Microbial Diversity of Soda Lake Habitats

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Contents

1. Introduction

1.1. The soda lake environment

Soda lakes and soda deserts represent the major types of naturally occurring highly alkaline environments, in which the indigenous microflora is subjected to a number of extreme ecological pressures. Such sites are widely distributed throughout the world (see Table 1.1) and most of the detailed biological analyses have been limnological rather than microbiological. The best studied soda lakes are those of the East African Rift valley which have been investigated since the early 1930s (Grant *et al*., 1990; Jenkin, 1932; Jones *et al*., 1998; Jones *et al*., 1994; Tindall, 1988), wherein detailed limnological and microbiological investigations have been carried out. Microbiological studies of Central Asian soda lakes have also been well documented (Zhilina & Zavarzin, 1994).

Table 1.1: World-wide distribution of soda lakes and soda desert

North America	
Canada	Manito
United States	Alkali Valley, Albert Lake, Lake Lenore, Soap Lake, Big Soda Lake, Owens Lake, Mono Lake, Searles Lake, Deep Springs, Rhodes Marsh, Harney Lake, Summer Lake, Surprise Valley, Pyramid Lake, Walker Lake
Central America	
Mexico	Texcoco
South America	
Venezuela	Langunilla Valley
Chile	Antofagasta
Europe	
Hungary	Lake Fehér
Yugoslavia	Pecena Slatina
Russia	Kulunda Steppe, Tanatar Lakes, Karakul, Araxes plain, Chita, Barnaul, Slavgerod
Asia	Central Asia: lakes of Tuva
Turkey	Van
India	Lake Looner, Lake Sambhar
China	Qinhgai Hu, Sui-Yian, Heilungkiang, Kirin, Jehol, Chahar, Shansi, Shensi, Kansu
Tibet	Soda lake
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 Elmentieta, Lake Magadi, Lake Simbi, Lake Sonachi
Tanzania	Lake Natron, Lake Embagi, Lake Magad, Lake Manyara, Lake Balangida, Basotu Crate lakes, Lake Kusare, Lake Tulusia, El Kekhooito, Momela lakes, Lake Lekandiro, Lake Reshitani, Lake Lgarya, Lake Ndutu, Lake Ruckwa North
Uganda	Lake Katwe, Lake Mahega, Lake Kikorongo, Lake Nyamunuka, Lake Munyanayange, Lake Murumuli, Lake Nunyampaka
Chad	Lake Bodu, Lake Rombou, Lake Djikare, Lake Momboio, Lake Yoan
Australia	Lake Corangamite, Red Rock Lake, Lake Werowrap, Lake Chidnup

The formation of the soda lake environment

The conditions necessary for the formation of a soda lake have much in common with those responsible for the generation of an athalassohaline (i.e., not derived from sea water) salt lake. However, in a soda lake carbonate or carbonate complexes become the major anions in solution (Jones *et al.*, 1994). The alkaline conditions in soda lakes were, for many years, known to be derived from the presence of unusually high levels of sodium carbonate. A number of theories concerning the source of the carbonate have been proposed (Abd-el-Malek & Rizk, 1963; Baker, 1958). The simplest and most favoured explanation was that this was due to a geological anomaly, where the rocks contained high levels of sodium carbonate, washed out by groundwater and accumulated in the lake basin (Baker, 1958). Detailed theories of the formation of alkaline, saline lakes have appeared elsewhere (e.g., Eugster & Hardie, 1978) and only a summary of the important features will be given here. The factors leading to the formation of the alkaline, saline deposits, such as those found at Lake Magadi, Kenya may be divided into climatic, geological, and topographical (Tindall, 1988): The influence of **climate** controls 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. Solution of carbon dioxide results in the formation of a weak acid, carbonic acid, which undergoes ion exchange with the surrounding rock, leaching the minerals. Consequently, the geochemistry of the region directly affects the ionic composition of the groundwater, although this may be further modified by precipitation of insoluble salts or replenishment of lost bicarbonate/carbonate by further solution of carbon dioxide. In the case of the alkaline environment, the most important factor is a lack of magnesium and calcium in the surrounding strata, which means an absence of rocks of sedimentary origin. Concentration of the ions in the groundwater through evaporation leads to a shift in the carbon dioxide/bicarbonate/carbonate equilibrium in favour of carbonate:

$$\underset{\text{carbon dioxide}}{CO_2 + H_2O} \rightleftharpoons \underset{\text{carbonic acid}}{H_2CO_3} \rightleftharpoons \underset{\text{bicarbonate}}{H^+ + HCO_3^-} \rightleftharpoons \underset{\text{carbonate}}{2H^+ + CO_3^{2-}}$$

An increase in carbonate ion concentration results in the precipitation of insoluble carbonates of, first, calcium, and, then, magnesium, removing these divalent cations from solution and allowing the more soluble carbonates of sodium and potassium to accumulate. The final stages in the formation of an alkaline brine are dependent on the **topography**, which allows the concentration of the salts in a shallow depression forming

a closed drainage basin with a high marginal relief, having sufficient rainfall to sustain streams entering the basin to produce a standing body of water. There is generally little or no outflow and water loss is by evaporation. In arid zones with high rates of evaporation exceeding inflow, salts accumulate.

In the case of Lake Magadi the presence of hot springs may also result in some recirculation of the brines, but there is no evidence that this recirculation adds further solutes to the brines or that other sources of sodium carbonate, such as the volcano Ol Doiny Lengai, which lies south of Lake Magadi, significantly contribute to the alkalinity and salinity of this lake, or the neighbouring Lake Natron (Baker, 1958; Eugster, 1970; Jones *et al.*, 1977).

An alternative method for the generation of alkalinity, involving bacterial sulfate reduction, has been proposed by Abd-el-Malek for the lakes of the Wadi Natrun depression in Egypt (Abd-el-Malek and Rizk, 1963), but this is unlikely to be a general mechanism.

The eastern or Gregory Rift Valley in East Africa contains a number of closed-basin lakes, stretching from Ethiopia to Tanzania (see Fig. 1.1). Some of these are fresh, like Naivasha, but others are quite saline, with sodium carbonate-bicarbonate comprising the major solute. Hydrologic closure is provided either by volcanic craters (e.g., Lake Bogoria) or by natural depressions in association with block faulting of the lavas forming on the floor of the Rift Valley. The salinities of these lakes range from approximately 5% total salts (w/v), in the case of the more northerly lakes (Bogoria, Nakuru, Elmenteita and Sonachi), to saturation (30% or greater) in the southern lakes (Magadi, Little Magadi and Natron), with roughly equal proportions of Na_2CO_3 and NaCl as the major salts (Grant, 1992). The pH values range from 9 to above 11.5 in the most concentrated lakes. The Rift Valley also exhibits active volcanism, with numerous hot springs (some boiling) on the shores of some of the lakes. These springs are more diluted and less alkaline than the lakes they feed but, where mixing occurs, complex pH-, temperature- and salinity-gradients occur, affording a range of different soda lake habitats (Duckworth *et al.*, 1996).

In the Rift Valley of Kenya-Tanzania, the chain of lakes occupying the rift floor depressions represents remnants of lakes of the pluvial period that occurred 10,000 years ago (Zavarzin *et al.*, 1999). The Rift is characterised by active volcanic activities supplying eruption material rich in carbonatites (carbonate igneous magmatic rock often associated with kimberlite and alkaline rock). The lakes are located in an area with an extremely large geothermal gradient and connected with subterranean hydrotherms forming gas-steam vents, as in Lake Bogoria, or thermal outlets in Lake Magadi.

Fig. 1.1: Map of the Rift Valley, Kenya, showing the position of the lakes in relation to the main topographical features of the Rift (Brown, 1973). All lakes in the Rift Valley are soda lakes, unless otherwise marked (fresh). The major geological features of the area, fault lines and volcanoes, are also illustrated. Lake Hannington is now called Lake Bogoria.

Lake Magadi

Lake Magadi is an extreme example of a highly saline, alkaline (soda) lake. It has been studied largely because of the vast trona deposits which are mined commercially (Baker, 1958). Lake Magadi is especially interesting in that, despite its unique features, it served as a model for the modern process of soda deposit formation (Eugster, 1986). Fig. 1.2 gives an idealised scheme of the processes taking place at Lake Magadi.

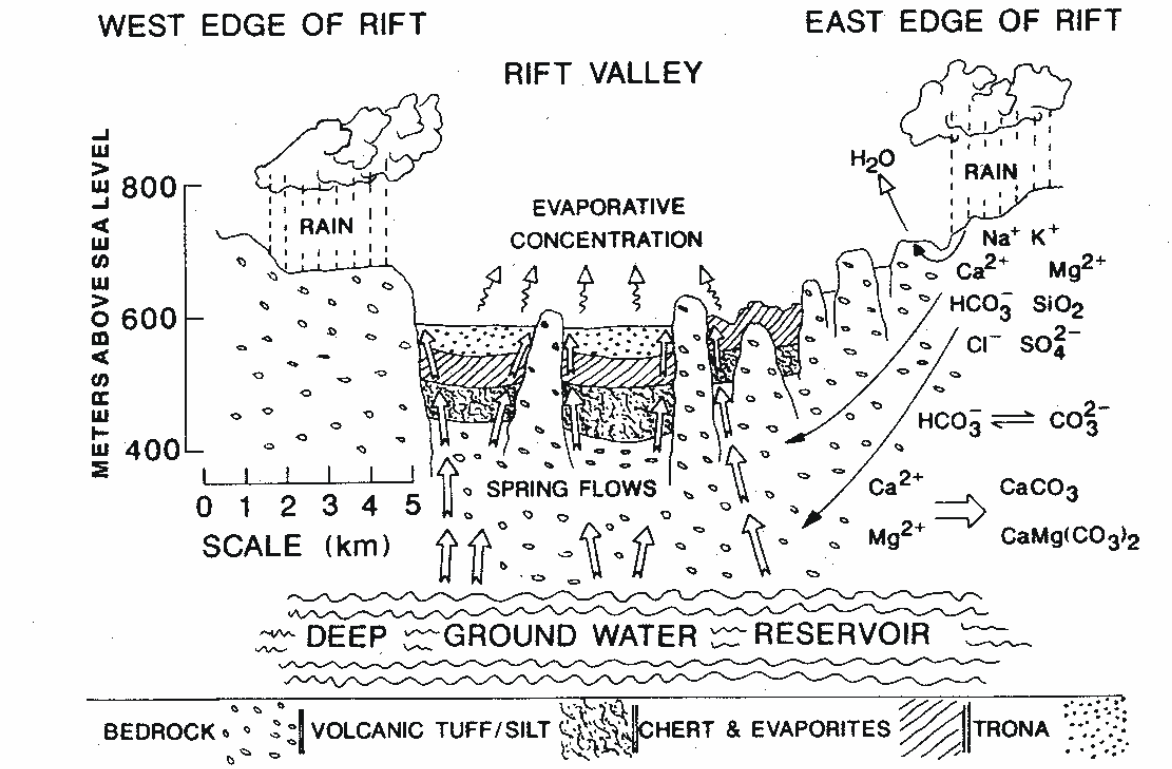

Fig. 1.2: Schematic representation of the possible mechanisms involved in the formation of Lake Magadi –an alkaline, saline lake (modified from Grant *et al.*, 1990; Jones *et al.*, 1977)).

Lake Magadi, Kenya lies in the southern part of the graben of the Gregory Rift Valley, close to the Tanzania border, between Lake Natron in the south and freshwater Lake Naivasha to the north (Fig. 1.1). Magadi is approximately 2°S of the equator, at an elevation of 600 m above sea level and lies in the lowest part of the trough in a naturally formed closed lake basin. Covering an area of 90 km^2, Magadi is one of the smaller rift lakes. Evaporation is intense during the dry season (3,500 mm/a), the range of temperatures being between 22°C and 34°C. The Loita Hills and the Mau Escarpment to the west shield the valley floor from rainfall, resulting in an annual total of approximately 500 mm of rainfall in the two rainy seasons (Behr & Röhricht, 2000). It has also been postulated that subterranean outflow from Lake Naivasha contribute to the brines, and are comparable in solute load to the saline groundwater (Eugster, 1970). The Magadi trough is approximately 40 km long and has several arms, each 3-4 km wide,

representing fault-bound grabens in the thick sequences of trachyte lava flows which filled the floor of the Rift Valley 1-1.9 million years ago (Baker *et al*., 1971). The development towards an alkaline saline lake (pH=9.0-12.5) occurred during the past 12,000 years, mainly, due to climatic changes. It was locally associated with increased tectonic activity that modified the local rift morphology and the local hydrological and climatic systems (Behr & Röhricht, 2000). Tectonic activity and seismicity in the Magadi depression continues presently. The rock formations are predominantly of volcanic origin and consist mainly of alkaline trachyte type lavas (Baker, 1958). The lake consists, for the most part, of an almost solid deposit of sodium chloride and sodium carbonate (usually as sodium sesquicarbonate, $Na_2CO_3 \cdot NaHCO_3 \cdot 2H_2O$, also known as trona), the upper layers forming a white surface, a few centimetres thick. The main body of the lake trough contains a deposit of bedded trona as thick as 50 m, called the Evaporite Series, which has accumulated since high Magadi time, dated at 12,000 to 7,100 years (Butzer *et al*., 1972). This deposit, which thins towards the edges, has a high porosity and contains a concentrated alkaline brine. The Magadi trough contains sediment deposited from two older, somewhat larger and more dilute lakes: Lake Oloronga dated by C^{14} at > 780, 000 years and High Magadi Lake dated at 12,000 to 7,100 years (Eugster & Hardie, 1978). The shore lines of High Magadi time are still visible some 15 m above the present lake level, while Oloronga must have been considerably deeper, because its sediments are covering some of the shallower trachyte horsts near the lake. The precursors of Lake Natron and Lake Magadi are suspected to have been interconnected during the period of the former Lake Oloronga. Trona continues to form at the present time. In all but exceptionally wet years, or during the rainy seasons, the lake water lies below the surface and surface water is usually only found around the edges of the crystalline deposits, where thermal springs feed the lake (Tindall, 1980). There are no permanent rivers entering the Magadi basin and solutes are supplied mainly by a series of alkaline springs, with temperatures as high as 86°C, which are located around the perimeter of the lake. The springs feed lagoons, which are perennial water bodies at the lake margins. Where the salinity is low enough, and the temperatures not too high, these peripheral lagoons support a thriving colony of fish *Tilapia grahami*, which can tolerate a pH of 10.5 and temperatures of 39°C. In the more saline lagoons, probably in the absence of *Tilapia*, mass accumulations of microorganisms may be observed (Tindall, 1988). The crystalline trona deposits of the lake itself are variously coloured off-white, red/orange, or red/purple. Closer examination of the surface trona deposits shows that, under appropriate conditions, a visible microbial stratification occurs which resembles stromatolitic formations found in other benthic saline environments (Tindall, 1980). The lower layer of the lake is a region of degrading organic matter rendered black by sulfate

reduction. In summary, in terms of the water chemistry and mineralogy, Lake Magadi is an example of a typical alkaline saline lake at the stage of maximum evaporite productivity. It is located in the rain shadow of mountains with a large catchment area. Bedrocks are largely volcanic, in addition to some metamorphic basement, and, hence, dilute inflow is of the Ca-Na-HCO_3 type. Evaporation is intense and no perennial overland flow reaches the lake.

Biotechnology

Microbial communities in natural alkaline environments such as soda lakes have attracted attention because of possible biotechnological use of enzymes and metabolites from such organisms. In a sense, extreme alkaliphiles and extremophiles, in general, are specialists since they have to be able to thrive under such harsh conditions.

Today, these organisms are of considerable industrial interest, particularly for the production of enzymes such as proteases for inclusion in laundry detergents or for application in the manufacture of leather, xylanases for use in the pulp paper industry and cyclodextrin glucanotransferase for cyclodextrin manufacture from starch, frequently used in foodstuffs, chemicals, cosmetics and pharmaceuticals (Grant *et al.*, 1990; Horikoshi, 1996). Studies on alkaline enzymes have concentrated largely on those organisms which have been easily observed in the natural environment. For example, large numbers of alkaliphilic *Bacillus* species have been isolated over the years, many due to the systematic work of Horikoshi and co-workers. Archaeal isolates have also been targeted for exploration. Their chemistry is distinct and they contain such lipids, where their stereo-configuration is different to that in Bacteria (Grant *et al.*, 1990). A haloalkaliphilic archaeon, *Natronococcus* sp. strain Ah-36, produces an extracellular maltotriose-forming amylase (Kobayashi *et al*., 1992). The gene encoding this enzyme has been cloned and expressed in *Haloferax volcanii* (Kobayashi *et al*., 1994). The majority of halobacteria examined to date have retinal-based pigments capable of the light-mediated translocation of ions across the cell membrane. Bacteriorhodopsin as a light-driven proton pump and halorhodopsin as an inward chloride pump became perfect models for energy conversion, opening interesting biotechnical perspectives for the use of these molecules in different applications, including holographic techniques and information storage (Oren, 1998). It has also been documented that the carotenoid pigments of halobacteria trap solar radiation, increasing the ambient temperature and evaporation rates in salterns, hastening the deposition of sea salt (Tindall, 1988). It can safely be assumed that the extremophilic organisms derived from the soda lake environment will have a great, yet-to-be-exploited, potential for biotechnological applications.

1.2. Microbial diversity of soda lakes

One of the most striking features of many soda lakes is their colour. Depending on the water chemistry of the individual lakes, they are likely to be green, pink, red or orange, due to massive permanent or seasonal blooms of microorganisms (Grant *et al.*, 1990). This is reflected in the extremely high primary productivity associated with some of these lakes. Despite the extreme nature of such environments, they are characterised by exceptionally rich productivity rates exceeding 10 g C m^{-2} day^{-1}, presumably because of the relatively high ambient temperatures, high light intensities, availability of phosphate and unlimited access to CO_2 in these carbonate-rich waters (Grant *et al.*, 1990; Melack & Kilham, 1974). They are also regarded as naturally eutrophic reservoirs and, like all eutrophic bodies of water, they feature considerable microbial diversity (Zavarzin *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 alkali-tolerant representatives of major bacterial and archaeal phyla (reviewed by Duckworth *et al.*, 1996; Grant *et al.*, 1999; Jones *et al.*, 1998; Zavarzin *et al.*, 1999). Anaerobic decomposition, which dominates in these lakes, has received less attention, with relatively few obligately alkaliphilic anaerobes, mainly of the clostridial line of descent, having been isolated (Grant *et al.*, 1999).

Phototrophic primary producers

A marked difference in procaryotic communities has been observed between the strongly hypersaline, alkaline brines of Lake Magadi and Lake Natron in the Rift Valley, Owens Lake in California and some of the lakes of the Wadi Natrun depression in Egypt with salt concentrations approaching saturation (30% w/v or greater) and the more dilute waters of lakes Elmenteita, Nakuru, Bogoria, etc., with salinities on the order of 5% w/v (Jones *et al.*, 1994 and 1998). Despite these differences, photosynthetic primary production appears to play an important role in the soda lake environment and, presumably, supports the rest of the microbial community (Grant *et al.*, 1999; Jones *et al.*, 1998). The less alkaline lakes are usually dominated by dense blooms of cyanobacteria while the hypersaline lakes, on occasion (i.e., after extensive rainfall causes dilution of the brine), support blooms of both cyanobacteria and alkaliphilic anoxygenic phototrophs belonging to the genera *Ectothiorhodospira* and *Halorhodospira* (Grant *et al.*, 1999; Jones *et al.*, 1998).

In the lakes of the East African Rift the blooms of cyanobacteria are usually dominated by planktonic *Spirulina* spp., serving as major organic matter producers (Zavarzin *et al.*, 1999). These cyanobacteria are the principle food of the vast flocks of Lesser Flamingo (*Phoeniconaias minor*) that inhabit the Rift valley soda lakes. In

different lakes and also depending on seasonal factors *Cyanospira* ssp. and unicellular forms, which might be *Synechococcus* or *Chroococcus*, may also be common (Jones *et al.*, 1998). From Lake Magadi various extreme alkaliphilic, moderate halophilic, benthic cyanobacteria were isolated that were identified, by morphology, to be *Synechocystis salina, Aphanothece stagnina, Chamaesiphon subglobosus, Rhabdoderma lineare, Synechococcus elongatus, Phormidium ambiguum, Phormidium foveolarum, Phormidium retzii, Oscillatoria splendida, Oscillatoria limnetica, Spirulina fusiformis* and *S. laxissima* (Dubinin *et al*., 1995). Unicellular cyanobacteria were observed to develop mostly at higher salinities (approximately 7% NaCl and 10% of the net soda), while trichomic forms were better suited to lower salinity and alkalinity (Zavarzin *et al.*, 1999). The cyanobacteria are not only essential for the fixation of nitrogen in this environment but they are also producers of O_2. Daytime rates of oxygen production in excess of 2 g O_2 m^{-2} h^{-1} have been recorded from *Spirulina* ssp. (Melack & Kilham, 1974).

At lower conductivity, phototrophic eukaryotes of the diatom genera *Nitzschia* and *Navicula* were observed, but their wider significance in this ecosystems is unclear and has received only little attention (Jones *et al.*, 1994). Recently, a eukaryotic microalgae was isolated from Lake Magadi water samples that was identified, by morphology, as *Chlorella minutissima* (Gerasimenko *et al*., 1999).

Organic matter is produced not only by cyanobacteria but also by anoxygenic phototrophic purple bacteria (Zavarzin *et al.*, 1999). Soda water bodies typically contain sulfur purple bacteria of the genera *Ectothiorhodospira* and *Halorhodospira*, which oxidise hydrogen sulfide with intermediate extra-cellular sulfur deposition. The most thoroughly studied now 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 and 1988). Recently, two new strictly anaerobic obligately phototrophic purple sulfur bacteria, *Thiorhodospira sibirica* and *Thioalkalicoccus limnaeus*, were isolated from low-saline soda lakes in the steppe of south-east Siberia (Bryantseva *et al*., 1999a and 2000a). Under anoxic conditions, these bacteria use hydrogen sulfide and elemental sulfur as photosynthetic electron donors.

Among the anoxygenic phototrophic bacteria that have been described from soda lakes, very few purple nonsulfur bacteria also were found in lakes with low mineralisation. The alkaliphilic alpha-proteobacterium *Rhodobaca bogoriensis,* isolated from Lake Bogoria, Kenya, is capable of both, phototrophic and chemotrophic growth, and so far it is the sole representative of purple nonsulfur bacteria described from soda lake environments (Milford *et al*., 2000). Additionally, two alkaliphilic heliobacteria *Heliorestis daurensis* and *H. baculata* that grow photo-heterotrophically (Bryantseva *et al.*, 1999a and 2000b) and an alkaliphilic Bacteriochlorophyll *a*-containing “aerobic

phototroph" *Roseinatronobacter thiooxidans* (Sorokin *et al*., 2000b) have been isolated from Siberian low-salt soda lakes.

Aerobic alkaliphiles

Although the soda lakes of the Rift Valley are eutrophic, relatively shallow, and, presumably oxygen limited, they maintain dense populations of non-phototrophic, aerobic organotrophic bacteria that utilise products of photosynthesis as well as products of anaerobic destruction. Viable counts (colony forming units, cfu) of aerobic organotrophs from a range of diluted lakes indicate 10^5-10^6 cfu ml^{-1} (Grant *et al.*, 1990). A number of aerobic chemoorganotrophic, alkaliphilic isolates obtained from several East African soda lakes were studied in detail (Duckworth *et al.*, 1996; Jones *et al.*, 1994). Regrettably, only a few of these isolates are published or available in any of the public culture collections.

