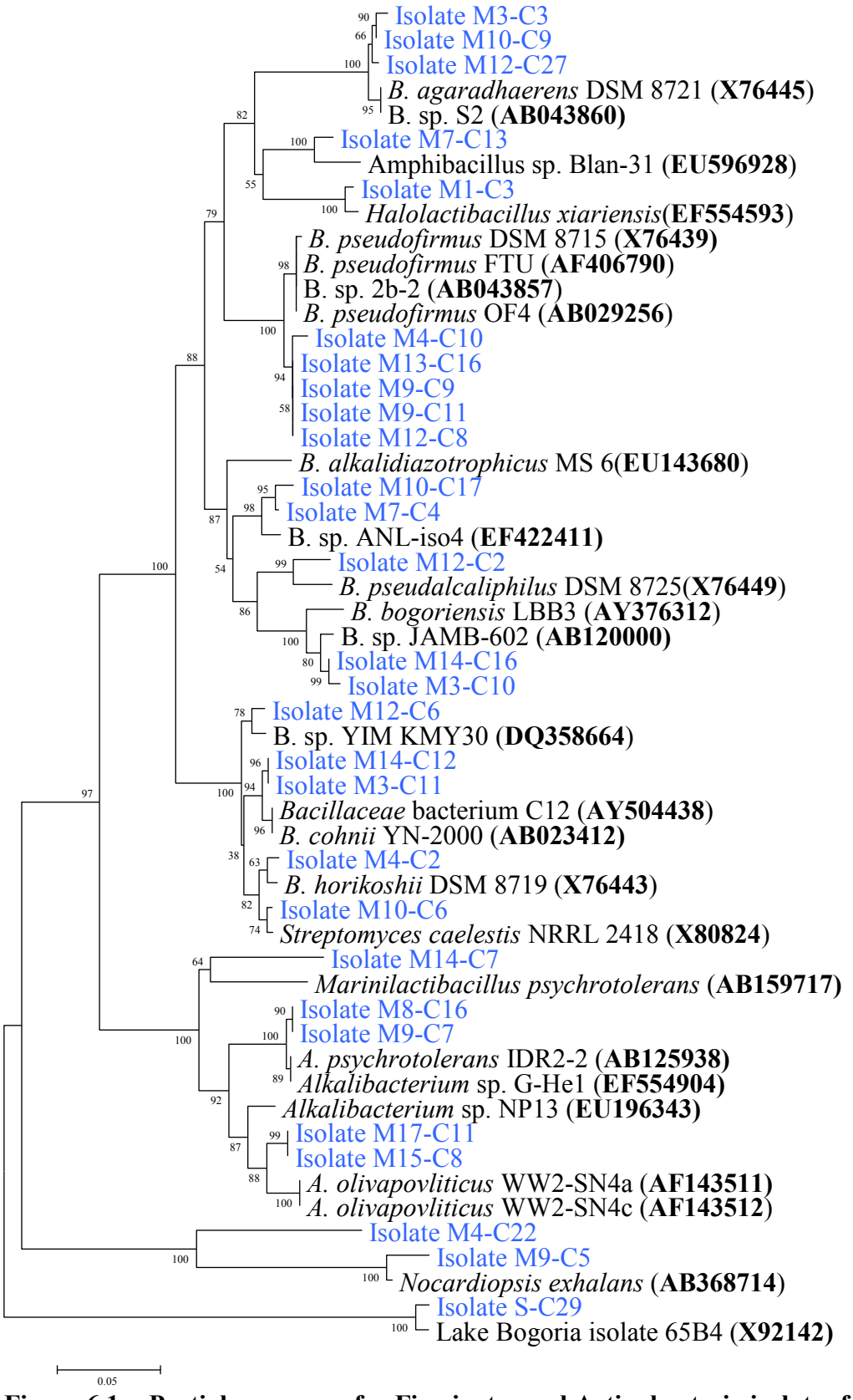

Figure 6.1a: Partial sequences for Firmicutes and Actinobacteria isolates from Lake Elmenteita

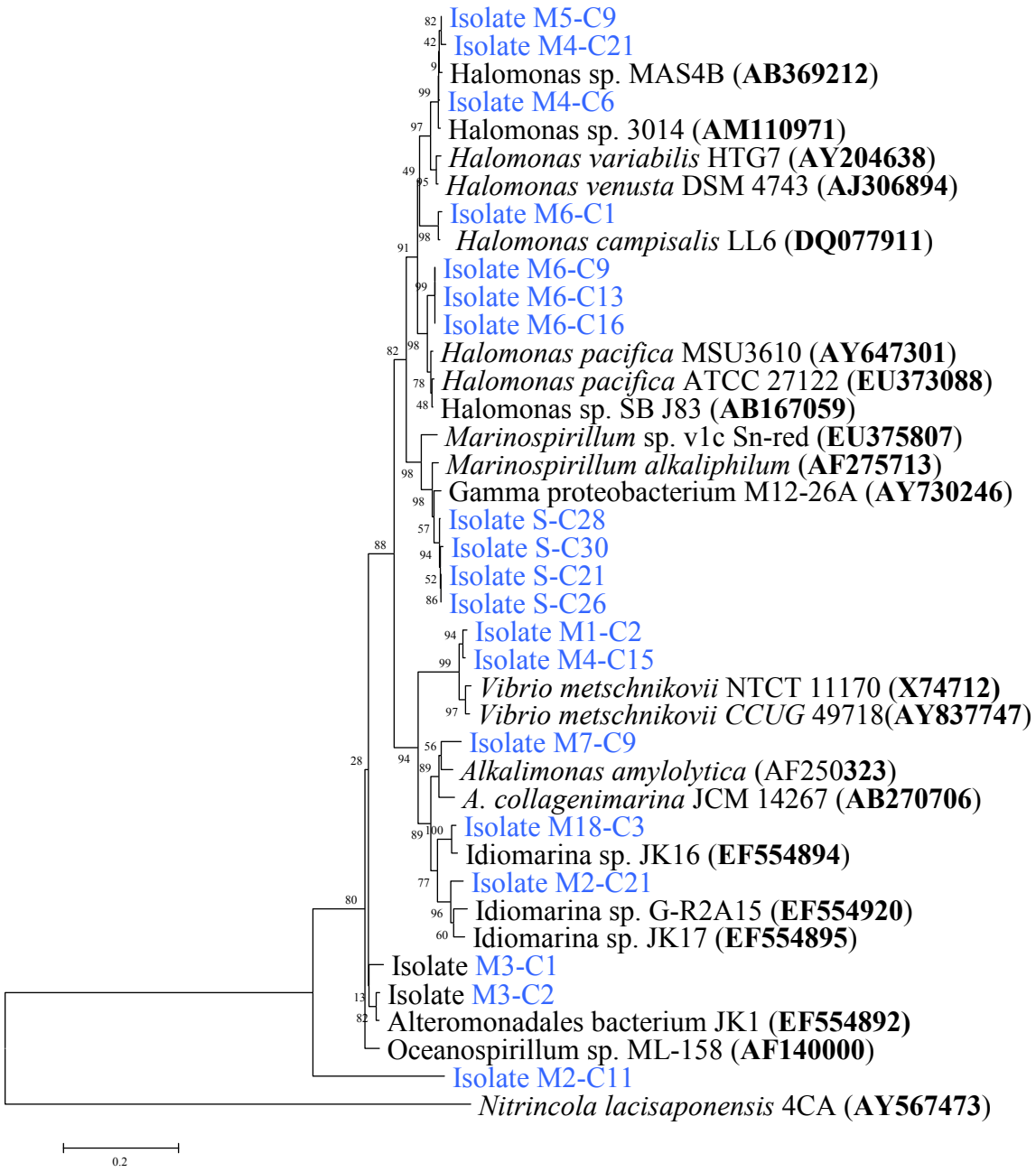

Figure 6.1b: Partial sequences for isolates in the *Gammaproteobacteria* from Lake Elmenteita

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Figure 6.1a and 6.1b show the phylogenetic affiliation of representative isolates to the closest relatives in the ARB database. The phylogenetic relationship is based on partial sequences generated using the sequencing primer 530r. The family *Halomonadaceae* belongs, together with the "*Alcanivoraceae*," and the "*Oceanospirillaceae*," to the "*Oceanospirillales*," an order within the Gammaproteobacteria (Garrity *et al.,* 2003) that consists mainly of marine species. In this study 68 isolates belonged to the Halomonas group whereas 20 isolates were affiliated to microorganisms belonging to the genus *Marinospirillum*. From the partial sequences analysis, fifteen isolates resembled *Idiomarina* species. A single isolate from the genus *Alkalimonas* was recovered and the almost complete sequence shows that the closest neighbour is *Alkalimonas delamerensis* (98%) which was also isolated from Lake Elmenteita.

Two isolates were affiliated to the family *Nocardiaceae* and one of the isolates was almost fully sequenced and was 99% similar to *Nocardiopsis exhalans*. A single isolate from the genus *Streptomyces* was recovered in this study. The almost complete sequence showed that it was 96% similar to *Streptomyces caelestis.* The unique feature of this strain is that it had an optimum growth temperature of 39^{o} C and an optimum pH of 8.4.

Two isolates from the Genus *Enterococcus* were recovered. The genus *Enterococcus* contains bacterial species associated with animals and plants. Currently there are 45 validly published species. A total of 58 isolates from the genus Bacillus were

recovered and this makes it the second most common group after the halomonads. In this study the seven isolates recovered were closely related to *Alkalibacterium olivoapovliticus* strain WW2-SN4a (AF143511) *A. olivoapovliticus* strain WW2-SN4c (AF143512), *A. psychrotolerans* (AB125938), Alkalibacterium sp. G-He1 (EF554904) and *Alkalibacterium* sp. NP13 (EU196343). One isolate from the genus *Amphibacillus* was recovered and it had a 97% similarity to *Amphibacillus sediminis*. A single isolate with 94% to *Marinilactibacillus piezzotolerans* was recovered. The Genus *Marinilactibacillus* (Ishikawa *et al.,* 2003) has only two described species and both are from the ocean. Five isolates from the genus Vibrio were recovered in this study and the closest Neighbour in BLAST search was *Vibrio metschinkovii*, isolated from the ocean.

Discussion

In this study a polyphasic approach was employed for the identification of various strains. The majority of the isolates were from the Gammaproteobacteria and the Bacilli group within the Firmicutes. This concurs with earlier reports that the majority of Gram-negative isolates and culture-independent bacterial clones retrieved from soda lakes belong to the class *Gammaproteobacteria* (Jones *et al.,* 1994, 1998; Ma *et al.,* 2004b), including strains related closely to typical aquatic bacteria such as *Aeromonas* and *Pseudomonas* (Duckworth *et al.,* 1996), moderate halophiles from the *Halomonas*/*Deleya* group, and marine bacteria, e.g. *Marinobacter* (Rees *et al.,* 2004).

The family *Halomonadaceae* was originally proposed by Franzmann *et al.* (1988) and it was later amended by Dobson and Franzmann (1996). A chemotaxonomic study of members of the family *Halomonadaceae* concluded that on the basis of respiratory quinone, polar lipid, and fatty acid compositions, no clear distinction existed at the genus level (Franzmann and Tindall, 1990). In this study, isolates from the genus *Halomonas* constituted 36.5 % of all the isolates. Species belonging to the family are chemoheterotrophic Gram-negative, straight or curved, rod-shaped bacteria which have been isolated from seawater, estuarine water, hypersaline soils, and bodies of hypersaline water, including Antarctic lakes, the Dead Sea, and several soda lakes of the Rift Valley, Kenya. They are aerobic, and some strains have the capacity for facultative anaerobic growth in the presence of nitrate. Members of *Halomonadaceae* have been shown to be of biotechnological importance in the production of compatible solutes as well as extracellular compounds such as exopolysaccharides and enzymes, and their use in environmental bioremediation processes.

Twenty isolates in this study were distantly affiliated to members of the genus *Marinospirillum* (Satomi *et al.,* 1998). All species that belong to this genus are helical, halophilic, Gram-negative, heterotrophic and motile by means of a single polar flagellum or bipolar flagella tuft and belong to the γ-subclass of the Proteobacteria. However the isolates retrieved here differed from the Genus *Marinospirillum* in the physiological, biochemical as well as the DNA G+C content and phylogenetically. This means they could represent a novel genus.

Fifteen isolates whose closest relatives as per BLAST analysis were from the genus *Idiomarina*. The genus *Idiomarina* was proposed by Ivanova *et al.* (2000a) and to date nine species are now validly published. *Idiomarina abyssalis* and *Idiomarina zobellii* (Ivanova *et al.,* 2000b) were isolated from water samples collected from a depth of 4000-5000 m in the north-western area of the Pacific Ocean. *Idiomarina baltica* (Brettar *et al.,* 2003) was isolated from the oxic water column of the Gotland Deep, a basin with anoxic deep water in the central Baltic Sea. *Idiomarina loihiensis* (Donachie *et al.,* 2003) was isolated from the Lo‘ihi submarine volcano, Hawaii at a depth of 1296 m while *Idiomarina fontislapidosi* was isolated from a soil sampled at Fuente de Piedra (Ma´laga, S. Spain), an inland, hypersaline wetland and *Idiomarina ramblicola* was isolated from a water sample taken in Rambla Salada, a hypersaline steep-sided river bed, normally dry but subject to flash flooding (Martı´nez-Ca´novas *et al.,* 2004). *Idiomarina seosinensis* (Choi & Cho, 2005) was isolated from hypersaline water of a solar saltern located in Seosin, in Korea while *Idiomarina salinarum* (Yoon *et al.,* 2007) was isolated from a marine solar saltern of the Yellow Sea in Korea.

Features that distinguish members of the genus *Idiomarina* from other marine bacteria are their high content of iso-branched fatty acids and their physiological properties, in particular their ability to grow within a broad range of temperatures, pH values and sodium chloride concentrations (Marti´nez-Ca´novas *et al.,* 2004). No species have been described so far from a soda lake environment. Although the isolates in this study

were affiliated to the Genus *Idiomarina* according to BLAST results, phylogenetically they form a distinct cluster hence they have been proposed to belong to a novel genus.

The members of the genus *Nocardia* are aerobic, Gram-positive, non-motile catalase positive actinomycetes that are typically acid alcohol-fast at some stages of the growth cycle. They form rudimentary to extensively branched substrate hyphae that often fragment in situ or, on mechanical disruption, disrupt into rod shaped to coccoid, nonmotile elements. The genus *Nocardia* presently includes 38 species, which form a well-delineated clade in the 16S rRNA *Corynebacterineae* gene tree. Nearly half of these species have been described within the last five years mainly to accommodate organisms isolated from clinical material (Gürtler *et al.,* 2001b; Hamid *et al.,*2001; Kageyama *et al.,*2003a; Kageyama *et al.,*2003b, Yassin *et al.,*2003) and environmental samples, notably soil (Albuquerque de Barros *et al.,*2003; Kämpfer *et al.,*2003; Saintpierre- Bonaccio *et al.,*2003; Zhang *et al.,*2004). The two isolates recovered in this study were identical phylogenetically though the morphology was different. However they could represent a new species from the soda lake environment. The closest neighbour in BLAST analysis was *Nocardiopsis exhalans* (Peltola *et al.,* 2001).

So far no isolate from the genus *Streptomyces* has been described from the East African soda lake environment. *Streptomycetes* are Gram-positive aerobic members of the order *Actinomycetales* within the class Actinobacteria (Stackebrandt *et al.,* 1997)

and have a DNA G+C content of 69 ± 78 mol%. The single isolate recovered in this study could represent a novel genus. However it is reported that Among 420 International Streptomyces Project (ISP) Streptomyces strains tested for their maximum growth pH, only six strains grew at pH 11.5. Of these six strains, three strains, *S. caeruleus* ISP 5103, *A. alborubidus* ISP 5465 and *S. autotrophicus* ISP 5011 contained meso-diaminopimelic acid (Kämpfer, 2006). Members of the family *Streptomycetaceae* are ubiquitous in nature. Only a few reports of alkaliphilic, acid-sensitive actinomycetes have been published (Mikami *et al.,* 1982; Mikami *et al.,* 1985). Members of the genus *Streptomyces* are involved in the biodegradation of various polymers abundant in soil owing to their ability to produce extracellular enzymes.

The *Enterococci* belong to the Firmicutes with low G+C content, the so-called clostridial branch. Two isolates were recovered in this study. The *Enterococci* are most often considered as components of the intestinal flora of humans and animals acting as opportunistic pathogens in different extra-intestinal compartments of the body. Phylogenetically the closest relative of the enterococci, but well separated from the latter, is the genus *Vagococcus* and next *Carnobacterium, Tetragenococcus, Aerococcus, Alloiococcus, Dolosigranulum, Facklamia, Globicatella* and *Abiotrophia* (Collins *et al.,* 1997). Others, notably the plant-associated yellow-pigmented *E. casseliflavus* and *E. mundtii* (Collins *et al.,* 1986; Vaughn *et al.,* 1979) may occur transiently in the intestines.

Bacillus species are Gram-positive, rod-shaped, endospore-forming bacteria with an aerobic or facultatively anaerobic metabolism and among the most commonly found aerobic, eubacterial alkaliphiles both in soda lakes and in less selective environments (Horikoshi and Akiba, 1982; Krulwich and Guffanti, 1983; Guffanti *et al.,* 1980; Guffanti *et al.,* 1986; Takami *et al.,* 1999). The *Bacilli* were the second group after the *Halomonas* in terms of diversity. A total of 58 isolates were retrieved. The *Bacilli* are grouped into two clusters of alkaliphiles and alkaline-tolerant isolates based on physiological and biochemical characteristics as well as DNA base composition, hybridization, and 16S rDNA analyses (Fritze *et al.,* 1990, Nielsen *et al.,* 1994; Nielsen *et al.,* 1995). Analysis of the 16S rDNA structure has shown that there are two distinct groups within the bacilli (Nielsen *et al.,* 1994; Jones *et al.,* 1998). *Bacillus alcalophilus* and associated strains are mainly found in mud at the shoreline or dry regions of soda soil where organisms are subjected to fluctuating water levels and concomitant fluctuations in pH and salt levels. Many of these strains require only low concentrations of Na+ for growth. The bacilli related to *Bacillus clarkii* (Nielsen *et al.,*1994; Nielsen *et al.,*1995), are thought to be more prevalent in sediments and waters that are subject to less variability and these alkaliphiles typically exhibit requirements for higher Na+ for growth (Jones *et al.,*1998).

The genus *Alkalimonas* was first proposed by (2004a) to describe two novel Alkaliphilic microorganisms described from Kenyan and Mongolian soda lakes respectively. A single isolate from the genus *Alkalimonas* was isolated and it is closely

related to *Alkalimonas delamerensis* isolated from the same lake (Ma *et al.,* 2004a). Members of the genus exhibit a superficial resemblance to representatives of the genera *Pseudoalteromonas* (Gauthier *et al.,* 1995), *Marinobacter* (Gauthier *et al.,* 1992) and *Marinobacterium* (Gonzalez *et al.,* 1997) of the family *Alteromonadaceae*, and the genus *Marinomonas* (Gauthier and Breittmayer, 1992) of the *Oceanospirillum* group. The strains can also be readily distinguished from close phylogenetic relatives by being alkaliphilic and halophilic meaning they are well suited for survival in Soda Lake conditions (Ma *et al.,* 2004b).