The majority of Gram-negative isolates were members of the gamma subdivision of the Proteobacteria, including many proteolytic organisms related to members of the genus *Halomonas* (Duckworth *et al.*, 2000). Other Gram-negative, lipolytic isolates were affiliated with members of the genera *Pseudomonas* sensu strictu (rRNA group1) and *Stenotrophomonas*. A few strains grouped with typical aquatic bacteria such as *Aeromonas, Vibrio* and *Alteromonas* (Duckworth *et al.*, 1996; Jones *et al.*, 1998). Recent studies on the low-saline Siberian soda lakes (Hadyn, Tsaidam, Low Mukei) and some lakes of the Kenyan Rift Valley (Bogoria, Crater lake Sonachi, Elmenteita, Nakuru and Magadi) revealed the presence of a diverse population of aerobic sulfur-oxidising bacteria of the genera *Thioalkalimicrobium* and *Thioalkalivibrio* (Sorokin *et al*., 2001). Recently, the new alkaliphilic aerobic heterotrophic bacterium *Alkalilimnicola halodurans* was isolated from sediments of Lake Natron, which was affiliated with members of the family *Ectothiorhodospiraceae* (Yakimov *et al*., 2001).

Furthermore, a few alkaliphilic methane-oxidising bacteria of the *Methylococcaceae* were isolated from several moderately saline soda lakes. The methanotroph "*Methylobacter alcaliphilus"* (whose name is not validly published), was isolated from soda lakes in Tuva, Central Asia (Khmelenina *et al*., 1997). Five strains of methanotrophic bacteria were isolated from sediments of south-eastern Transbaikal soda lakes and were named *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 oxidise ammonia to nitrite at pH 10-10.5 and is also capable of oxidising organic sulfur compounds at high pH.

Lithotrophic, nitrite-oxidising bacteria (*Nitrobacter alkalicus*) of the alpha subdivision of Proteobacteria were 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 chemolithotrophic nitrifying bacteria play an important role in biological nitrogen cycling by converting reduced inorganic nitrogen compounds to nitrate.

Gram-positive aerobic isolates of both the high G+C and low G+C divisions have been found. The low G+C isolates were associated mainly with members of the diverse *Bacilli* taxon, especially *Bacillus alcalophilus* (rRNA group 6, according to Nielsen *et al.* 1994 and 1995) and *Bacillus clarkii* ("group 7"), (Duckworth *et al.*, 1996). Within the high G+C divisions of the Gram-positive lineage two alkaliphilic organotrophic isolates from a moderately saline and alkaline East African soda lake (Lake Oloiden) were 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).

Examination of a number of alkaline, highly saline lakes from different geological locations, including Lake Magadi (Kenya), the lakes of the Wadi Natrun (Egypt), and Owens Lake (California), indicated that haloalkaliphilic archaea of the family *Halobacteriaceae* (so called "halobacteria"), are found in all such lakes (Tindall, 1988). 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 C_{50} 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).

Anaerobic microorganisms

In contrast to the aerobic habitats, the anaerobic alkaline saline environment has received less attention (Jones *et al.*, 1998; Zavarzin *et al.*, 1999). The predominant biological process in soda water bodies is sulfate reduction (sulfidogenesis). It is responsible not only for the final steps of organic matter degradation but also for generating alkaline conditions as a result of transformation of sulfate to sulfide (Zavarzin *et al.*, 1999). The first alkaliphilic SRB (sulfate reducing bacterium) *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 sulfate reducer of the delta subdivision of Proteobacteria, *Desulfonatronum lacustre* Z-7951, was obtained from Lake Khadyn (Tuva), an oligotrophic alkaline lake with low mineralisation (Pikuta*, et al.*, 1997 and 1998). Hydrogen-utilising, 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 & Zavarzin, 1994) and in mixed samples from Tuva lakes (Zavarzin *et al.*, 1999) but have not been fully characterised. Also, hydrogen acetogenesis provides a available hydrogen sink. A representative of the homoacetogenic bacteria (strain Z-7937) was isolated from lake Magadi samples (Zhilina & Zavarzin, 1994). Yet 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).

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 (Kevbrin *et al*., 1997; Zhilina & Zavarzin, 1994). The methylotrophic methanogen *Methanohalophilus oregonense* was isolated from an anoxic aquifer near Alkali Lake, an hypersaline, alkaline desert lake in south central Oregon in the United States (Liu *et al*., 1990).

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). Phylogenetically, this species is associated with members of the *Halobacteroidaceae* (order *Halanaerobiales*) within the Gram-positive lineage of bacteria. Recently *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).

Also, organisms fermenting amino acids, called acetogenic ammonifiers, were isolated from soda lakes. Both strains, *Natronincola histidinovorans* (Zhilina *et al*., 1998) and the non-spore-forming bacterium *Tindallia magadiensis* (Kevbrin *et al*., 1998), were derived 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 were 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 and colleagues also isolated saccharolytic anaerobes from Lake Magadi and from the slightly mineralised Lake Nizhee Beloe in the south-eastern Transbaikal region (Tourova *et al*., 1999; Zhilina *et al.* 2001b). The strains obtained from Lake Magadi were either facultative anaerobes and related to members of the genus *Amphibacillus* or obligately anaerobic strains clustering within the order *Halanaerobiales*. However, the complete taxonomic description of both, the strains isolated by the group of Zhilina and those isolated by Jones and colleagues are not yet available. In an anaerobic community saccharolytic spirochetes act as dissipotrophs (Zavarzin *et al.*, 1999), utilising sugars and a limited range of polysaccharides to produce acetate, lactate, ethanol and H_2. 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).

Alkaliphiles are not confined exclusively to soda lakes but may be found in almost any environment, even in environments where the overall pH may not be particularly alkaline (Grant *et al.*, 1990). For instance, many alkaliphilic or alkalitolerant members of the “Bacilli” are fairly ubiquitous. However, is seems probable that some organisms are unique to soda lakes, especially the haloalkaliphiles found in the hypersaline lakes such as Lake Magadi (Jones *et al.*, 1998). Although present-day soda lakes are geologically quite recent, they are supposed to be habitats of relict microbial communities and are regarded as possible centres of the origin of microbial diversity (Zavarzin, 1993). Microbial communities in soda lakes have recently attracted attention as potential sources of industrially potent enzymes (Horikoshi, 1996) and as a new perspective on microbial diversity (Duckworth *et al.*, 1996; Jones *et al.*, 1998; Zavarzin *et al.*, 1999). However, the diversity of haloalkaliphiles, particularly those inhabiting the hypersaline soda lakes, has not yet been adequately explored and most attention has been paid to a few separate groups of microorganisms. The major obstacle in understanding the soda lake microbial communities may be the ability to culture the organisms (Jones *et al.*, 1998). In this regard, the application of molecular techniques could provide new insights into the microbial composition of the soda lake environments, and the information revealed could serve as starting-point for the development of new cultivation techniques for yet uncultivated microorganisms.

Table 1.2: Taxonomic groups containing procaryotes isolated from soda lakes.

Bacteria	
Cyanobacteria	(Dubinin *et al.*, 1995)
Firmicutes	
Bacilli	
Bacillales	
Bacillaceae	
Amphibacillus	
Amphibacillus fermentum	(Zhilina *et al.*, 2001b)
Amphibacillus tropicus	(Zhilina *et al.*, 2001b)
***Bacillus* (group 6)**	(Duckworth *et al.*, 1996)
***Bacillus* (group 7)**	(Duckworth *et al.*, 1996)
Clostridia	
Clostridiales	
Clostridiaceae	
Clostridium (cluster XI)	(Jones *et al.*, 1998)
Natronincola	
Natronincola histidinovorans	(Zhilina *et al.*, 1998)
Tindallia	
Tindallia magadiensis	(Kevbrin *et al.*, 1998)
Heliobacteriaceae	
Heliorestis	(Bryantseva *et al.*, 1999a)
Halanaerobiales	
Halobacteroidaceae	
Halonatronum	
Halonatronum saccharophilum	(Zhilina *et al.*, 2001a)
Halanaerobiaceae	
Natroniella	
Natroniella acetigena	(Zhilina *et al.*, 1996a)
Actinobacteria	
Actinomycetales	
Dietziaceae	
Dietzia	(Duckworth *et al.*, 1998)
Micrococcaceae	
Arthrobacter	(Duckworth *et al.*, 1996)
Intrasporangiaceae	
Terrabacter	(Duckworth *et al.*, 1996)
Proteobacteria	
Alpha-proteobacteria	
Rhizobiales	
Bradyrhizobiaceae	
Nitrobacter	(Sorokin *et al.*, 1998)
Rhodobacterales	
Rhodobacteraceae	
Paracoccus	(Zavarzin *et al.*, 1999)
Rhodobaca	(Milford *et al.*, 2000)
Roseinatronobacter	(Sorokin *et al.*, 2000b)
Gamma-proteobacteria	
Chromatiales	
Chromatiaceae	
Thioalkalicoccus	(Bryantseva *et al.*, 2000a)

Ectothiorhodospiraceae
Alkalilimnicola
Alkalilimnicola halodurans (Yakimov *et al.*, 2001)
Ectothiorhodospira
Ectothiorhodospira vacuolata (Imhoff *et al*., 1981; Tindall 1980)
Halorhodospira
Halorhodospira abdelmalekii (Imhoff *et al.*, 1979)
Halorhodospira halochloris (Imhoff *et al.*, 1979)
Halorhodospira halophila (Grant & Tindall, 1986)
Thiorhodospira (Bryantseva *et al*., 1999b)
Thioalkalivibrio
Thioalkalivibrio nitratis (Sorokin *et al.*, 2001)
Thioalkalivibrio versutus (Sorokin *et al.*, 2001)
Thiotrichales
Piscirickettsiaceae
Thioalkalimicrobium (Sorokin *et al.*, 2001)
Methylococcales
Methylococcaceae
Methylobacter (Khmelenina *et al.*, 1997)
Methylomicrobium (Kaluzhnaya *et al.*, 2001)
Oceanspirillales
Halomonadaceae
Halomonas
Halomonas magadiensis (Duckworth *et al.*, 2000)
Pseudomonadales
Pseudomonadaceae
Pseudomonas (Duckworth *et al.*, 1996)
Xanthomonadales
Xanthomonadaceae
Stenotrophomonas (Duckworth *et al.*, 1996)
Aeromonadales
Aeromonadaceae
Aeromonas (Duckworth *et al.*, 1996)
Alteromonadales
Alteromonadaceae
Alteromonas (Duckworth *et al.*, 1996)
Vibrionales
Vibrionaceae
Vibrio (Duckworth *et al.*, 1996)

Delta-proteobacteria
Desulfonatronovibrionales
Desulfohalobiaceae
Desulfonatronovibrio
D. hydrogenovorans (Zhilina *et al.*, 1997b)
Desulfonatronumaceae
Desulfonatronum
Desulfonatronum lacustre (Pikuta *et al*., 1998)

Spirochaetes
Spirochaetales
Spirochaetaceae
Spirochaeta
Spirochaeta africana (Zhilina *et al.*, 1996b)
Spirochaeta alkalica (Zhilina *et al.*, 1996b)

Archaea	
Euryarchaeota	
Halobacteria	
Halobacteriales	
Halobacteriaceae	
Halorubrum	
Halorubrum vacuolatum	(Mwatha & Grant, 1993)
Natrialba	
Natrialba magadii	(Tindall *et al*., 1984)
Natronobacterium	
Natronobacterium gregoryi	(Tindall *et al.*, 1984)
Natronococcus	
Natronococcus amylolyticus	(Kanai *et al*., 1995)
Natronococcus occultus	(Tindall *et al.*, 1984)
Natronomonas	
Natronomonas pharaonis	(Soliman & Trüper, 1982; Tindall *et al*., 1984)
Natronorubrum	
Natronorubrum bangense	(Xu *et al*., 1999)
Natronorubrum tibetense	(Xu *et al.*, 1999)
Methanococci	
Methanosarcinales	
Methanosarcinaceae	
Methanohalophilus	
Methanohalophilus oregonense	(Liu *et al.*, 1990)
Methanohalophilus zhilinae	(Boone *et al.*, 1986; Mathrani *et al.*, 1988)
Methanohalophilus sp. Z-7936	(Kevbrin *et al.*, 1997; Zhilina & Zavarzin, 1994)

Taxonomic groups were named according to *Bergey's Manual Trust* "species outline" (Garrity *et al.* , 2001). Genera and species that were found in the hypersaline soda lakes such as Lake Magadi and Natron (Kenya), the lakes of the Wadi Natrun (Egypt), and Owens Lake (California), are printed in bold-face.

1.3. Ribosomal RNA(-genes) and molecular microbial ecology

With developments in molecular biology, the ideas of Zuckerkandl and Pauling (1965) to deduce the phylogenetic history of organisms by comparing the primary structures of macromolecules became applicable. The first molecules to be analysed for this purpose were cytochromes and ferredoxins (Zuckerkandl & Pauling, 1965; Ambler *et al.*, 1979). Subsequently, Carl Woese and co-workers demonstrated the usefulness of small subunit (SSU) ribosomal RNA (rRNA) as a universal phylogenetic marker (Fox *et al*., 1977; Woese & Fox, 1977) and articulated the recognised primary lines of evolutionary descent, currently termed "domains": Eucarya (eukaryotes), Bacteria (initially called eubacteria) and Archaea (initially called archaebacteria) (Woese *et al*., 1990). The comparative sequence analyses further allowed the definition of the major lineages (phyla or divisions) within the three primary domains (Woese, 1987). These studies suggested natural relationships between microorganisms on which a procaryotic systematics could be based (Ludwig & Klenk, 2001) and justified the use of the current 16S rRNA systematics as a backbone for the structuring of the second edition of *Bergey's Manual of Systematic Bacteriology*.

The nature of rRNA

The ribosomal RNAs are ubiquitous in all life forms, they exhibit functional constancy, change slowly in sequence, and they are experimentally tractable. Moreover, 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). However, there are different interpretations of whether ribosomes are subject to horizontal gene transfer (Wang & Zhang, 2000). Therefore, the rRNA molecules, particularly the 16S rRNA and to lesser extent 23S rRNA, have been chosen as the molecular basis for phylogenetic reconstruction at least in the procaryotic world. The three types of rRNA are classified by their sedimentation rates during ultracentrifugation as 23S, 16S and 5S. They have lengths of approximately 3,300, 1,650 and 120 nucleotides, respectively. The rRNA sequences can be obtained from the rRNA itself or from the encoding genes located in the chromosomal DNA (also referred to as rDNA). Their primary structures are alternating sequences of invariant, moderately conserved to highly variable regions. The latter characteristic results from different functional selective pressures acting upon the independent structural elements (Ludwig & Klenk, 2001). The frequencies of compositional changes at different positions in the molecule vary greatly (Gutell *et al*., 1994; Van de Peer *et al*., 1996). Invariant and highly conserved positions are essential for the recognition and alignment of homologous sequences, slowly evolving positions confer valuable information on the deepest groups, sites of higher variability are useful for the elucidation of more recent branching (Ludwig & Schleifer, 1994). For example, signature sequences for the Archaea, Bacteria, and Eucarya have been recognised (Giovannoni *et al*., 1988a; Stahl & Amann, 1991; Woese, 1987) as well as many short stretches of sequences characteristic of a number of the bacterial divisions and subdivisions (OPD, Oligonucleotide Probe Database, http://www.cme.msu.edu/OPD/).

The 23S rRNA molecule is a larger information unit than the 16S and, in many cases, has higher resolving power for phylogenetic reconstruction (Ludwig *et al*., 1998). However, due to its length, its sequencing has not been as popular as that of 16S and the number of 23S rRNA sequences in the databases is much smaller. Meanwhile, the 16S rRNA approach is one of the most widely used standard techniques in microbial taxonomy. Consequently, a comprehensive sequence data set (approximately 30,000 entries in 2002) is available in widely accessible databases (Maidak *et al*., 2001; Stoesser *et al*., 2002; Van de Peer *et al*., 2000), and the number of entries is permanently increasing. It is also widely accepted to apply the rRNA technology as an integrated part of a polyphasic approach for new descriptions of bacterial species or higher taxa (Ludwig *et al.*, 1998; Stackebrandt & Goebel, 1994).

Unfortunately, the resolving power of the 16S rRNA has been recognised to be insufficient to guarantee correct delineation of bacterial species (Fox *et al*., 1992; Martinez-Murzia *et al*., 1992). Consequently, organisms sharing identical SSU rRNA sequences may be more divergent at the genome level than others which contain rRNAs differing at a few variable positions. This has been shown by comparison of 16S rRNA sequence and genomic DNA-DNA hybridisation data (Stackebrandt & Goebel, 1994). Therefore, Ludwig has pointed out, that in the interpretation of phylogenetic trees the branching patterns at the periphery of the tree cannot reliably reflect phylogenetic reality (Ludwig & Klenk, 2001). 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 & Goebel, 1994). According to the current species concept, genomic DNA similarity of approximately 70% and higher is shared by strains of a species (Wayne *et al*., 1987). However, there is no recognised threshold value of 16S rRNA sequence similarity for species recognition (Stackebrandt & Goebel, 1994).

Furthermore, the importance of choosing outgroup reference organisms in the reconstruction of trees has been discussed (Stackebrandt & Ludwig, 1994). It turns out that the branching order in reconstructed phylogenetic trees depends not only on differences in base composition and selection of sequence stretches analysed, but also on the number of organisms and the selection of reference organisms. The pros and cons of different treeing methods are reviewed by Ludwig *et al.* (1998).

A detailed comparison of duplicate rRNA sequences present in the GenBank database revealed high levels of intraspecies variation (within and between strains) of 16S rRNA sequences (Clayton *et al*., 1995). Such variability has been proposed to represent interoperon variation within a single strain, strain-to-strain variation within a species, misidentification of strains, or sequencing errors. Interoperon differences of up to 5% in 16S rRNA gene sequences have been noted, (Mylvaganam & Dennis, 1992; Nübel *et al*., 1996; Rainey *et al*., 1996). These findings would call the application of rRNA sequences for phylogenetic and identification purposes into question (Vandamme *et al*., 1996).

Alternatively, several other macromolecules have been examined for their potential as molecular markers. Among others, the beta subunit of ATPase, elongation factor Tu, chaperonin, various ribosomal proteins, RNA polymerases, and tRNAs have been shown to be valuable molecular chronometers in bacterial systematics (Vandamme *et al.*, 1996). The congruence between global tree topologies derived from 16S rRNAs and different phylogenetic markers, such as 23S rRNA, ATPase subunits, elongation factors and RNA

polymerases, has been tested and resulted in very similar tree topologies (Ludwig *et al.*, 1998). Also the aminoacetyl-tRNA synthetase trees in aggregate suggest the same major taxonomic groupings within each domain, as does the rRNA tree (Woese *et al*., 2000). On the other hand, phylogenetic analyses of more than 60 proteins revealed that universal phylogenetic trees inferred from many of them do not fundamentally agree with the rRNA-based universal phylogenetic tree (Brown & Doolittle, 1997). With the advent of genomics, genome-wide phylogenetic analysis could become an important method for studying the ancient diversification of life on earth, as recent whole-genome assessments indicate (Fitz-Gibbon & House, 1999; Graham *et al*., 2000; Snel *et al*., 1999) and it was found that "there is a strong signal within the genomes reflecting the evolutionary histories of the organisms despite horizontal gene transfer, gene duplication and gene loss" (Fitz-Gibbon & House, 1999).

Nevertheless, at present, the vast majority of bacterial taxonomists accept that 16S rRNA sequence analysis provides a framework for procaryotic systematics and it appears that 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 & Amann, 2001).

"Molecular phylogenies will be of immense scientific interest, but they will not themselves give the desired information on phenotype, behaviour, ecology, etc., that will be needed for the taxonomic data banks and information systems of the future" (Sneath, 1995).

Analysis of microbial communities by 16S rDNAs

Knowledge of microorganisms in the environment has depended in the past mainly on studies of pure cultures. Today it is generally known that culture-dependent surveys suffer from the "great plate count anomaly" (Staley & Konopka, 1985) and studies of several types of environments estimate that more than 99% of organisms seen microscopically are not cultivated by routine techniques (Amann *et al*., 1995).

The application of molecular biological methods to study the diversity and ecology of microorganisms in natural environments has emerged since the mid-1980s. Since that time many new insights into the composition of uncultivated microbial communities have been gained (Head *et al*., 1998). Norman Pace and co-workers were among the first to appreciate Woese's new phylogeny with molecular biology, and began what is now recognised as molecular microbial ecology (Olsen *et al*., 1986; Pace, 1996). The ribosomal RNA genes were obtained by cloning DNA isolated directly from the environment. Initially, "shotgun libraries" of random DNA fragments served as a source

of rRNA, as well as other genes, but required sorting of rRNA genes from the others. The first application of this approach was the characterisation of a marine picoplankton sample (Schmidt *et al*., 1991). Currently, the most widely used approach to survey the constituents of microbial ecosystems is through the use of PCR (Mullis & Floona, 1987; Saiki *et al*., 1988; Weisburg *et al*., 1991). The highly conserved nature of rRNA allows for the synthesis of "universal" PCR primers. In principle, PCR carried out with these primers amplifies the rRNA genes of "all" types of organisms present in the environmental sample. Individual types of genes in the mixture are separated by a cloning step and then sequenced. This approach was first applied in an analysis of Sargasso Sea picoplankton (Giovannoni *et al*., 1990). Subsequently, this technique has been applied also to a number of different marine, soil, and extreme (thermal, hypersaline etc.) natural environments, resulting in the detection of many novel types of rRNA sequences, often representing major new lineages only distantly related to known ones (e.g., Barns *et al*., 1994 and 1996; Benlloch *et al*., 1995 and 2001; DeLong *et al*., 1993; Fuhrman *et al*., 1993; Grant *et al*., 1999; Liesack & Stackebrandt, 1992; Munson *et al*., 1997; Stackebrandt *et al*., 1993; Takai & Horikoshi, 1999; Torsvik *et al*., 1990). To complete the picture, it should be remarked in passing, that cloning of cDNA transcribed from 16S rRNA with the enzyme reverse transcriptase also allows the selective retrieval of rRNA sequence information, and was first applied to study a cyanobacterial mat (Ward *et al*., 1990; Weller & Ward, 1989). Several methods were developed for the assessment of genetic diversity which include sequence analysis of randomly picked clones, hybridisation with taxon specific probes (Amann *et al*., 1990b; DeLong *et al*., 1989), restriction fragment length polymorphism (RFLP) (Moyer *et al*., 1994), and amplified ribosomal DNA restriction analysis (ARDRA) of clones (Massol-Deya *et al*., 1995) or separation of amplified rDNA by temperature or denaturing gradient gel electrophoresis (TGGE or DGGE) (Muyzer *et al*., 1993; Nübel *et al*., 1996), or single strand conformation polymorphism (SSCP) (Schwieger & Tebbe, 1998).

Application of molecular methods to the study of bacterial diversity, advantages and limitations

While we have gained much new and valuable knowledge using the molecular techniques described, as with all methods, there are important limitations that must be recognised. Each physical, chemical and biological step involved in the molecular analysis of an environmental sample is a potential source of bias which could dramatically affect the outcome of the microbial community analysis (Rainey *et al*., 1994b; Von Wintzigerode *et al*., 1997).

The first crucial step for all subsequent analyses lies in the sample collection and handling. Comparing different sample handling procedures, Rochelle and co-workers reported significant variations in 16S rRNA gene types and diversity from anaerobic deep marine sediments (Rochelle *et al*., 1994).

Furthermore, the validity of using molecular techniques in environmental studies depends on obtaining representative extracts of nucleic acids from entire microbial communities. Nucleic acid extraction methods, however, suffer from compounded inefficiencies in the individual component steps, including incomplete cell lysis, DNA sorption to particles, co-extraction of enzymatic inhibitors, and loss, degradation, or damage of DNA (Miller *et al*., 1999). Since the composition of environmental samples is mostly unknown, it is difficult to assess the efficiency of recovery by any extraction technique, but it is likely that the same lysis method may give different results with different types of samples such as water, sediment, or soil. For example, comparisons of methods for lysis of indigenous soil bacteria indicate that the portion of bacteria lysed by a particular method depends greatly on the method employed and the types and sizes of cells in the sample (Moré *et al*., 1994; Zhou *et al*., 1996). It was found that smaller and coccoid bacterial cells (0.3-1.2 µm) were more resistant to lysis. This clearly has implications for the recovery of sequences from environmental samples where many cells may be in a state of starvation and, hence, are likely to be small. Numerous comparative studies of DNA extraction methods have indicated that these techniques can introduce biases of their own (Bürgmann *et al*., 2001; Courtois *et al*., 2001; Frostegard *et al*., 1999; Krsek & Wellington, 1999; Leff *et al*., 1995; Miller *et al.*, 1999; Moré *et al.*, 1994; Rochelle *et al*., 1992; Steffan *et al*., 1988; Tien *et al*., 1999; Zhou *et al.*, 1996).

Since most of the community studies are conducted using PCR to amplify rDNA from extracted environmental DNA, the question arises as to whether molecular analyses accurately reflect the division-level diversity that occurs in the environment. It is well established that PCR-associated artefacts such as differential amplification of rDNA templates (Reysenbach *et al*., 1992; Suzuki & Giovannoni, 1996), sensitivity to rRNA gene copy number (Farrelly *et al*., 1995), PCR primer specifity (Weisburg *et al*., 1991), sensitivity to template concentration (Chandler *et al*., 1997), amplification of contaminant rDNA (Tanner *et al*., 1998), and formation of chimeric sequences (Kopczynski *et al*., 1994; Wang & Wang, 1996) may skew the assessment of microbial diversity. It was also reported, that the cloning system also has an influence on the distribution of taxon-specific clones in 16S rDNA clone libraries (Rainey *et al.*, 1994b).

Finally, the analysis of 16S rRNA sequence data creates additional problems to researchers in microbial ecology. The quality of results obtained by comparative sequence analyses depends greatly on the available data set. Although today a

comprehensive, permanently growing sequence data set is available, including full and partial 16S rRNA (-gene) sequences of cultivated microorganisms and environmental clones, this number reflects only a minor part of the expected microbial diversity. 16S rRNA genes retrieved from environmental samples often exhibit only low sequence similarities to sequences of known organisms and the question is posed whether environmental sequences represent uncultured, novel microorganisms or whether they cannot be assigned to known taxa due to the fact that for many cultivated microorganisms 16S rRNA sequences are not available or are of low quality (Von Wintzigerode *et al.*, 1997).

Despite its limitations, the rRNA approach is permitting major advances in the understanding of microbial ecology and evolution. The sequences of the rDNA genes retrieved from the environment give an estimate of the microbial diversity present (Benlloch *et al.*, 1995). They are snapshots of organisms, representatives of different types of genomes, or targets for further characterisation if they seem interesting or useful (Pace, 1996). The potential biases associated with PCR clearly indicate that we can never confidently extrapolate from sequence composition in a clone library to a quantitative population composition in an environmental sample. However, clonal analyses probably include the most abundant (metabolically active) bacterial sequence types in the samples analysed, likely representing the members of the communities that are involved in the principal metabolic activities, such as carbon cycling (Hugenholtz *et al*., 1998).

Some researchers claimed that the molecular assessment of uncultivated organisms could provide insights into many of the properties (e.g., physiology, putative ecological role) of the otherwise unknown organisms through comparison with its studied relatives (Barns *et al.*, 1994; Benlloch *et al.*, 1995; Pace, 1996 and 1997). However, the utility of sequence data as predictors of the physiological attributes of newly described phylotypes seems to be more limited considering the fact that “many phylogenetically coherent procaryotic lineages, for example the proteobacteria, often encompass a bewildering array of physiological and metabolic diversity” (Stein *et al*., 1996). On the other hand it is likely that differences in rRNA sequence types probably are reflected in substantial physiological differences. With respect to defining a functional role in their particular ecosystems, organisms catalogued only by sequence will permit assessment of diversity only. “It is quite clear that 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). Thus, the role of classical microbiology should not be underestimated. On the contrary, applications of molecular techniques in microbial ecology have an important role as guides for the isolation and characterisation of new procaryotic taxa (Palleroni, 1997).

1.4. Aim of the project

This doctoral project focused on the application of cultivation-independent molecular techniques for the detection of new procaryotic (*Bacteria* and *Archaea*) diversity in an extreme ecosystem, the hypersaline soda lake environment. As outlined in the previous sections, the diversity of haloalkaliphiles, particularly those inhabiting the hypersaline soda lakes, has not been adequately explored and most attention has been paid to a few separate groups of procaryotes. Hence, the application of molecular techniques was expected to provide new insights into the composition of the soda lake environment. Suitable strategies and methodologies had to be established for this molecular approach, including the generation of a ligation independent cloning (LIC) vector and the development of a new non-radioactive colony-hybridisation method.

To expand the restricted amount of archaeal 16S rDNA sequences available in the public databases at the outset of this study, 39 haloalkaliphilic archaeal isolates from different saline and alkaline habitats, such as the Wadi Natrun (Egypt) and Owens Lake (California), were analysed by molecular characterisation. Additionally, various bacterial isolates from Lake Magadi, including a unicellular cyanobacterial culture, as well as fourteen aerobic sporeforming bacterial strains, were included in the molecular analysis.

2. Materials and methods

2.1. Samples

Sediment samples

Lake Magadi in a dry period is covered by a white precipitate of trona ($NaHCO_3$-Na_2CO_3-$2H_2O$) approximately 1-2 cm thick, below which a purple layer of bacteria develops, below which there is a black zone of trona mixed with mud. Samples were collected in a dry period from sediment under the trona (M. M. Yakimov and W. R. Abraham, personal communication). The pH and temperature at the collecting site were recorded as 11.0 and 57°C, respectively. Samples were stored in 250 ml bottles (Schott glass). The DNA was stabilised by adding 1/3 volume of dichloromethane to the sediment samples (M. M. Yakimov, personal communication).

Archaeal and bacterial strains

The 39 haloalkaliphilic archaeal strains isolated from Owens Lake, California, and Wadi Natrun, Egypt, as well as all reference strains of halobacteria included in the ARDRA analysis, were provided by Dr. B. J. Tindall at the DSMZ, Braunschweig. All strains were grown at 37°C on agar plates containing DSM medium 371 ("Natronobacteria medium").

The 14 aerobic sporeforming strains, isolated from sediment samples of Lake Magadi and Lake Natron, Kenya, were provided by Dr. M. M. Yakimov at the GBF. The strains were grown at 37°C on agar plates containing a combination of DSM medium 61 ("Clostridium thermohydrosulfuricum medium"), without Resazurin , and DSM medium 31 (Na-sesquicarbonate solution).

The unicellular cyanobacterial culture, Z-9405, was grown, with shaking at 37°C, in "M medium" (Dubinin *et al.*, 1995) of the following composition (g/l): Na_2CO_3, 50.0; $NaHCO_3$, 8.0; NaCl, 100.0; KCl, 2.0; Na_2SO_4, 1.4; KNO_3, 2.0; K_2HPO_4, 0.5; Na-Mg-EDTA, 0.0005; $FeCl_3$, 0.0003; pH 10.0, and the vitamin complex A5 + Co: H_3BO_3, 2.86; $MnCl_2*4H_2O$, 1.81; $ZnSO_4*7H_2O$, 0.22; $CuSO_4*5H_2O$, 0.08; $Na_2MoO_4*2H_2O$, 0.39; $Co(NO_3)_2*6H_2O$, 0.049.

2.2. Extraction of DNA

Several protocols have been developed and described for the preparation of genomic DNA from microorganisms. Most based upon the methods of Kirby and Marmur (Kirby, 1957; Marmur, 1961), which generally comprise: a) chemical cell disruption by enzymic digestion and detergent lysis; b) extractions with organic solvents and c) selective recovery of the DNA. The methods used in these studies have been optimised specifically for the application of isolated DNA in the polymerase chain reaction, for the amplification of targeted 16S rRNA genes.

2.2.1. Preparation of genomic DNA from pure cultures of bacteria

A protocol used in these studies for preparation of genomic DNA from pure cultures of bacteria is a modified version of the protocol of Wilson (Wilson, 1987). Liquid cultures (1.5 ml), grown to mid-log phase, were harvested by centrifugation (Eppendorf microfuge) at 10,000 x g. The cell pellets were resuspended with 564 µl TE buffer, and approximately 10 µg crystaline lysozyme were added. During incubation at 37°C for 60 min, the cell suspension was mixed thoroughly by inverting the Eppendorf tube several times. After addition of 6 µl Proteinase-K (10 mg/ml) and 30 µl SDS or SLS (10%), the cell suspension was incubated again at 37°C for 60 min. Subsequently, 100 µl NaCl (5M) were added and the suspension was incubated for 2 min at 65°C. After addition of 80 µl preheated (65°C) CTAB-solution (10% CTAB in 0.7 M NaCl) the suspension was incubated for 10 min at 65°C. The cell suspension was extracted three times with organic solvents to remove proteins and cell debris: first, with an equal volume chloroform:isoamyl alcohol (24:1) solution, and centrifuged 5 min at 15,000 x g. The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Finally, the resulting aqueous phase was extracted with an equal volume chloroform:isoamyl alcohol (24:1). To precipitate extracted nucleic acids, 0.7 volumes isopropanol were added to the aqueous phase, followed by 30 min centrifugation at 15,000 x g. The DNA pellets were washed with 500 µl EtOH (70%) and microfuged another 15 min. The pellets were resuspended in 40 µl TE buffer and stored at 10°C.

2.2.2. Rapid preparation of genomic DNA from bacterial colonies

A simple protocol, being much more rapid than methods employing organic extractions, for preparing DNA from bacteria, was used in this study when small amounts of DNA were required for subsequent analysis, e.g., for PCR amplifications. The protocol used in this study, an extension of a method published by Holmes and Quigley for the

preparation of plasmid DNA (Holmes & Quigley, 1981), was applied to the extraction of DNA from individual bacterial colonies (Güssow & Clackson, 1989; Moore *et al*., 1999).

Individual colonies (1-5 colonies were usually adequate for generating sufficient PCR-template DNA) were picked from an agar plate, using a sterile pasteur pipette, and resuspended in 100 µl TE buffer. The cell suspension was incubated for 10 min in a 97°C waterbath and the resulting cell lysate was centrifuged 5 min at 15,000 x g. An aliquot (1.0 µl) of the supernatant was added directly to a PCR, without any additional purification steps.

2.2.3. Extraction of DNA from sediment samples

Total DNA was extracted from Kenyan sediment samples, according to the direct DNA extraction method described by Mau (Mau, 1997). This protocol represents a modified version of the protocol of Wilson (Wilson, 1987), optimised for the extraction of genomic DNA from sediments.

One gram of sediment sample (wet weight) was distributed equally in eight, sterile, 2 ml Eppendorf tubes. Firstly, the sediment aliquots were washed once with 500 µl TE buffer (pH 8.0), to remove the dichloromethan fixative and salt residue of the sediment, both of which would be expected to inhibit enzymatic DNA extraction procedures. The sediment aliquots were resuspended then with 560 µl TE buffer (pH 8.0) and approximately 10 µg crystaline lysozyme were added to each tube. During incubation, at 37°C for approximately 60 min, the compounds were mixed thoroughly by inverting the Eppendorf tubes several times. After addition of 6 µl Proteinase-K (10 mg/ml), and 30 µl SDS (10%), the mixtures were incubated again, at 37°C for approximately 60 min. Subsequently, 100 µl NaCl (5M) were added, and the tubes were incubated for 2 min at 65°C. After addition of 80 µl preheated CTAB-solution (10% CTAB in 0.7 M NaCl), the tubes were incubated another 10 min at 65°C, followed by a series (three times) of alternating freezing, in liquid nitrogen, and thawing, at 65°. Sediment, proteins, cell debris and, to some extent, humic acids were removed during extraction with organic solvents: first, with an equal volume of chloroform:isoamyl alcohol (24:1) solution, and centrifuged 5 min at 15,000 x g. The upper aqueous phases were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and, finally, with an equal volume chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated by the addition of 0.3 volumes ammonium acetate and 0.7 volumes isopropanol to the aqueous phase, followed by 30 min centrifugation at 15,000 x g. DNA pellets were resuspended in TE buffer and combined in 100 µl. DNA was precipitated a second time by adding 0.1 volumes 3M sodium acetate (pH 5.2) and 2.5 volumes ethanol

(absolute). The DNA pellet was washed with 500 µl ethanol (70%), and microfuged another 10-15 min. After removing the ethanol, the pellet was dried briefly (2-3 min) in a speed vac and resuspended in a final volume of 40 µl 10 mM Tris buffer (pH 8.0).

Gel purification of crude DNA extracts:

The crude DNA extract (40 µl) was purified by agarose gel (1.0 %, w/v) electrophoresis, using Low-Melting-Point (LMP) agarose (Gibco), which melts, due to its additional hydroxyethyl groups, at 65°C; i.e., below the melting temperature of large molecular weight nucleic acids. After staining with an ethidium bromide solution (10 mg/ml), chromosomal DNA was visualised and excised under UV irradiation. Subsequently, an aliquot (5 µl, corresponding to 500-900 ng DNA) of the melted (at 65°C) agarose block was added directly to a hot-start PCR.

2.2.4. Determination of DNA concentration

The concentration of extracted DNA in suspension was estimated by spectrophotometric measurement at A_{260}. For double-stranded DNA suspensions, an OD of 1.0 at a wavelength of 260 nm and using a cuvette with 1 cm light path, is equal to a concentration of 50 µg/ml. The quality of the DNA was evaluated by measurement of the A_{260}/A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0. Ratios less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA. The A_{230}/A_{260} ratio should be 0.3-0.9. Ratios greater than 0.9 indicate the presence of salts or humic acids.

2.3. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) (Mullis & Floona, 1987) provides a rapid and highly sensitive method for the primer-mediated enzymatic amplification of specific target sequences in genomic DNA resulting in the exponential increase of target DNA copies. Denatured target DNA is incubated together with thermostable DNA polymerase (Saiki *et al.*, 1985 and 1988), isolated from *Thermus aquaticus*, dNTPs and two primers, initialising the synthesis of the complementary DNA strands. Each cycle comprises three steps: in an initial step, the duplex DNA strands are denatured by heat. The second step, the so-called primer annealing, involves the hybridisation of copious primers and complementary DNA strands. Finally, the complementary strands are synthesised by the thermostable *Taq*-polymerase, starting at the 3´-end of the primers.

The standard PCR amplification was carried out using the protocol listed in the appendix A. The amplification of 16S rDNA from total extracted DNA, from

environmental samples, was carried out in triplicate using a hot-start PCR protocol (D' Aquila *et al*., 1991), which has proven to be effective in minimising unspecific annealing of primers to non-target DNA: with the exception of the *Taq*-polymerase (*AmpliTaq*-DNA-polymeraseLD) the PCR reaction mixture was covered with mineral oil. The enzyme was added to the reaction mixture after the initial denaturation step (one minute at 96°C). Subsequently, the amplification was performed, with a Landgraf thermocycler (Landgraf, Langenhagen), using thirty incubation cycles, each consisting of 96°C for 60 sec, 55°C for 30 sec, and 72°C for 180 sec, terminating with a single step of 72°C for 600 sec.

The amplification of 16S rDNA from isolates, was carried out with a GenAmp 9600 thermocycler (Perkin-Elmer-Cetus), using 30 cycles, each consisting of 94°C for 60 sec, 55°C for 60 sec, and 72°C for 120 sec, and including a final incubation step for 600 sec at 72°C. Successful amplifications were confirmed by agarose gel (08-1.0% w/v) electrophoresis and ethidium bromide staining.

Dilution of template DNA

PCR amplifications of 16S rRNA genes (rDNA) from environmental samples were carried out, using gel-purified community DNA which was serially-diluted by factors of ten (in pre-warmed TE buffer). Therefore, concentrations of template DNA, extracted from environmental samples, varied from 0.5-1 µg DNA (for the undiluted DNA extract) to 50-100 pg (for the 10^{-4} dilution) per 100 µl PCR volume.

For isolates, the amplification of the 16S rRNA genes was performed using 0.5-1.0 µl of a total 40 µl DNA extract or, alternatively, 0.3-1.0 µl of a boiled preparation extracted from a single colony, as template DNA.

Reamplification of cloned 16S rDNA PCR products

Crude lysates of clones were obtained according to the protocol in chapter 2.2.2.. Cloned PCR fragments were reamplified by PCR with primers T3 and T7 binding symmetrically on both sides of the vectors multicloning side. Reamplified 16S rDNA inserts were used directly for ARDRA (amplified rDNA restriction analysis), without any additional purification steps.

2.3.1. Purification of PCR products

Amplified 16S rDNA was purified, for sequence determination and dot blot preparations, using the QIAquick PCR purification kit (Qiagen), following the instructions of the

manufacturer. Purified PCR products were eluted from the purification columns by the addition of 50 µl 10 mM Tris buffer (pH 8.0).

PCR products, as they resulted from amplification of total DNA from environmental samples, were purified by agarose gel (0.8 %) electrophoresis prior to cloning. After staining with ethidium bromide, a defined band was visualised under UV irradiation and excised. Besides removing surplus primers, nucleotides, and salts, this method possessed the advantage that incomplete (shorter) amplification fragments are also removed prior to cloning. Subsequently, the DNA was extracted from the gel matrix material, using the QIAEX gel extraction kit (Qiagen), whereby the DNA is bound to silica gel particles, in the presence of high salt concentrations. Purified PCR products were eluted with 40 µl TE buffer (pH 8.0). In this manner, purified PCR products were applied directly to the T4-DNA-polymerase treatment.

2.3.2. Oligonucleotide primers for PCR

The nearly entire genes encoding 16S rRNA were amplified for *Archaea* and *Bacteria* using the following primer pairs: The forward amplification primers were 16F27 for *Bacteria* (Lane, 1991), and 16F23A for *Archaea* (Barns *et al.*, 1994), while in both cases, the reverse amplification primer was 16R1492 (Lane, 1991). The name of a primer includes information about the type of ribosomal target molecule ("16" indicates 16S rRNA/rDNA), the direction of synthesis ("F" indicates the forward or sense primer, "R" indicates the reverse or anti-sense), and the next number defines the 3'-terminal nucleotide of the primer, according to the *Escherichia coli* 16S rRNA gene sequence numbering (Brosius *et al.*, 1978). The melting temperature (T_m), defined as the temperature corresponding to the mid-point in the transition from helix to random coil, was estimated using the simple equation described by Suggs *et al.*, (1981), which sums the contributions of GC and AT pairs:

$$T_m = 4N_{G+C} + 2N_{A+T}$$

Where N_{G+C} and N_{A+T} are the numbers of G and C and of A and T.

The oligonucleotide primers were synthesised by Gibco, BRL and sequences are listed in appendix B.

2.4. Cloning of amplified 16S rDNA

The PCR products generated from total, environmental DNA, were cloned using the restriction independent PCR DIRECT ^{TM} Cloning System, following the manufacturer's protocol (Clontech, Palo Alto, USA). Alternatively, a reamplified Clontech-vector was applied, since the company did not provide the PCR DIRECT ^{TM} Cloning kit anymore. Transformation was carried out using MAX Efficiency DH5α^{TM} Competent Cells (GIBCO, BRL, Eggenstein) with the genotype: Fϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ *thi*-1 *gyr*A96 *rel*A1.

2.4.1. Blue/white screening for recombinant plasmids

After transformation with the plasmid/insert DNA hybrid mixture, the ($LacZ^-$) DH5α bacterial host cells were plated on 2XTY medium containing 100 µg/ml ampicillin, for selection of transformants. X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid. The cloning site in the pDIRECT vector is located in the middle of the plasmid's lacZαgene; if no insert is present, functional β-galactosidase is produced, and the transformed bacterial colony is blue. These few blue colonies occur due to the presence of supercoiled vector molecules which have escaped linearisation. However, if the host cell receives a recombinant plasmid containing a 16S rDNA insert in the lacZαgene, the resulting transformant colony is white ($LacZ^-$). Occasionally, some faint blue colonies were observed containing DNA inserts similar to those plasmids from white colonies.

2.4.2. Size screening for recombinant plasmids

Clones containing approximately 1.5-kb 16S rDNA inserts were identified by PCR screening, using the rapid protocol for preparation of template DNA from single bacterial colonies (see chapter 2.2.2.), and T3/T7 plasmid primers. The amplification products were checked by agarose gel (0.8-1.0% w/v) electrophoresis.

Alternatively, cell lysates of clones containing the superhelical plasmids were compared directly by agarose gel (1.0% w/v) electrophoresis (Le Gouill & Déry, 1991). In this protocol, individual colonies were picked from an agar plate, using a sterile pasteur pipette, and resuspended in 16 µl lysis-loading buffer containing 30 volumes of H_2O, 18 volumes of 5X loading dye, 8 volumes of NaOH (1N), and 4 volumes of SDS (10%). Proteins and chromosomal DNA were precipitated by addition of 4 µl potassium acetate (3M) and centrifugation for 4 min at 15,000 x g. Subsequently, the entire supernatant was loaded directly onto an agarose gel (1% w/v) for electrophoresis.

2.4.3. Storage of clones

Clones were stored as glycerol (15% w/v) stock solutions. Individual colonies were picked from agar plates, using a sterile pasteur pipette, and were resuspended in 850 µl 2XTY medium containing 100 µg/ml ampicillin. After 6 hours of incubation at 37°C, 150 µl glycerine (absolute) were added and suspensions were mixed by vortexing. The glycerol stock suspensions were stored at –70°C.

2.4.4. Preparation of plasmid DNA

Plasmid DNA was isolated and purified, using the QIAwell 8 plasmid kit (Qiagen), following the protocol of the manufacturer. The protocol is based on the alkaline lysis method described by Birnboim and Doly (1979), followed by binding of plasmid DNA to Qiagen resin particles that have been immobilised in an inert polytetrafluoroethylene membrane matrix. The resin selectively adsorbs plasmid DNA from a cleared lysate while RNA, proteins, and low-molecular-weight impurities are removed by a medium-salt wash. The plasmid DNA was eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. The plasmid DNA obtained was used for sequencing and dot blot analysis.

2.5. 16S rDNA sequencing

PCR products and plasmids were sequenced using the Applied Biosystems 373A or 377 DNA Sequencers, as described in the protocols recommended by the manufacturer (Perkin Elmer Biosystems) for *Taq* Dye Deoxy Terminator Cycle Sequencing kit. This sequencing method is based upon the prototypal chain-terminating method, using T7-Polymerase established by Sanger (Sanger *et al*., 1977). Instead of T7-Polymerase (Sequenase) the Cycle-Sequencing technique provided by Applied Biosystems employs the thermostable AmpliTaq-Polymerase,FS isolated from *Thermus aquaticus*. This enzyme is a mutant from the wildtype AmpliTaq-Polymerase and has, essentially, no 5'-3' nuclease-activity and reduced discrimination for didesoxynucleotides. The labelling of the DNA fragments is accomplished through the incorporation of fluorescence-dye-labelled didesoxy-nucleotides, causing a termination of the DNA chain synthesis. Subsequently, the labelled DNA fragments are separated by denaturing polyacrylamide gel electrophoresis. The detection system consists of an argon laser with main emissions of 488 nm and 514,5 nm wavelength. Labelled DNA fragments emit fluorescent ray while passing the area hidden by the laser directed to the separation system through a mirror.

The sequencing primers (Barns *et al.*, 1994; Edwards *et al*., 1989; Lane, 1991) were synthesised by Gibco, BRL and are listed in appendix A.

2.5.1. Thermocycler protocol

Sequencing reactions were carried out with a GenAmp 9600 thermocycler (Perkin-Elmer-Cetus) using 25 incubation cycles, each consisting of 15 sec denaturing at 96° C, 15 sec primer annealing at 60°C, and 240 sec primer extension at 60°C.

2.5.2. Purification of extension products

Unincorporated terminators were removed by ethanol precipitation: initially, 80 µl H_2O were added to the 20 µl reaction mixture. The entire contents of the reaction tubes were transferred to 0.5 µl Eppendorf tubes, and mixed by vortexing with 10 µl 3M NaAc (pH 4.8) and 250 µl absolute Ethanol (RT). The reactions were microfuged for 20-30 min at 15,000 x g. The ethanol was removed and pellets were washed by adding 300 µl 70% ethanol and microfuging for 10 min at 15,000 x g. After removing the ethanol, pellets were dried in a vacuum centrifuge.