Member of the genus *Alkalibacterium* are found in the soda lake and in this study seven isolates were recovered. An isolate designated as WN16 from Lake Nakuru was isolated in a previous study by Duckworth *et al.,* (1996). Isolates were recovered from the other Kenyan soda lakes namely Crater Lake Sonachi, Lake Bogoria and Lake Nakuru (This study).

The genus *Amphibacillus* was first proposed by Niimura *et al.,* (1990) and the genus currently comprises four recognized species, *Amphibacillus xylanus* (Niimura *et al.,*1990), *Amphibacillus fermentum* and *Amphibacillus tropicus* (Zhilina *et al.,*2001) and *Amphibacillus sediminis* (Sun-Young *et al.,*2007). Members of the genus Amphibacillus are moderately alkaliphilic and facultatively anaerobic (Zhilina *et al.,* 2001) and lack isoprenoid quinones and oxidase activity (Niimura *et al.,* 1990). The strains are capable of both anaerobic growth (at the expense of fermentation of glucose

and certain mono- and disaccharides with the formation of formate, ethanol, and acetate) and aerobic growth. The single isolates recovered in this study could represent a new species within the genus. A single isolate was recovered that was 94% similarity value to *Marinilactibacillus piezzotolerans*. The Genus has only two described species and both are from the ocean. Isolation and taxonomic studies of lactic acid bacteria from marine environments to date are few and have generally been confined to those from cultured fish (Ringø and Gatesoupe, 1998; Gatesoupe, 1999). Therefore the new species could represent a novel genus of lactic acid bacteria from the soda lake.

The name *Vibrionaceae* was formally proposed (Véron, 1965) as a convenient grouping for fermentative bacteria that have polar flagella and a positive oxidase reaction. Five isolates were recovered in this study and the closest Neighbour in BLAST search was *Vibrio metschinkovii*, isolated from the ocean. Species of the family *Vibrionaceae* occupy many different ecological niches (Campbell, 1957; Baumann and Baumann, 1981; Sakazaki and Balows, 1981; Simidu and Tsukamoto, 1985). They cause disease of humans and animals and also occur in the environment. Many factors probably govern their distribution, but four of the most important are: the particular animal or plant hosts, temperature, salinity, and depth below the surface for the species that are found in the ocean (Simidu and Tsukamoto, 1985).

6.5 Conclusion

The generic affiliation of cultured microbes reflects the sampling methods and culture conditions used in the isolation process and this has a major effect on the range of

types encountered in the laboratory. Those methods that match most closely the physiology of members of a microbial community will be enriched and subsequently isolated. In this study the media used for enrichment and isolation was from one poor in nutrients to one rich in nutrients. Some of the media used also targeted detection of useful extracellular enzymes produced by the alkaliphiles. We here note that using this approach, novel microorganisms not isolated before were retrieved.

CHAPTER SEVEN

7.0 Description of new species belonging to *Salinospina* gen. nov. a group of novel haloalkaliphilic members of the Gammaproteobacteria.

ABSTRACT

Five bacterial strains designated as E–292, E-408, E–433, E–436, E–438 were isolated from Lake Elmenteita, an alkaline saline lake within the East African Rift Valley. The strains are alkaliphilic and differ from the closest relatives in their morphology, substrate utilization, temperature, pH, and salt tolerance. The isolates do not require growth factors but NaCl is required for growth. Growth occurs at a pH range between 5.5 and 12.0 with the optimum lying between 9.0 and 10.5. Salt is required for optimum growth between 5 and 10%. Temperature range for growth is from 15.8 – 43.5°C, with optimum growth occurring between 30 and 38 °C. No growth was detected at below 15.8 °C. Major cellular fatty acids are the 16:1 ω7c/15 iso 2OH, 16:0, and the 18:1 ω7c. The major isoprenoid quinone is Ubiquinone (Q-8). The strains have a DNA G+C content between 53.1 – 53.5 mol %. The 16S rRNA gene sequences analysis shows they form a distinct phylogenetic cluster. The isolates represent novel haloalkaliphilic members of the Gamma Proteobacteria. Based on physiological, biochemical and chemotaxonomic traits and comparative 16S rRNA gene sequence analysis, it is proposed that the isolates represent a novel genus for which the name *Salinospina* gen nov. is proposed.

7.0 Introduction.

The genus *Marinospirillum* was created to accommodate *Oceanospirillum minutulum*, originally classified as *Spirillum minutulum*, and a new isolate *Marinospirillum megaterium* (Watanabe, 1959; Satomi *et al.,* 1998). The genus *Spirillum* could be differentiated from the *Oceanospirillum* based on Phenetic characteristics (Terasaki, 1972; Carney *et al.,* 1975), DNA – rRNA hybridization (Pot *et al.,* 1989), fatty acid composition (Sakane and Yokota, 1994) and on the basis of the 16S rRNA gene sequence as the type species of a new genus, *Marinospirillum* along with the new isolate *M. megaterium* (Satomi *et al.,* 1998). Among the four validly species in the genus, only *Marinospirillum alkaliphilum* is from an alkaline soda lake in China (Zhang *et al.,* 2002). All species that belong to this genus are helical, halophilic, Gram-negative, heterotrophic and motile by means of a single polar flagellum or bipolar flagella tuft and belong to the γ-subclass of the Proteobacteria. In this study five isolates distantly related to *Marinospirillum alkaliphilum* are described. *Marinospirillum minutulum* (DSM 6287), *Marinospirillum alkaliphilum* (DSM 21637) and *Marinospirillum insulare* (LMG 28102) were used as reference strains in this study.

7.1 Materials and Methods

7.1.1 Sampling and isolation.

The isolates described here were recovered during microbial diversity studies on Lake Elmenteita. The lake is situated 0°27’ S, 36°15’ E on the floor of the East African rift valley at 1776 m above sea level. At the time of sampling the water surface

temperature was recorded as 28.3^{o} C and the ambient temperature was 31^{o} C. Unless otherwise stated, enrichment and isolation of the strains was done on different media prepared using filter sterilized Lake water. Solid media was prepared by addition of 15g/l Agar and all the media were supplemented with 100mg/l Cycloheximide to inhibit the growth of Fungi. The strains were easily identified from the other microbial flora due to their swarming morphology on solid media. The stains were purified on Dilute Tryptic Soy Broth supplemented with 15g Bacto Agar (Difco), 3.5% NaCl and 1% $NaCO_3$. The strains were stocked in Tryptic soy Broth supplemented with 3.5% NaCl, 1% $NaCO_3$ and 20 % (v/v) glycerol.

7.1.2 Phenotypic characterization

Standard methods for characterization were performed to determine the closeness or differences between the strains and the type strains. The following physiological and biochemical properties were examined: oxidation/ fermentation of glucose; Arginine dihydrolysis; Tyrosine decarboxylation, cell pigmentation and cell morphology. The strains were tested for their ability to hydrolyze gelatine, DNase activity, starch utilization and Tween-80. Chitinase activity was tested by use of 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide. Motility was tested with cells from 2-day-old liquid cultures on soft agar incubated for 48hrs at 28^{o} C. Gram-stain was done using the aminopeptidase and KOH reactions, catalase activity was tested using H_2O_2 Cytochrome c oxidase was determined by adding a few drops of Tetramethyl-phenylenediamine solution to a 2-day-old slant of each strain.

Utilization of glucose, sucrose, fructose, lactose and mannitol as a sole carbon source was performed on a basal media containing per litre 1g yeast extract (Difco), 1g KH_2PO_4, 0.1g $MgSO_4.7H_2O$,0.05g $CaCl.2H_2O$, 4% NaCl and 1% $NaCO_3$ and the respective sugar to a final concentration of 1%. Salt tolerance tests were done on Diluted nutrient broth (Difco) supplemented with 1% $NaCO_3$ and the salt concentration was varied from 0% to 20%. The optimum temperature for growth was determined on trypticase soy broth containing 4% (w/v) NaCl and 1% $NaCO_3$ using a temperature gradient incubator (model TN-3 Toyo Kagaku Sangyo) with the lowest value of 9.4^{o} and the highest 49.3^{o} C . The optical density was recorded after 18 hrs of growth using a Spectrophotometer at 620 nm. The pH range for growth was determined in dilute nutrient Broth with the pH adjusted using phosphate Buffer and a salt concentration of 4%. To test for the pH optima, pH values of separate batches of media raged from 6.0 to 13.

The ability to oxidize or utilize organic substrates was also investigated using BIOLOG-GN plates as recommended by the manufacturer except that the optical density was adjusted to a transmittance of 52 in 0.4 M NaCl solution. Microplates were inoculated with 150µl cell suspension per well for each strain and incubated at 28 °C. The results were read using the BIOLOG Microplate reader after incubation for 6hrs, 24hrs, 48hrs, and 72 hrs and after 5 days of growth. Carbon assimilation tests were also determined using the commercial API 20E and API 50 CH systems (BioMe´rieux). Tests were read after 6hrs, 24, 48, and 72hrs and after 5 days of

growth. Electron micrographs of negatively stained cells, using 1.25 % uranyl acetate, were obtained using a Zeiss EM 10 CA electron microscope (80 kV).

7.1.3 Cellular fatty acid analysis

The strains and the reference strains were subjected to fatty acid methyl ester analysis to confirm membership to the genus. Cultures of all strains were grown on the modified trypticase soy broth (TSB; Difco) 28°C for 48h. *Marinospirillum minutulum* and *Marinospirillum insulare* could not grow in the alkaline media hence were grown on marine broth (Difco). The cell mass was harvested from the broth cultures via centrifugation. Saponification, methylation and extraction were done as recommended by the Sherlock Microbial Identification System (MIDI; http://www.midi-inc.com). The samples were analyzed on an Agilent Technologies 6890N gas chromatograph. The data were analyzed for taxonomic information by the TSBA40 and TSBA50 method of the Sherlock MIS software. The individual fatty acids were expressed as percentages of the total Fatty Acids.

7.1.4 Respiratory Quinone analysis

Respiratory lipoquinones and polar lipids were extracted from freeze-dried cell material (100 mg) using a two-stage method (Tindall, 1990a, b) and separated into their structural classes (menaquinones and ubiquinones) by thin layer chromatography. Lipoquinones were separated by TLC and UV-absorbing bands were removed from the plate and analysed further by reverse-phase HPLC. Polar lipids were separated by two-dimensional, silica-gel TLC; total lipid material and specific functional groups

were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate–Schiff (α-glycols), Dragendorff (quaternary nitrogen) and anisaldehyde–sulfuric acid (glycolipids) as described previously (Tindall, 1990a, b). The mol% G + C content of the strains was determined as described Mesbah *et al.,* (1989).

7.1.4 PCR amplification and sequencing

DNA extraction was carried out using the SEQLAB Bacteria-DNA-Kit and PCR amplification of the 16S rRNA genes was carried out at SeqLab (Göttingen, Germany). The sequences were assembled with the Sequencer software, version 4.1 for Macintosh (Genes Codes, Ann Arbor, MI). The sequences were automatically aligned according to the SILVA database release 94 (Ribocon, Bremen, Germany) using the FAST aligner tool of the ARB program (Ludwig *et al.,* 2004). Phylogenetic relationship was determined using neighbour-joining (Felsenstein, 1993) and maximum-likelihood analyses (Olsen *et al.,* 1994). The resultant tree topologies were evaluated in bootstrap analyses (Felsenstein, 1985) of the Neighbour joining method based on 1000 resamplings. Phylogenetic analyses were conducted in MEGA 4 (Tamura *et al.,* 2007). The sequences were deposited under the accession numbers FJ764782, FJ764783, FJ764784, FJ764785 and FJ764786

7.2 RESULTS

7.2.1 Phenotypic characterization of isolates

Phenotypic characterization shows that the cells were Gram-negative, aerobic, oxidase and catalase positive and non-spore forming helical rods. The cells were about 5 µm in length and motility was via bipolar tufts of flagella. In liquid broth the cells were highly motile. When grown on solid media the strains formed colonies that were translucent but colour changed to ivory with aging. On solid media the colonies were flat, irregular shaped and there was rapid swarming when streaked or point-inoculated on freshly prepared solid media. Organic factors such as vitamins were not required for growth and the pH range for growth was noted to be between 6.5 and 11.0 with the optimum lying between 9.0 and 11.0. Growth was recorded in media with a salt concentration between 0 and 20%. The salt optimum was between 6 and 8%. Temperature for growth was from 19.8 – 46.1°C, with optimum growth occurring between 28.1 and 34.7°C and no growth was detected below 15.8°C. All the strains were able to hydrolyse gelatine. All the strains were positive for dextrin and acetic acid on the BIOLOG GN plate; Arbutin, Esculin, D-Turanose, Potassium-5-ketogluconate on the API CH system and on the API 20E gelatinase and Indole were produced. Casein was not hydrolyzed; DNase and Tyrosine decarboxylase were absent. Starch, Cellulose, Xanthan, Xylan and Lipids were not utilized. The major isoprenoid quinone was Ubiquinone (Q-8). Table 7.1 below shows the results of selected Biochemical tests differentiating member of the Genus *Salinospina* from one another and from Members of the Genus *Marinospirillum*.

Table 7.1 Selected phenotypic characteristics differentiating the isolates in the Genus *Salinospina* from one another and from *Marinospirillum minutulum* (DSM 6287), *Marinospirillum* alkaliphilum (DSM 21637) and *Marinospirillum insulare* (LMG 28102). ± Data on *Marinospirillum minutulum* and *Marinospirillum alkaliphilum (*Zhang *et al.,* 2002) and *Marinospirillum insulare* Satomi *et al.,* 2004).

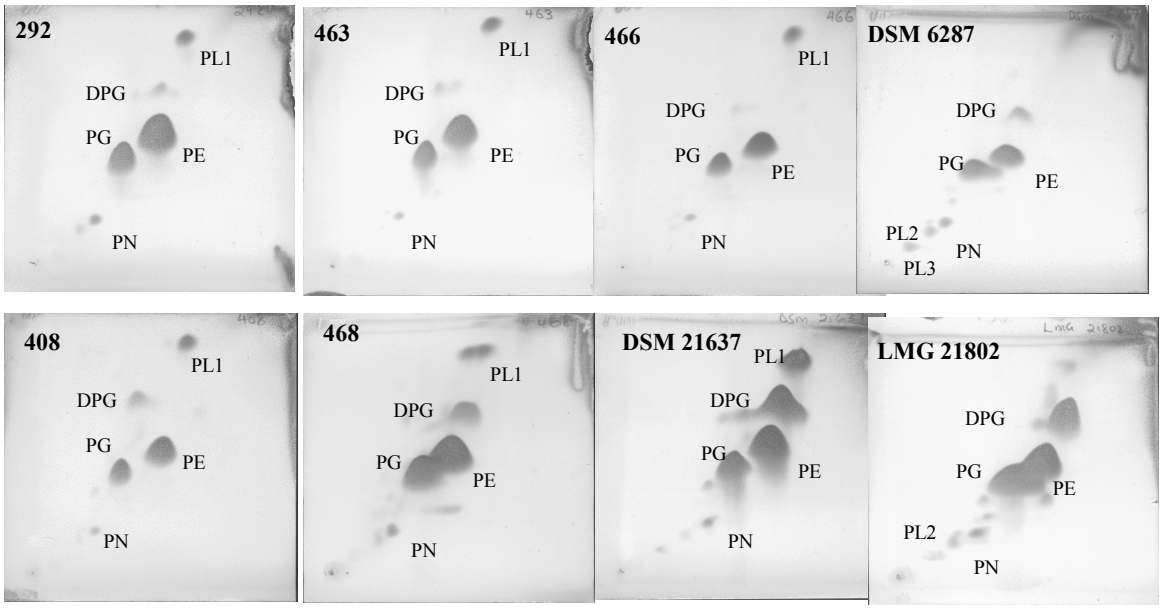
		E-292	**E-408**	**E-463**	**E-466**	**E-468**	**DSM 21637±**	**DSM 6287±**	**LMG 21802±**
Oxygen requirement		Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Catalase		+	+	+	+	+	+	+	+
Nitrate Reduction		-	-	-	-	-	+	-	+
pH Tolerance	Range	6.0 - 11.5	6.0 - 11.5	7.0 - 11.0	6.0 - 11.0	7.5 - 11	7.0 - 11	7.0 - 10.5	6.5 – 10.0
	Optimum	10.5	9.5	11.0	9.5	10.5	9.5	9.0	7.5 – 8.0
Salt Tolerance	Range	0 - 20%	0 - 8%	0 - 10%	0 - 12%	0 - 16%	0.2 - 5.0	0.2 - 8.0	0.5 – 10.0
	Optimum	6	7	8	8	8	2.0	ND	2.0 – 3.0
Temperat.	Range	19.8 - 42.0	19.8 - 42.0	15.8 - 40.4	22.3 - 46.1	23.1 - 45.2	8.0 - 49	11.0 - 37.0	4.0 - 37.0
	Optim.	29.8	29.8	28.1	34.7	33.1	37.0	30.0	25–30
Quinone		Q8	Q8	Q8	Q8	Q8	Q8	Q8	Q8
GC Content		53.1	53.5	53.2	53.3	53.3	46.8	42.5	42.1

Table 7.2: Biochemical features differentiating putative species of *Salinospina* gen. nov. from two comparative species in the genus Marinospirillum: *Marinospirillum minutulum* DSM 6287 (Terasaki, 1972), *Marinospirillum alkaliphilum* DSM 21637 (Zhang *et al.,* 2002). + means positive, - for negative and W for weak

	Test Substrate	E-292	E-408	E-463	E-466	E-468	DSM 21637	DSM 6287
BIOLOG GN	Dextrin	+	+	+	+	+	+	+
	Glycogen	-	-	-	-	-	-	**W**
	Tween 40	+	-	+	+	+	+	-
	Tween 80	+	-	+	-	+	-	-
	N-Acetyl-D-Galactosamine	-	-	-	-	+	-	-
	L-Arabinose	-	-	-	-	-	+	**W**
	Pyruvic Acid Methyl Ester	-	-	-	-	-	-	+
	Succinic Acid Methyl-Ester	-	-	-	-	-	-	+
	Acetic Acid	+	+	+	+	+	-	+
	Cis-Aconitic Acid	-	-	-	-	-	-	**W**
	Citric Acid	+	-	-	-	-	-	
	α-Hydroxybutyric Acid	-	-	-	-	-	-	+
	β-Hydroxybutyric Acid	-	-	-	-	-	-	+
	α-Keto Butyric Acid	-	-	-	-	-	-	+
	α-Keto Glutaric Acid	+	-	+	+	+	-	+
	α-Keto Valeric Acid	-	-	-	-	-	-	+
	D,L-Lactic Acid	+	-	-	-	-	-	+
	Propionic Acid	-	-	-	-	-	-	**W**

	Succinic Acid	+	-	-	-	-	-	**W**
	Bromosuccinic Acid	+	-	-	-	-	-	**W**
	Succinamic Acid	-	-	-	-	-	-	**W**
	D-Alanine	+	-	-	-	-	-	**W**
	L-Alanine	+	-	-	-	-	-	**W**
	L-Asparagine	+	-	-	-	-	-	
	L-Glutamic Acid	+	-	-	-	-	-	**W**
	Hydroxy-LProline	-	-	-	-	-	-	**W**
	L-Proline	+	-	+	+	-	-	
	L-Threonine	-	-	-	-	-	-	**W**
	N-AcetylGlucosamine	-	-	-	-	-	-	+
	Amygdalin	-	-	-	-	-	-	+
	Arbutin	+	+	+	+	+	+	+
	Esculin/ferric citrate	+	+	+	+	+	+	+
API 50CH	D-Cellobiose	-	-	**W**	-	-	-	-
	D-Maltose	-	-	-	+	-	-	
	D-Turanose	+	+	+	+	+	+	+
	potassium 5-KetoGluconate	+	+	+	+	+	-	-
API 20E	Indole production	+	+	+	+	+	+	+
	Gelatinase	+	+	+	+	+	-	-

Plate 7.1 shows the polar lipid profiles of the strains described in this study in comparison to three type strains belonging to the genus Marinospirillum.