2.5.3. Analysis of sequence data

Sequence data were aligned manually with reference 16S rRNA sequences (Maidak *et al*., 2001; Stoesser *et al.*, 2002), based on conserved primary sequence and secondary-structure considerations (Gutell *et al*., 1985), using the Olsen sequence editor (Olsen, 1991, personal communication). Sequences were submitted to the CHECK_CHIMERA program of the RDP (Maidak *et al.*, 2001), in order to detect the presence of possible chimeric artefacts generated by PCR. Similarities were calculated for nearly complete 16S rDNA sequences using only unambiguously determined nucleotide positions. Sequence dissimilarities were converted to evolutionary distances according to Jukes and Cantor (Jukes & Cantor, 1969). Phylogenetic trees were constructed from pairwise distances, using the least-squares algorithm of Fitch and Margoliash (Fitch & Margoliash, 1967) included in the software programs of the PHYLIP package (Felsenstein, 1989).

2.6. DNA hybridisation

2.6.1. Dot blotting of DNA

A portion of 0.1 to 1.0 µg of plasmid-DNA or PCR-product, in a volume of approximately 5 µl, was mixed with one volume NaOH (0.4 N) for denaturation. After 5 min incubation at room temperature, the mixture was neutralised with two volumes HCl (0.2 N). Subsequently, the denatured nucleic acids were bound to positively-charged

nylon membrane Hybond^{TM}-N^{+} (Amersham, Braunschweig), using a vacuum dot blot apparatus (Bio-Rad, Munich). Each well was washed with 20µl of 2X SSC buffer (0.3 m NaCl; 30 mM sodium citrate; pH 7.0). The nucleic acids were immobilised onto the membrane by UV-crosslinking (Stratalinker, Stratagene), allowing the formation of covalent bonds between thymine residues and positively-charged residues on the nylon membrane. Blots were used immediately or were stored at –20°C.

2.6.2. Dot blot pre-hybridisation and hybridisation

A pre-hybridisation step prepares the membrane for probe hybridisation by blocking non-specific nucleic acid binding sites on the membrane and by equilibration of the membrane in the conditions (ion, temperature) required for hybridisation and, ultimately, effectively lowers background reactions. Generally, pre-hybridisation conditions are identical to those of hybridisation although the reaction time is shorter. Many different pre-hybridisation and hybridisation solutions have been described in the literature. The important components of a pre-hybridisation and hybridisation buffer and their functions should be outlined briefly. Detergents and blocking agents help to reduce non-specific binding of probe to membrane. Blocking agents include Denharts reagent, dried milk and heparin. The most commonly used detergents are sodium dodecyl sulfate (SDS) and N-laurylsarcosine (SLS). Urea or formamide can be used to depress the melting temperature of the hybrid.

Initially, blotted DNA was pre-wetted in 2X SSC buffer. The membranes were pre-hybridised in a solution (10 ml per 86 cm^2 of membrane surface area) containing 6X SSC (0.9 M NaCl plus 0.09 M sodium citrate, pH 7.0), 2% blocking reagent (added from 10% stock solution), 0.1% N-laurylsarcosine, 0.02% sodium dodecyl sulfate (SDS), and 30-50% formamide, and incubated in hybridisation tubes for at least 2 hours at the hybridisation temperature (Hoeltke *et al*., 1992). The probe was added directly to the pre-hybridisation solution, to a concentration of 10-20 ng/ml, and allowed to hybridise overnight at the pre-hybridisation temperature.

2.6.3. Stringency washing

The post hybridisation washes removed unbound probe, that would cause higher background signals if not removed. Usually, the temperature and/or salt concentrations during the washing are more stringent than during hybridisation. The optimal washing conditions were determined using a constant temperature with different salt concentrations. The membranes were washed twice, 15 min per wash, in 20 ml washing buffer containing 20 mM Tris (pH 8.0), 0.01% SDS, and 20-200 mM NaCl, at the

hybridisation temperature. Subsequently, washed membranes were employed for chemiluminescense detection or stored at –20°C.

2.6.4. Chemiluminescense detection

Biotin-labelled hybrids were detected, using peroxidase-conjugated streptavidin (Dianova, Hamburg): non-specific binding sites of the membrane were blocked by immersing the membrane in 5% blocking reagent in Tris-buffered saline-Tween (TBS-T) for one hour at room temperature, with shaking. Peroxidase-conjugated streptavidin was diluted 1:1,000 with 5% blocking reagent in Tris-buffered saline-Tween (TBS-T), to a final concentration of 1 µg/ml. The membrane was incubated with 2-5 ml of this solution in a sealed plastic bag for 1 hour at room temperature. The membrane was washed successively, 10 min per wash, in TBS-T, TBS-T containing 1.5 M NaCl, and TBS-T containing 0.5% Triton-X-100, to remove unbound streptavidin. The detection of peroxidase activity was performed using the ECL-kit (Amersham, Braunschweig). The light emission was detected by exposure (1-10 min) of X-ray film (Biomax, Kodak).

2.6.5. Rehybridisation

A “stripping” protocol removes the original probe, and is often useful for reusing a blot with different probes. There have been many described stripping protocols in the literature. The procedure, proven to be suitable for removing the probe from DNA dot blot and colony hybridisations, includes an initial washing step in H_2O for 1 min, followed by two incubations of the membrane in alkaline probe-stripping buffer, containing 0.2 NaOH and 0.1% SDS, for 10 min at 37°C. The membrane can be used directly for pre-hybridisation and hybridisation or can be stored at –20°C.

2.6.6. Oligonucleotide probes

The 5’ Biotin-labelled oligonucleotide probes were synthesised by Gibco, BRL. The sequences of oligonucleotide probes are listed in the appendix B.

2.7. Amplified ribosomal DNA restriction analysis (ARDRA)

In this study, a standardised method for amplified ribosomal DNA restriction analysis (A. Bennasar, personal communication) was employed to screen cloned 16S rDNA amplification products from environmental samples and from isolates. Five to ten microlitres (depending on the yield and corresponding to, roughly, 1 µg DNA) of PCR-amplified 16S rDNA were digested with 5 U of the tetrameric endonuclease *Taq*I (MBI)

in a final volume of 20 µl containing the appropriate restriction buffer. The reaction mixtures were covered with mineral oil and incubated at 65°C for 3 hours. After digestion of amplified 16S rDNA and addition of 4 µl of 5-fold loading buffer, the resulting products of restriction fragment length polymorphism (RFLP) were separated by agarose gel electrophoresis. Ten microlitres of the mixtures were loaded on an 3% (w/v) agarose gel (1.5% LE-agarose, Roth, and 1.5% NuSieve 3:1, FMC Bioproducts). Of the overall 20 wells per gel, 17 were used for 16S rDNA restriction fragments and three for the molecular weight marker (1-kb DNA ladder, Gibco). Electrophoresis was performed in TBE buffer (pH 8.0) at 100V for 3 h. The gels were stained with ethidium bromide, and DNA fragments were visualised by UV translumination and photographed using the Enhanced-Analysis-System (Herolab).

The digitised gel images were stored as TIFF (Tagged Image File Format) files and were analysed with the GelCompar version 4.0 software (Applied Maths, Kortrijk, Belgium) in three steps: conversion, normalisation and numerical analysis. The conversion program was utilised to mark the different tracks on the gel image, calculate densitometric curves and to save the patterns with descriptive information. After conversion of the gel images to the standard track format, the raw track data were normalised. Normalisation of the patterns is of importance to achieve reliable databases by which an objective comparison of patters can be performed. The normalisation of a gel included the alignment of reference patterns by aligning their corresponding bands and by subsequent interpolation of the intermediate values. The non-reference tracks were then aligned gradually according to their closest neighbouring reference tracks. By defining one standard reference pattern and further aligning the bands of all reference tracks from any other gel to the corresponding bands of that single pattern, all gels became compatible with one another. All ARDRA patterns were normalised using the molecular weight marker (1-kb DNA ladder) as reference profile. Every band in the ARDRA patterns larger than 75 bp were included in the numerical analysis. A similarity matrix between each pair of combined patters was calculated using the Dice similarity coefficient (S_D) according to the formula $2n_{AB}/(n_A + n_B)$ with n_{AB} the number of bands in pattern A and B, n_A the total number of bands in pattern A, and n_B the total number of bands in pattern B. Dendrograms were reconstructed using the UPGMA (unweighted pair group method using arithmetic averages) clustering algorithm, showing the clustering of the 16S rDNA sequences based on the similarities of their combined ARDRA patterns.

3. Results and discussion

3.1. Screening of 16S rDNA molecules

New molecular techniques have revealed extensive microbial diversity that was previously undetected with cultivation-dependent methods, although exhaustive inventories of microbial communities still remain impractical. As a result, researchers must rely on samples to estimate the actual diversity of microbial communities. Among the most significant obstacles facing studies of microbial diversity are those in obtaining reliable and comprehensive assessments of the existing diversity in a given sample. Generally, the number of different types of organisms observed in any community, increases with the sampling effort until all types are observed (Hughes *et al.*, 2001). The use of appropriate screening strategies, such as DNA sequencing, fingerprinting and hybridisation probing, contribute to reducing the analytical effort necessary for the assessment of complex communities. The following chapters provide an overview of the screening methods used in this study to analyse the community structure of Lake Magadi sediment samples.

3.1.1. DNA sequencing

The partial and complete sequence analysis of 16S rDNA genes has been proven to be a reliable strategy for the screening of limited numbers of isolates and 16S rDNA clones. The first step in such an analysis consisted of a partial sequence determination, comprising approximately 500 nucleotides of the entire 16S rDNA molecule. In this study, the 5' end of the PCR-amplified 16S rDNA was chosen as the starting-point, since this part of the molecule, generally, possesses a higher degree of variability, compared to that of the 3' end, and allows for higher resolution differentiation of sequence types. If the partial sequences exhibited less than 95% similarity to a reference sequence, the corresponding isolates or clones were selected for subsequent (almost complete) sequence analysis. However, nucleic acid sequence analysis is relatively expensive and requires specialised equipment and can present difficulties for the routine application of this technique to rapid analysis of large numbers of isolates or clones.

3.1.2. Fingerprinting analysis

An alternative approach for screening large numbers of clones or isolates comprises restriction enzyme studies, such as amplified rDNA restriction analysis (ARDRA) (Vaneechoutte *et al.*, 1992), in which the amplified ribosomal RNA gene (rDNA) is subjected to restriction endonuclease digestion. The resulting restriction fragment pattern

is then used as a "fingerprint" for a differential characterisation. During the last few years, studies using amplified 16S rDNA restriction analysis have been applied for the characterisation of a variety of bacterial isolates such as *Acinetobacter* (Dijkshoorn *et al*., 1998), *Bacillus* and *Paenibacillus* (Heyndrickx *et al*., 1996) *Lactobacillus* (Giraffa *et al*., 1998), *Saccharomonospora* (Yoon *et al*., 1997), *Veillonella* (Sato *et al*., 1997), *Xanthomonas* and *Stenotrophomonas* (Nesme *et al*., 1995). Furthermore, the ARDRA approach has been used for analysing mixed bacterial populations (Moyer *et al.*, 1994; Ovreas & Torsvik, 1998; Urakawa *et al*., 1999; Zhou *et al*., 1997).

An important advantage of the ARDRA over 16S rRNA(-gene) sequencing is its faster throughput of samples, since 16S rDNA sequence characterisation by ARDRA can be performed relatively easily and using standard molecular biology equipment. However, as with any other method, there are limitations to the ARDRA fingerprinting approach that are often underestimated. The reliability of restriction fragment data for assessments of phylogenetic relationships is not recommended, primarily because such data violate the assumption of independence among characters (Swofford *et al*., 1996). In the case of restriction enzyme analysis, fragments of identical mobility tend to be homologous for sequences from closely related individuals. However, whether used with isolates or communities, matching ARDRA band patterns cannot be used to unequivocally establish *identities* of organisms, since different but closely related strains may have the same band patterns (Massol-Deya *et al.*, 1995). Furthermore, the likelihood of two samples having fragments of the same size, although produced by different cleavage sites, increases as sequences become more different (Upholt, 1977). Conversely, fragments of different mobility may actually be homologous (Dowling *et al*., 1996; Swofford *et al*., 1996).

Therefore, we applied the ARDRA approach as a screening method at the outset of our study to obtain indicative 16S rDNA sequence information, that was used to estimate sequence diversity, and to select strains or clones for detailed taxonomic studies. The selection of the restriction enzyme *Taq*I (a four base site-specific restriction endonuclease cleaving T/CGA) was based on theoretically simulated digestions of nearly complete bacterial and archaeal 16S rDNA sequences available from the database. Moreover, the ARDRA approach using *Taq*I has been applied for analysing a mixed bacterial community from sediment samples (Bennasar, personal communication), although this method was not yet applied to archaeal populations. Firstly, the resolving power of the *Taq*I-ARDRA method was evaluated based on a comparative study of ARDRA fingerprints and 16S rDNA sequences, as determined for 50 *Archaea* clones and 40 haloalkaliphilic archaeal isolates. The resolving capacity of the *Taq*I-ARDRA approach was estimated to correspond with 95 to 97% 16S rDNA sequence similarities,

thus allowing a differentiation between 16S rDNA sequences belonging, approximately, to different genera (Amann *et al.*, 1995; Devereux *et al.*, 1990; Fry *et al.*, 1991) Subsequently, the *Taq*I-ARDRA method was applied for screening archaeal and bacterial 16S rDNA clone libraries: clonal types were initially grouped on the basis of their amplified rDNA restriction patterns. The sequences from representatives of different *Taq*I-ARDRA fingerprinting groups were then determined and compared with those available from the DNA databases.

Although fragment analysis offers less resolving power than nucleotide sequencing in some cases, it is a rapid and cost effective alternative where large numbers of clones or isolates are being screened. ARDRA fingerprinting proved itself to be a powerful complement to sequence analysis.

3.1.3. Non-radioactive colony hybridisation

The aim of this experimental section was to optimise existing colony hybridisation protocols for their application in non-radioactive hybridisation experiments with single end-labelled oligonucleotides for rapid screening of 16S rDNA clone libraries.

The technique of colony hybridisation was first reported in 1975 (Grundstein & Hogness, 1975) and comprises lysis of bacterial colonies on filters, followed by denaturation and immobilisation of cellular DNA, allowing the hybridisation to DNA or RNA probes. Initially, the method was developed for screening *Escherichia coli* transformants in cloning experiments, for identifying colonies containing a specific gene. Subsequently, many variations of the original method have been described, appropriate for different applications, including analysis of environmental isolates. The main benefit of colony hybridisation is that it allows a rapid screening of bacterial recombinant libraries for specific target DNA sequences.

In the original protocol, bacterial cells were grown up on nitrocellulose membrane filters laid over nutrient agar. Alkaline treatment served to lyse the colonies, releasing cellular DNA, which was simultaneously denatured. After neutralising the filter and washing to remove loosely-bound cellular debris, single-stranded DNA was immobilised by baking, and filters were applied for hybridisation with DNA probes. Subsequent modifications of the method included transferring the colonies from agar to filters by blotting (colony lifts) and using more robust, neutral or positively-charged nylon membranes for binding DNA, RNA or protein. Before the development of non-radioactive probing systems, most applications used ^{32}P-labelled DNA or RNA probes. Methods using DNA labelled with ^{32}P are very sensitive, but the lack of stability, safety and waste disposal problems associated with radiation have provoked efforts to develop

and improve alternatives to radioactive labels. The use of biotin-labelled DNA probes for colony hybridisation was reported in 1986 (Haas & Flemming, 1986) and there are now a variety of alternative non-radioactive systems for labelling and detection available. However, because the detection of non-radioactive probe bound to the target DNA usually relies on antibody binding, there may be significant background where cellular debris and proteins remain on the filter. This poses a problem, particularly when single 3'- or 5'-end labelled oligonucleotides are used during hybridisation, whereas tailed oligonucleotides or PCR probes enable a compensation of the background effect because of their relatively high sensitivity during detection. However, a drawback of such probes is their relatively low specificity.

These problems of high background could be overcome by using probes directly labelled with enzymes, e.g., alkaline phosphatase (Jablonski *et al*., 1986; Wright *et al*., 1993) or horse radish peroxidase (Schönhuber *et al*., 1999; Thorpe *et al*., 1985), or, as was done in this work, by optimising the standard colony lift protocols (Anonymous, 1995; Hirsch, 1995; Sambrook *et al*., 1989) to reduce colony debris on the filter.

Experimental approach

Initially, the question arose as to whether the background is caused due to non-specific binding of probe or to the detection system (antibody) reacting with colony debris remaining on the filter. Therefore, a radioactive and a non-radioactive approach were combined in a single colony hybridisation experiment: *Escherichia coli* DH5α clones containing target and non-target (single mismatch) 16S rDNA were transferred to uncharged nylon membranes according to: a) a standard colony lift protocol (Sambrook *et al.*, 1989); and b) a second protocol including a Proteinase-K treatment (Anonymous, 1995). Hybridisation was performed using the standard hybridisation and washing conditions (chapter 2.6.2. and 2.6.3.) and employing a double-labelled oligonucleotide probe (5'-biotinylated and 3'-phosphorylated). The hybrids were detected in succession, firstly, by autoradiography and, subsequently, by chemiluminescense . The colony blots generated using the colony lift protocol, without Proteinase-K treatment, lead to satisfactory results after autoradiography, that is, positive controls produced strong positive signals and negative controls gave no signals. When these blots were applied to chemiluminescense detection, target clones gave weak positive signals while non-target clones also produced signals of same intensity. These results lead to the conclusion that low signal and high background occur due to non-specific binding of antibody to colony debris on the filter and not to non-specific hybridisation of probe. Notably, no signals, neither for autoradiography nor for chemiluminescence detection, were obtained for colony blots generated using a colony lift protocol including a Proteinase-K treatment.

This result raised the question of whether the applied colony lift protocol was appropriate for binding a sufficient amount of DNA to the membrane. Probably, the Proteinase-K treatment, subsequent to the UV-binding treatment, generally causes a digestion of proteins as well as the removal of immobilised DNA, which may form stable complexes with proteins after UV-fixation.

Optimised procedures for colony blotting

The optimised method, described in detail below, was observed to work for colony hybridisation with non-radioactive end-labelled oligonucleotide probes, combined with commercial chemiluminescense detection systems (ECL-kit, Amersham, or CDP-Star, Boehringer).

Preparation of plates

Transformed *Escherichia coli* DH5α cells were plated on 2X TY agar medium containing ampicillin and incubated overnight at 37°C. The colonies were not allowed to grow greater than 2 mm in diameter to prevent smearing, but were at a stage where new growth subsequent to the colony lift was possible. A colony density of 100-150 colonies per plate (of 82 mm diameter) has been proven to be optimal for accurate selection of clones. Petri dishes were pre-cooled for, at least, 60 minutes at 4°C prior to colony lift.

Choice of membrane

There is a considerable range of different membranes available, possessing distinct properties, which may be difficult to differentiate, since some companies supply membranes manufactured by another company, under a different name. Nitrocellulose filter membranes have been reported to give good results with low background signal, although they were not included in the series of tests, as they are fragile and also unstable in alkaline conditions. The main benefit of nylon membranes, apart from their strength, is that they bind released DNA covalently, with a higher binding capacity than nitrocellulose. Therefore they can be "stripped" and re-hybridised several times without significant loss of bound DNA. However, a drawback of nylon membranes is that they can give high background noise, especially with positively-charged nylon membranes. Hybond-N nylon membranes (Amersham, Braunschweig) achieved the best results with the lowest background:signal ratio for non-radioactive hybridisations. These membranes are uncharged and have a pore size of 0.45 μm. Membranes were autoclaved to be sterile in case the plate from which they are taken was to be kept.

Colony lift

Membrane discs were carefully placed onto the agar surface without producing air bubbles. The membrane was allowed to sit on the surface for approximately 1 minute. The orientation of the membrane to the plate was marked, to be able to identify colonies after detection. The membrane disc was removed carefully with blunt-ended forceps and blotted briefly, colonies side upwards, on dry Whatman paper.

Colony lysis

The DNA was released from the bacteria, denatured and neutralised. This was achieved by placing the discs, bacterial side upwards, on Whatman paper saturated with the following solutions. The first stage was a separate lysis step, using 10% SDS for 3 minutes, followed by a transfer to dry filter paper to remove excess liquid. Liberation and denaturation of DNA were achieved in one step by placing the membrane for 5 minutes on filter paper soaked with denaturation solution (0.5 N NaOH, 1.5 M NaCl), followed by a transfer to dry paper to remove excess alkaline solution. The membrane discs were placed for 5 minutes onto filter paper soaked with neutralisation solution (1 M Tris-HCl pH 8.0), then transferred to dry paper. The membrane discs then were placed for 10 minutes onto filter paper soaked with 2X SSC buffer.

Proteinase-K treatment

A protease treatment was done prior to the UV-crosslinking step, in order to remove proteins that interfere with probe hybridisation and detection. The membrane was placed in a sterile petri dish and 0.5 ml of 2 mg/ml Proteinase-K in 2X SSC buffer was added to the surface of the membrane disc (82 mm diameter). The petri dish was closed with parafilm and incubated for 1 h at 37°C. Subsequently, the membrane disc was placed on two Whatman glass microfibre filters soaked with 2X SSC, and this stack was applied to a filtration unit (Millipore). A vacuum of 200-250 mbar was applied and filters were washed three times by pipetting 1 ml 2X SSC buffer on the surface. Membranes were blotted between Whatman paper fully wetted with sterile H_2O and a rolling tube was used to apply pressure. Remaining cellular debris stuck to the filter paper.

Fixation and blot storage

Transferred DNA was crosslinked with UV-light (Stratalinker). Membranes were stored between two sheets of Whatman paper sealed in a plastic bag at –20°C.

3.2. Cultivation-independent analysis of microbial diversity

3.2.1. Extraction of DNA

A DNA extraction and purification method was established to obtain high molecular genomic DNA from Lake Magadi sediment that can be amplified by PCR. The DNA yield was estimated by spectrophotometric measurement at A_{260} and approximately 34 µg total DNA of 23 kb in size were obtained per gram wet weight of extracted sediment sample (Fig. 3.1). The A_{260}/A_{280} and A_{260}/A_{230} ratios of the extracted DNA were approximately 1.7 and 1.2, respectively, the latter significantly lower than for pure DNA, indicating the presence of humic acids. Hence, the crude DNA extract was purified using gel electrophoresis with low-melting-point agarose (chapter 2.2.3.) and, subsequently, an aliquot of the melted agarose block was added directly to the PCR. The main benefits of this DNA purification method are the complete removal of PCR-inhibitory compounds present in the crude extract, combined with a size selection of genomic DNA without the risk of shearing or losses of DNA.

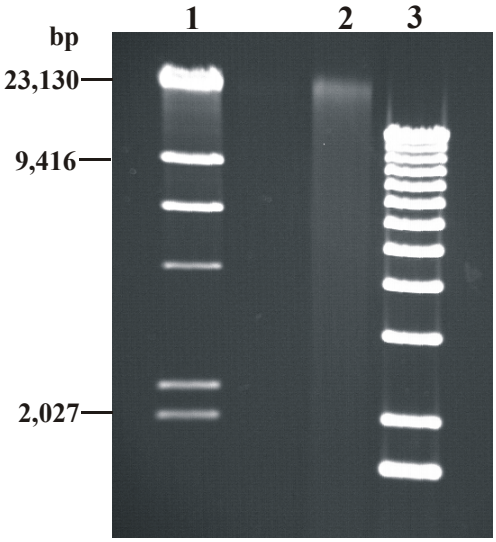

Fig. 3.1: Agarose gel (0,6% w/v) electrophoresis and ethidium bromide staining of genomic DNA prepared from 1 g (wet weight) of Lake Magadi sediment sample (lane 2). Lanes: 1) Lamda DNA *Hind* III fragments; 3) 1-kb DNA ladder (Gibco-BRL).

Numerous DNA extraction methods have been developed and evaluated, but all methods yield crude extracts that are contaminated to varying degrees with substances that inhibit key enzymatic processes in molecular biological methods (Frostegard *et al*., 1999; Krsek & Wellington, 1999; Leff *et al*., 1995; Miller *et al*., 1999; Moré *et al*., 1994; Steffan *et al*., 1988; Tien *et al*., 1999; Zhou *et al*., 1996). Humic acids are the most widely reported contaminants in soil and sediment nucleic acid extracts (Wilson, 1997),

but other constituents, such as metals and polysaccharides, can also inhibit molecular reactions (Tsai & Olson, 1992).

The amount of humic acids in soil is known to have a dramatic effect on the quality (purity) of the DNA obtained, particularly when a direct lysis method is employed (Holben, 1994). Humic acids are composed of higher molecular weight materials containing aromatic rings, nitrogen in cyclic forms, and in peptide chains formed by polycondensation of similar but not identical constituents created during the decomposition of organic matter in soil or sediments (Paul & Clark, 1989). The humic materials present in soil and sediment have a similar molecular weight and net charge to DNA and, thus, are readily co-purified. Humic contaminants interfere with subsequent enzymatic reactions of DNA, such as restriction digestion or PCR (Ogram *et al.*, 1987; Holben *et al.*, 1988; Steffan *et al.*, 1988). Humic contaminants also confound precise quantification of the recovered DNA because they exhibit substantial absorbance of light at 260 nm, the measure of which is generally used to quantitate DNA (Holben, 1994).

Furthermore, besides from the removal of PCR-inhibiting substances, such as humic acids, the most difficult and uncertain step in obtaining DNA from microbial cultures is the disruption of the cells, since many microorganisms are known to be extremely resistant to cell disruption due to their thick cell walls of polysaccharide or pseudopeptidoglycan. These include, particularly, some members of the Gram-positive phylum (*Mycobacterium*, *Peptococcus*, *Rhodococcus*, etc.), as well as some Archaea (e.g., methanogens), and many species of fungi, algae and cyanobacteria. The chemical lysis of cells generally requires one agent for disrupting the cell wall, and a second agent for destroying the cytoplasmatic membrane. Usually lysozyme is used to disrupt the cell wall by cleaving the β-1,4-glycosidic linkage between the N-acetymuramic acid and N-acetylglucosamine repeating unit of the murrein sacculus, comprising a part of the peptidoglycan layer of the cell walls of most species of bacteria. Generally, lysozyme is used in combination with a metal-chelating agent (e.g., EDTA) which reduces the stabilising effect of divalent cations, particularly magnesium ions, on bacterial cell walls and membranes. Additionally, EDTA inhibits nucleases which may act against released DNA. Proteinase-K, a serine protease, is frequently used to cleave the peptide bonding, including those comprising the peptide cross-linking interbridges of the peptidoglycan layers of the cell walls of bacteria.

The addition of detergent provides a disruption of the cytoplasmatic membrane by binding strongly to proteins and causing irreversible denaturation. Sodium dodecyl sulfate (SDS) is an anionic detergent, binding to cellular proteins and lipoproteins, forming SDS-polypeptide complexes, and further SDS inhibits nucleases. For the cell

disruption of bacteria which produce capsular slimes (e.g., cyanobacteria etc.), N-laurylsarcosine (SLS) can be more effective in denaturing cellular polysaccharide material than SDS. Also, cetyltrimethyl ammonium bromide (CTAB), a cationic detergent, has been proven to be effective in preparation of nucleic acids from microorganisms producing high amounts of polysaccharide cell wall layers, by denaturing and precipitating cell wall lipopolysacharides and proteins. Furthermore, CTAB has been shown to be effective in removing humic acids during DNA preparation from sediment and soil sample, by catalysing the exchange of heterocyclic compounds from the aqueous to the organic phase (Janusczkiewicz & Alper, 1983).

For microorganisms whose cell walls are not particularly sensitive to enzymic and detergent treatments, cell disruption may be achieved by physical methods, employing a bead beater, a French press, or a sonicator. Unfortunately, the DNA obtained using these methods is often sheared and may not be appropriate for use in further molecular applications. An application which has been shown to be effective for generating high molecular weight DNA, is the freeze (using liquid nitrogen) and thaw (at 95-98°C) technique, which is frequently used for extracting nucleic acids directly from environmental samples, such as sediment and soil (Mau, 1997; Tsai & Olson, 1991).

3.2.2. PCR amplification

Crude and gel-purified community DNA, extracted from Lake Magadi sediment, were used as template DNA in PCR assays with 16S rDNA primers specific for *Bacteria* or *Archaea*. No visible products were obtained when 0.45 µl, corresponding to approximately 380 ng of crude DNA extract, were used as template DNA in a 100 µl PCR assay. However, visible PCR-amplification products were obtained for 5 µl of melted LMP-agarose, corresponding to 380 ng DNA (Fig 3.2). Inhibitory effects of LMP-agarose were not observed until 0.25 % (w/v) agarose were added to the PCR mixture (data not shown). The results demonstrate that PCR-inhibitory compounds present in the crude extracts were completely removed using the low-melting point agarose DNA purification method without the need of dilution of the DNA extract.

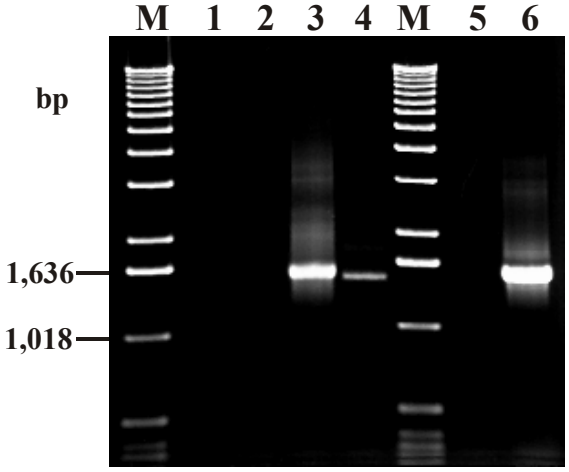

Fig 3.2: Agarose gel (1% w/v) electrophoresis and ethidium bromide staining of 16S rDNA PCR-amplification products of DNA extracted from Lake Magadi sediment. No products were obtained of undiluted, crude DNA extract, using primers specific for *Bacteria* (lane 1) and *Archaea* (lane 2). Products of gel-purified template DNA, using primers specific for *Bacteria* (lane 3) and *Archaea* (lane 4). Lane 5: negative control, i.e., without template DNA. Lane 6: positive control. M: 1-kb DNA ladder.

3.2.3. Cloning

3.2.3.1. Generation of 16S rDNA clone libraries from serial-diluted DNA

The 16S rRNA gene (rDNA) clone libraries were generated using a "semi-quantitative" PCR approach. That is, PCR-amplification, using primers specific for *Bacteria* or *Archaea,* targeting entire 16S rRNA genes, included amplification reactions utilising template DNA diluted to extinction, to obtain indicative information about the microorganisms which are predominant within the environmental samples. This PCR-technique is based on simple dilution of the template, and was formerly applied to characterise the bacterial population of sediment samples from a highly polluted river (Mau, 1997). Hypothetically, PCR amplifications using undiluted template DNA is expected to reveal higher sequence diversity than PCR amplifications using diluted template DNA, i.e., in the latter case rare sequence types will be thinned out as a result of dilution (Degrange & Bardin, 1995; Féray *et al*., 1999; Pillai *et al*., 1991; Sykes *et al*., 1992).

Community DNA extracted from Lake Magadi sediment samples was gel-purified and serially diluted (in TE-buffer) by a factor of ten. PCR amplification of 16S rRNA genes was carried out, in triplicate per dilution, according to the hot-start PCR protocol and using primers specific for *Bacteria* or *Archaea*. Fig. 3.3 shows the amplification products from PCR reactions using serially diluted template DNA.

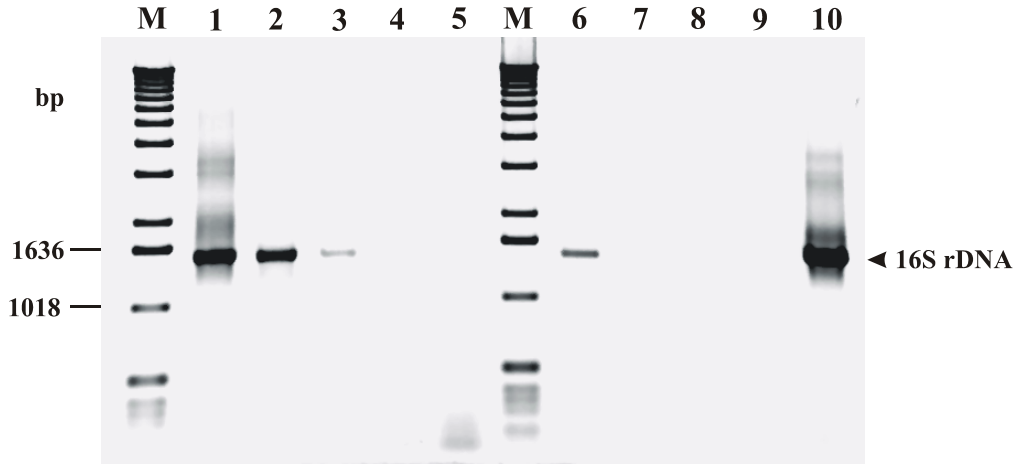

Fig. 3.3: 16S rDNA PCR-amplification products of DNA extracted from Lake Magadi sediment and diluted to extinction. Lanes 1 to 5: products of template DNA diluted tenfold and primers specific for *Bacteria*. Lanes 6 to 8: products of template DNA diluted tenfold and primers specific for *Archaea*. Lane 9: negative control, i.e., without DNA. Lane 10: positive control; M: 1-kb DNA ladder.

Amplification products generated from each dilution were pooled together and purified by agarose gel-electrophoresis prior to cloning. PCR-products were cloned, using restriction-independent ligation, for creating libraries containing cloned 16S rDNA sequences from representatives of the species existing within the environmental samples. *Bacteria* 16S rDNA clone libraries were generated using PCR products from amplification reactions utilising 10^0 and 10^{-2} dilutions of the DNA extract, corresponding to approximately 0.5-1 µg and 5-10 ng template DNA, respectively. The PCR amplification using primers specific for *Archaea* revealed detectable products only when undiluted DNA concentrations, corresponding to approximately 0.5-1 µg of mixed community DNA, were used as template DNA. Consequently, a single clone library was created containing 16S rDNA sequences from *Archaea*.

3.2.3.2. Regeneration of a ligation-independent cloning (LIC) vector

The aim of this approach was the regeneration of the pDirect ligation-independent cloning vector (Clontech), in order to generate a high-efficiency cloning vector.

Ligation-independent cloning (LIC) was developed for the directional cloning of PCR products without subsequent application of restriction enzyme digestion or ligation reactions (Aslanidis & de Jong, 1990; Haun *et al*., 1992). LIC vectors are created by treating linearised double-stranded plasmid DNA with T4 DNA polymerase in the presence of only one dNTP. In the absence of the necessary dNTPs, the exonuclease activity of the T4 DNA polymerase removes nucleotides in the 5'→3' direction from the ends of the template until the enzyme reaches the first nucleotide position corresponding

to the single dNTP present in the reaction mix. The presence of an excess amount of this dNTP in the reaction mix ensures that the exonuclease activity will not remove any base beyond the corresponding dNTP, since the polymerase activity will replace the dNTP as quickly as it is removed effectively preventing further excision. Plasmid DNA adjacent to the site of linearisation are typically designed to produce specific non-complementary 12 to 14 base single-stranded overhangs in the vector. PCR products with complementary overhangs are created by incorporating appropriate 5'-extensions into the amplification primers. The PCR product is purified to remove unincorporated dNTPs and then treated with T4 DNA polymerase in the presence of the appropriate dNTP to generate the specific vector-compatible overhangs. The enzymatic activity is killed subsequently by heat-inactivation and the insert is annealed to the linearised vector and transformed into competent cells.

The commercially available pDirect vector (Clontech), allowed the ligation-independent cloning of PCR products that were treated with T4 DNA polymerase. The two 5'-ends of the pDIRECT vector have different single stranded tails, 12- and 13-nucleotides long, respectively, which are not complementary to each other. Thus, the vector cannot recircularise itself, and the insert is cloned unidirectionally. The PCR product to be cloned is amplified by PCR, using primers containing the specific nucleotide sequence corresponding to the complementary sequence of the vector overhangs. In the pDirect vector, no thymidine residues are included in the single-stranded region and dTTP must be included in the T4 DNA polymerase reaction to block exonuclease activity at the first thymidine in the insert. The pDirect vector has the T3 and T7 phage promoter region flanking the cloning region, allowing priming sites for sequencing or amplification of the insert. The cloning site in the vector is located in the middle of the plasmid's lacZα gene, allowing a blue/white screening for recombinant plasmids. Until 1996, this vector was supplied as a linear, T4 DNA polymerase-treated DNA molecule with the appropriate buffers and T4 DNA polymerase. After 1996, this vector was no longer commercially available.

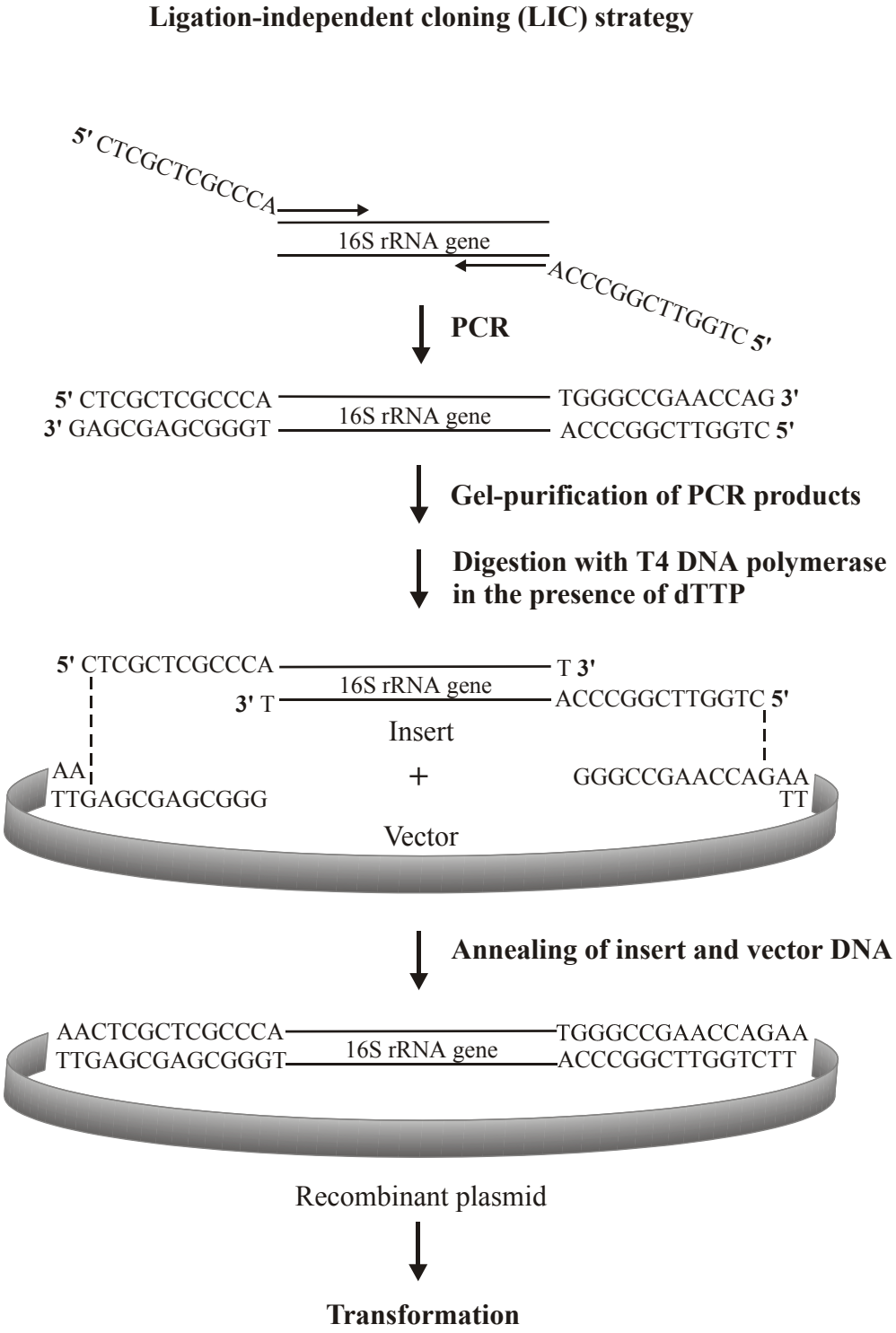

Fig. 3.4: **Schematic diagram of the PCR-Direct cloning system**. PCR primers with PCR-Direct sequences at the 5'-end were used to amplify the desired target DNA. The PCR product was gel-purified and then treated with T4 DNA polymerase in the presence of dTTP. The insert was annealed with the linear vector and the recombinant plasmid was used to transform competent cells (adapted from Clontech product protocol for PCR-Direct cloning system).

Experimental approach

The pDirect vector was amplified by PCR using a plasmid miniprep of a 16S rDNA clone as template DNA and primers complementary to regions of the multicloning site. A proofreading enzyme was used for PCR amplification of the pDirect vector, to minimise base misinsertions that might have occurred during polymerisation (Saiki *et al.*, 1988).

Choice of polymerase

In general, proof-reading enzymes possess an associated 3'→5' exonuclease activity, allowing the excision of misincorporated nucleotides and, therefore, exhibiting higher replication fidelity than non-proof-reading DNA polymerases (Kunkel, 1992). The estimated error rate (misinsertions per nucleotide per cycle) of *Taq* polymerase varies from 2×10^{-4} during PCR (Saiki *et al.*, 1988) to 2×10^{-5} for nucleotide substitution errors produced during a single round of DNA synthesis of the *lacZα* gene (Lundberg *et al*., 1991), while the error rates exhibited by proof-reading enzymes range from 10^{-6} to 10^{-7} (Kunkel, 1992). Additionally, it has been shown that, in contrast to *Taq* polymerase, proof-reading polymerases generate PCR products with blunt ends (Skerra, 1992), thus permitting their direct application in the subsequent T4 polymerase treatment without additional modification steps. During the last ten years, several thermostable proof-reading DNA polymerases have been introduced for high fidelity PCR amplification (Cline *et al*., 1996; Lundberg *et al.*, 1991). It has been reported that they exhibit a variation in fidelity, which may be attributed to differences in the rate of mispair excision, the level of discrimination between mispaired and correctly paired bases, the rate of mispair extension and/or the efficiency of shuttling the 3'-primer terminus between the polymerase and exonuclease active sites (Cline *et al.*, 1996; Flaman *et al*., 1994). The UlTma DNA polymerase (Perkin Elmer) isolated from *Thermotoga maritima*, as well as two *Pfu* DNA polymerases isolated from *Pyrococcus furiosus*, manufactured by Stratagene (Lundberg *et al.*, 1991) and Promega, respectively, were used to PCR-amplify the pDirect vector. PCR was performed in 100 µl reactions under conditions recommended by the suppliers. The amplification products were visualised by agarose (1%) gel electrophoresis (data not shown). The best results were achieved with UlTma DNA polymerase, leading to consistent amplification results with high yields and low amounts of undesirable secondary products. When the *Pfu* polymerases were used, the amount of PCR product dropped considerably, while the amount of secondary products markedly increased. Therefore, all further amplification steps were performed using the UlTma DNA polymerase (Perkin Elmer).

PCR primers

The following primers were used for amplification of vector DNA:
vector primers PDBX (5'-*GGGCCGAACCAGT*TGGATCCTCTAGAG-3') and PAEEH (5'-*GGGCGAGCGAGT*TGAATTCGATATCAAG-3'). Nucleotides belonging to the 11 to 12 base pair overhangs are depicted in italics and underlined.

Amplification of vector DNA

The PCR conditions recommended by the manufacturer were further optimised to achieve the highest product yield and specificity. Optimisation parameters included annealing temperature, extension time, and concentrations of enzyme, primers, $MgCl_2$, and dNTPs. All PCRs were performed in 100 µl reaction mixtures, using the following optimised conditions: 10 mM Tris-HCl (pH 8.8), 10 mM KCl, 0.002% Tween 20 (v/v), 1.5 mM $MgCl_2$, 40 µM of each dNTP, 0.2 µM of each primer, and 3 units of UlTma DNA polymerase. The vector was amplified using 85 ng plasmid miniprep (Qiagen) DNA of an archaeal 16S rDNA clone as template nucleic acid. Amplification was carried out with a GenAmp 9600 thermocycler (Perkin-Elmer-Cetus), using an initial denaturation step at 94°C for 60 seconds, and 30 cycles, each consisting of 94°C for 60 seconds, 55°C for 60 seconds, 72°C for 240 seconds, and including a final incubation step of 720 seconds at 72°C. Products from PCRs were analysed by agarose (1%) gel electrophoresis and ethidium bromide staining.

Purification of amplified vector DNA

Initially, ten 100 µl PCRs were pooled and nucleic acids were precipitated by adding 0.1 volumes of 3M NaAc, and 2 volumes of ethanol, cooled over night at –20°C in 1.5 ml Eppendorf tubes, followed by a 30 minute centrifugation at 15,000 x g. The DNA pellets were washed with 300 µl EtOH (70%), and microfuged another 15 minutes. Air dried pellets were resuspended in a final volume of 100 µl TE buffer (pH 8.0). The concentrated amplified vector was purified by agarose (0.8%) gel electrophoresis in TAE buffer. The vector fragment was then recovered from the agarose gel using a glass powder suspension (Geneclean, Qbiogene) as recommended by the supplier. The concentration of DNA in suspension was estimated by spectrophotometric measurement at A_{260}. Since it is known that glass milk partially interferes with spectrophotometric measurements, the DNA suspensions were microfuged briefly prior to measurements.

T4 DNA polymerase treatment

Approximately 300 ng purified vector DNA were treated with 3 units of T4 polymerase (Clontech) in a 10 µl reaction volume containing 33 mM Tris acetate (pH 8.0), 66 mM

potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol and 100 µg/ml bovine serum albumin, in the presence of 2.5 mM dATP, for the generation of the single stranded tails. After incubation for 30 minutes at 37°C, the reaction was stopped by adding 2 µl SE solution (2.5 M NaCl, 100 mM EDTA). The polymerase was heat-inactivated by incubating the reaction mixture at 75°C for 15 minutes.