Marinospirillum

DPG = Diphosphatidylglycerol
PG = Phosphatidylglycerol
PE = Phosphatidylethanolamine
PL = Phospholipid
PN = Aminophospholipid
PL1-PL3 = Phospholipids

Plate 7.1 Polar lipid profiles of strains E -292, E – 408, E – 463, E – 466, E – 468 and three reference strains; *Marinospirillum minutulum* (DSM 6287), *Marinospirillum* alkaliphilum (DSM 21637) and *Marinospirillum insulare* (LMG 28102) after separation by TLC.

Table 7.3 shows the fatty acid profiles are expressed as percentages. Note that strain DSM 21637 is the closest in terms of phylogenetic relationship. However there are salient differences between the strains described here and the comparative strains. This shows that the strains described here are a different phylogenetic group from the *Marinospirillum*

Table 7.3 Fatty Acid Profiles of the strains isolated in this study and the *Marinospirilla.* Strains: E -292; E – 408; E – 463; E – 466; E – 468; *Marinospirillum alkaliphilum* (DSM 21637); *Marinospirillum minutulum* (DSM 6287); and *Marinospirillum insulare* (LMG 28102).

	E -292	**E-408**	**E-463**	**E-466**	**E-468**	**DSM 21637**	**DSM 6287**	**LMG 21802**
12:0	0.75	1.90	1.82	0.75	1.16	-	4.18	8.34
12:1 3OH	-	-	-	-	-	1.54	-	0.12
12:0 30H	3.01	3.23	2.76	2.87	2.60	1.17	4.94	8.38
14:0	0.86	1.55	1.37	0.84	-	-	-	3.89
14:0 20H	-	-	-	-	-	-	-	1.06
14:0 30H/16:1 ISO I	2.84	2.46	2.54	2.91	2.45	-	-	0.19
16:1 ω7c/15 ISO 2OH	12.64	21.79	20.62	10.82	18.31	21.95	26.23	26.18
16:0	23.68	27.94	27.54	22.65	24.85	27.19	32.30	27.03
17:0	1.45	0.55	1.20	3.58	1.93	-	-	0.19
18:1 ω9c	-	-	-	-	-	-	3.08	0.48
18:1 ω7c	50.56	37.43	38.03	51.34	43.64	41.20	26.90	21.69

Plate 7.2 below shows the relative size of *Marinospirillum alkaliphilum* (DSM 21637) in relation to three isolates in the genus *Salinospina*. The former cells are much smaller in size and thinner.

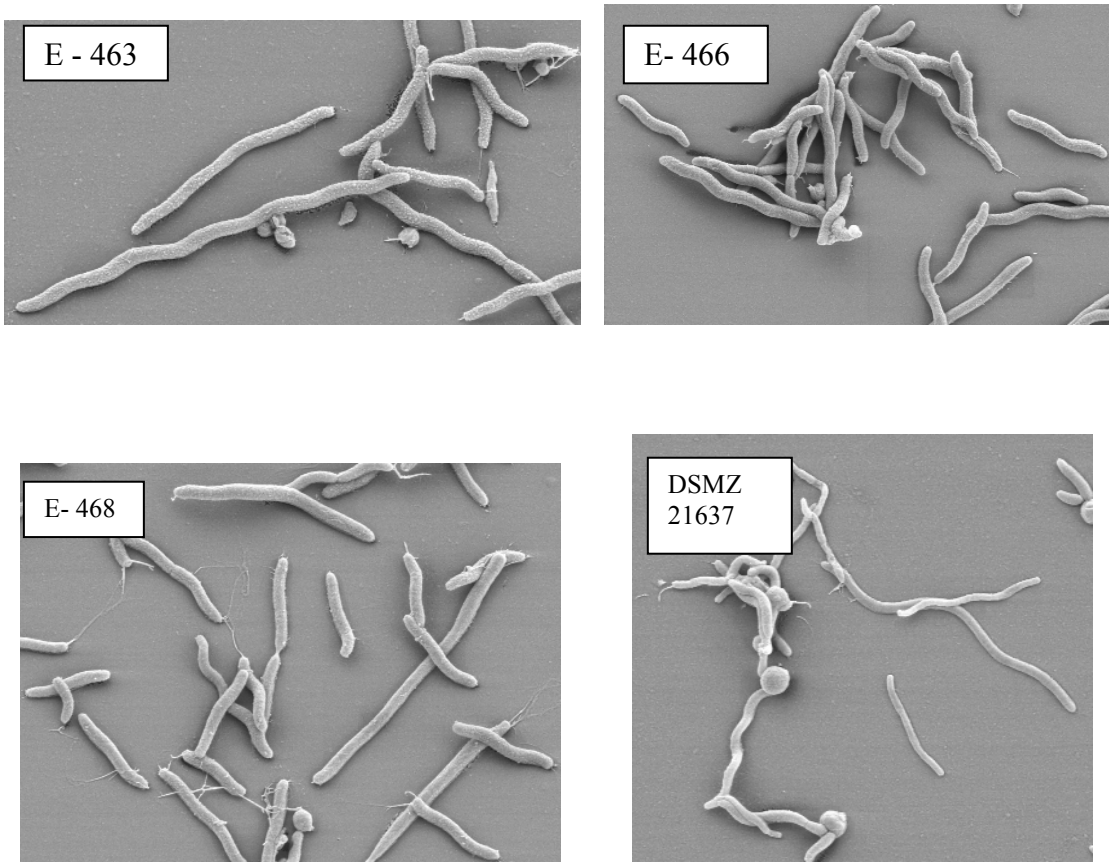


Plate 7.2: Electromicrographs (×5000) of 3 isolates and a comparative strain DSMZ 21737

7.2.2 Phylogenetic analysis

Phylogenetic analysis showed that strains: E-292; E–408; E–463; and E–468; had 97% sequence similarity with the 16S rRNA of members of the Genus *Marinospirillum alkaliphilum* whereas Strain E–466 had 98% sequence similarity to *Marinospirillum alkaliphilum*. Figure 7.1 shows the phylogenetic relationship of the strains described here in relation to the *Marinospirilla*.

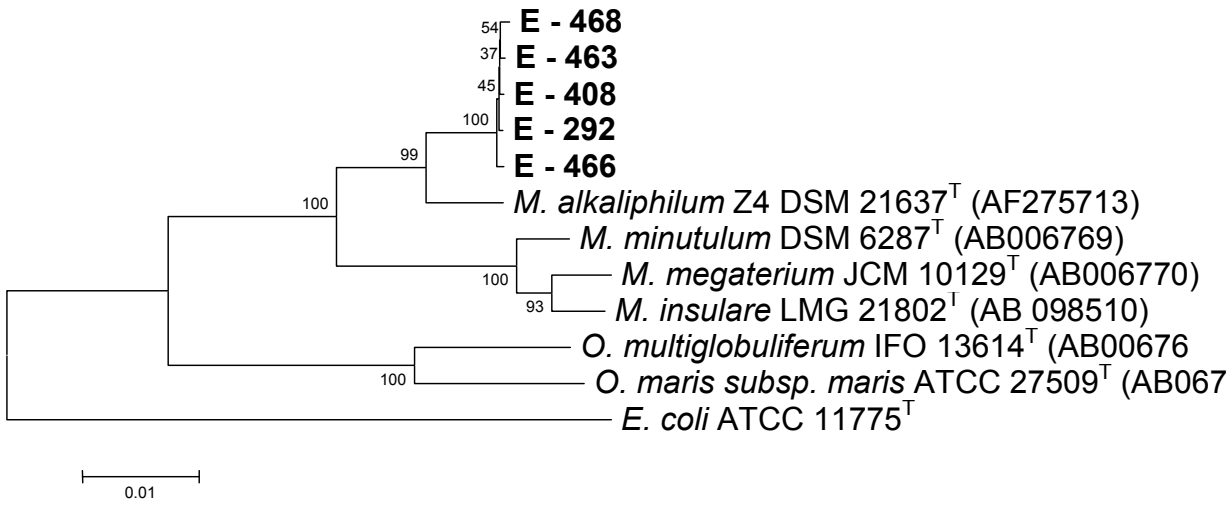


Figure 7.1: Neighbour-joining tree showing the position of the strains described in this study and the closest neighbours on the basis of 16S rRNA gene sequences. Numbers at branch points indicate bootstrap confidence values (%) of 1000 resamplings. Bar shows 0.01 substitutions per nucleotide position.

7.3 Discussion

The genus *Marinospirillum* was established in 1998 (Satomi *et al.,* 1998) to accommodate helical spirilla that were previously assigned to the genus *Oceanospirillum* on the basis of phenotypic characteristics (Krieg, 1984). Amongst all the four validly published species, only *Marinospirillum alkaliphilum* was isolated from an alkaline soda lake in China (Zhang *et al.,* 2002). The five strains described here form a distinct phylogenetic group that can be recognized as a novel taxon within the gamma-3 subclass of the Proteobacteria. The percentage 16s rRNA similarity values (to *Marinospirillum*) were 97 % except for E-466 which had a value of 98%. Physiological and chemotaxonomic differentiate the new strains from *Marinospirillum* (Table 7.1). None of the strains described here could reduce nitrate unlike the closest relative, *Marinospirillum alkaliphilum*. *Marinospirillum alkaliphilum* could however not liquefy gelatine. The new strains grow optimally in the presence of sodium ions; have a higher minimum temperature for growth and higher salt tolerance which probably reflects the particular conditions of the soda lake environment that they inhabit. Optimum growth was realized at a salt concentration between 6 and 8%. This coupled with the high pH for growth indicated that the strains described in this study are haloalkaliphilic. Furthermore the new strains can be readily distinguished from close phylogenetic relatives by the higher G+C content. Besides the G+C content of *Marinospirillum* is much lower (46.8%) as compared to the strains described in this study (Table 7.1). The physiological data clearly show that the new strains are adapted to soda lake conditions. Based on physiological, biochemical and chemotaxonomic

traits and comparative 16S rRNA gene sequence analysis, it is demonstrated that the isolates represents a novel Genus within the Gamma proteobacteria, for which the name *Salinospina* sp. nov. is proposed. The type strain is *Salinospina alkaliphilus.*

N.L. adj. *salinus*, saline; L. fem. n. *spina*, spine; N.L. fem. n. *Salinospina*, a salt (loving) spine (long thin rod).

7.4 Description of the Genus *Salinospina*

The cells are Gram-negative, aerobic, oxidase and catalase positive and non-spore forming helical rods. The cells are about 5 µm in length. Motility is via bipolar tufts of Flagella. Colonies are clear but colour changes to ivory with aging. When grown on solid media the colonies are flat, irregular shaped and swarms when streaked or point-inoculated on freshly prepared solid media. Organic factors are not required for growth and strains grow at a pH range between 6.5 and 11.0 with the optimum lying between 9.0 and 11.0. Salt is required for optimum growth between 6 and 18%. Temperature for growth ranges from 19.8–46.1°C, with optimum growth occurring between 28.1 and 34.7°C. No growth was detected at below 15.8 °C. Gelatinase is present. All the isolates utilize dextrin and acetic acid on the BIOLOG GN plate; Arbutin, Esculin, D-Turanose, Potassium-5-ketogluconate on the API CH system on the API 20E Gelatinase and Indole are produced. Casein is not hydrolyzed; DNase and Tyrosine decarboxylase are absent. Starch, Cellulose, Xanthan, Xylan and Lipids are not utilized. The major isoprenoid quinone is Ubiquinone (Q-8). Major features differentiating the strains from the *Marinospirillum alkaliphilum* are that members of

the *Salinospina* have low levels of Diphosphatidylglycerol (Plate 7.1), cannot utilize L-Arabinose, but have ability to utilize acetic acid and gelatine. Unlike the *Marinospirilla*, members of the novel genus *Salinospina* have high tolerance to salt and a much higher G+C content value. The major cellular fatty acids common in the Genus *Salinospina* are 16:1 ω7c/15 ISO 2OH, 16:0, and the 18:1 ω7c The strain also has low levels of 14:0 30H/16:1 ISO I and 12:1 3OH is lacking. The type strain is *Salinospina alkaliphilus*

7.5 Description of *Salinospina alkaliphila* sp. nov. (Strain E-468)

In addition to the description given above for the genus, this species grows at pH between 7.5 – 11 and an optimum pH of 10.5 and the optimum growth was observed at a salt concentration of 8%. Temperature for growth ranges from 23.1–45.2 °C and an optimum of 33.1°C. The following substrates gave positive results on BIOLOG GN plates: Dextrin, Tween 40, Tween 80, Acetic Acid and α-Keto Glutaric Acid. D-Alanine, L-Alanine, L-Glutamic Acid, maltose and all the other substrates are not utilized. Arbutin, Esculin/ferric citrate, D-Turanose, potassium 5-KetoGluconate gave a positive result on API 20NE whereas Indole production and Gelatine liquefaction were positive on API 20E. The strain had a DNA G+C content of 53.3 mol%. The proposed species name is *Salinospina alkaliphilum* Salient feature differentiating this species from the other species in the genus is the ability to utilize N-Acetyl-D-Galactosamine, inability to utilize L-Proline and growth above a pH of 7.5.

7.6 Description of *Salinospina citratiphilum* (strain E-292)

In addition to the description given above for the genus, this species grows at pH between 6 – 11 and an optimum pH of 10.5 and the optimum growth was observed at a salt concentration of 6%. Temperature for growth ranges from 16 – 43.5 °C and an optimum of 29.8° C. Dextrin, Tween 40, Tween 80, Acetic Acid , Citric Acid, α-Keto Glutaric Acid, D-Lactic acid, Succinic Acid, Bromosuccinic Acid, D-Alanine, L-Alanine, Asparagine, L-Glutamic Acid, L- Proline are utilized on BIOLOG GN plates and all the other substrates are not utilized. Arbutin, Esculin/ferric citrate, D-Turanose and potassium 5-KetoGluconate were positive on API20 NE. Tests for Indole and Gelatinase were positive on API 20E. The strain had a DNA G+C content of 53.1 mol%. Features that differentiate *Salinospina citratiphilum* from the other strains are the ability to utilize Citric Acid, D-Lactic acid, Succinic acid, Bromosuccinic acid, D-Alanine, L-Alanine, Asparagine, L-Glutamic Acid and L- Proline. The strain also has a lower optimum salt concentration of 6%.

7.7 Description of *Salinospina mwathaensis* sp. nov. (Strain E-408)

Isolate E-408 grows at pH between 6.0 – 11.5 and an optimum pH of 9.5, salt concentration of 7% and at a temperature of 29.8 °C. Only Acetic acid and Dextrin are utilized according to BIOLOG GN test. All the other substrates are not utilized. Arbutin Esculin/ferric citrate, D-Turanose, and potassium 5-KetoGluconate are utilized on the API CH. API 20E test results indicate that Gelatinase and Indole are produced and all the other substrates gave a negative result. The strain does not reduce

Nitrate. No pigment is produced after prolonged growth. DNA G-C content of 53.5 mol%. Features that differentiate *Salinospina mwathaensis* from the other strains are the inability to utilize α-Keto Glutaric acid, L- Proline, Tween 40 and Tween 80.

7.8 Description of *Salinospina elmenteitii* sp. nov. (Strain E-463)

Isolate E-463 grows at pH between 7 and 11 and an optimum pH of 11.5. The strain has an optimum salt concentration of 8%. Temperature for growth ranges from 15.8–40.4°C and an optimum of 28.1°C. Dextrin, Tween 40, Tween 80, Acetic Acid, α-Keto Glutaric Acid, Succinic Acid, Bromosuccinic Acid, D-Alanine, L-Alanine, Asparagine, L-Glutamic Acid, L- Proline are utilized BIOLOG GN test results. All the other substrates are not utilized. Arbutin, Esculin/ferric citrate, D-Turanose, potassium 5-KetoGluconate, Indole and Gelatinase positive on API 20E. The strain has a DNA G+C content of 53.2 mol%. Features that differentiate *Salinospina elmenteitii* from the other strains are its ability to utilize Cellobiose and a much lower limit of the temperature of growth of 15.8 °C.

7.9 Description *Salinospina maltophila* sp. nov. (Strain E-466)

In addition to the description given above for the genus, this species grows at pH between 6 – 11 and an optimum pH of 9.5 and the optimum growth was observed at a salt concentration of 8%. Temperature for growth ranges from 22.3–46.1 °C and an optimum of 34.7°C. Dextrin, Tween 40, Acetic Acid, α-Keto Glutaric Acid and L-Proline were utilized on BIOLOG GN test results and all the other substrates gave a

negative result. On the API 20NE strip Arbutin, Esculin/ferric citrate, D-Turanose and Potassium 5-KetoGluconate gave positive results. Indole production and gelatine liquefaction were positive on API 20E. The strain has a DNA G+C content of 53.3 mol%. Salient Features that differentiate *Salinospina maltophilum* from other strains described here are the utilization of Maltose, highest amount of 18:1 ω7c fatty acid and the inability to hydrolyze Tween 80.

CHAPTER 8

8.0 Description of new species of *Halalkalibacter* gen. nov. a group of novel haloalkaliphilic members of the Gamma Proteobacteria.

ABSTRACT

Five bacterial strains designated as E-34, E–36, E–113, E–116, E–410 were isolated from Lake Elmenteita, an alkaline saline lake within the East African Rift Valley. The strains are alkaliphilic and differ in their morphology, substrate utilization Temperature, pH, salt tolerance and 16S rRNA gene sequences. The strains do not require growth factors, and growth occurs at a pH ranges between 5.5 and 12.0 with the optimum lying between 9.0 and 10.5. Optimum growth was observed on media with a salt concentration between 5 and 10%. The temperature for growth was between 15.8 – 43.5°C, with optimum growth occurring between 30 and 38 °C. No growth detected at below 15.8 °C. Major cellular fatty acids are the Iso-branched 15:0, 17:0 and the ISO 17:1 ω9c. DNA G-C content is between 49.0 – 52 mol%. The major isoprenoid quinone is Ubiquinone (Q-8). The isolates represent novel haloalkaliphilic members of the Gamma Proteobacteria. Based on physiological, biochemical and chemotaxonomic traits and comparative 16S rRNA gene sequence analysis, it is proposed that the isolates represents a novel Genus within the Gamma proteobacteria, for which the name *Halalkalibacter* gen. nov. sp. nov. is proposed. The type strain is *Halalkalibacter chitinolytica* (Strain E-410)

8.0 Introduction

They *Gammaproteobacteria* (Stackebrandt *et al.,* 1988) consists of manly marine heterotrophic Gram-negative aerobic or facultatively anaerobic bacteria affiliated to the genera *Alteromonas* (Baumann *et al.* 1972), *Colwellia* (Deming *et al.,* 1988), *Pseudoalteromonas* (Gauthier *et al.,* 1995), *Glaciecola* (Bowman *et al.* 1998c) and *Idiomarina* (Ivanova *et al.,* 2000b). They share similar phenotypic, genotypic and phylogenetic characteristics. These bacteria occur in very diverse habitats including coastal and open water areas, deep-sea and hydrothermal vents, and marine sediments. Phylogenetic analyses have shown that the genera of *Alteromonas*, *Pseudoalteromonas* and *Idiomarina* represent a distinct cluster within the γ-subclass of the class Proteobacteria. Since the description of the first two marine bacteria belonging to the Alteromonad group by Ivanova *et al.,* (2000). The genus *Idiomarina* now consist of nine validly published species. No species have been described so far from a soda lake environment. In this study we describe 5 isolates from Lake Elmenteita in Kenya, whose closest relatives are from the genus *Idiomarina*. However the species form a distinct phylogenetic cluster and it is proposed that they belong to a new genus.