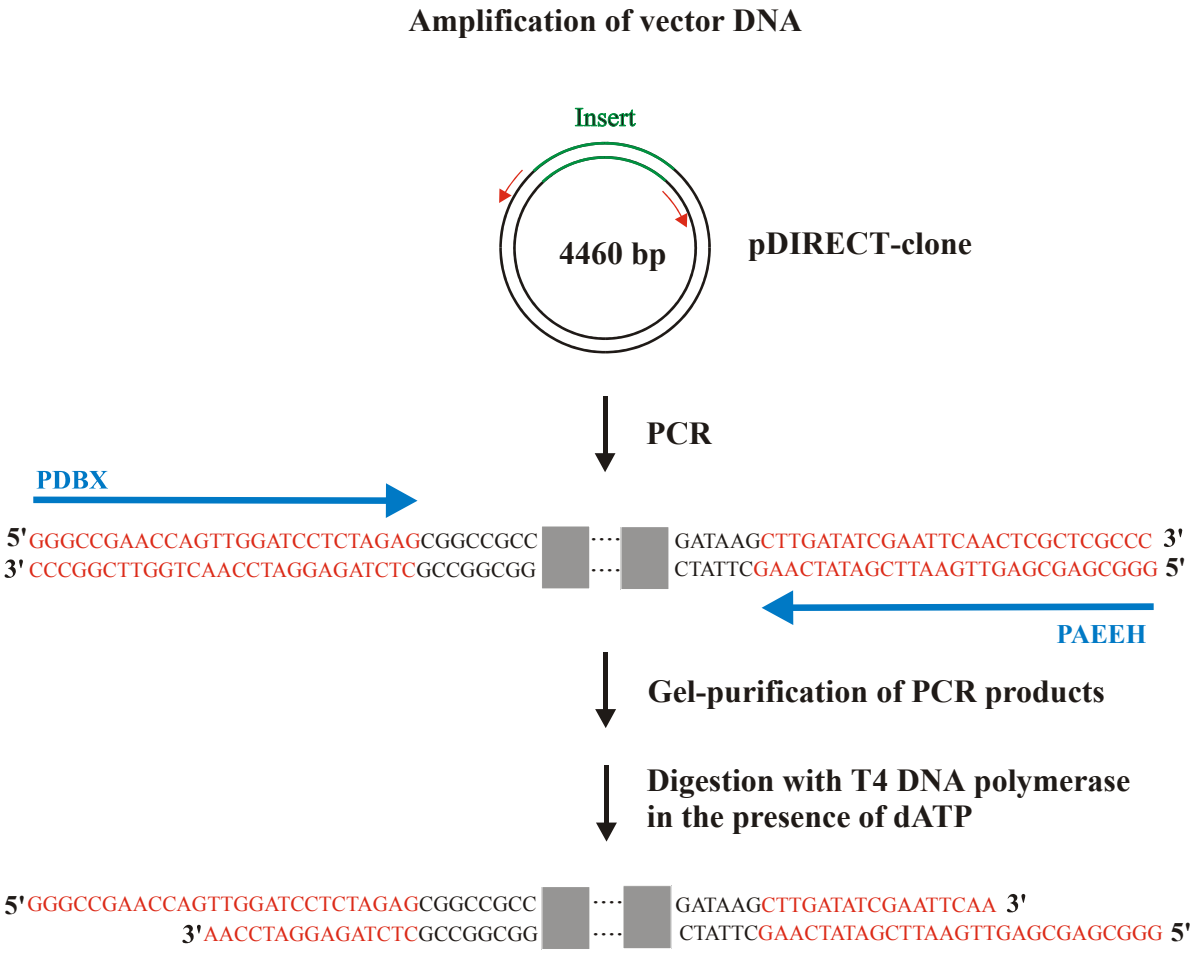

Fig. 3.5: **Regeneration of pDirect vector**. The pDirect vector was amplified by PCR using a plasmid miniprep of a 16S rDNA clone as template DNA and primers complementary to regions of the multicloning site. The PCR product was gel-purified and treated with T4 DNA polymerase in the presence of dTTP.

Cloning and transformation

A 2 µl aliquot of T4 DNA polymerase treated vector DNA (approximately 50 ng) was combined with 12 µl (approximately 100 ng) of T4 DNA polymerase treated 16S rDNA amplification product, generated from mixed community DNA of Lake Magadi sediment samples. For the generation of non-covalently linked hybrid products, the reaction

mixture was incubated at room temperature for four hours prior to transformation. Subsequently, 5 µl of the hybridisation mixture were used to transform 100 µl DH5α™ Competent Cells (GIBCO), using Clontech recommended procedures. 2 x TY medium (900 µl) was added and the bacterial cell suspension was incubated at 37°C for 1 hour. Aliquots of 50 to 200 µl of the bacterial suspension were mixed with 25 µl of 20 mg/ml IPTG and 25 µl of 24 mg/ml X-Gal, and plated on 2 x TY plates containing 100 µg/ml of ampicillin.

Screening of transformants

Transformants were checked for the presence of recombinant plasmids by PCR amplification of the insert, using a boiling preparation of template DNA from individual colonies (see chapter 2.2.2.), and T3 and T7 primers. The size of the insert DNA was checked by agarose (0.8%) gel electrophoresis and ethidium bromide staining. Furthermore, cloned PCR products were analysed by ARDRA fingerprinting using *Taq*I.

Results and interpretation

The transformation resulted in approximately 400 white clones, while no blue or faint blue clones occurred. However, the absence of any blue transformants cannot be automatically attributed to good transformation efficiency. If traces of any conformation of the circular plasmid, previously used as template DNA for the PCR, and possessing an archaeal 16S rDNA insert located in the middle of the plasmid's lacZα gene, have been co-purified during agarose gel electrophoresis, the transformation of these original plasmids would likewise lead to white colonies. Approximately 100 clones were checked by PCR and subsequent agarose gel electrophoresis. In all tested transformants, amplification of the expected 1.6 kb fragment occurred. Further characterisation of these PCR products by restriction analysis, using *Taq*I restriction enzyme, lead to many distinct band patterns, although one pattern occurred frequently (in 65 of 100 analysed clones). Partial sequence determination of three PCR products possessing the dominant *Taq*I restriction pattern revealed three identical archaeal 16S rDNA sequences, indicating that minute amounts of circular plasmid DNA survived PCR and gel purification during the generation procedure of the vector, and now cause an undesirable background. This was confirmed by comparison of *Taq*I restriction patterns from clones and from circular vector, possessing identical band patterns. Moreover, a control transformation using only T4 DNA polymerase-treated amplified vector (50 ng) and no insert, resulted in approximately 200 white clones per 100 µl competent cells. The frequent occurrence (65%) of these "background clones" could be explained by the increased transformation capability of supercoiled plasmid in comparison to the open circle conformation,

generated during the hybridisation reaction. This undesirable effect of circular vector on transformation was formerly reported by the originators of the LIC-cloning method (Aslanidis & de Jong, 1990). To eliminate the non-recombinant background, Aslanidis *et al.* suggested linearising the vector DNA prior to PCR amplification or to reduce the quantity of circular plasmid used as template DNA for the amplification. The background was eliminated by linearising residues of circular vector with the restriction endonuclease *Sma*I prior to the T4 DNA polymerase treatment. *Sma*I, a six base-specific restriction endonuclease cleaving CCC/GGG, cuts the archaeal 16S rDNA insert, but not the vector sequence itself and, thus, prevents the transformation of circular vector used for PCR amplification.

LIC-cloning versus other cloning strategies

For many analyses of microbial ecosystems, amplified 16S rRNA genes have to be separated prior to subsequent sequencing and/or hybridisation as they constitute a heterogeneous mixture of sequences (Von Wintzigerode *et al.*, 1997). Therefore, cloning into *E. coli* is still a widely used strategy to separate PCR products similar in length but different in sequence. A variety of methods have been developed to clone the DNA products of PCR amplification (Costa *et al*., 1994). However, the influence of cloning systems on the composition of 16S rRNA gene libraries generated from environmental samples is poorly investigated. Nevertheless, Rainey and colleagues (Rainey *et al.*, 1994b) reported a changing distribution of taxon-specific clones in 16S clone libraries which were derived from the same batch of DNA but generated using different cloning systems.

One common approach, the blunt-end cloning, enzymatically treats the PCR product to produce blunt ends and then ligates it into a plasmid vector linearised with a restriction endonuclease that also generates blunt ends (Liu & Schwartz, 1992). This technique is less efficient than other cloning procedures, since the ligation of blunt ends favours the direct recircularisation of the vector, and requires higher concentrations of PCR product in the ligation reaction. The blunt-end cloning approach was used for 16S rDNA sequence comparisons, e.g., in Atlantic an Pacific bacterioplankton communities (Mullis *et al*., 1995) and in Sargasso Sea bacterioplankton communities (Britschgi & Giovannoni, 1991).

Another common strategy is to incorporate restriction sites into the amplification primers (Scharf *et al*., 1986). Digestion of the DNA with the appropriate enzyme or enzymes will yield an insert with compatible ends to the desired vector. The vector and insert are then ligated and transformed into competent cells. However, restriction sites close to the ends of PCR products can be very difficult to cleave (Kaufmann & Evans,

1990), resulting in reduced cloning efficiency. Additionally, this strategy could pose a problem for unknown sequence types, since the PCR-product may contain one of the restriction sites utilised in the primers. This would lead to internal cleavage of the product, which would be eliminated by subsequent size fractionation electrophoresis techniques. Several researchers used this approach for the analysis of PCR-amplified 16S rRNA genes from complex microbial ecosystems (Barns *et al.*, 1994; Gray & Herwig, 1996; Liesack & Stackebrandt, 1992; Reysenbach *et al*., 1994).

One method for the direct cloning of PCR products utilises linearised vectors containing a single 3'-terminal thymidine (T) overhang at the insertion site and inserts with a single 3'-deoxyadenosine. PCR products can be ligated directly into the vectors without further enzymatic modification or, if necessary, the insert DNA can be gel-purified before ligation. This system is called "T/A cloning" and takes advantage of the "extendase" activity that several of the DNA polymerases have (Clark, 1988). Extendase activity is defined as the template-independent addition of a single nucleotide (generally an adenosine residue) at the 3'-ends of the amplified fragments. However, the added nucleotide differs according to the terminal nucleotide in the template-dependent product (Hu, 1993). Currently, this technique enjoys great popularity for analysis of PCR-amplified 16S rRNA genes from complex microbial ecosystems (Borneman *et al*., 1996; Buckley *et al*., 1998; Godon *et al*., 1997; Grosskopf *et al*., 1998; Henckel *et al*., 1999; Massana *et al*., 1997; Munson *et al.*, 1997; Pukall *et al*., 1999).

3.2.4. Analysis of the predominant bacterial 16S rDNA sequence types

Previous studies, based on partial sequence determinations and subsequent cluster analysis of cloned 16S rDNA of a *Bacteria* clone library generated from diluted (10^{-2}) template DNA, allowed the "identification" of those microorganisms expected to be predominant within the environmental sample (Baumgarte, 1996). Subsequently, the sequences were determined for representative cloned sequence types of the observed 16S rDNA clusters. Based upon the results of the partial and complete sequence determinations of the cloned 16S rDNA, specific oligonucleotide probes were generated, targeting sequences detected in high frequency in the clone library, as well as sequences of selected reference species. In the present study, these oligonucleotide probes were applied for the screening of 16S rDNA clone libraries, prepared from diluted, as well as from undiluted, template DNA (see chapter 3.2.3.1.), allowing the detection of cloned sequence redundancy. Cloned 16S rDNA sequences which did not hybridise with any of the applied probes were further analysed by partial and complete sequence determinations.

The analysis of the clone library generated from diluted template DNA extracted from Lake Magadi sediment samples, comprising 95 clones, revealed 16S rDNA sequence types clustering, predominantly, with the taxa of cyanobacteria (40 clones = 42%), the Gram-positive bacteria with low G+C-content (37 clones = 39%), i.e., *Bacillus* and *Clostridia*, members of the order *Halanaerobiales*, as well as alpha- (3 clones), gamma- (11 clones), and delta- (2 clones) subclasses of the *Proteobacteria* and the "*Cytophaga-Flavobacter-Bacteroides*" (CFB) phylum (1 clone). A single clone (B80) could not be assigned to any described taxon. Table 3.1 summarises the clones sequenced. Approximately 55% of the sequences found in this clone library were highly similar (greater than 95% sequence similarity) to other database entries, while 18% showed only slight relationships (90-95% sequence similarity), and approximately 27% of the sequences showed less than 90% similarity to other known sequences. All sequences were checked for possible chimera formation, using the CHECK_CHIMERA program of the RDP (Maidak *et al.*, 2001) and with consideration of the secondary structure of the 16S rDNA molecule. In all determined sequences, the beginning and the end of the cloned gene sequences demonstrated identifications which correlated with each other. Thus, chimera formation was not indicated.

Table 3.1: Nearest neighbours of SSU rDNA clones generated using diluted (10^{-2})DNA extracted from Lake Magadi sediment.

Taxonomic group	Clone	similarity (%)	Nearest neighbours	Accession no.	No. of clones
Cyanobacteria					
Chroococcales					
	B12	98.4	*Euhalothece sp.* MPI95AH10	AJ000709	40
Firmicutes (Gram-positives with low G+C content of DNA)					**37**
Bacilli					**11**
Bacillales					
	B10	94.4	*Bacillus alcalophilus* DSM 485^T	X76436	7
	B30	93.1	*Bacillus pseudofirmus* DSM 8715	X76439	1
	B55	93.5	*Bacillus smithii* DSM 4216	Z26935	1
	B75	92.6	*Bacillus agaradhaerens* DSM 8721^T	X76445	1
	B23	98.4	*Bacillus silvestris*	AJ006086	1
Clostridia					**26**
Clostridiales					**14**
	B72	90.0	*Clostridium felsineum*	Y77851	1
	B45	90.0	*Tindallia magadiensis*	Y15626	1
	B3	86.6	*Thermobrachium celer* DSM 8682^T	X99238	11
	B11	86.2	*Lake Magadi isolate M12/2	AJ271450	
		85.9	*Thermobrachium celer* DSM 8682^T	X99238	
	B66	88.3	*Lake Magadi isolate M14/4	AJ271451	1
		85.3	*Desulfotomaculum geothermicum* DSM 3669	Y11567	
Thermoanaerobacteriales					**1**
	B46	84.6	*Thermoanaerobacter ethanolicus* ATCC 33223	L09164	1
Halanaerobiales					**11**
	B1	87.3	*Halothermothrix orenii* DSM 9562^T	L22016	6
	B83	87.8	*Halocella cellulosilytica* DSM 7362^T	X89072	3
	B69	96.9	*Natroniella acetigena* DSM 5156^T	X95817	2
Proteobacteria					**16**
Alpha-Proteobacteria					**3**
Rhizobiales					**2**
	B35	94.9	*Chelatococcus asaccharovorans* DSM 6462	AJ294349	1
	B65	96.4	*Methylobacterium mesophilicum* JCM 2829	D32225	1
Rhodobacterales					**1**
	B135	94.5	*Rhodobacter sphaeroides* IF012203	D16425	1

Taxonomic group	Clone	similarity (%)	Nearest neighbours	Accession no.	No. of clones
Gamma-Proteobacteria					**11**
Chromatiales					
	B24	94.4	**Alkalispirillum mobilis*	AF114783	1
		94.1	*Alkalilimnicola halodurans* DSM13718^T	AJ404972	
	B44	92.9	*Alkalilimnicola halodurans* DSM13718^T	AJ404972	1
	B118	95.9	*Thioalkalivibrio nitratus* str. ALJ12^T	AF126547	1
Oceanspirillales					
	B19	97.3	*Bacterial sp. (Lake Magadi isolate 27M1)	X92137	8
		96.8	*"Halomonas salina"* ATCC 49509^T	X87217	
Delta-Proteobacteria					**2**
Desulfuromonadales					
	B20	82.3	*Desulforomusa kysingii* DSM 7344^T	X79414	2
Bacteroidetes (CFB phylum)					
Flavobacteria					
Flavobacteriales					
	B21	88.9	*Flavobacterium ferrugieum* ATCC 13524	M62798	1
Ungrouped					
	B80	85.4	*Uncultured bacterium	AF172914	1

Cloned 16S rRNA genes were sequenced (*E. coli* 16S rRNA gene sequence positions 27 to 1491). The most similar sequence from an organism whose name is validly published was determined using the FASTA search tool (Pearson, 1990) available in the EBML (Stoesser *et al.*, 2002). In some cases, indicated by "*", higher similarities were found with environmental rDNA clones, or uncharacterised strains. The total number of clones belonging to the same phylogenetic group based on 16S rDNA sequence determination and/or hybridisation probing, is indicated.

3.2.4.1. Predominance of sequence types of the *Cyanobacteria*

The 16S rDNA insert of clone B12 was sequenced completely (1,410 nucleotides), as a representative of 10 cyanobacterial sequences showing at least 98 % identity at their 3'-ends (400 nucleotides were determined). A FASTA analysis for clone B12 revealed a maximum of 98.4 % identity with sequences of the strains MPI 95AH10, MPI 95AH11, and ATCC 43922 *Aphanothece halophytica*, all belonging to a phylogenetically coherent group within the *Cyanobacteria*. Recently, 16S rDNA sequence analysis of 13 strains of unicellular cyanobacteria from hypersaline environments demonstrated that these organisms are distinct from any other known marine or freshwater unicellular cyanobacteria, forming a monophyletic cluster, for which the authors proposed the name "Halothece" (Garcia-Pichel *et al.*, 1998). The degree of 16S rDNA sequence diversity observed within this cluster supported the existence of two subclusters that may be

divergent at the generic level, one encompassing 12 strains (spanning less than 5% 16S rRNA gene sequence divergence and named "Euhalothece" subcluster), and a single, distinct, deep-branching isolate. The 16S rDNA sequences belonging to this group were observed to be 84.6-91.0% similar to sequences from other cyanobacteria. Furthermore, it was shown that the "Halothece" cluster could be defined simply, and independently from molecular analyses, on the basis of morphology and physiology and encompassing the unicellular, extremely halotolerant cyanobacteria. Common features of strains comprising this cluster include growth at 45°C (moderate thermophiles), the presence of β-carotene, echinenone, and L-fucose-myxol, the ability to synthesise shinorine as the principal MAA, the absence of phycoerythrin and phycoerythrocyanin as light-harvesting phycobiliproteins, and formation of markedly pale cells. Other known marine and freshwater unicellular cyanobacteria, as well as described filamentous, halotolerant strains, failed to demonstrate extreme halotolerance (no growth above 13% salinity) and their 16S rRNA gene sequences did not cluster within the "Halothece". Notably, the "Halothece" cluster, as defined above, includes strains that would be classified under different generic epithets according to both, the botanical and the bacteriological system, and it contains strains that would be classified traditionally under different orders, according to *Bergey's Manual of Systematic Bacteriology* (*Pleurocapsales* and *Chroococcales*) (Garcia-Pichel *et al.*, 1998). The hypothesis that unicellular, extremely halotolerant cyanobacteria form a monophyletic group among cyanobacteria was recently supported by amplified 16S rDNA restriction analysis of 12 strains of coccoid cyanobacteria of different hypersaline origins (Margheri *et al*., 1999).

Similarity values between 16S rDNA sequences of clone B12 and members of the "Euhalothece" cluster were observed to range from 95.6 to 98.4%, while the values between clone B12 and other cyanobacteria range from 87.7 to 91.7%, indicating that the sequence of clone B12 could be assigned to the "Euhalothece" cluster. The dendrogram based on 16S rRNA gene sequence analysis shows the inferred phylogenetic position of clone B12 within the radiation of the "Halothece" cluster and other cyanobacteria. All cyanobacteria within this group were isolated from halophilic environments (see Table 3.3) and, until now, no organisms from haloalkaliphilic environments were found to cluster within this group. Therefore, the 16S rDNA sequence of clone B12 represents the first evidence for an organism from a haloalkaliphilic environment (Lake Magadi) grouping within the "Halothece" cluster.

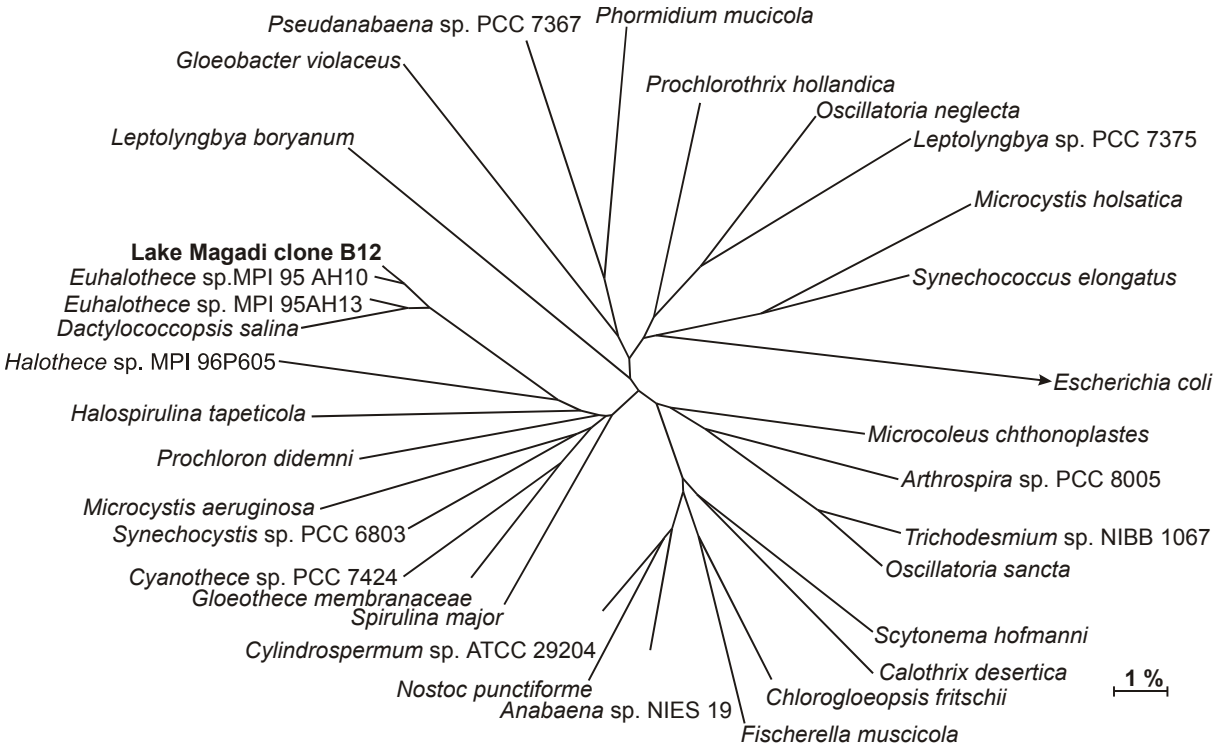

Fig. 3.6: Reconstructed phylogenetic tree showing the inferred relationships of Lake Magadi clone sequence B12 with representative species of the *Cyanobacteria*, based on their 16S rRNA gene sequences. The tree was constructed on the basis of 1323 unambiguously determined nucleotide positions, corresponding to positions 38 to 1455 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.*, 1978), that were common to all of the 33 almost complete 16S rDNA sequences used in the analysis. The 16S rRNA gene sequence of *Escherichia coli* was used as an outgroup sequence. The scale bar represents one inferred nucleotide change per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.4.

The cyanobacteria form a monophyletic group of Gram-negative, aerobic procaryotes possessing chlorophyll *a* and performing oxygenic photosynthesis (Woese, 1987). Within the oxygenic photosynthetic bacteria, two groups are distinguished, traditionally by their photosynthetic pigment composition, the cyanobacteria and the "prochlorophytes". All cyanobacteria contain chlorophyll *a* and phycobiliproteins as their primary photosynthetic pigments while the "prochlorophytes" contain chlorophylls *a* and *b* and lack phycobiliproteins. However, recent progress in the study of cyanobacterial and "prochlorian" phylogeny (Wilmotte, 1994) and the photosynthetic apparatus (Matthijs *et al*., 1994) led to the proposal that the order *Prochlorales* (Florenzano *et al*., 1986) should be rejected (Pinevich *et al*., 1997).

The cyanobacteria are morphologically (and developmentally) one of the most diverse groups of procaryotes. They are widely distributed in nature in terrestrial, freshwater, and marine habitats. In general, they are tolerant to environmental extremes

and are often the dominant or sole photosynthetic organisms in hot springs, saline lakes, and other extreme environments. Many members are found on the surfaces of rocks or soils. Traditionally, this group of phototrophic procaryotes has been classified with the algae. As is the case for other algae, the classical taxonomy of the cyanobacteria is based on morphological features and their nomenclature is ruled by the Botanical Code (Stafleu *et al*., 1972). Therefore, several names are still used for these organisms, including "cyanophyceae", "cyanophytes", and "blue-green algae". Phycologists have developed a system of classification for these organisms based on their morphological, developmental, and ecological characteristics, as determined not on pure cultures but on natural samples. The determination key published by Geitler is still the basis of numerous taxonomic works (Geitler, 1932). This system, which contains approximately 150 genera and 1,000 species, has proven to be applicable for the classification of cyanophytes in natural material but is inadequate, in many instances, for the classification of cyanobacteria maintained in axenic culture. It has become apparent that the number of species was overestimated because of the existence of ecophenes, i.e., organisms sharing the same genotype but expressing distinct morphologies under the influence of environmental factors (Wilmotte, 1994).

In the 1970s, Stanier and colleagues advocated, because cyanobacteria are procaryotic, that their taxonomy and nomenclature should be treated according to the Bacteriological Code (Stanier *et al*., 1978). Consequently, the reference for each species became a pure culture instead of a herbarium specimen and the taxonomy of cyanobacteria was revised based upon the study of chemical, genotypic and phenotypic characters determined with axenic cultures. The basis of the bacteriological taxonomy of the cyanobacteria was published by Rippka and colleagues (Rippka *et al*., 1979) and still relies largely on morphology. More recently, efforts have been made to alter the system of classification for cyanobacteria through a compromise of the bacteriological and the classical botanical system (Anagodistis & Komárel, 1985; Castenholz & Waterbury, 1989). The cyanobacteria studied in pure culture are currently placed in five orders (Rippka *et al.*, 1979) that correspond closely to the five groups (sections) used by Stanier and his collaborators (Castenholz & Waterbury, 1989). The Prochlorales, *sensu* Lewin, have been recognised as a sixth group (Castenholz & Waterbury, 1989; Lewin, 1989). However, genotypic analyses suggest that these six morphologically-based groups are not natural, phylogenetic groups (Honda *et al*., 1999; Turner *et al*., 1999; Urbach *et al*., 1998; Wilmotte, 1994).

Table 3.2: Taxonomy of cyanobacteria

I.	*Chroococcales*	unicellular cyanobacteria that reproduce by binary fission or by budding
II.	*Pleurocapsales*	unicellular cyanobacteria that reproduce by multiple fission
III.	*Oscillatoriales*	filamentous non-heterocystous cyanobacteria that divide in only one plane
IV.	*Nostocales*	filamentous heterocystous cyanobacteria that divide in only one plane
V.	*Stigonematales*	filamentous heterocystous cyanobacteria that divide in more than one plane
VI.	*Prochlorales*	unicellular and filamentous cyanobacteria that perform oxygenic photosynthesis and contain chlorophyll *a* and chlorophyll *b*

The cyanobacteria form one of the major *Bacteria* phyla, as shown by the analysis of 16S rRNA sequences (Woese, 1987), 23S rRNA and protein sequences (Schleifer & Ludwig, 1989). The first analyses of 16S rRNA sequences of cyanobacteria are attributed to Carl Woese's oligonucleotide catalogue data. A more detailed genotypic study comprising 29 partial 16S rRNA sequences, and including representatives of the five Rippka sections, was published by Giovannoni and collaborators (Giovannoni *et al*., 1988b). Their results revealed that three of the five taxonomic sections defined by Rippka *et al.* (sections II [pleurocapsalean], IV [heterocystous, filamentous, nonbranching], and V [heterocystous, filamentous, branching]) were genotypically coherent, while species comprising the other two sections (I [unicellular] and III [nonheterocystous, filamentous]) were intermixed among different genotypic clusters. Furthermore, Giovannoni and colleagues found that the rRNA sequence diversity within the cyanobacteria is considerably less than the diversity that separates other major bacterial taxa, suggesting that relatively close genotypic relationships underlie extensive morphological diversity occurring within the cyanobacteria. Additionally, many of the cyanobacterial lineages have similar branching depths indicating that the modern groups probably arose from an expansive radiation. Alternatively, SSU rRNA gene sequences may simply lack the information necessary to infer detailed relationships among deep-branching events (Turner *et al.*, 1999). Giovannoni suggested that the rise of the oxygen concentration in the precambrian atmosphere allowed the colonisation of new biotopes and probably led to the extensive divergence of the cyanobacteria. The 16S rRNA(-gene) sequence analyses have also demonstrated that the "prochlorophytes" are not genotypically close relatives but belong to two or three lineages (Urbach *et al*., 1992).

Wilmotte proposed a grouping system for cyanobacteria, comprising eight monophyletic groups, based on the reconstruction of 16S rRNA sequence trees (Wilmotte, 1994). Another proposal for the taxonomic reconstruction of cyanobacteria was made by Honda and collaborators, who proposed seven evolutionary lineages based on 16S rRNA gene sequence analysis and corresponding to phylogenetic relationships based on other genes, e.g., *psbA*, *rbcL*, *rnpB*, *rpoC*, and *tufA* (Honda *et al.*, 1999). Turner reported a detailed molecular phylogeny for 16S rRNA gene sequences of cyanobacteria. Using several analysis methods, he was able to detect 10 monophyletic groups (Turner *et al.*, 1999).

Recently, the relatively limited amount of data was extended to approximately 200 cyanobacterial 16S rRNA(-gene) sequences that are available in the database. However, more than 50% of these sequences are incomplete (partial sequences), a problem that is attributed to the general difficulties in obtaining pure cyanobacterial cultures. Moreover, the data set comprises mainly 16S rRNA sequences derived from axenic cultures and does not reflect the diversity of cyanobacteria in nature since, so far, only a small fraction of cyanobacteria occurring in nature can be maintained in culture (Castenholz, 1992). The analyses of 16S rRNA(-gene) sequences indicated that the oxygenic-phototrophic bacteria are not yet classified in harmony with their evolutionary relationships, because the evaluation of phenotypic and genotypic similarities does not yield congruent results. It is hoped that a polyphasic taxonomy integrating genotypic and phenotypic characteristics once will overcome the contradictory systematics of cyanobacteria.

Table 3.3: Origin of halotolerant, coccoid cyanobacterial strains comprising the "Halothece" cluster.

Strain	**Origin**	**Reference**
PCC 7418, *Cyanothece*	Plankton of Solar Lake, Sinai, Egypt	(Cohen *et al*., 1975)
PCC 8305, *Dactylococcopsis*	Plankton of Solar Lake, Sinai, Egypt	(Walsby *et al*., 1983)
ATCC 43922, *Aphanothece halophytica*	Solar evaporation ponds of the Leslie Salt Co., San Francisco, California, USA	(Yopp *et al*., 1978)
MPI 95AH10, MPI 95AH11, MPI 95AH13	Benthic gypsum crusts in solar evaporation ponds in Eilat, Israel	(Garcia-Pichel *et al.*, 1998)
MPI 96P402, MPI 96P605	Solar evaporation ponds of Exportadora de Sal, Guerrero Negro, Baja California Sur, Mexico	(Garcia-Pichel *et al.*, 1998)
MPI 96AL03, MPI 96AL06	Benthic gypsum crusts in the solar evaporation ponds of Salinas del Cabo de Gata, Almeria, Spain	(Garcia-Pichel *et al.*, 1998)
SYN CI P22, *Synechococcus*	Benthic microbial mats on Christmas Island	(Y. Cohen)
MPI 96N303, MPI 96N304	Hypersaline pool in an upper tidal channel, Laguna Ojo de Liebre, Guerrero Negro, Baja California Sur, Mexico	(Garcia-Pichel *et al.*, 1998)
CE 4, CE 9	Saltwork of Cervia, Emilia Romagna, Italy	(Margheri *et al.*, 1999)
TP 5, TP 8	Saltwork of Trapani, Sicily, Italy	(Margheri *et al.*, 1999)
CH 1	Saltwork, Greece	(Margheri *et al.*, 1999)
16Som2	Saltwork, Getzira, Republic of Somalia	(Margheri *et al.*, 1999)
CA3	Hypersaline pond, Sardinia, Italy	(Margheri *et al.*, 1999)
VI 13, VI 22	Tidal pool, Sardinia, Italy	(Margheri *et al.*, 1999)
PE 14	Tidal pool, Cape Malea, Greece	(Margheri *et al.*, 1999)
IR 20	Dead Sea, Israel	(Margheri *et al.*, 1999)

Table 3.4: Cyanobacterial taxa used for reconstruction of 16S rRNA tree, with classifications and SSU rRNA accession numbers.

Species	Strain	Classification			Accession no.	Reference
		A	**B**	**C**		
Anabaena cylindrica	NIES 19 (PCC 7122^T)	IV		NOST	AF091150	(Beltran & Neilan, 2000)
Arthrospira sp.	PCC 8005	III	group 2	OSC	X70769	(Nelissen *et al*., 1994)
Calothrix desertica	PCC 7102^T	IV		NOST	AF132779	(Turner *et al.*, 1999)
Chlorogloeopsis fritschii	PCC 6718	V		NOST	AF132777	(Turner *et al.*, 1999)
Cyanothece sp.	PCC 7424	I	group 5		AF132932	(Turner *et al.*, 1999)
Cylindrospermum stagnale	ATCC 29204 (PCC 7417^T)	IV		NOST	AF132789	(Turner *et al.*, 1999)
Dactylococcopsis salina	PCC 8305^T	I		S/P/M	AJ 000711	(Garcia-Pichel *et al*., 1996)
Euhalothece sp.	MPI 95AH13	I			AJ 000710	(Garcia-Pichel *et al.*, 1996)
Euhalothece sp.	MPI 95AH10	I			AJ 000709	(Garcia-Pichel *et al.*, 1996)
Fischerella muscicola	PCC 7414^T	V		NOST	AF132788	(Turner *et al.*, 1999)
Gloeobacter violaceus	PCC 7421^T	I		GBACT	AF132790	(Turner *et al.*, 1999)
Gloeothece membranaceae	PCC 6501^T	I	group 5		X78680	(Nelissen *et al*., 1995)
Halospirulina tapeticola	CCC Baja-95 Cl.2^T				Y18791	(Nübel *et al*., 2000)
Halothece sp.	MPI 96P605	I			AJ 000724	(Garcia-Pichel *et al.*, 1996)
Leptolyngbya boryanum	PCC 73110	III	group 4	LEPT	AF132785, X84810	(Nelissen *et al*., 1996; Turner *et al.*, 1999)
Leptolyngbya sp.	PCC 7375	III	group 4	PHOR	AF132786	(Turner *et al.*, 1999)
Microcoleus chthonoplastes	PCC 7420^T	III	group 2	OSC	X70770	(Nelissen *et al.*, 1995)
Microcystis aeruginosa	PCC 7941^T	I	group 5	S/P/M	U40340	(Neilan *et al*., 1997a)
Microcystis holsatica	NIES 43	I	group 6		U40336, D89036	(Neilan *et al.*, 1997a)
Nostoc punctiforme	PCC 73102^T	IV		NOST	AF027655	(Miao *et al*., 1997)
Oscillatoria neglecta	IAM M-82	III		PHOR	AB003168	(Ishida *et al*., 1997)
Oscillatoria sancta	PCC 7515^T	III		OSC	AF132933	(Turner *et al.*, 1999)
Phormidium mucicola	IAM M221	III	group 3	PSAN	AB003165	(Ishida *et al*., 1997)
Prochloron didemni		VI	group 5	S/P/M	X63141	(Urbach *et al.*, 1992)
Prochlorothrix hollandica		VI		PHOR	AF132792	(Turner*, et al.*, 1999)
Pseudanabaena sp.	PCC 7367	III		PSAN	AF091108	(Turner, 1997)
Scytonema hofmanni	PCC 7110^T	IV		NOST	AF132781	(Turner *et al.*, 1999)
Spirulina major	PCC 6313^T	III	group 5	S/P/M	X75045	(Nelissen *et al.*, 1994)
Synechococcus elongatus (*Anacystis nidulans*)	PCC 6301^T	I	group 6	SO	X03538, AF132776	(Tomioka & Sugiura, 1983; Turner *et al.*, 1999)
Synechocystis sp.	PCC 6803	I	group 5	S/P/M	D90916 AB001339	(Kaneko *et al*., 1996)
Trichodesmium sp.	NIBB 1067	III	group 2	OSC	X70767	(Wilmotte *et al*., 1994)

A) classifications according to Castenholz and Waterbury (1989).
B) SSU rRNA sequence groups, as defined by Honda (1999).
C) SSU rRNA sequence groups, as defined by Turner (1999): GBACT–*Gloeobacter* sequence group; LEPT-*Leptolyngbya* sequence group; NOST-*Nostoc* sequence group; OSC-*Oscillatoria* sequence group; PHOR-*Phormidium* sequence group; PSAN-*Pseudanabaena* sequence group; SO-*Synechococcus* sequence group; S/P/M-*Synechocystis/Pleurocapsa/Microcystis* sequence group. Type strains are denoted by "^{T}".

3.2.4.2. Sequence types related to those of the *Firmicutes*

The second most abundantly represented group of sequences (37 clones) was affiliated with the cluster of Gram-positive bacteria with low G+C content (Firmicutes), principally *Bacillus* and *Clostridium* relatives, as well as members of the order *Halanaerobiales*.

3.2.4.2.1. Sequence types related to those of species of the *Bacilli*

The Lake Magadi cloned sequence types clustering within the class of *Bacilli* were found to be affiliated with three distinct lineages within this group and showed a substantial degree of relatedness (92.6–98.4%) to reference 16S rRNA sequences of *Bacillus* in the database. Notably, most *Bacillus* clones showed the highest similarity values with sequences of obligate alkaliphilic or alkalitolerant organisms, simultaneously tolerating moderate to high NaCl concentrations (Fig. 3.7 and Table 3.6).

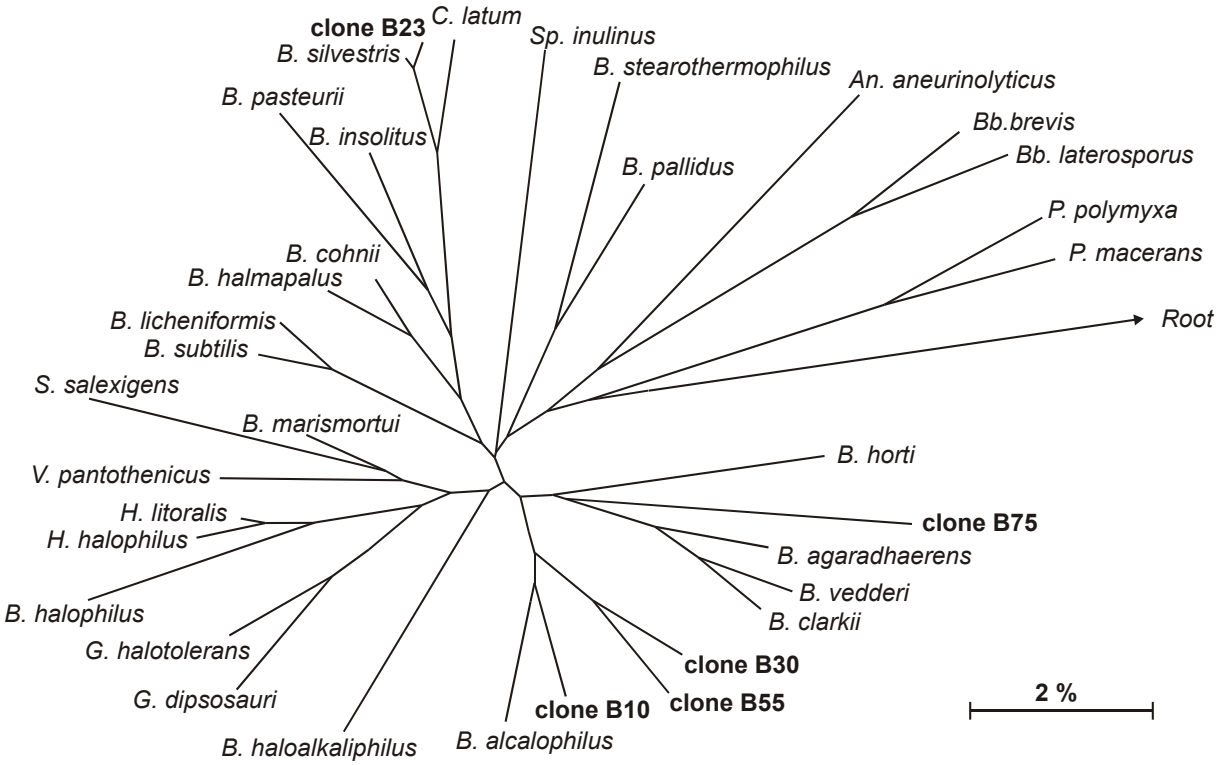

Fig. 3.7: Reconstructed phylogenetic tree showing the inferred relationships of Lake Magadi clone sequences to members of the genus *Bacillus* and related organisms based on 16S rDNA sequence data. Abbreviations: *An., Aneurinibacillus; B., Bacillus; Bb., Brevibacillus; C., Caryophanon; G., Gracilibacillus; H., Halobacillus; P., Paenibacillus; S., Salibacillus; Sp., Sporolactobacillus; V., Virgibacillus.* The tree was rooted with *Alicyclobacillus acidocaldarius*. The scale bar represents two nucleotide substitutions per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the analysis are indicated in Table 3.5.

A single sequence (Lake Magadi clone B75) was found to be most closely associated (90.9-92.3% similarity) with sequences of organisms forming a deep branching group of obligately alkaliphilic *Bacillus* strains, comprising the soil isolates, *B. agaradhaerens* and *B. clarkii* (Nielsen *et al*., 1994 and 1995), as well as *B. vedderi* (Agnew *et al*., 1995), the latter isolated from bauxite waste. Interestingly, both soil isolates show high sodium ion requirement for growth (see Table 3.6). Recently, a new alkalitolerant *Bacillus* species, *B. horti* (Yumoto *et al*., 1998) was described, being loosely associated (90.8-92.0% 16S rRNA sequence similarity) with the group consisting of *B. agaradhaeerens*, *B. clakii* and *B. vedderi*. The soil isolate *B. horti* grows at pH 7, but optimally at pH 8-10 and also tolerates high NaCl concentrations. The sequences of *B. horti* and clone B75 showed a similarity value of 90.0% to each other. The 16S rDNA sequences of clone B75, *B. agaradhaerens, B. clarkii* and *B. horti* contain a characteristic insertion between *E. coli* 16S rRNA gene sequence positions 70 and 100 (Brosius *et al.*, 1978), which was first observed by Nielsen *et al*. (1994), and is missing in the sequence of *B. vedderi*.

Three 16S rDNA sequences (Lake Magadi clones B10, B30 and B55, Fig. 3.8) were closely related to each other (93.8-96.3% similarity), forming a single cluster, associated with sequences in the rRNA group 6 of *Bacillus* (Ash *et al.*, 1991; Nielsen *et al.*, 1994).

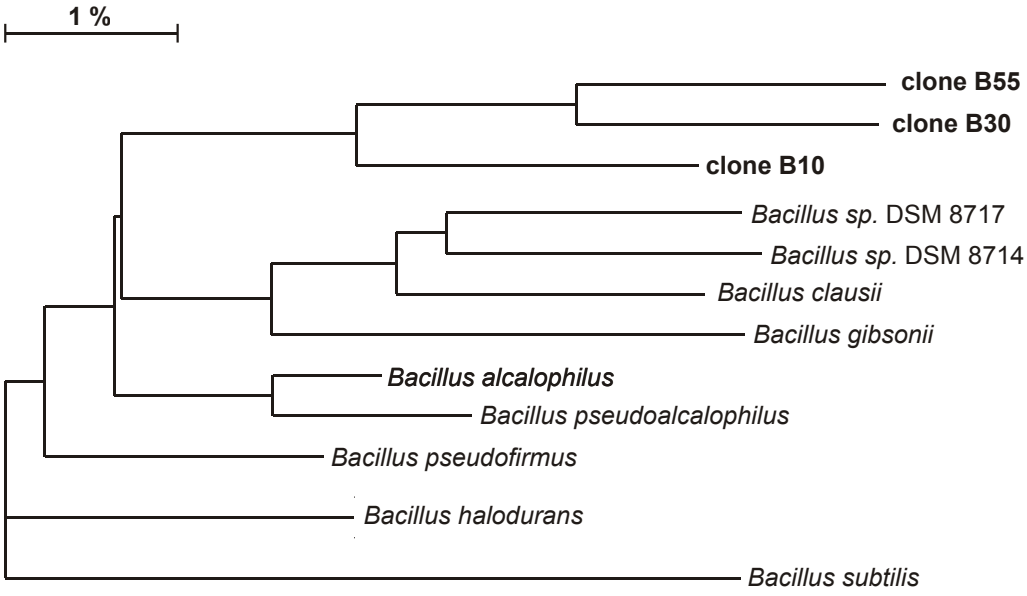

Fig. 3.8: Inferred phylogenetic positions of Lake Magadi clone sequences clustering within the rRNA group 6 of *Bacillus*. The tree was rooted with *Bacillus subtilis*. The scale bar represents one nucleotide substitution per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.5.

The Lake Magadi clone B23 (see Fig. 3.7) showed the highest simililarity value (98.4%) to the sequence of *Bacillus silvestris*, a forest soil isolate, belonging to the *Bacillus* rRNA group 2 (Ash *et al.*, 1991). The rRNA group 2 of the genus *Bacillus*, comprises round-spore-forming members of the genus *Bacillus*, possessing murein based on L-Lysine or D-Ornithine (Stackebrandt *et al*., 1987). Analyses of 16S rRNA(-genes) of Gram-positive bacteria from other genera indicated that this *Bacillus* group also contains non-*Bacillus*-type organisms, such as members of *Caryophanon* and *Planococcus* (Farrow *et al.*, 1994; Stackebrandt *et al.*, 1987), *Filibacter* (Clausen *et al*., 1985) and *Sporosarcina* (Farrow *et al.*, 1992).

The genus *Bacillus*, as defined traditionally, comprises Gram-positive, rod-shaped, endospore-forming bacteria with an aerobic or facultatively anaerobic metabolism. The taxonomy of the genus *Bacillus* has long been recognised to be unsatisfactory. Comprehensive studies of 16S rRNA(-gene) sequences have shown that the genus consists of several highly divergent phylogenetic lineages and is in need of taxonomic revision (e.g., Ash *et al*., 1991; Farrow *et al*., 1992 and 1994; Nielsen *et al*., 1994; Rainey *et al*., 1994a). These findings provided the basis for the consecutive dissection of the genus *Bacillus* and the description of several new genera, including former *Bacillus* species, such as *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Aneurinibacillus* (Shida *et al*., 1996), *Brevibacillus* (Shida *et al.*, 1996), *Gracilibacillus* (Wainø *et al*., 1999), *Halobacillus* (Spring *et al*., 1996), *Paenibacillus* (Ash *et al.*, 1993) and *Virgibacillus* (Heyndrickx *et al*., 1999).

Table 3.5: Bacterial strains, origins and accession numbers of sequences used in the 16S rRNA (-gene) analysis.