8.1 MATERIALS AND METHODS

8.1.1 Sampling and isolation.

The isolates described were recovered during microbial diversity studies on Lake Elmenteita. Unless otherwise stated enrichment and isolation of the strains was carried out on different media prepared using filter sterilized Lake water. Several media were used in this study : **M2**: 1% starch,0.4 % yeast extract and 0.2 % peptone, **M7**: only 15 g Agar, **M8**: 7.5 g Casamino acids (Difco), 10g Yeast extract (Difco), 3 g Trisodium Citrate, 0.3 g $MgSO_4.7H_2O$, 2 g Potassium Chloride, traces of iron and manganese. Solid media was prepared by addition of 15 g/l Agar and all the media were supplemented with 100 mg/L Cycloheximide to inhibit the growth of fungi.

The isolates were purified on dilute Tryptic Soy Broth supplemented with 15 g Bacto Agar (Difco), 3.5% NaCl and 1% Na_2CO_3. The stains were stocked in Tryptic soy Broth supplemented with 3.5% NaCl, 1% Na_2CO_3 and 20 % (v/v) glycerol. *Idiomarina abyssalis (*DSM 15222*)* and *Idiomarina homiensis (*DSM 17923*)* were used as reference strains.

8.1.2 Phenotypic characterization

The strains were subjected to cultural, biochemical and morphological analyses. Standard methods for characterization of *Idiomarina* species were performed as described Ivanova *et al*. (2000). The following physiological and biochemical properties were examined: oxidation/ fermentation of glucose; Arginine dihydrolase; Tyrosine decarboxylase, cell pigmentation and cell morphology. The strains were

tested for their ability to hydrolyze gelatine, DNase activity, starch utilization and Tween-80. Chitinase activity was tested by use of 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide. Motility was tested with cells from 2-day-old liquid cultures on soft agar incubated for 48 h at 28^{o} C. Gram-staining was done using the aminopeptidase and KOH reactions, catalase activity was tested using H_2O_2 test as described by Zimmermann *et al.,* (1990). Cytochrome C oxidase was determined by adding a few drops of Tetramethyl-phenylenediamine solution to a 2-day-old slant of each strain.

Utilization of Glucose, sucrose, fructose, lactose and mannitol as sole carbon sources was tested on a basal media containing per litre 1g Yeast Extract (Difco), 1g KH_2PO_4, 0.1g $MgSO_4.7H_2O$,0.05g $CaCl.2H_2O$, 4% NaCl and 1% $NaCO_3$ and the respective sugar to a final concentration of 1%. Salt tolerance tests were done on Diluted Nutrient Broth (Difco) supplemented with 1% $NaCO_3$ and the salt concentration was varied from 0% to 20%. The optimum temperature for growth was determined on TSB containing 4% (w/v) NaCl and 1% $NaCO_3$ using a temperature gradient incubator model TN-3 (Toyo Kagaku Sangyo) with the lowest value of 9.4^{o} C and the highest 49.3^{o}C. The optical density was recorded after 18hours of growth using a Spectrophotometer at 620 nm. The pH range for growth was determined in dilute nutrient Broth with the pH adjusted using phosphate Buffer and a salt concentration of 4%. To test for the pH range for optimum growth, pH values of separate batches of media ranged from 6.0 to 13.

The ability to oxidize or utilize organic substrates was investigated using BIOLOG-GN plates as recommended by the manufacturer except that the optical density was adjusted to a transmittance of 52 in 0.4 M NaCl solution. Microplates were inoculated with 150µl cell suspension per well for each strain and incubated at 28 °C. The results were read using the BIOLOG Microplate reader after incubation for 6 h, 24 h, 48 h, and 72 h and after 5 days of growth. Carbon assimilation tests were also determined using the commercial API 20E and API 50 CH systems (BioMe´rieux). Tests were read after 6 h, 24 h, 48 h, and 72 h and after 5 days of growth. Electron micrographs of negatively stained cells, using 1.25 % uranyl acetate, were obtained using a Zeiss EM 10 CA electron microscope (80 kV).

8.1.3 Fatty acid analysis

The strains and the reference strains were subjected to fatty acid methyl ester analysis to confirm membership to the genus. Cultures of all strains were grown on the modified Trypticase Soy Broth (TSB; Difco) 28 °C for 48 h. *Idiomarina abyssalis* and *Idiomarina homiensis* could not grow in the alkaline media hence were grown on Marine Broth (Difco). The cell mass was harvested from the broth cultures via centrifugation. Saponification, methylation and extraction were done as recommended by the Sherlock Microbial Identification System (MIDI; http://www.midi-inc.com). The samples were analyzed on an Agilent Technologies 6890N gas chromatograph. The data were analyzed for taxonomic information by the TSBA40 and TSBA50

method of the Sherlock MIS software. The individual Fatty acids were expressed as percentages of the total Fatty Acids.

8.1.4 Respiratory Quinone and polar lipid analysis

Respiratory lipoquinones and polar lipids were extracted from freeze-dried cell material (100 mg) using a two-stage method (Tindall, 1990a, b) and separated into their structural classes (menaquinones and ubiquinones) by thin layer chromatography. Lipoquinones were separated by TLC and UV-absorbing bands were removed from the plate and analysed further by reverse-phase HPLC. Polar lipids were separated by two-dimensional, silica-gel TLC; total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate–Schiff (α-glycols), Dragendorff (quaternary nitrogen) and anisaldehyde–sulfuric acid (glycolipids) as described previously (Tindall, 1990a, b). The mol% G + C content of the strains was determined as described Mesbah *et al.,* (1989).

8.1.5 PCR amplification and sequencing

DNA extraction was carried out using the SEQLAB Bacteria-DNA-Kit and PCR amplification of the 16S rRNA genes was carried at SeqLab (Göttingen, Germany). Partial gene sequences were obtained using the 530 reverse primer (5′-GKATTACCGCGGCKGCTG-3′). The partial sequences were first analyzed by BLAST (Altschul *et al.,* 1997). Almost complete sequences were generated by SeqLab

(Göttingen, Germany). The sequences were assembled with the Sequencer software, version 4.1 for Macintosh (Genes Codes, Ann Arbor, MI). The almost complete sequences were automatically aligned according to the SILVA database release 94 (Ribocon, Bremen, Germany) using the FAST aligner tool of the ARB program (Ludwig *et al.,* 2004)). Phylogenetic relationship of almost complete sequences was determined using neighbour-joining (Felsenstein, 1993) and maximum-likelihood analyses (Olsen *et al.,* 1994). The resultant tree topologies were evaluated in bootstrap analyses (Felsenstein, 1985) of the Neighbour Joining method based on 1000 resamplings. Phylogenetic analyses were conducted in MEGA 4 (Tamura *et al.,* 2007). The almost complete sequences were deposited under the accession numbers FJ764787, FJ764788, FJ764789, FJ764790 and FJ764791.

8.2 RESULTS

8.2.1 Phenotypic characteristics

The isolates are Gram-negative, aerobic, oxidase and catalase positive and non-spore forming rods. The cells are about 2 μm in length and occur singly or in pairs. Motility is via a monopolar Flagellum. No organic factors are required for optimum growth. Table 8.1 below shows various phenotypic characteristics differentiating the strains from one another and from the closest relatives. The pH range for growth is between 5.5 and 12.0 with the optimum lying between 9.0 and 10.5. Salt is required for optimum growth between 5 and 10%. Temperature range is from 15.8 – 43.5°C, with optimum growth occurring between 30 and 38 °C. However no growth is detected at below 15.8 °C.

8.2.2 Substrate Utilization

The strains were subjected to an array of biochemical tests. Table 8.2 gives a summary of the relevant tests. All the strains described here are able to hydrolyze Gelatine and DNase activity was detected on the respective media. Casein is hydrolyzed and Tyrosine decarboxylase is also present. Dextrin, Arbutin, D-Turanose and potassium 5-KetoGluconate are used as carbon sources. Starch, Cellulose, Xanthan, Xylan and Lipids are not utilized.

Table 8.1 Selected phenotypic characteristics differentiating the Isolates in this study: **E -34**, **E – 36**, **E – 113**, **E – 116**, **E – 410**) from one another and from *Idiomarina abyssalis* DSM 15222 (Ivanova *et al.,* 2000) and *Idiomarina homiensis* DSM 17923 (Kwon *et al.,* 2006)

		E - 34	**E - 36**	**E - 113**	**E - 116**	**E - 410**	**DSM 15222**	**DSM 17923**
Flagellum		Single, polar	Single, polar	Single, polar	Single, polar	Single, polar	Single, polar	Single, polar
pH Tolerance	Range	6.5 - 12.0	7.0 - 12.0	6.5 - 13.0	6.5 - 11.5	7.5 - 11	5.5 - 9.5	6.0 - 9.0
	Optimum	10.5	10.5	10.0	9.5	9.0	7.5 - 8	ND
Salt Tolerance	Range	0.5 - 20%	0 - 16%	0.5 - 20%	0 - 20%	2.0 - 20%	0.6 – 15.0	1.0 - 15.0
	Optimum	10	12	6	5	8	3-6	3.0 - 5.0
Temperature	Range	15.8 - 42	16.2 - 40.4	15.8 - 41.9	16.2 - 41.9	16.2 - 43.3	4.0 - 30.0	4.0 - 45
	Optimum	33.1	37.5	31.1	34.7	32.3 - 34.7	20 - 22	25 - 30
Ubiquinones		Q8	Q8	Q8	Q8	Q8	Q7 & Q8	Q8, Q9
Menaquinones							MK7, MK8	
G+C Content		49	50	51	50	52	50.00	45.1

Table 8.2 Selected biochemical characteristics differentiating the isolates in this study from one another and from *Idiomarina abyssalis* DSM 15222 (Ivanova *et al.,* 2000) and *Idiomarina homiensis* DSM 17923 (Kwon *et al.,* 2006). W- Weakly positive

	E-34	E-36	E-113	E-116	E-410	DSM 15222	DSM 17923
API CH							
D-Xylose	-	W	-	-	-	-	-
D-Glucose	-	-	-	-	+	-	-
D-Mannose	-	W	-	-	-	-	-
L-Sorbose	-	W	-	-	-	-	-
D-Sorbitol	-	W	-	-	-	-	-
Methyl-α-D-Mannopyranoside	-	-	-	-	+	-	-
Amygdalin	-	-	-	-	+	-	-
Arbutin	W	W	W	+	+	W	+
Esculin/ferric citrate	+	+	+	+	-	W	+
D-Melibiose	-	-	-	-	W	-	-
D-Saccharose (sucrose)	-	-	-	-	W	-	-
Inulin	-	-	-	-	W	-	-
D-Melezitose	-	-	-	-	W	-	-
Glycogen	-	-	-	-	W	-	-
D-Turanose	+	+	+	+	+	-	+
potassium 2-KetoGluconate	-	-	-	-	W	-	-
potassium 5-KetoGluconate	+	+	+	+	+	+	-
BIOLOG							
Dextrin	+	+	+	+	+	+	+
Tween 40	-	-	-	-	+	+	-
Acetic Acid	-	-	-	-	+	-	-
API 20E							
Beta-Galactosidase	-	-	-	-	+	-	-
Gelatinase	+	+	+	+	+	+	+

8.2.3 Respiratory quinone analysis

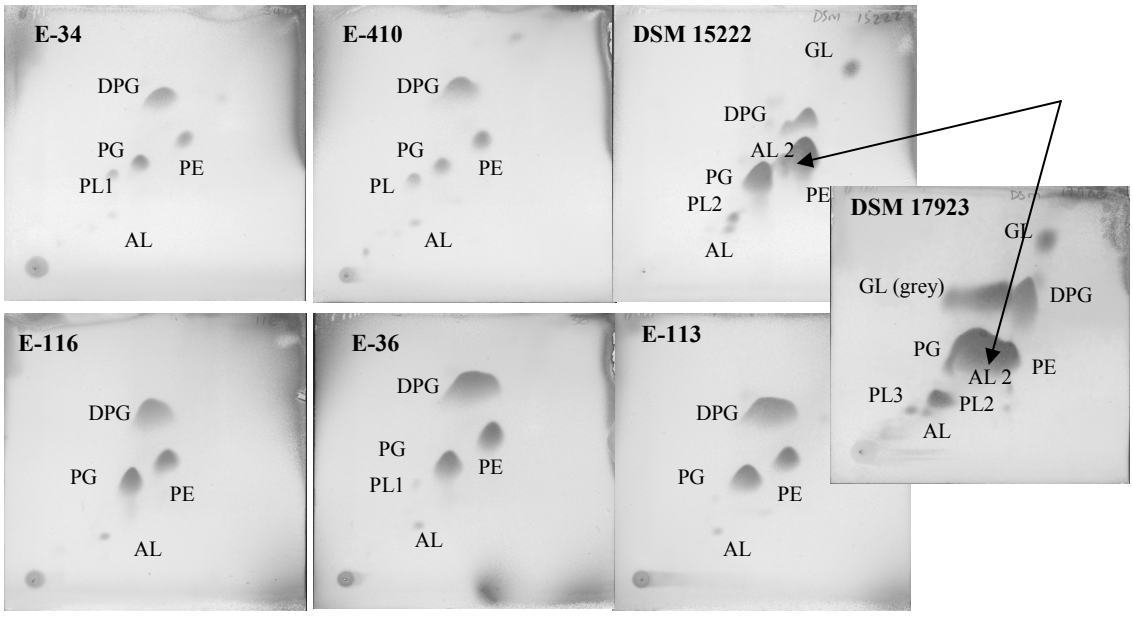
Results from respiratory quinone analysis indicated that the major respiratory lipoquinone is Ubiquinone 9 (Q9) compositions (Table 8.1). This differentiates the strains described here from the Idiomarina. *Idiomarina abyssalis* (Ivanova *et al.,* 2000) has Ubiquinone 7 and 8 whereas *Idiomarina homiensis* (Kwon *et al.,* 2006) has Ubiquinone 8 and 9. In addition *Idiomarina homiensis* has Menaquinone 7 and 8. All the strains have major cellular fatty acids as the Iso-branched 15:0, 17:0 and the ISO 17:1 ω9c.

8.2.4 Polar lipid analysis

The polar lipid profiles (Plate 8.1) reveal the differences between the strains and *Idiomarina* species; all the strains lack glycolipids present in the *Idiomarina* species and also there are larger amounts of Phosphatidylglycerol, presence of extra Aminolipids and Glycolipids. In addition *Idiomarina homiensis* has more Phospholipids marked as PL2 and PL3 on Plate 8.1.

Table 8.3 Fatty acid composition of strains: E -34, E – 36, E – 113, E – 116, E – 410, *I. abyssalis* DSM 15222 (Ivanova *et al.,* 2000) and *Idiomarina homiensis* DSM 17923 (Kwon *et al.,* 2006) -ve sign means not detected or less than 1%.

Fatty Acid	E - 34	E - 36	E - 113	E - 116	E - 410	DSM 15222	DSM 17923
11:0 ISO	-	-	-	-	-	**2.32**	**2.27**
10:0 30H	-	-	-	-	-	-	**1.47**
11:0 ISO 3OH	2.28	2.81	2.29	2.16	2.54	3.64	5.36
13:0 ISO	1.49	1.31	1.36	1.55	0.63	1.40	1.05
12:0 30H	-	-	-	-	-	-	1.06
13:0 ISO 30H	-	-	-	-	-	3.32	2.51
14: O	-	-	-	-	1.06	-	1.07
15:1 ISO F	4.66	3.91	4.17	4.73	2.09	2.00	1.69
15:0 ISO	**31.66**	**31.72**	**43.67**	**42.85**	**29.25**	**25.34**	**24.31**
16:0 ISO	-	-	-	-	1.09	-	-
16:1 ω7c/15 ISO 2OH	1.14	0.54	0.44	0.71	2.84	4.25	5.53
16: O	1.77	1.50	1.28	1.90	2.71	6.76	**10.31**
15:0 ISO 3OH	3.69	2.98	2.70	2.44	3.85	-	-
ISO 17:1 ω9c	**21.96**	**25.74**	**18.09**	**21.07**	**13.05**	**17.45**	9.81
17:0 ISO	**25.00**	**25.61**	**23.21**	**18.69**	**15.70**	**13.94**	**11.29**
17:1 ω8c	0.13	-	-	0.10	1.50	0.98	0.32
17:0 Cycl.	-	-	-	-	1.38	0.95	2.01
17: O	0.67	0.45	0.31	0.46	5.37	1.25	0.54
18:1 ω9c	-	-	-	-	-	1.35	1.78
18:1 ω7c	0.68	0.89	0.44	0.61	2.78	6.79	8.03
18: O	0.81	0.44	0.25	0.34	4.35	2.67	3.28
19:0 ISO	0.46	0.40	0.19	0.23	0.21	0.33	0.36
19:0 Cycl.	-	-	-	-	4.89	0.35	0.68



Idiomarina

DPG = Diphosphatidylglycerol
PG = Phosphatidylglycerol
PE = Phosphatidylethanolamine
PL1, PL2 = Phospholipid
AL = Aminolipid
GL= Glycolipid

Plate 8.1 Polar lipid profiles of strains E -34, E – 36, E – 113, E – 116, E – 410 and two reference strains; *I. abyssalis* DSM 15222 (Ivanova *et al.,* 2000) and *Idiomarina homiensis* DSM 17923 (Kwon *et al.,* 2006) after separation by two-dimensional TLC.

8.2.5 Phylogenetic analysis

The strains described here had a DNA G+C content of between 49.0 – 52 mol %. Analysis of the 16s rRNA sequences shows that the strains form a phylogenetic cluster distinct from the *Idiomarina*. Figure 8.1 shows the phylogenetic relationship of the strains described here in relation to the *Idiomarina*.

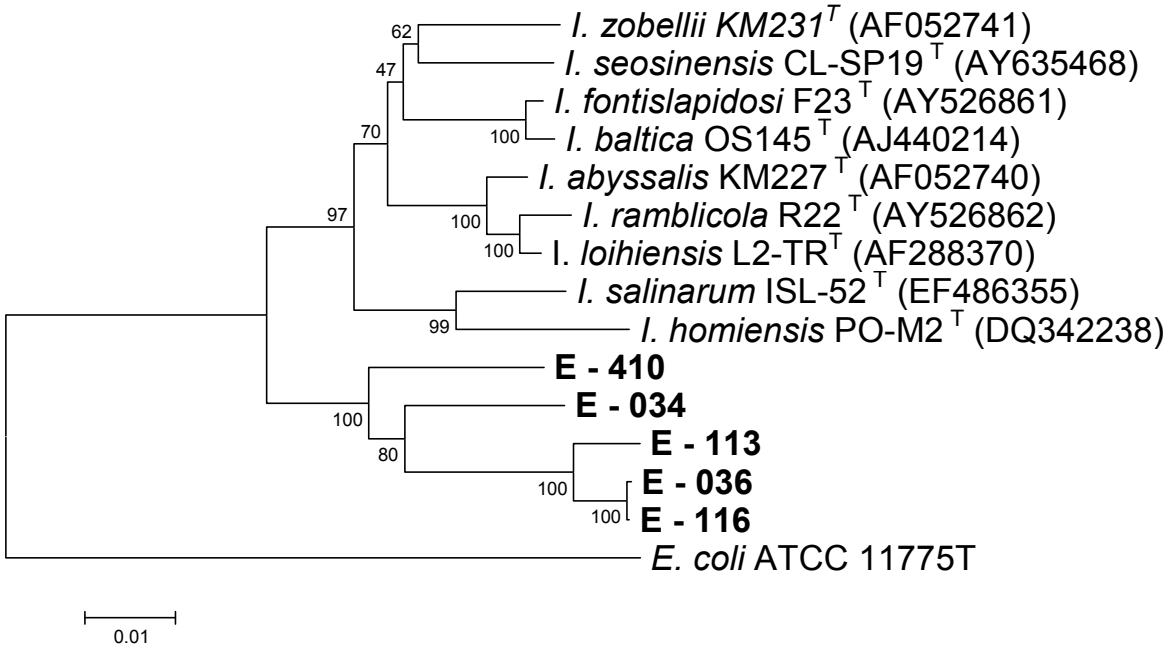


Figure 8.1 Neighbour-joining tree showing the position of strain the strains described in this and *Idiomarina* species on the basis of 16S rRNA gene sequences. Numbers at branch points indicate bootstrap confidence values (%) of 1000 resamplings. The Bar represents 0.01 substitutions per nucleotide position.

8.3 Discussion

The genus *Idiomarina* now consists of nine validly published species. Of these; two species have been described from aquatic/saline environments. *Idiomarina seosinensis* (Choi and Cho, 2005) was isolated from hypersaline water of a solar saltern located in Seosin, in Korea while *Idiomarina salinarum* (Yoon *et al.,* 2007) was isolated from a marine solar saltern of the Yellow Sea in Korea. However the Phylogenetic, physiological and biochemical features indicate that the strains described in this study

differ from *Idiomarina* and other Alteromonads. The two comparative strains used in this study could only grow in marine broth. The strains described here form a phylogenetic cluster (Figure 8.1) distinct from the closest relatives which belong to the genus *Idiomarina*. The strains also have high salt and pH optima indicating they are haloalkaliphilic. These could be a reflection of the alkaline saline conditions prevalent in the soda lakes environment. Besides the fatty acid profile (Table 6.3) confirm that the strains described here differ from the *Idiomarina* species described so far. This differentiates the strains described here from the *Idiomarina*. *Idiomarina abyssalis* (Ivanova *et al.,* 2000) has Ubiquinone 7 and 8 whereas *Idiomarina homiensis* (Kwon *et al.,* 2006) has Ubiquinone 8 and 9. In addition *Idiomarina homiensis* has Menaquinone 7 and 8.

The major cellular fatty acids are the Iso-branched 15:0, 17:0 and the ISO 17:1 ω9c. The strains lack the following fatty acids found in *Idiomarina*: 13:0 ISO 30H, 18:1 ω9c and 11:0 ISO. However the *Idiomarina* spp. also lack fatty acids 15:0 ISO 3OH which is common in all the strains described in this study. All the strains lack α-glycolipids present in the *Idiomarina* species and also there are larger amounts of Phosphatidylglycerol, presence of extra Aminolipids and Glycolipids. Based on the phenotypic and biochemical characteristics (Table 6.1; 6.2) and polar lipid patterns (Plate 6.1) it can be concluded that the strains represent a new genus of haloalkaliphiles from the soda lake environment for which the genus name *Halalkalibacter* gen. nov. sp. nov. is proposed.

Gr. n. *hals*, salt; Arabic n. *al-qaliy*, the ashes of saltwort; L. n. *bacter*, rod; N.L. masc. n. *Halalkalibacter*, salt and alkaline loving rods

8.4 Description of the genus *Halalkalibacter* gen. nov.

The isolates are Gram-negative, aerobic, oxidase and catalase positive and non-spore forming rods. The cells are about 2 μm in length and occur singly or in pairs. Motility is via a monopolar flagellum. Organic factors are not required for growth. The pH range for growth is between 5.5 and 12.0 with the optimum lying between 9.0 and 10.5. Salt is required for optimum growth between 5 and 10%. Temperature range is from 15.8 – 43.5°C and the optimum growth occurs between 30 and 38 °C. All the strains described here are able to hydrolyze Gelatine and DNase positive. Casein is hydrolyzed and Tyrosine decarboxylase is also present. Dextrin, Arbutin, D-Turanose and potassium 5-KetoGluconate are used as carbon sources. Starch, Cellulose, Xanthan, Xylan and Lipids are not utilized. The major respiratory lipoquinone is Ubiquinone 9 (Q9).

8.5 Description of *Halalkalibacter chitinolytica* (Strain E- 410)

In addition to the description given above for the genus, this species grows at pH between 7 – 11 and an optimum pH of 9.0, salt concentration of 8%. Temperature for growth ranges from 16 – 43.5 °C and an optimum between 32 and 35°C. Dextrin, Tween 40 and Acetic acid are utilized on the BIOLOG GN test results and all the other substrates are not utilized. Arbutin, D-Melibiose, D-Saccharose, Inulin, D-

Melezitose, Glycogen, D-Turanose, potassium 2-KetoGluconate, potassium 5-KetoGluconate are utilized on API CH. ONPG and Gelatinase positive. No pigment is produced after prolonged growth. The DNA G+C content is 52 mol%. *Halalkalibacter chitinolytica* differs from the other members of the genus in that Chitinases lacking in other species are present and it grows in media with salt between 2 – 20%. The strain utilizes acetic acid, D-Glucose, Methyl-α-D-Mannopyranoside, Amygdalin and Tween 40 is hydrolysed. The strain gives positive results for Esculin/Ferric citrate on API CH. The strain has cellular fatty acids lacking in the other species mainly 14:O, 16:0 ISO, 17:1 ω8c, 17:0 Cyclopropene, 18:1 ω7c, 18:0 and 19:0 Cyclopropene. The strain produces a brown pigment as well as exopolysaccharides after prolonged growth. The strain has been named *H. chitinolytica* since it is the only species in the genus that hydrolyses chitin

8.6 Description of *Halalkalibacter kikopeyii* (Strain E-34).

In addition to the description given above for the genus, this species grows at pH between 6.5 – 12 and an optimum pH of 10.5, salt concentration of 10% and at a temperature of 33.1 °C. Only L-Alanine and Dextrin are utilized according to BIOLOG GN test results. Arbutin Esculin/ferric citrate, D-Turanose, and potassium 5-KetoGluconate are utilized on the API CH. API 20E test results indicate that Gelatinase is produced and all the other substrates gave a negative result. The strain lacks Nitrate reductase. No pigment is produced after prolonged growth. DNA G-C content of 49 mol%. The only feature that differentiates *Halalkalibacter kikopeyii*

from the other strains is that optimum growth occurs at 10% salt and a pH of 11.5. The name **kikopey** is a Maasai name for a small town adjacent to Lake Elmenteita.

8.7 Description of *Halalkalibacter saccharovorans* **(**strain E-36**)**.

In addition to the description given above for the genus, this species grows at pH between 7 – 12 and an optimum pH of 10.5, salt concentration of 12%. Temperature for growth ranges from 16 – 42 °C and an optimum of 31.1 °C. Only Dextrin is utilized according to BIOLOG GN test results. The strain is negative for all the other substrates. D-Xylose, D-Mannose, L-Sorbose, D-Sorbitol, Arbutin, Esculin/ferric citrate, D-Turanose, potassium 5-KetoGluconate are positive on the API 50CH. Tests on API 20E indicate that Gelatinase is produced and all the other substrates are negative. Nitrate reductase is present. No pigment is produced after prolonged growth and the strain lacks 14:0 ISO-branched cellular fatty acids. The DNA G-C content is 50 mol%. Features that differentiate strains of *Halalkalibacter saccharovorans* from the other strains is the ability to utilize D-Xylose, D-Mannose, L-Sorbose and D-Sorbitol. The species name depicts the ability of the strain to utilize the various sugars.

8.8 Description of *Halalkalibacter salina* (strain E-113).

In addition to the description given above for the genus, this species grows at pH between 6.5 – 13 and an optimum pH of 10, salt concentration between 0.5 - 20% and the optimum is 6%. Temperature for growth ranges from 16 – 42 °C and an optimum of 31.1 °C. Only Dextrin is utilized on BIOLOG GN test plate. Arbutin Esculin/ferric

citrate, D-Turanose, and potassium 5-KetoGluconate are utilized on the API CH. API 20E test results indicate that Gelatinase is produced and all the other substrates gave a negative result. Nitrate is reduced hence the strain has Nitrate reductase. A brown pigment is produced after prolonged growth. ISO-branched 14:0 fatty acids are lacking. The strain has a DNA G+C content of 50 mol%. Features that differentiate *Halalkalibacter salina* from the other strains in the genus is the high amounts of ISO branched fatty acid (15:0 ISO) as shown on Table 8.3.

CHAPTER 9: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS.

9.0 General Discussion

Application of molecular techniques in microbial ecology has an important role as a guide for the isolation and characterization of new prokaryotic taxa (Palleroni, 1997). It is due to the power of clone libraries to give an *a priori* idea of the diversity before isolation studies. Results from the clone library analysis show that clones possessed a higher similarity to other environmental clones than to cultured microorganisms. A comparison of the results from this study with previous attempts by Rees *et al.,* (2004) to assess the microbial diversity on several East African soda lakes shows that there may be much higher diversity within Lake Elmenteita. The higher diversity as reported in the clone libraries described in this study could be as a result of the DNA extraction method and that no purification was done using columns. This in turn could have minimized the loss of extracted DNA. It can be postulated that the soda lakes are a home to much higher diversity than already reported. This is confirmed by the fact that of the 655 clone sequences selected for analysis, 525 (80.15%) were related to uncultured members of the Domain Bacteria. More than 90% of the clones had similarity values below 80% to both cultured and uncultured microorganisms. Furthermore results from isolation using different media led to the retrieval of novel microorganisms not detected in the soda lakes before (Table 6.2).

Results from this study point to a complex microbial community which drives the various geochemical cycles within the soda lake environment. The four dominant Phyla in the clone libraries are the Cyanobacteria, Firmicutes, Proteobacteria and the Bacteroidetes. The finding that Cyanobacteria clones predominate concurs with earlier reports that the less alkaline lakes such as Lake Elmenteita are usually dominated by dense blooms of cyanobacteria while the hypersaline lakes occasionally support blooms of both cyanobacteria and alkaliphilic anoxygenic phototrophs belonging to the genera *Ectothiorhodospira* and *Halorhodospira* (Jones *et al.,* 1998; Grant *et al.,* 1999; Ballot *et al.,* 2004). The four orders of Cyanobacteria were represented with clones in the genus *Synechococcus* being the most common (Table 4.3). Cyanobacteria groups reported so far from Lake Elmenteita include *Arthrospira, Arthrospira fusiformis, A. arnoldii, Synechococcus* sp., *Synechocystis* sp., *Spirulina subtilissima*, *Spirulina subsalsa* and *Pseudanabaena* sp. (Iltis, 1969; Vareschi, 1982; Melack, 1988; Jones *et al.,* 1998; Kebede and Willén, 1998; Grant *et al.,* 1999 Ballot *et al.,* 2004). Clones related to *Arthrospira*, *Calothrix*, *Gloethece*, *Leptolyngbya* and *Microcystis* were detected only in the hotspring samples. This indicates that the unique hotspring environment supports more diversity that other areas within the lake. More studies using culture dependent approach would shed more light on the diversity. Therefore it can be concluded that the Cyanobacteria are behind the high rates of primary productivity in Lake Elmenteita.

In terms of diversity the Proteobacteria come third (Table 4.1). The *Betaproteobacteria* have not been previously reported from the East African soda lakes or the other soda lakes such as Wadi al-Natrun in Egypt (Mesbah *et al.,* 2004). However there was no overlap between the clone library and the isolation studies. The majority of the isolates retrieved were from the *Gammaproteobacteria*. This concurs with earlier reports that he majority of Gram-negatives isolated so far are members of the *Gammaproteobacteria* mainly from the genus *Halomonas* (Duckworth *et al.,* 2000), *Aeromonas, Vibrio and Alteromonas* (Duckworth *et al.,* 1996; Jones *et al.,* 1998). It has been reported that the majority of Gram-negative isolates and culture-independent bacterial clones retrieved from soda lakes belong to the Gamma Proteobacteria (Jones *et al.,* 1994, 1998; Ma *et al.,* 2004b). Two strains have so far been isolated from the Kenyan soda lake: *Halomonas magadiensis* (Duckworth *et al.,* 2000) and *H. kenyensis* (Boltyanskaya *et al.,* 2007). The ability to oxidize thiosulfate to tetrathionate seems widely distributed among the haloalkaliphilic *Halomonas* (Dimitry *et al.,* 2005). Two groups are recognized as haloalkaliphilic; *H. desiderata* and *H. magadiensis*. Several members of *Halomonas* spp. are able to grow and concomitantly oxidize sulphide and polysulfide to elemental sulphur anaerobically under denitrifying conditions. Due to the high degree of halophily of halomonads, it can be anticipated that this group is important in the provision of the acetate sink in the alkaliphilic microbial community. The possible ecological role of haloalkaliphilic halomonads also consists in their involvement in the nitrogen cycle, including N_2O transformation. Thus, there is a potential for the natural utilization of this greenhouse

gas in soda lakes. Members of *Halomonadaceae* have been shown to be of biotechnological importance in the production of compatible solutes as well as extracellular compounds such as exopolysaccharides and enzymes, and their use in environmental bioremediation processes.

Gram-negative heterotrophic bacteria whose closest relatives are in the genera *Marinospirillum* and *Idiomarina* were recovered. These have been assigned to new genera as described earlier. The groups have not been detected in the East African soda lakes before. A single isolate from the genus *Alkalimonas* was isolated and it is closely related to *Alkalimonas delamerensis* isolated from the same lake (Ma *et al.,* 2004a). Isolates from the Genus *Alkalibacterium* isolates have also been recovered from the other Kenyan soda lakes namely Crater Lake Sonachi, Lake Bogoria and Lake Nakuru (This study). Members of the genus *Vibrio* have not been detected in the soda lakes before. Five isolates were recovered and their close similarity to isolates from the ocean may point to an evolutionary adaptation. All of the organisms included in the family *Vibrionaceae* belong to the eubacterial kingdom and to the "purple bacteria" branch of this kingdom (Woese, 1985). Species of the family *Vibrionaceae* occupy many different ecological niches (Campbell, 1957; Baumann and Baumann, 1981; Sakazaki and Balows, 1981; Simidu and Tsukamoto, 1985). They cause disease of humans and animals and also occur in the environment. Many factors probably govern their distribution, but four of the most important are: the particular animal or

plant hosts, temperature, salinity, and depth below the surface for the species that are found in the ocean (Simidu and Tsukamoto, 1985).

Anoxygenic phototrophic purple bacteria also participate in primary production (Zavarzin *et al.,* 1999). Purple sulphur bacteria of the genera *Ectothiorhodospira* and *Halorhodospira* oxidize hydrogen sulphide with intermediate extra-cellular sulphur deposition (Baumgarte, 2003). In this study clones affiliated to *Ectothiorhodospira shaposhnikovii* and *Ectothiorhodosinus mongolicum* were retrieved. Hence it can be deduced that members of these genera contribute to primary production within the soda lakes. All strains of the family *Ectothiorhodospiraceae* produce external globules of elemental sulphur during photosynthetic sulphide oxidation (Imhoff, 1984). The sulphur cycle in these lakes utilizes sulphur and sulphate presumably generated by *Ectothiorhodospira* and *Halorhodospiria* sp. (the link between the carbon and sulphur cycles), and also aerobic sulphur-oxidizers. Sulfuroxidizing bacteria belong to two groups, those similar to the non-lithotrophic *Halomonas deleya* and those assigned to the novel genus *Thioalkalovibrio* (Sorokin *et al.,* 2001). Sulphate-reducing bacteria then complete the cycle; *Desulphonatronovibrio hydrogenovorans* and *Desulphonatrum lacustre* (Zhilina *et al.,* 1997; Pikuta *et al.,* 1998) have been isolated from Siberian soda lakes. The alkaliphilic alpha-proteobacterium *Rhodobaca bogoriensis* isolated from Lake Bogoria in Kenya is a representative of purple nonsulfur bacteria described from soda lake environments (Milford *et al.,* 2000). The occurrence of 35 clones belonging to the order *Rhodobacterales* confirms that bacteria

in the genera *Rhodobaca* and *Rhodobacter* play an important role of primary production in the soda lake environment.

Clones affiliated to the phyla Chlorobi (19 clones) and Chloroflexi (one clone) were retrieved. Members of these phyla participate in primary production. Of the 19 clones in the phylum Chlorobi, 12 were from the hotspring sediment samples. Further more *Chlorobium tepidum* is the only thermophilic member of the genus *Chlorobium* isolated from a hotspring (Wahlund *et al.,* 1991). Phylum Chlorobi consists of green sulphur bacteria which are anoxygenic photosynthetic bacteria. The Phylum Chloroflexi consists of green gliding bacteria and the members are metabolically versatile. The members grow as heterotrophs or photosynthetically and carbon fixation is through a special cycle. The two isolates in the phylum Chloroflexi were isolated from hotsprings in Japan. Therefore the presence of clones from these two phyla indicates the presence of thermophilic members of these phyla in the hotsprings found in the soda lakes.

Chemoorganotrophic microorganisms play an important role in the soda lake ecosystem by breaking down the organic materials from primary production. Clones from the orders *Bacillales*, *Clostridiales*, *Dethiobacter*, *Lactobacillales*, *Thermoactinomycete* and *Thermoanaerobacter* were retrieved. From isolation studies, Gram-positive aerobic isolates of both the high G+C and low G+C divisions have been found. From the low G+C isolates represented are especially *Bacillus*

alcalophilus (rRNA group 6, according to Nielsen *et al.,* 1994; 1995) and *Bacillus clarkia* group, (Duckworth *et al.,* 1996). Overlap between the clone library and isolates retrieved was evident in the genus *Lactobacillus* and *Bacillus*. The *Enterococci* belong to the Firmicutes with low G+C content, the so-called clostridial branch. The *Enterococci* are most often considered as components of the intestinal flora of humans and animals acting as opportunistic pathogens in different extra-intestinal compartments of the body. The plant-associated yellow-pigmented *E. casseliflavus* and *E. mundtii* (Collins *et al.,* 1986; Vaughn *et al.,* 1979) may occur transiently in the intestines. It is not clear if their occurrence in the soda lake is due to pollution.

The occurrence of isolates distantly related to the Genus *Amphibacillus* and *Marinilactibacillus* in the soda lake environment is interesting and the low similarity values mean they could represent novel genera. The isolates of the Genus *Amphibacillus* (Niimura *et al.,* 1990) are moderately alkaliphilic and facultatively anaerobic (Zhilina *et al.,* 2001) at the expense of fermentation of glucose and certain mono- and disaccharides with the formation of formate, ethanol, and acetate. The Genus *Marinilactibacillus* has only two described species and both are from the ocean. Isolation and taxonomic studies of lactic acid bacteria from marine environments to date are few and have generally been confined to those from cultured fish (Ringø and Gatesoupe, 1998; Gatesoupe, 1999).

The occurrence of clones with low similarity values to cultured isolates indicated that there are more novel uncultured groups within the *Actinobacteria* in the soda lake environments. So far no isolate from the genus *Streptomyces* has been described from the East African soda lake. The single isolate recovered in this study has a similarity value of 95% to *Streptomyces caelestis* hence could represent a novel genus. Interesting is that the strain grows optimally at 39 ^{o}C and a pH of 8.4. The members of the genus *Nocardia* are aerobic, Gram-positive, non-motile catalase positive actinomycetes that are typically acid alcohol-fast at some stages of the growth cycle. Two isolates from this genus were recovered indicating that the genus is ubiquitous. However no clones were retrieved.

The order *Clostridiales* was widely represented in the clone library with a total of 84 clones. No isolates were retrieved in this study and this could be due to the method of enrichment and isolation. However organisms fermenting amino acids, called acetogenic ammonifiers have been isolated from soda lakes. Two strains, *Natronincola histidinovorans* (Zhilina *et al.,* 1998) and the non-spore-forming bacterium *Tindallia magadiensis* (Kevbrin *et al.,* 1998) were isolated from Lake Magadi, Kenya, and were found to be related with members of Group XI of the *Clostridium* taxon (Collins *et al.,* 1994) including *Clostridium felsineum*, *C. formicoaceticum,* and the more distant *C. halophilum*. A number of alkaliphilic saccharolytic *Clostridia* strains have been isolated from Lakes Elmenteita, Bogoria and Magadi (Jones *et al.,* 1998; Zhilina *et al*., (2001b).

Members of the order *Clostridiales* play an important role in biogeochemistry. The nitrogen cycle in these lakes for example involves the production of ammonia by fermentative anaerobes such as *Tindallia magadii* (Kevbrin *et al.,* 1998). Ammonia is utilized by methanotrophs and nitrifiers, producing nitrate. Nitrate, in turn, is utilized by the chemoorganotrophs, creating a link between the nitrogen and carbon cycles. Clone S1-111 was closely related to members of the genus *Thioalkalivibrio,* which comprises obligately alkaliphilic and obligately chemolithoautotrophic sulphur oxidising bacteria that use nitrate or oxygen as electron acceptors (Sorokin *et al.,* 2001).

The phylum Bacteroidetes encompasses the three new classes *Bacteroidetes*, *Flavobacteria* and *Sphingobacteria* (Ludwig and Klenk, 2001). Of the three, *Sphingobacteriales* and *Flavobacteriales* consist mainly of aerobic organisms while known strains of order *Bacteroidales* are all anaerobic (Kirchman, 2002; Reichenbach, 1991). The classes of bacteria in this Phylum are widely distributed in the environment, including in soil, in sediments, sea water and in the guts of animals. The distribution of the clones retrieved is shown on Table 4.1. However due to the shallow depths of Lake Elmenteita, it may be possible that mixing of the water tilts the distribution of the phylotypes.

Other phyla detected in the clone libraries include members of the Phyla Fusobacteria, Acidobacteria and Spirochete. The Acidobacteria are common in soils and sediments but few have been isolated. The Phylum consists of aerobic genus Acidobacterium, the

homoacetogenic bacteria Holophaga and the iron reducing bacteria of the genus Geothrix. In an anaerobic community saccharolytic spirochetes utilize sugars and a limited range of polysaccharides to produce acetate, lactate, ethanol and Hydrogen (Zavarzin *et al.,* 1999). Two haloalkaliphilic strains *Spirochaeta alkalica* and *S. africana* have been isolated from Lake Magadi, and an alkaliphilic species *S. asiatica* from Lake Khatyn, Central Asia (Zhilina *et al.,* 1996b).

All known Archaea belong to the Phyla *Crenarchaeota*, *Euryarchaeota*, *Korarchaeota* and *Nanoarchaeota*. In this study all the clones retrieved were from the phylum Euryarchaeota. This concurs with what has been recovered from other alkaline environments (Rees *et al*., 2004; Mesbah *et al.,* 2007). It is possible that the environmental conditions determine the distribution of the various Phyla. The members *Methanocalculus* group obtain their metabolic energy via reduction of CO_2 to methane whereas H_2 and formate are electron donors. Their distinguishing feature is tolerance to high salt concentration. *Methanosaeta* is a genus consisting of obligate anaerobes which use acetate as only energy source. Methanotrophic methanogens isolated from several soda lakes are related with members of the *Methanosarcinaceae* within the Euryarchaeota.

Members of the order *Halobacteriales* require high salt for growth and are chemo-organotrophic, may be aerobic or facultatively anaerobic. They are ubiquitous where salt concentration is high. Members of the Genus *Natronococcus* are alkaliphilic and

require a pH of at least 8.5 for growth. Members of this group have been previously reported from Lake Elmenteita (Rees *et al*., 2004). DNA from the *Halobacteriales* has been extracted from the Dead Sea, solar salterns, Antarctic hypersaline lakes, alkaline African hypersaline lakes, and Solar Lake, Sinai (Benlloch *et al.,* 2001; Cytryn *et al.,* 2000; Moune´, *et al.,* 2003; Oren, 2002). Saline soda lakes support blooms of halobacteria and harbour alkaliphilic representatives of the genera *Natronobacterium* and *Natronococcus, Natronomonas, Natrialba, Natronorubrum* and *Halorubrum*. Functionally, they have a specific trophic position and flourish on the organic matter concentration arising from evaporation of brine and the death of its microbial population (Zavarzin *et al.,* 1999).

Even with the advantages of applying molecular tools to study microbial diversity, it is important to realize that libraries of PCR-amplified 16S rRNA and 16S rRNA genes may not represent a complete or accurate picture of the bacterial community since species diversity is so great (Torsvik *et al.,* 1990, Curtis *et al.,* 2002; Gans *et al.,* 2005). Even all of the currently published sequences combined would seem to constitute an incomplete census of all of the 16S rRNA genes on earth (Schloss and Handelsman, 2004). In addition, there may be biases in the contributions of the various bacterial groups to libraries. The efficiencies of nucleic acid extraction may be different for different bacteria, the number of copies of 16S rRNA or 16S rRNA gene copy per cell varies, and there may be preferential amplification of some sequence

types relative to others by PCR (Embley and Stackebrandt. 1997, von Wintzingerode *et al.,* 1997, Frostegård *et al.,* 1999).

The resolving power of the 16S rRNA has been recognized to be insufficient to guarantee correct delineation of bacterial species (Fox *et al.,* 1992; Martinez-Murzia *et al.,* 1992). Assigning physiologies and functions to the hosts of 16S rRNA gene sequences is complicated in many cases by the lack of characterized close relatives (Dojka *et al.,* 2000, Hugenholtz *et al.,* 2001, Mummey and Stahl, 2003) and by the diversity of phenotypes among close relatives in some groups (Achenbach and Coates, 2000; Saito *et al.,* 1998). It was realized that some of the clones in this study could not be assigned to any phylogenetic group. Some, but not all, of these biases may be overcome as metagenomic data sets accumulate (Liles *et al.,* 2000; Quaiser et al 2003; Tringe *et al.,* 2005). Given the low phylogenetic resolving power at the levels of close relatedness (above 97% 16S rRNA sequence similarity), it is highly recommended to support conclusions based on SSU rRNA sequence data analysis by alternative comparative data, such as genomic DNA reassociation studies (Stackebrandt and Goebel,1994).

Isolation and cultivation still remains an important component of microbial ecology as it was in the last century. Organisms catalogued by rRNA gene sequences only permit assessment of phylogenetic diversity. Pure isolates help to define and understand the functional role of the microorganisms in their particular ecosystems. Much of the

versatility (and therefore of the global biological diversity) of bacteria and archaea resides in properties that only can be observed in living organisms (Palleroni, 1997). Cultured microbes reflect the sampling methods and culture conditions used in the isolation process and this has a major effect on the range of types encountered in the laboratory. In this study the media used for enrichment and isolation ranged from one poor in nutrients to one rich in nutrients. Some of the media used also targeted detection of useful extracellular enzymes produced by the alkaliphiles. Using this approach, novel microorganisms not isolated before were retrieved.

One of the challenges of studying the microbial diversity of soda lakes is to retrieve a diverse and higher portion of culturable microorganisms. It was proposed that *in situ* isolation would give better yields (Mwatha, 1991). In this study nutrient rich media yielded mostly members of the Gamma subdivision of Proteobacteria mainly from the Genus *Halomonas*. This concurs to what has previously reported (Wagner *et al.,* 1994). Though the majority of the isolates were from Firmicutes and the Gamma proteobacteria, this may be due to an enrichment bias or the media used. A comparison between the clone libraries and the isolates shows that the isolates so far are only a small fraction of the actual diversity within the soda lake environment.

Conclusions

- **The application of both culture dependent and culture independent approaches gives a better overview of the microbial diversity within the soda lake environment than the restriction to only one of them**.

- **The majority of the groups of bacteria in the soda lakes are yet to be cultured.** The clone library shows that 80.15% sequences were related to uncultured members of the Domain Bacteria.

- **The Cyanobacteria form the dominant group, most likely playing the crucial role as primary producers in these lakes.** They are involved not only in primary productivity but also in nitrogen fixation and evolution of oxygen.

- **The use of different media in isolation could help retrieve novel groups not isolated in the soda lakes before.**

Recommendations

1. Studies should be carried out to determine if changes in microbial composition correlates with changes in seasons. The Soda lakes undergo cycles of dilution (during the rains) and concentration (dry season). This could help understand how the microorganisms adapt to changes in salt and pH fluctuations.
2. Intensive study focusing on archaeal taxa needs to be performed. Most of the studies have reported only the presence of the Euryarchaeota and it is not known if there are other groups of Archaea in the soda lake environment.
3. Intensive studies on anaerobes within the soda lake environment should be carried out as non-culture studies highlight their presence.
4. It is well known that a diverse isolation regime will enhance the recovery of more diversity. Studies so far have been done in laboratories abroad and on samples frozen for years.
5. Construction of metagenomic libraries will help to discover novel functional genes which could lead to discovery of novel microbial metabolites with biotechnological potential.

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Appendix I: Summary of Clones related to β-Proteobacteria

Phylotype	Family	GenBank Acc. No.	*n*	Closest Relative in Blast Search		% Similarity
	Macromonas					
	Clone _W2-078	FJ764273		Uncultured bacterium Clone 124b1	EF459884	98
	Hydrogenophaga					
	Clone_W1- 079	FJ764257		Uncultured bacterium Clone	DQ088755	95
	Clone_W1- 120	FJ764260		Uncultured bacterium G1Clone47	EF149060	98
	Clone _W1- 010	FJ764270		*Malikia granosa* 16S rRNA gene	AJ627188	97
	Clone _W4- 044	FJ764278		Uncultured Hydrogenophaga sp. Clone DS062	DQ234146	97
	Uncultured					
	Clone _W1- 044	FJ764253		Uncultured bacterium Clone CJRC170	DQ202200	97
Burkoholderiales	**Aquabacterium**					
	Clone _W2 – 021	FJ764271		Uncultured bacterium Clone 261ds10	AY212711	98
	Alcaligenes *et rel.*,					
	Clone _W4- 032	FJ764276		Uncultured bacterium Clone RH.204i-20	DQ458382	92
	Clone _W4- 019	FJ764284		Uncultured bacterium Clone SS-4 16S	AY945899	96
	Clone _W4- 064	FJ764280		Uncultured betaproteobacterium Clone TK-SE4	DQ463713	94
	Clone _W4- 018	FJ764283		Uncultured betaproteobacterium Clone TK-NE3	DQ463723	98
	Clone _W1- 003	FJ764269		Uncultured betaproteobacterium Clone TH1-69	AM690866	95
	Lautropia					
	Clone _W4- 034	FJ764277		Uncultured soil bacterium Clone W4Ba40	DQ643704	94
	Clone _W1- 042	FJ764252		Uncultured beta proteobacterium Clone LiUU-30-393	EF066723	96
Methylophillales	*Methylophilaceae*					
	Clone _W1-130	FJ764471		Uncultured bacterium Clone Lgja-64	AY381286	95
	Clone _W1- 121	FJ764470		Uncultured betaproteobacterium Clone BIfci36	AJ318116	95
	Clone _W1- 026	FJ764211		Uncultured bacterium Clone HKT448	DQ439598	94
	Clone _W1- 077	FJ764215		Uncultured betaproteobacterium Clone HKT909	DQ989447	94

Appendix I: Summary of Clones related to Gamma Proteobacteria

Phylotype	Genus	GenBank Acc. No.	*n*	Closest Relative in Blast Search		% Similarity
	Rhodanobacter *et rel.,*					
	Clone _S2- 072	FJ764623		Uncultured bacterium Clone J56-814	EF174287	91
	Clone _S2- 159	FJ764625		Uncultured gammaproteobacterium Clone HF071	EF417660	91
Xanthomonadales						
	Thioalklaivibrio					
	Clone _S1 – 111	FJ764309		Uncultured gammaproteobact. Clone WN-FSB-157	DQ432081	97
	Thiothrix					
	Clone _S2- 160	FJ764618		Uncultured bacterium Clone ORCA-17N122	DQ823225	89
	Clone _S2- 147	FJ764617		Uncultured bacterium gene for 16S rRNA,	AB255060	94
Thiotricales						
	Piscirckettsiaceae					
	Clone _S2- 114	FJ764465		Uncultured bacterium Clone H49-814	EF174279	93
	Clone _S2 - 179	FJ764468		Uncultured bacterium gene for 16S rRNA,	AB187922	91
	Clone _S1- 136	FJ764310		Uncultured gammaproteobacterium Clone B1-29	AM229469	97
Chromatiales	*Ectothiorhodospiraceae*					
	Clone _W2 – 059	FJ764314		*Ectothiorhodosinus mongolicum* strain M9	AY298904	98
	Clone _D5 – 037	FJ764308		Phototrophic bacterium 16S rRNA gene (DSM 2111)	X93478	87
	Clone _W1- 056	FJ764312		Uncultured gammaproteobact. Clone ML1218M-31	AF452606	97
	Clone _W2 – 053	FJ764313		Uncultured gammaproteobact. Clone WN-FSB-157	DQ432081	97
	Clone _S2- 136	FJ764311		Uncultured bacterium Clone EV1E04	EF470447	93
	Clone _S2- 116	FJ764466		Uncultured bacterium Clone CJRC49	DQ202169	88
	Methylococcus					
Methylococcales	Clone _S2 - 170	FJ764467		Uncultured bacterium Clone ORCA-17N112	DQ823216	87

Appendix I: Summary of Clones related to Alpha Proteobacteria

Phylotype	Genus	GenBank Acc. No.	Closest Relative in Blast Search		% Similarity
	Phyllobacteriaceae				
	Clone _S1- 022	FJ764496	*Mesorhizobium* sp. NH-14 complete Uncultured	AB196496	98
Rhizobiales	*Methylocystaceae*				
	Clone _S1- 098	FJ764495	bacterium Clone 175up	AY212626	98
	Rhodobacteraceae				
	Clone _W4 - 087	FJ764532	Uncultured bacterium Clone BCM3S-3B	AY102910	95
	Clone _W1 - 054	FJ764516	Uncultured alphaproteobacterium Clone MU042	AM157606	90
Rhodobacterales	Clone _S5 - 052	FJ764515	Uncultured alpha proteobact. Clone WN-FWB-38	DQ432440	96
	Clone _W2 - 033	FJ764526	*Rhodobaca bogoriensis* strain LBB2	AF384205	99
	Clone _W4 - 033	FJ764529	*Rhodobaca bogoriensis* 16S ribosomal RNA gene	AF248638	99
	Clone _W1 - 063	FJ764517	Uncultured bacterium Clone CCSD_DF1080_B15	AY820696	97
	Clone _D5 - 055	FJ764502	Uncultured *alphaproteobac*t. Clone WN-FSB-111	DQ432069	97
	Clone _S1 - 003	FJ764506	Uncultured alphaproteobact. Clone WN-HSB-248	DQ432298	97
	Clone _S1 - 138	FJ764508	Uncultured alphaproteobact Clone WN-HSB-177	DQ432280	97
	Clone _W1 - 119	FJ764519	Uncultured alphaproteobacterium B30	EF655643	95
	Clone _W1- 093	FJ764518	Uncultured bacterium Clone M35.1	AJ517872	97
	Clone _W1-179	FJ764525	Uncultured bacterium Clone TFOa21	AY351636	97
	Clone _S2- 051	FJ764512	Alphaproteobacterium IMCC1702	EF220748	97
	Clone _S2- 152	FJ764514	Uncultured bacterium Clone EV1A06	EF470427	94
	Clone _D5 - 027	FJ764494	Uncultured alphaproteobact. Clone WN-HWB-29	DQ432334	98
	Clone _S2-108	FJ764513	Uncultured organism Clone ctg_CGOAA42	DQ395484	85
	Erythrobacteraceae				
	Clone _W4- 036	FJ764607	Uncultured Alphaproteobact. Clone WN-FSB-117	DQ432070	99
Spingomonadales	Clone _W2 - 036	FJ764605	Uncultured Alphaproteobacterium Clone MU059	AM157616	98
	Sphingomonadaceae				
	Clone _W2-067	FJ764606	Uncultured bacterium Clone EV1H09	EF470461	97
	Clone _W1-099	FJ764604	*Novosphingobium nitrogenifigens* strain Y88	DQ448852	94
	Clone _S2- 032	FJ764556	Uncultured bacterium Clone 7_06_MAT_RED_	DQ984469	97

Appendix IV: Summary of Clones related to Delta Proteobacteria

Phylotype	Genus	GenBank Acc. No.	*n*	Closest Relative in Blast Search		% Similarity
Myxococcales	*Polyangiaceae*					
	Clone _S2- 056	FJ764472		Polyangium sp. 16S rRNA gene, complete sequence	M94280	97
Bdellovibrionales	*Bacteriovoracaceae*					
	Clone _D5 - 095	FJ764245		Uncultured bacterium Clone FS140-91B-02	DQ513018	90
	Clone _W4- 075	FJ764247		Uncultured proteobacterium Clone SIMO-842	AY712379	85
	Desulfobulbaceae					
	Clone _D5 – 036	FJ764404		Uncultured bacterium isolate SSCP band LS1	DQ463287	92
Desulfobacterales	Desulfobacteraceae					
	Clone _S1- 119	FJ764405		Uncultured deltaproteobacterium Clone PA2	AY771978	95
	Clone _S4 - 078	FJ764407		Uncultured bacterium Clone Phe4A	EF467179	91
	Clone _S2- 085	FJ764411		Uncultured bacterium Clone T-OTU8	AY369164	88
	Clone _S4 – 042	FJ764246		Uncultured deltaproteobacterium Clone GA23	DQ408757	90
Desulfovibrionales	*Desulfonatronumaceae*					
	Clone _S1- 134	FJ764402		*Desulfonatronum lacustre* DSM 10312	AF418171	97

Appendix V: Summary of Clones related to Bacteroidetes, Chlorobi and Verrucomicrobia

Phylotype		GenBank Acc. No.	Closest Relative in Blast Search		% Similarity
	Uncultured				
Bacteroidetes	Clone _S2 – 173		Uncultured bacterium Clone Amb_16S_599	EF018245	87
	Clone _S2- 035		Uncultured Bacteroidetes bact. Clone WN-FSB-7	DQ432086	90
	Clone _D5 – 085		Uncult. Bacteroidetes bact. Clone WN-FWB-9	DQ432435	94
	Clone _S5 – 051		Uncult. Bacteroidetes bact. Clone WN-FWB-125	DQ432448	90
	Clone _D5 – 091		Uncult. Bacteroidetes bact. Clone WN-FWB-107	DQ432444	96
	Clone _W2 – 005		Uncultured bacterium Clone biogas-DE-b44	DQ419642	99
	Clone _W1- 113		Uncult. Bacteroidetes bacterium Clone MBAE28	AJ567587	84
	Clone _S3 – 059		Uncultured bacterium Clone ELB19-204	DQ015831	92
	Clone _W1- 015		Uncultured bacterium Clone:R1D21-25	AB205617	97
	Sphingobacteriaceae				
	Clone _S5 – 004		Uncultured bacterium Clone Asc-w-19	EF632711	94
	Clone _S1- 081		Uncultured Bacteroidetes Clone WN-HSB-94	DQ432272	93
	Clone _S2- 110		Uncultured bacterium Clone ORSFES_g07	EF393472	89
	Clone _W2 - 030		Uncultured bacterium Clone 1-1	AY548930	86
	Flavobacteriaceae				
	Clone _W4- 089		Uncultured bacterium Clone PL-23B1	AY570585	88
	Clone _W4- 060		Uncultured Bacteroidetes Clone MSB-5A10	DQ811911	95
	Clone _S1- 100		Uncultured bacterium Clone DSBR-B018	AY302118	94
	Cryomorphaceae				
	Clone _W1- 112		Uncultured bacterium Clone GZKB119	AJ853611	89
	Clone _W4- 052		Uncultured bacterium Clone ELB25-042	DQ015777	89
	Saprospiraceae				
	Clone _S2- 041		Uncultured bacterium Clone DTB123	EF205530	93
	Clone _W1- 135		Uncult. Bacteroidetes bact. Clone WN-USB-58	DQ432188	91
	Clone _S2 - 062		Uncultured bacterium Clone ML-7-75.2	DQ520178	86
	Flexibacteraceae				
	Clone _W2 – 080		Uncultured bacterium Clone W1038	AM259168	89
	Clone _W2 – 061		Uncultured *Flexibacteraceae* Clone MB-11	DQ507161	90
	Clone _W1- 025		*Flexibacter tractuosus* 16S ribosomal RNA	M58789	87

Appendix V: Summary of Clones related to Bacteroidetes, Chlorobi and Verrucomicrobia cont…….

	Flexibacteraceae				
	Clone _W1-178	FJ764594	*Chimaereicella alkaliphila* type strain AC74	AJ717393	95
	Clone _S2- 109	FJ764569	*Algoriphagus* sp. 10.1	AY695071	93
Bacteroidetes	Clone _W4- 068	FJ764600	*Flectobacillus ruber*	AY264064	94
	Clone _W1- 088	FJ764587	Uncultured Bacteroidetes bact. Clone WN-FSB-75	DQ432102	93
Chlorobi	*Uncultured*				
	Clone _S1- 033	FJ764289	Uncultured bacterium Clone:LS4-176	AB234252	90
	Clone _S1 – 082	FJ764280	Uncultured bacterium Clone TTMF84	AY741703	90
	Clone _S2- 026	FJ764295	Uncultured Chlorobi bacterium Clone MU048	AM157602	88
Verrucomicrobia	*lentisphaerae*				
	Clone _S3 - 002	FJ764292	Uncult. Verrucomicrobia bact. Clone ML316M-1	AF454310	95

Appendix VI: Summary of Clones related to Planctomyces

Phylotype		GenBank Acc. No.	Closest Relative in Blast Search		% Similarity
	Pirellula				
Planctomycetales	Clone _W1- 053	FJ764485	Planctomycete str. 449	AJ231177	98
	Clone _W4- 062	FJ764491	Uncultured bacterium isolate JH12_C66	AY568904	92
	Blastopirellula				
	Clone _S1 – 102	FJ764478	Uncultured planctomycete Clone Sylt 14	AM040110	89
	Clone _W1- 094	FJ764486	Uncultured bacterium Clone CCSD_DF1080_B6	AY820691	98
	Clone _S2 – 182	FJ764482	Uncultured *Pirellula* Clone 5H12	AF029076	89
	Clone _W2 - 020	FJ764489	Uncultured organism Clone ctg_NISA088	DQ396010	91
	Rhodopirellula		Planctomycete str. 337	AJ231171	95
	Clone _W1- 134	FJ764487			
	Uncultured				
	Clone _S1- 088	FJ764474	Uncultured bacterium Clone CD4F5	AY038495	90
	Clone _S2 – 165	FJ764481	Uncultured bacterium Clone 7_06_MAT_RED	DQ984461	96
	Clone _S1- 004	FJ764202	Uncult. forest soil bacterium Clone DUNssu137	AY913344	88

Appendix VII: Summary of Clones related to Firmicutes

Phylotype	Genus	GenBank Acc. No.	*n*	Closest Relative in Blast Search		% Similarity
	Clostridiaceae					
	Clone _S2- 021	FJ764360		Bacterium Te20A	AY587782	99
	Clone _S1- 051	FJ764286		Uncultured bacterium Clone OIV32	AY780039	87
	Clone _S4 – 048	FJ764377		Uncultured bacterium Clone AA94	EF016617	92
	Clone _W4- 079	FJ764394		Uncultured bacterium Clone HC21_9	AY508256	100
Clostridia	Clone _S4 – 012	FJ764372		Uncultured Clostridium sp. Clone TM2_12	DQ279397	98
	Clone _S1- 150	FJ764354		Uncultured bacterium Clone ORSPEP_h05	EF393547	98
	Clone _S1- 031	FJ764341		*Alkalliphilus auruminator*	AB037677	98
	Clone _S1- 001	FJ764336		*Clostridium mesophilicum*	AY682207	98
	Clone _D4 – 063	FJ764316		Uncultured bacterium Clone PL-3B11	AY570631	97
	Clone _S3 – 033	FJ764366		Uncultured bacterium Clone R35	AF407690	90
	Clone _D4 - 040	FJ764397		Uncult. low G+C Gram-pos. bact. Clone WN-FWB-18	DQ432432	97
	Clone _D5 – 092	FJ764329		Uncult. low G+C Gram-pos. bact. Clon WN-FWB-140	DQ432451	91
	Clone _D5 - 056	FJ764322		Uncult. low G+C Gram-pos. bact. Clon WN-FWB-117	DQ432446	92
	Clone _D5 - 086	FJ764327		*Clostridium elmenteitii* isolate E2SE1-B	AJ271453	96
	Clone _S1- 074	FJ764333		*Clostridium bogorii* 16S rRNA gene, isolate B8NS1-C	AJ271457	90
	Clone _S3 – 054	FJ764368		Uncultured bact. partial 16S rRNA gene, Clone M69.1	AJ517874	91
	Clone _D5 – 068	FJ764325		*Clostridium aminovorans*, isolate B7FT-A	AJ271455	93
	Clone _W4- 049	FJ764393		Bacterium str. 77003	AF227847	99
	Clone _D5 – 087	FJ764328		*Clostridium hastiforme* strain DSM 5675	X80841	92
	Clone _D5 – 050	FJ764321		Uncultured soil bacterium Clone M50_Pitesti	DQ378266	92
	Clone _S3 - 036	FJ764367		Uncult. low G+C Gram-pos. bact. Clone WN-HSB-258	DQ432330	96
	Desulfitibacter					
	Clone _S3 – 055	FJ764401		Uncult. low G+C Gram-pos. bact. Clon WN-FWB-126	DQ432449	98
	Clone _S5 - 036	FJ764389		Uncult. low G+C Gram-pos. bact. Clone WN-FSB-22	DQ432093	97
	Natronoanaerobium					
	Clone _S3 – 068	FJ764370		Uncult. low G+C Gram-pos. bact. Clone WN-USB-21	DQ432172	90
	Clone _S3 – 024	FJ764364		Uncult. low G+C Gram-pos. bact. Clone WN-FWB-29	DQ432438	90
	Clone _S1- 044	FJ764331		Uncult. low G+C Gram-pos. bact. Clone ML635J-34	AF507889	87

	Syntrophomonadaceae Clone _S1- 021 FJ764360		Uncultured bacterium Clone LCKS40-B3	DQ129947	93
Dethiobacter	*Uncultured*				
	Clone _S5 – 022	FJ764417	Uncult. low G+C Gram-positive bact. Clone ML635J-4	AF507887	94
	Clone _S5 – 032	FJ764418	*Dethiobacter alkaliphilus* strain AHT1	EF422412	97
	Clone _D5 – 088	FJ764412	Uncultured bacterium Clone LC2.42.E12	AY396023	96
	Alkalibacteria				
Bacillales-Mollicutes	Clone _D5 - 079	FJ764464	*Alkalibacterium* sp. G-He1	EF554904	98
	Clone _D4 - 015	FJ764454	Bacterial sp. (Lake Nakuru isolate WN16)	X92169	98
	Bacilli				
	Clone _S2 - 189	FJ764229	*Bacillus arbutinivorans* 16S ribosomal RNA gene,	AF519469	88
	Clone _D3 – 017	FJ764223	Uncultured bacterium Clone ZB-Z54	AY162956	98
	Clone _D3 - 020	FJ764224	*Bacillus pseudofirmus* 16S rRNA, complete sequence	AB029256	98
	Exiguobacteria				
	Clone _S1- 013	FJ764228	*Exiguobacterium* sp. BPC-C1/20-2	DQ999990	99

Appendix VIII: Summary of Clones related to Actinobateria, Spirochete, and Acidobacteria

Phylotype	Genus	GenBank Acc. No.	*n*	Closest Relative in Blast Search		% Similarity
	Uncultured					
	Clone _W1- 154	FJ764		Uncultured actinobacterium Clone A-B4-1C2	AY307895	98
	Clone _W1 – 023	FJ764210		Uncultured actinobacterium Clone ML817J-5	AF448181	98
Actinobacteria	Clone _W1- 068	FJ764213		Uncultured actinobacterium Clone ML602M-18	AF448182	98
	Clone _W1- 055	FJ764203		Uncultured actinobacterium Clone ADK-SGe02-63	EF520366	89
	Clone _S1- 030	FJ764288		Uncultured bacterium Clone AKIW776	DQ129254	92
	Clone _W4- 013	FJ764204		Uncultured actinobacterium Clone ML817J-9	AF448198	97
Spirochaete	*Spirochaetaceae*					
	Clone _D5 - 006	FJ764608		Uncultured spirochete Clone WN-FWB-135	DQ432450	97
Acidobacteria_4	Uncultured					
				Uncultured bact. Clone 7_06_GRN_MAT_16S_11	DQ984444	92
	Clone _S2- 082	FJ764538				

Appendix IX: Summary of Clones related to Cyanobacteria

Phylotype	Genus	GenBank Acc. No.	*n*	Closest Relative in Blast Search		% Similarity
	Cyanobium					
	Clone _W1- 176	FJ764		Uncultured bacterium clone S25_1631	EF575287	93
	Clone _W1- 008	FJ764731		Cyanobium sp. JJNV	AM710351	94
	Clone _W1- 132	FJ764638		Cyanobium sp. JJM10D4	AM690850	95
	Clone _W1- 018	FJ764		Cyanobium sp. JJ27STR	AM710383	98
	Clone _S1- 092	FJ764		Uncultured cyanobacterium clone GPENV80	DQ512825	95
Chroococcales						
	Gloeothece			Uncultured bacterium gene for 16S rRNA,	AB179527	92
	Clone _S2- 101	FJ764639				
	Microcystis					
	Clone _W2 -027	FJ764655		*Microcystis aeruginosa* strain SPC 777 1	EF121241	98
	Synechococcus					
	Clone _W4- 085	FJ764748		Synechococcus sp. 0BB26S03	AJ639899	98
	Clone _W1- 048	FJ764698		Uncultured cyanobacterium clone TH3-10	AM690909	97
	Clone _S1 – 060	FJ764690		Uncultured Cyanobium sp. clone EC49	DQ889923	94
	Clone _S1- 128	FJ764		Uncultured Synechococcus sp. clone Dpcom	AY664645	98
	Clone _S1- 064	FJ764		Synechococcus sp. 0BB26S03	AJ639899	99
	Clone _W4- 055	FJ764		Uncultured cyanobacterium clone GPENV80	DQ512825	96
	Clone _W1- 103	FJ764		Synechococcus sp. 0BB22S0	AJ639898	94
	Clone _W1- 125	FJ764		Uncultured cyanobacterium clone GPENV2	DQ512804	96
	Synechocystis					
	Clone _D4 – 072			Uncultured cyanobacterium clone GPENV75	DQ512824	97
	Clone _D5 – 003			Uncultured cyanobacterium clone GPENV152	DQ512833	97
	Clone _S1- 036			Uncultured cyanobacterium clone GPENV34	DQ512817	94
	Anabaena					
Nostocales	Clone _W2 - 079			Uncultured bacterium clone SILK9	EF467518	96
	Clone _S2- 054			Uncultured bacterium clone CI5cm.45	EF208676	91
	Clone _W1- 123			*Anabaena cylindrica*	AF091150	92
	Nodularia					
	Clone _W4- 054			*Nodularia sphaerocarpa* strain BECID36	AJ781147	98

Appendix IX: Summary of Clones related to Cyanobacteria cont…..

	Nostoc			
	Clone _W2 - 049	Uncultured bacterium clone YCB40	EF205475	91
	Clone _S2- 085	Uncultured bacterium clone T-OTU8	AY369164	88
	Clone _W2 - 032	Uncultured soil bacterium clone W4Ba81	DQ643743	88
	Calothrix			
	Clone _W2 - 003	Uncultured bacterium clone EV3G10	AM230683	98
	Arthrospira			
	Clone _S2 - 074	*Arthrospira platensis* CG590	EF222475	94
	Clone _S2- 040	*Arthrospira platensis* strain PCC 9223	DQ393285	94
Oscillatoriales	*Leptolyngbya*			
	Clone _S2 – 178	Leptolyngbya sp. Greenland_7	DQ431002	93
	Clone _S2 - 004	thermophilic cyanobacterium tBTRCCn 407	DQ471447	93
	Clone _S2 - 029	Uncultured cyanobacterium clone OB05	EF429514	99
	Clone _W2 - 046	Uncultured bacterium clone BT60PA10BC2	AF365825	85
	Oscillatoria			
	Clone _D5 - 007	Uncultured Antarctic cyanobacterium clone SalP03	AY541529	98
	Clone _D5 – 096	*Oscillatoria acuminata* gene for 16S rRNA,	AB039014	97
	Clone _D5 - 090	Unidentified bacterium DGGE band 5	AJ314574	96
	Spirulina			
	Clone _W1- 033	*Spirulina laxissima* strain SAG 256.80	DQ393278	98
	Clone _S2 – 205	Uncultured Cyanobacterium clone LrhB04	AM159315	95
	Clone _W2 – 039	*Spirulina subsalsa* 16S ribosomal RNA gene,	AF329394	99
	Clone _W2 - 009	Uncultured Cyanobacterium clone LrhB04	AM159315	94
	Trichodesmium			
	Clone _S1- 039	Uncultured Antarctic cyanobacterium clone FreP07	AY541565	91
Stigonematales	*Stigonema*			
	Clone _D5 - 025	Uncultured cyanobacterium clone SC3-19	DQ289927	91

Appendix X: Summary of Clones related to Archaea

Phylotype	Family	GenBank Acc. No.	*n*	Closest Relative in Blast Search		% Similarity
	Halobacteriaceae					
	Clone _S5 - 005	FJ746865		*Halorubrum* sp. strain EN-2	AM268115	93
	Clone _D5 -109	FJ746861		Uncultured haloarchaeon clone TX4CA_12	EF690567	93
	Clone _D5 - 130	FJ746862		Uncultured haloarchaeon clone TX4CA_65	EF690620	97
Halobacteriales	Clone _D3 - 042	FJ746849		Uncultured haloarchaeon clone TX4CA_75	EF690630	99
	Clone _D5 - 001	FJ746851		Uncultured haloarchaeon clone TX4CA_74	EF690629	98
	Clone _W3 – 004	FJ746872		Uncultured haloarchaeon clone TX4CA_47	EF690602	96
	Clone _D3 017	FJ746847		Uncultured haloarchaeon clone TX4CA_19	EF690574	98
	Clone _W4 012	FJ746874		*Halalkalicoccus tibetensis* strain DS12	AF435112	94
	Clone _S2 019	FJ746878		Uncultured haloarchaeon clone TX4CA_33	EF690588	95
	Clone _W4 046	FJ746877		*Halobiforma lacisalsi* strain AJ5	AY277582	96
	Clone _S5 058	FJ746866		*Halorubrum* sp. strain EN-2	AM268115	94
	Clone _W4 050	FJ746876		Uncultured archaeon clone WN-FWA-21	DQ432499	94
	Clone _D3 002			*Natronococcus amylolyticus*	D43628	94
	Clone _D3 007	FJ746846		*Natronococcus xinjiangense*	AF251285	98
	Clone _D5 045	FJ746856		Uncultured haloarchaeon clone TX4CA_19	EF690574	97
	Clone _S1 001			Uncultured archaeon clone MD2902-A25	EU048605	86
	Genera incertae sedis					
	Clone _W1 087	FJ746887		*Methanocalculus pumilus* gene for 16S rRNA,	AB008853	98
Methanomicrobiales	Clone _S5 050	FJ746884		Uncultured archaeon clone LR-100 16S rRNA	DQ302461	97
	Methanospirillaceae					
	Clone _W1 009	FJ746885		Uncultured Methanospirillaceae archaeon	AB236096	97
	Methanosaetaceae					
	Clone _W4 058	FJ746889		Uncultured archaeon Arc No. 5	AF395423	91
	Clone _W1 031	FJ746888		*Methanosaeta* sp. 16S rRNA gene	AJ133791	94
Uncultured	Clone _W1 025	FJ746891		Uncultured euryarchaeote clone VII-D1	EF376988	97
	Clone _W1 001	FJ746881		Uncultured archaeon, clone BCMS-10	AJ579730	97
	Clone _W1 022	FJ746890		Uncultured archaeon clone SBAK-shallow-27	DQ640153	95
	Clone _W1 093	FJ746883		Uncultured euryarchaeote clone VII-D1	EF376988	97

Appendix XI: Summary of Isolates

Class	Order	**Closest Relative in Blast Search**		**% Similarity**
Gamma Proteobacteria	*Chromatiales*			
	M18-C7	*Alkalimonas collagenimarina* gene for 16S rRNA,	AB270706	96
	Alteromonadales			
	M2-C21	Idiomarina sp. G-R2A15 16S ribosomal RNA gene,	EF554920	96
	M3-C2	Alteromonadales bacterium JK1 16S rRNA gene	EF554892	97
	M18-C3	Idiomarina sp. JK16 16S ribosomal RNA gene,	EF554894	98
	M7-C14	Idiomarina sp. JK17 16S ribosomal RNA gene	EF554895	97
	M3-C1	*Nitrincola lacisaponensis* strain 4CA	AY567473	99
	Vibrionales			
	M1-C2	*V.metschnikovii* (NCTC 11170) gene for 16S	X74712	97
	M4-C15	*Vibrio metschnikovii* 16S ribosomal RNA gene,	AY837747	98
	Oceanospirillales			
	M6-C1	*Halomonas campisalis* strain LL6 16	DQ077911	98
	M6-C9	*Halomonas pacifica* 16S ribosomal RNA gene	AY647301	99
	M6-C13	*Halomonas pacifica* 16S ribosomal RNA gene	EU373088	98
	M5-C9	Halomonas sp. 3014 partial 16S rRNA gene	AM110971	98
	M4-C6	Halomonas sp. MAS4B gene for 16S rRNA,	AB369212	98
	M6-C16	Halomonas sp. SB J83 gene for 16S rRNA gene	AB167059	99
	S-C21	*Halomonas variabilis* strain HTG7 16S rRNA gene	AY204638	98
	M4-C21	*Halomonas venusta* partial 16S rRNA , DSM 4743	AJ306894	90
	M2-C11	Oceanospirillum sp. ML-158 16S rRNA gene,	AF140000	99
	S-C30	*Marinospirillum megaterium* gene for 16S rRNA	EU375807	97
	S-C26	Gamma proteobacterium M12-26A 16S	AY730246	96
	M7-C9	Gamma proteobacterium N10 16S rRNA gene	AF250323	99
	S-C28	Unidentified Hailaer soda lake bacterium Z4	AF275713	97

Appendix XI: Summary of Isolates cont……..

	Lactobacillales			
	M17-C11	*Alkalibacterium olivoapovliticus* strain WW2-SN4a	AF143511	98
	M15-C8	*Alkalibacterium olivoapovliticus* strain WW2-SN4c	AF143512	98
	M8-C16	*Alkalibacterium psychrotolerans* gene for 16S	AB125938	98
	M9-C7	Alkalibacterium sp. G-He1 16S ribosomal RNA gene	EF554904	98
	M9-C16	Alkalibacterium sp. NP13 16S ribosomal RNA gene	EU196343	97
	M7-C13	Amphibacillus sp. Blan-31 16S rRNA gene	EU596928	99
	M1-C3	*Halolactibacillus xiariensis* strain H-5 16S	EF554593	98
	M14-C7	*Marinilactibacillus psychrotolerans*	AB159717	96
	Bacillales			
	M14-C12	Bacillaceae bacterium C12 16S rRNA gene,	AY504438	99
	M3-C3	*Bacillus agaradhaerens* DSM 8721,	X76445	99
	M10-C17	*Bacillus alkalidiazotrophicus* strain MS 6	EU143680	94
	M14-C16	*Bacillus bogoriensis* strain LBB3	AY376312	95
	M3-C11	*Bacillus cohnii* 16S ribosomal RNA gene	AB023412	99
	M4-C2	*Bacillus horikoshii* gene for 16S rRNA	X76443	99
	M10-C6	*Bacillus horikoshii* strain Oct1	DQ363137	99
	M12-C2	*Bacillus pseudalcaliphilus* DSM 8725	X76449	97
	M9-C11	*Bacillus pseudofirmus* DSM 8715, 16S rRNA gene	X76439	99
	M9-C9	*Bacillus pseudofirmus* gene for 16S	AF406790	99
	M4-C10	*Bacillus pseudofirmus* gene for 16S...	AB029256	99
	M12-C8	*Bacillus pseudofirmus* strain FTU 16S rRNA gene	AF406790	98
	M13-C16	Bacillus sp. 2b-2 gene for 16S rRNA	AB043857	99
	M7-C4	Bacillus sp. ANL-iso4 16S ribosomal RNA gene,	EF422411	96
	M3-C10	Bacillus sp. JAMB-602 gene for 16S rRNA	AB120000	99
	M10-C9	Bacillus sp. S2 gene for 16S rRNA	AB043860	99
	M12-C6	Bacillus sp. YIM KMY30 16S ribosomal RNA gene,	DQ358664	99
	M12-C27	Bacillus sp. ZBAW6 16S ribosomal RNA,	AY453415	98
	S-C29	Bacterial sp. 16S rRNA gene (Lake Bo...	X92142	98
Actinobacteria	M9-C5	*Nocardiopsis exhalans* gene for 16S rRNA,	AB368714	99
	M4-C22	*Streptomyces purpureus* partial 16S rRNA	X53170	96

Firmicutes

APPENDIX XII: Summary of the sequenced isolates from lake Elmenteita

S-Lab	Isolate ID	Bp	Accession	Next Relative	% similarity
361	M17-C11	567	FJ763885	*Alkalibacterium olivoapovliticus* strain WW2-SN4a	98
299	M15-C8	562	FJ763876	*Alkalibacterium olivoapovliticus* strain WW2-SN4c	98
399	M17-C52	569	FJ763887	*Alkalibacterium olivoapovliticus* strain WW2-SN4c	98
129	M8-C16	593	FJ763857	*Alkalibacterium psychrotolerans*	98
405	M18-C2	501	FJ763888	Alkalibacterium sp. G-He1	99
145	M9-C7	509	FJ763860	Alkalibacterium sp. G-He1	98
154	M9-C16	505	FJ764159	Alkalibacterium sp. NP13	97
410	M18-C7	490	FJ764178	*Alkalimonas collagenimarina*	96
48	M3-C12	491	FJ763897	Alteromonadales bacterium JK1	97
45	M3-C9	490	FJ763896	Alteromonadales bacterium JK1	98
44	M3-C8	490	FJ763895	Alteromonadales bacterium JK1	97
38	M3-C2	490	FJ763894	Alteromonadales bacterium JK1	97
104	M7-C5	492	FJ763898	Alteromonadales bacterium JK1	98
27	M2-C14	531	FJ763893	Alteromonadales bacterium JK1	98
240	M13-C7	546	FJ763900	Alteromonadales bacterium JK1	98
112	M7-C13	517	FJ763901	Amphibacillus sp. Blan-31	96
278	M14-C12	569	FJ764013	Bacillaceae bacterium C12	99
204	M11-C19	508	FJ763958	*Bacillus agaradhaerens* DSM 8721,	99
213	M12-C7	508	FJ763964	*Bacillus agaradhaerens* DSM 8721,	99
222	M12-C16	510	FJ763970	*Bacillus agaradhaerens* DSM 8721,	98
39	M3-C3	490	FJ763905	*Bacillus agaradhaerens* DSM 8721,	99
207	M12-C1	508	FJ763961	*Bacillus agaradhaerens* DSM 8721,	98
188	M11-C3	509	FJ763954	*Bacillus agaradhaerens*	99
190	M11-C5	507	FJ763955	*Bacillus agaradhaerens*	99
172	M10-C17	500	FJ763948	*Bacillus alkalidiazotrophicus* strain MS 6	94
282	M14-C16	560	FJ763989	*Bacillus bogoriensis* strain LBB3	95
47	M3-C11	496	FJ763907	*Bacillus cohnii* 16S	99
50	M4-C2	497	FJ763909	*Bacillus horikoshii*	99

161	M10-C6	494	FJ763938	*Bacillus horikoshii* strain Oct1	99
162	M10-C7	503	FJ763939	*Bacillus horikoshii* strain Oct1	99
160	M10-C5	533	FJ763937	*Bacillus horikoshii* strain Oct1	99
208	M12-C2	506	FJ763962	*Bacillus pseudalcaliphilus* DSM 8725	97
277	M14-C11	504	FJ763985	*Bacillus pseudalcaliphilus* DSM 8725	97
166	M10-C11	504	FJ763943	*Bacillus pseudalcaliphilus* DSM 8725	97
56	M4-C8	561	FJ763912	*Bacillus pseudalcaliphilus* DSM 8725	97
179	M10-C24	576	FJ763950	*Bacillus pseudalcaliphilus* DSM 8725	97
55	M4-C7	533	FJ763911	*Bacillus pseudalcaliphilus* DSM 8725	96
163	M10-C8	586	FJ763940	*Bacillus pseudalcaliphilus* DSM 8725	97
287	M14-C21	544	FJ763993	*Bacillus pseudalcaliphilus* DSM 8725	97
149	M9-C11	534	FJ763933	*Bacillus pseudofirmus* DSM 8715	99
147	M9-C9	501	FJ763932	*Bacillus pseudofirmus*	99
58	M4-C10	501	FJ763913	*Bacillus pseudofirmus*	99
121	M8-C8	498	FJ763924	*Bacillus pseudofirmus* strain FTU	98
241	M13-C8	505	FJ763975	*Bacillus pseudofirmus* strain FTU	99
214	M12-C8	489	FJ763965	*Bacillus pseudofirmus* strain FTU	98
153	M9-C15	504	FJ763934	*Bacillus pseudofirmus* strain FTU	99
255	M13-C22	522	FJ763979	Bacillus sp. 27-1	100
249	M13-C16	544	FJ763978	Bacillus sp. 2b-2	99
242	M13-C9	548	FJ763976	Bacillus sp. A-11	100
196	M11-C11	506	FJ763956	Bacillus sp. ANL-iso4	97
170	M10-C15	505	FJ763946	Bacillus sp. ANL-iso4	97
103	M7-C4	540	FJ763922	Bacillus sp. ANL-iso4	96
291	M14-C25	564	FJ763996	Bacillus sp. JAMB-602	99
46	M3-C10	504	FJ763906	Bacillus sp. JAMB-602	99
49	M4-C1	547	FJ763908	Bacillus sp. S2	99
269	M14-C3	509	FJ763994	Bacillus sp. S2	100
247	M13-C14	521	FJ763977	Bacillus sp. S2	100
61	M4-C13	548	FJ763914	Bacillus sp. S2	99
206	M11-C21	522	FJ763960	Bacillus sp. S2	100
68	M4-C20	517	FJ763917	Bacillus sp. S2	99

76	M5-C6	515	FJ763918	Bacillus sp. S2	99
180	M10-C25	520	FJ763951	Bacillus sp. S2	100
167	M10-C12	571	FJ763944	Bacillus sp. S2	99
165	M10-C10	510	FJ763942	Bacillus sp. S2	99
164	M10-C9	572	FJ763941	Bacillus sp. S2	99
157	M10-C2	560	FJ763935	Bacillus sp. S2	99
62	M4-C14	529	FJ763914	Bacillus sp. S2	99
205	M11-C20	521	FJ763959	Bacillus sp. S2	100
212	M12-C6	490	FJ763963	Bacillus sp. YIM KMY30	99
219	M12-C13	501	FJ763968	Bacillus sp. YIM KMY30	99
220	M12-C14	496	FJ763969	Bacillus sp. YIM KMY30	99
233	M12-C27	516	FJ763973	Bacillus sp. ZBAW6	98
232	M12-C26	540	FJ763972	Bacillus sp. ZBAW6	98
467	S-C29	486	FJ764166	Bacterial sp. (Lake Bo...	98
19	M2-C6	485	FJ764056	Bacterial sp. (Lake Bogoria isolate 65B4	99
88	M6-C5	493	FJ764074	Bacterial sp. (Lake Bogoria isolate 65B4)	99
305	M15-C14	481	FJ764111	Bacterial sp. (Lake Bogoria isolate 65B4)	99
364	M17-C14	538	FJ764135	Bacterial sp. (Lake Bogoria isolate 65B4)	98
312	M15-C21	454	FJ764139	Bacterial sp. (Lake Bogoria isolate 65B4)	99
93	M6-C10	493	FJ764077	Bacterial sp. (Lake Bogoria isolate 65B4)	99
156	M10-C1	515	FJ764005	*Enterococcus faecalis* strain 47-3	100
33	M2-C20	515	FJ764004	*Enterococcus saccharolyticus*	100
462	S-C24	480	FJ764036	Gamma proteobacterium M12-26A	99
464	S-C26	476	FJ764038	Gamma proteobacterium M12-26A	99
458	S-C20	480	FJ764034	Gamma proteobacterium M12-26A	99
473	S-C35	479	FJ764041	Gamma proteobacterium M12-26A	99
191	M11-C6	482	FJ764016	Gamma proteobacterium M12-26A	99
192	M11-C7	491	FJ764017	Gamma proteobacterium M12-26A	99
374	M17-C25	465	FJ764027	Gamma proteobacterium M12-26A	99
461	S-C23	477	FJ764035	Gamma proteobacterium M12-26A	98
360	M17-C10	459	FJ764026	Gamma proteobacterium M12-26A	100
296	M15-C5	467	FJ764022	Gamma proteobacterium M12-26A	100

108	M7-C9	488	FJ764770	Gamma proteobacterium N10	97
3	M1-C3	518	FJ764048	*Halolactibacillus xiariensis* strain H-5	98
84	M6-C1	486	FJ764073	*Halomonas campisalis* strain LL6	99
17	M2-C4	480	FJ764055	*Halomonas campisalis* strain LL6	99
7	M1-C7	501	FJ764052	*Halomonas campisalis* strain LL6	99
16	M2-C3	521	FJ764054	*Halomonas campisalis* strain LL6	99
71	M5-C1	525	FJ764067	*Halomonas campisalis* strain LL6	99
376	M17-C27	445	FJ764142	*Halomonas campisalis* strain LL6	100
159	M10-C4	485	FJ764097	*Halomonas campisalis* strain LL6	99
377	M17-C28	469	FJ764143	*Halomonas campisalis* strain LL6	99
381	M17-C32	479	FJ764146	*Halomonas campisalis* strain LL6	100
382	M17-C33	479	FJ764147	*Halomonas campisalis* strain LL6	99
383	M17-C34	479	FJ764148	*Halomonas campisalis* strain LL6	99
98	M6-C15	495	FJ764082	*Halomonas campisalis* strain LL6	100
395	M17-C47	393	FJ764153	*Halomonas campisalis* strain LL6	99
105	M7-C6	494	FJ764086	*Halomonas campisalis* strain LL6	100
118	M8-C5	493	FJ764090	*Halomonas campisalis* strain LL6	100
115	M8-C2	495	FJ764088	*Halomonas campisalis* strain LL6	99
133	M8-C20	490	FJ764091	*Halomonas campisalis* strain LL6	100
324	M16-C5	445	FJ764120	*Halomonas campisalis* strain LL6	100
294	M15-C3	445	FJ764104	*Halomonas campisalis* strain LL6	100
295	M15-C4	480	FJ764105	*Halomonas campisalis* strain LL6	100
297	M15-C6	445	FJ764106	*Halomonas campisalis* strain LL6	100
301	M15-C10	386	FJ764109	*Halomonas campisalis* strain LL6	99
306	M15-C15	470	FJ764112	*Halomonas campisalis* strain LL6	99
308	M15-C17	445	FJ764113	*Halomonas campisalis* strain LL6	99
309	M15-C18	417	FJ764114	*Halomonas campisalis* strain LL6	100
358	M17-C8	447	FJ764132	*Halomonas campisalis* strain LL6	99
316	M15-C25	446	FJ764118	*Halomonas campisalis* strain LL6	99
370	M17-C20	446	FJ764138	*Halomonas campisalis* strain LL6	99
342	M16-C23	420	FJ764126	*Halomonas campisalis* strain LL6	100
365	M17-C15	364	FJ764136	*Halomonas campisalis* strain LL6	100

363	M17-C13	445	FJ764134	*Halomonas campisalis* strain LL6	100
355	M17-C5	476	FJ764129	*Halomonas campisalis* strain LL6	100
356	M17-C6	468	FJ764130	*Halomonas campisalis* strain LL6	100
362	M17-C12	469	FJ764133	*Halomonas campisalis* strain LL6	99
357	M17-C7	470	FJ764131	*Halomonas campisalis* strain LL6	99
380	M17-C31	485	FJ764145	*Halomonas campisalis* strain LL6	99
91	M6-C8	520	FJ764075	*Halomonas campisalis* strain LL6	99
404	M18-C1	486	FJ764155	*Halomonas campisalis* strain LL6	99
394	M17-C46	490	FJ764152	*Halomonas campisalis* strain LL6	99
390	M17-C41	487	FJ764151	*Halomonas campisalis* strain LL6	99
74	M5-C4	530	FJ764068	*Halomonas campisalis* strain LL6	99
353	M17-C3	490	FJ764128	*Halomonas campisalis* strain LL6	99
184	M10-C29	530	FJ764099	*Halomonas campisalis* strain LL6	99
350	M16-C31	486	FJ764127	*Halomonas campisalis* strain LL6	99
302	M15-C11	496	FJ764110	*Halomonas campisalis* strain LL6	99
409	M18-C6	526	FJ764156	*Halomonas campisalis* strain LL6	99
428	M20-C1	523	FJ764160	*Halomonas campisalis* strain LL6	99
334	M16-C15	521	FJ764122	*Halomonas campisalis* strain LL6	98
335	M16-C16	504	FJ764123	*Halomonas campisalis* strain LL6	98
387	M17-C38	521	FJ764149	*Halomonas campisalis* strain LL6	99
300	M15-C9	495	FJ764108	*Halomonas campisalis* strain LL6	98
92	M6-C9	493	FJ764076	*Halomonas pacifica*	98
96	M6-C13	486	FJ764080	*Halomonas pacifica*	98
90	M6-C7	525	FJ764764	*Halomonas pacifica* LL6	97
429	M20-C2	527	FJ764161	*Halomonas pacifica* LL6	97
79	M5-C9	526	FJ764072	Halomonas sp. 3014	98
54	M4-C6	488	FJ764064	Halomonas sp. MAS4B	99
99	M6-C16	532	FJ764083	Halomonas sp. SB J83	98
459	S-C21	490	FJ764165	*Halomonas variabilis* strain HTG7	90
339	M16-C20	520	FJ764125	*Halomonas variabilis* strain HTG7	99
69	M4-C21	503	FJ764066	*Halomonas venusta* DSM 4743	97
34	M2-C21	498	FJ764171	Idiomarina sp. G-R2A15	96

406	M18-C3	520	FJ764177	Idiomarina sp. JK16	98
113	M7-C14	493	FJ764789	Idiomarina sp. JK17	97
25	M2-C12	493	FJ764170	Idiomarina sp. JK17	99
303	M15-C12	490	FJ764176	Idiomarina sp. JK17	95
116	M8-C3	494	FJ764174	Idiomarina sp. JK17	98
122	M8-C9	523	FJ764175	Idiomarina sp. JK17	96
36	M2-C23	531	FJ764788	Idiomarina sp. JK38	100
273	M14-C7	538	FJ764780	*Marinilactibacillus psychrotolerans*	89
463	S-C25	484	FJ764037	*Marinospirillum megaterium*	96
494	S-C56	492	FJ764045	*Marinospirillum megaterium*	96
496	S-C58	490	FJ764047	*Marinospirillum megaterium*	96
468	S-C30	494	FJ764040	*Marinospirillum megaterium*	96
472	S-C34	491	FJ764167	*Marinospirillum megaterium*	96
476	S-C38	490	FJ764042	*Marinospirillum megaterium*	96
292	M15-C1	513	FJ764782	Marinospirillum sp. v1c_Sn-red	93
352	M17-C2	528	FJ764025	Marinospirillum sp. v1c_Sn-red	92
38	M3-C1	470	FJ764760	*Nitrincola lacisaponensis* strain 4CA	99
148	M9-C10	472	FJ764191	*Nocardiopsis exhalans*	100
143	M9-C5	518	FJ764180	*Nocardiopsis exhalans*	99
24	M2-C11	322	FJ764058	Oceanospirillum sp. ML-158	97
18	M2-C5	483	FJ764	Pseudomonas sp. G-R2A7	99
70	M4-C22	535	FJ764793	*Streptomyces purpureus* partial	96
466	S-C28	479	FJ764785	Unidentified Hailaer soda lake bacterium Z4	99
2	M1-C2	495	FJ764182	*V.metschnikovii* (NCTC 11170)	98
150	M9-C12	494	FJ764192	*V.metschnikovii* (NCTC 11170)	96
142	M9-C4	497	FJ764190	*Vibrio metschnikovii*	98
63	M4-C15	502	FJ764187	*Vibrio metschnikovii*	98
65	M4-C17	499	FJ764189	*Vibrio metschnikovii*	98