Species	**Strain**	**Accession no.**	**rRNA group***	**Reference**
Alicyclobacillus acidocaldarius	DSM 446^T	X60742		
Aneurinibacillus aneurinolyticus	DSM 5562^T	X94194		(Shida *et al*., 1994)
Bacillus agaradhaerens	DSM 8721^T	X76445	u	(Nielsen *et al.*, 1995)
Bacillus alcalophilus	DSM 485^T	X76436	6	(Vedder, 1934)
Bacillus clarkii	DSM 8720^T	X76444	u	(Nielsen *et al.*, 1995)
Bacillus clausii	DSM 8716^T	X76440	6	(Nielsen *et al.*, 1995)
Bacillus cohnii	DSM 6307^T	X76437	1	(Spanka & Fritze, 1993)
Bacillus gibsonii	DSM 8722^T	X76446	6	(Nielsen *et al.*, 1995)
Bacillus halmapalus	DSM 8723^T	X76447	1	(Nielsen *et al.*, 1995)
Bacillus haloalkaliphilus	DSM 5271^T	X72876	6	(Fritze, 1996)
Bacillus halodurans	ATCC 27557^T	AB021187	6	(Nielsen *et al.*, 1995)
Bacillus halophilus	DSM 4471^T	AB021188	u	(Ventosa *et al*., 1989a)
Bacillus horikoshii	DSM 8719^T	X76443	1	(Nielsen *et al.*, 1995)
Bacillus horti	JCM 9943^T	D87035	u	(Yumoto *et al.*, 1998)
Bacillus licheniformis	NCDO 1772	X60623	1	(Chester 1901)
Bacillus marismortui	DSM 12325^T	AJ009793	u	(Arahal *et al*., 1999)
Bacillus pallidus	DSM 3670^T	Z26930	5	(Scholz *et al*., 1988)
Bacillus pasteurii	NCIMB 8841^T	X60631	2	(Gibson, 1935)
Bacillus pseudoalcaliphilus	DSM 8725^T	X76449	6	(Nielsen *et al.*, 1995)
Bacillus pseudofirmus	DSM 8715^T	X76439	6	(Nielsen *et al.*, 1995)
Bacillus silvestris	DSM 3670^T	AJ006086	2	(Rheims *et al*., 1999)
Bacillus sp.	DSM 8714	X76438	6	(Nielsen *et al.*, 1995)
Bacillus sp.	DSM 8717	X76440	6	(Nielsen *et al.*, 1995)
Bacillus stearothermophilus	ATCC 12980^T	X57309	5	(Baker *et al*., 1960)
Bacillus subtilis	NCDO 1769^T	X60646	1	(Cohn, 1972)
Bacillus vedderi	DSM 9768^T	Z48306	u	(Agnew *et al.*, 1995)
Brevibacillus brevis	NCIMB 9372^T	X60612	4	(Migula, 1900)
Brevibacillus laterosporus	DSM 25^T	X57307	4	(Laubach *et al*., 1916)
Caryophanon latum	NCIMB 9533^T	X70314		(Peshkoff, 1939)
Gracilibacillus dipsosauri	DSM 11125^T	X82436		(Lawson *et al*., 1996; Wainø *et al*., 1999)
Gracilibacillus halotolerans	DSM 11805^T	AF036922		(Wainø *et al.*, 1999)
Halobacillus halophilus	NCIMB 925^T	X62174		(Claus *et al*., 1983)
Halobacillus litoralis	DSM 10405^T	X94558		(Spring *et al.*, 1996)
Paenibacillus macerans	ATCC 8244^T	X57306	3	
Paenibacillus polymyxa	IAM 13419^T	D16276	3	
Salibacillus salexigens	DSM 11483^T	Y11603		(Garabito *et al*., 1997)
Sporolactobacillus inulinus	JCM 6014^T	D16283		(Kitahara & Suzuki, 1963)
Virgibacillus pantothenicus	IAM 11061^T	D16275		(Heyndrickx *et al*., 1999; Proom & Knight, 1950; Suzuki & Yamasato, 1994)

*rRNA groups of the genus *Bacillus* as defined by Ash *et al.* (1991) and Nielsen *et al.* (1994). "u": ungrouped. Type strains are denoted by "^{T}".

Table 3.6: Tolerance for pH, temperature and salts of selected strains of the genus *Bacillus* and related taxa.

Species	**Gr***	**Source**	**pH Tolerance**	**T [°C]**	**NaCl**
Bacillus cohnii	1	horse meadow soil	**obligate alkaliphilic**	10-47	5%
Bacillus halmapalus	1	soil	pH_{opt} 8.0,pH 7	10-40	< 5%
Bacillus horikoshii	1	soil	**obligate alkaliphilic**	10-45	17%
Bacillus marismortui	u	Dead Sea water	6.0-9.0, pH_{opt} 7.5	15-50	5-25%
Salibacillus salexigens		solar salterns and saline soils	6.0-11.0, pH_{opt} 7.5	15-45	20%
Virgibacillus pantothenicus		soil	n.d.	15-50	10%
Bacillus silvestris	2	forest soil	n.d.	10-40	5%
Bacillus pallidus	5	waste water	8.0-8.5	30-70	n.d.
Bacillus agaradhaerens	u	soil	**obligate alkaliphilic**	10-45	16%
Bacillus clarkii	u	soil	**obligate alkaliphilic**	15-45	16%
Bacillus horti	u	soil, Japan	alkalitolerant	15-40	10%
Bacillus vedderi	u	bauxite waste	**obligate alkaliphilic**	45-50	7.5%
Bacillus alcalophilus	6	soil and faeces	**obligate alkaliphilic**	10-40	8%
Bacillus clausii	6	garden soil	alkalitolerant	15-50	10%
Bacillus gibsonii	6	soil	pH 7, pH_{opt} 8.0	10-37	12%
Bacillus haloalkaliphilus	6	brine/mud, Wadi Natrun	**obligate alkaliphilic**	15-40	25%
Bacillus halodurans	6	soil	**obligate alkaliphilic**	15-55	12%
Bacillus halophilus	u	rotting wood, Japan	pH 6-8, pH_{opt} 7.0	n.d.	30%
Bacillus pseudoalcaliphilus	6	soil	**obligate alkaliphilic**	10-40	10%
Bacillus pseudofirmus	6	lake bank soil	**obligate alkaliphilic**	10-45	17%
Bacillus sp. DSM 8714	6	river bank soil	alkalitolerant	n.d.	10%
Bacillus sp. DSM 8717	6	horse and elephant manure	alkalitolerant	42	10%
Gracilibacillus dipsosauri		desert iguana	pH_{opt} 7.5	28-50	15%
Gracilibacillus halotolerans		Great Salt Lake, Utah	5-10, pH_{opt} 7.5	6-50	20%
Halobacillus halophilus		salt marsh soil	7.0-9.0	15-37	15%
Halobacillus litoralis		sediment, Great Salt Lake, Utah	6.0-9.5, pH_{opt} 7.5	10-43	25%

*rRNA groups of the genus *Bacillus* as defined by Ash *et al*. (1991) and Nielsen *et al.* (1994); u: ungrouped; n.d.: no data available.

3.2.4.2.2. Sequence types related to those of species of *Clostridia*

Peculiarly, 16S rDNA sequence similarities of Lake Magadi clones clustering within the radiation of the Clostridia were relatively low, ranging from 84.6 to 90.0% for members of the *Clostridiales* and *Thermoanaerobacteriales*, and from 87.3 to 96.9% for the *Halanaerobiales*.

Clostridiales* and *Thermoanaerobacteriales

The 16S rDNA sequence analysis of Lake Magadi clone sequences B45 and B72 showed that these two sequences were closely related to each other (95.1% similarity), forming a single cluster near the taxonomically heterogeneous cluster XI of the Gram-positive bacteria with low G+C content, as defined by Collins *et al.* (1994). The 16S rDNA similarity values between cloned sequences B45 and B72 and members of cluster XI were in the range of 83.7 to 90.0%.

Lake Magadi clone sequence B72 showed the highest similarity values for the sequences of the clostridial species *Clostridium felsineum* (90.0%) and *C. formicoaceticum* (88.8%). These two species are closely related to each other (level of 16S similarity, approximately 97%), forming a distinct subgroup within cluster XI, presumably at the genus level. Lake Magadi clone sequence B45 was loosely affiliated (90.0% sequence similarity) with a recently described alkaliphilic species, *Tindallia magadiensis* Z-7934^{T}, isolated from soda deposits of Lake Magadi, Kenya (Kevbrin *et al.*, 1998). *Tindallia magadiensis* also groups within cluster XI of Clostridia with similarity values in the range of 86.8 to 89.2% when compared with its closest relatives including *Clostridium felsineum*, *C. formicoaceticum*, and *C. halophilum*. Moreover, Lake Magadi clone sequences B45 and B72 showed some relationship (88.5 to 89.9%, and 87.4 to 89.5%, respectively) with 16S rDNA sequences of anaerobic, halotolerant strains, isolated from Kenyan soda lakes Elmenteita and Bogoria, clustering within group XI of the Clostridia (Jones *et al.*, 1998). These strains have not been further characterised.

Other frequently observed Lake Magadi cloned sequence types (e.g., B3 and B11) were loosely affiliated with reference 16S rDNA sequences of the genera *Caloramator* (Collins *et al.*, 1994) and *Thermobrachium* (Engle *et al*., 1996), both being peripherally related to members of the Clostridia cluster I as defined by Collins and colleagues. The highest levels of 16S sequence similarity for clones B3 and B11 were found to each other (92.5%), and with the thermophilic and alkalitolerant species, *Thermobrachium celere* (86.6 and 85.9%) and *Caloramator indicus* (85.7 and 85.0%), the first isolated from a hot spring environment in Ohinimutu, New Zealand, and the second species isolated from the deep-sea, non-volcanically heated waters of an Indian artesian aquifer (Engle *et al.*, 1996;

Christostomos *et al*., 1996). Based on 16S rDNA analysis *Caloramator indicus* and *Thermobrachium celere* are almost identical (99.75%). However, the genus *Caloramator* was established prior to the genus *Thermobrachium* (Collins *et al.*, 1994; Plugge *et al*., 2000). Moreover, clone sequences B3 and B11 showed a remote relationship (84.6 to 86.2 % similarity) to 16S rDNA sequences of three haloalkaliphilic isolates from Lake Magadi, designated strains M12/2, M14/4 and M16/4, and forming a separate group between clostridial clusters VIII and IX (Jones *et al.*, 1998). Similarity values within this cluster ranged from 92.3 to 97.7%.

Likewise, Lake Magadi clone sequence B66 was found to be loosely affiliated with members of the above mentioned cluster of haloalkaliphilic Lake Magadi isolates, by exhibiting the highest 16S rDNA similarity values with these species, ranging from 87.7 to 88.3%. However, sequence similarities, calculated for clone B66 with clones B3 and B11, were relatively low (84.5 and 84.4%, respectively). The highest degrees of relatedness of this clone (ranging from 83.0 to 85.3 % sequence similarity) were observed with members of the genus *Desulfotomaculum,* currently comprising 22 validly published species names of organisms, defined by sulfate reduction, Gram-positive cell walls and the presence of spores. The 16S rDNA sequences of the main *Desulfotomaculum* species cluster adjacent to members of the genera *Moorella, Thermoanaerobacterium*, and *Thermoanaerobacter* (Stackebrandt *et al*., 1997).

A single Lake Magadi clone sequence (B46) showed low similarities to any 16S rDNA sequence in the databases and a FASTA analysis revealed maximal 84.6% sequence similarity with known members of the clostridial group. The highest 16S sequence similarity values (83.0 to 84.6%) were observed with sequences of members of the genus *Thermoanaerobacter* (Lee *et al*., 1993), belonging to cluster V as defined by Collins *et al*. (1994). Moreover, similarity values calculated for B46 and other Lake Magadi clone sequences indicated a remote affiliation to cloned sequences B3 and B11 (81.1 and 87.6%, respectively).

The results of sequence analysis suggest that most of the clostridial Lake Magadi clone sequences probably represent novel groups of 16S rDNA sequences within the Clostridia, possibly at the genus level.

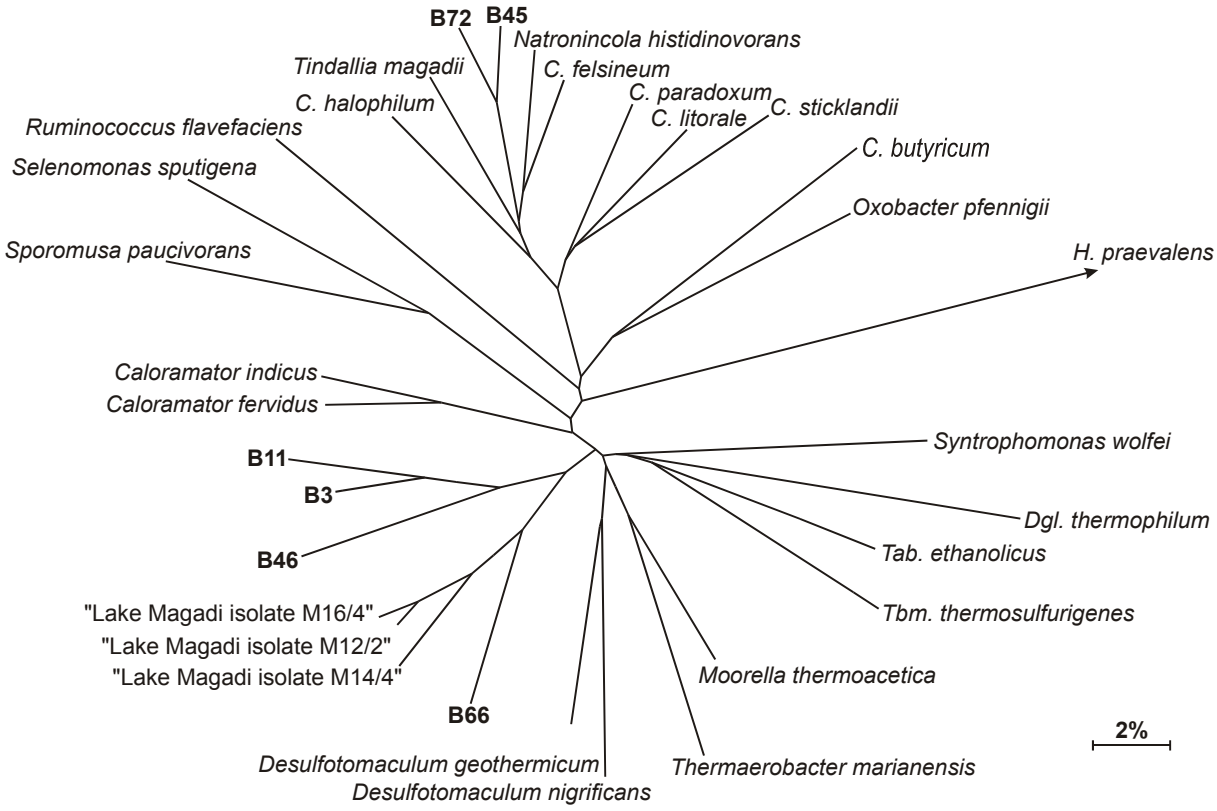

Fig. 3.9: Reconstructed tree showing the inferred phylogenetic relationships of Lake Magadi clone sequences (printed in bold) to members of the clostridia assemblage, based on their 16S rRNA gene sequences. The tree was constructed on the basis of 1214 unambiguously determined nucleotide positions, corresponding to positions 60 to 1431 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.*, 1978), that were common to all sequences used in the analysis. The 16S rRNA gene sequence of *Halanaerobium praevalens* (AB022034) was used as an outgroup sequence. The scale bar represents two inferred nucleotide changes per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.7. Abbreviations: *C.*, *Clostridium*; *Dgl.*, *Dictyoglomus*; *H.*, *Halanaerobium*; *Tab.*, *Thermoanaero-bacter*; *Tbm.*, *Thermoanaerobacterium*.

The natural interrelationships within the anaerobic genus *Clostridium* is still more fragmented than those among the aerobic bacilli. The genus *Clostridium*, currently comprising more than 146 validly published species names (Stackebrandt *et al*., 1999), is genotypically heterogeneous, with many species intermixed phylogenetically with other spore-forming and non-spore-forming genera. The major taxonomic revision of the genus *Clostridium* was proposed by Collins and collaborators on the basis of phenotypic criteria and 16S rDNA analyses on a large number of *Clostridium* species and related taxa (Collins *et al.*, 1994). As a result of this study, a hierarchical system for these organisms was proposed, taking into consideration the phenotypical heterogeneity and the estimated phylogenetic diversity, by defining 19 clusters and five new genera (i.e., *Caloramator, Filifactor, Moorella, Oxalophagus, Oxobacter)*. Since a considerable number of clostridial species, including *Clostridium butyricum*, the type species of the genus, were

observed to belong to a phylogenetically well-defined cluster (designated cluster I and equivalent to the rRNA group I of Johnson & Francis, 1975), the authors proposed that the homology group I of Johnson and Francis should form the basis of a redefined genus *Clostridium*. However, the remaining non-group I clostridia were observed to exhibit considerable degrees of phylogenetic diversity and to form numerous 16S rDNA clusters. Although several taxa have been described for former *Clostridium* species with distinct phenotypic properties, the majority of *Clostridium* species, which are not members of the core cluster I, can presently not be reclassified as long as taxon-specific, phenotypic properties, needed to circumscribe clusters defined by phylogenetic uniqueness, are not available (Stackebrandt *et al.*, 1999). The fact is, that many of the phenotypic properties and criteria used traditionally in the classification of Clostridia (such as morphology and spore formation) are not appropriate for reflecting the high degree of phylogenetic separateness.

Table 3.7: Bacterial strains, references and accession numbers of sequences used in the 16S rRNA(-gene) analysis including similarity calculations and generation of trees.

Clostridia	**Strain**	**Accession no.**	**Reference**
Caloramator fervidus	ATCC 43204	L09187	(Rainey *et al*., 1993)
Caloramator indicus	ACM 3982	X75788	(Christostomos *et al*., 1996)
Clostridium felsineum	DSM 794^T	X77851	(Collins *et al*., 1994)
Clostridium butyricum	ATCC 19398^T	M59085	Woese, unpublished
Clostridium halophilum	DSM 5387^T	X77837	(Collins*, et al.*, 1994)
Clostridium litorale	DSM 5388^T	X77845	(Collins*, et al.*, 1994)
Clostridium paradoxum	DSM 7308^T	L06838	(Li *et al*., 1993)
Clostridium sticklandii	n.i.a.	M26494	(Zhao *et al*., 1990)
Desulfotomaculum geothermicum	DSM 3669^T	Y11567	(Stackebrandt *et al*., 1997)
Desulfotomaculum nigrificans	NCIMB 8395^T	X62176	(Farrow *et al*., 1992)
Dictyoglomus thermophilum	DSM 3960^T	X69194	(Love *et al*., 1993)
Moorella thermoacetica	ATCC 39073^T	M59121	Woese, unpublished
Natronincola histidinovorans	DSM 11416^T	Y16716	(Zhilina *et al*., 1998)
Oxobacter pfennigii	DSM 3222^T	X77838	(Collins*, et al.*, 1994)
Ruminococcus flavefaciens	ATCC 129208^T	X85087	(Rainey & Janssen, 1995)
Selenomonas sputigena	ATCC 35185^T	AF373023	(Schleifer *et al*., 1990)
Sporomusa paucivorans	DSM 3637^T	M59117	Woese unpublished
Syntrophomonas wolfei	"LYB"	AF022248	(Hansen *et al*., 1999)
Thermaerobacter marianensis	DSM 12885^T	AB011495	(Takai *et al*., 1999)
Thermoanaerobacter ethanolicus	ATCC 31550^T	L09162	(Rainey*, et al.*, 1993)
Thermoanaerobacterium thermosulfurigenes	ATCC 33743^T	L09171	(Rainey*, et al.*, 1993)
Tindallia magadii	DSM 10318^T	Y15626	(Kevbrin *et al*., 1998)
"Lake Magadi isolate M12/2"		AJ271450	(Jones *et al*., 1998)
"Lake Magadi isolate M14/4"		AJ271451	(Jones*, et al.*, 1998)
"Lake Magadi isolate M16/4"		AJ271452	(Jones*, et al.*, 1998)

Environmental clones were included, indicated by quotation marks. Type strains are denoted by "^{T}". "n.i.a." means, no information available.

Halanaerobiales

The 16S rDNA sequence analysis demonstrated, that the Lake Magadi clone sequences B1 and B83 belonged to the phylogenetic group of "halanaerobes" as representatives of the family *Halanaerobiaceae*, by exhibiting similarity values with representatives of this family ranging from 82.8 to 87.3% (with *Halothermothrix orenii* as closest relative) for clone sequence B1, and from 85.5 to 87.8% (with *Halocella cellulosilytica* as closest relative) for sequence B83. By comparison, the similarity values of clone sequences B1 and B83 with representatives of the family *Halobacteroidaceae* ranged from 76.9 to 82.4%, and from 77.5 to 80.9%, respectively.

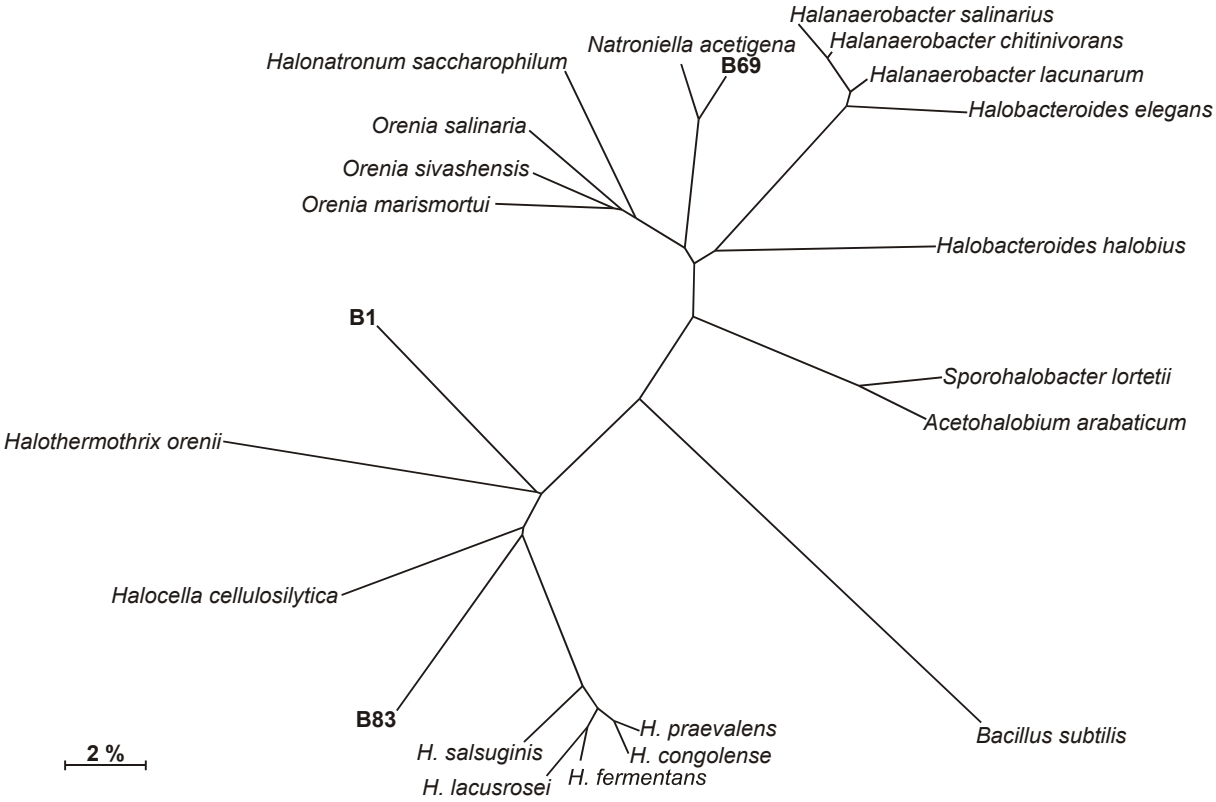

Fig. 3.10: Reconstructed tree showing the inferred phylogenetic relationships of Lake Magadi clone sequences (printed in bold) with members of the order *Halanaerobiales*, based on their 16S rRNA gene sequences. The tree was constructed on the basis of 1253 unambiguously determined nucleotide positions, corresponding to positions 45 to 1368 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.*, 1978), that were common to all 16S rDNA sequences used in the analysis. The 16S rRNA gene sequence of *Bacillus subtilis* (X60646) was used as an outgroup sequence. The scale bar represents two inferred nucleotide changes per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.8. Abbreviation: *H., Halanaerobium.*

Clone sequence B69 was most closely affiliated with 16S rDNA reference sequences of the family *Halobacteroidaceae* by exhibiting similarity values of 85.0 to 96.9% with members of this family, whereas the values calculated for members of the *Halanaerobiaceae* were in the range of 80.3 to 82.5%. The closest match (96.9% sequence similarity) was found with the 16S rDNA sequence of the Lake Magadi isolate *Natroniella acetigena* (Zhilina *et al.*, 1996a).

Peculiarities of cloned 16S rDNA sequences

All of the above analysed Lake Magadi clone sequences possessed the target region for the oligonucleotide probe (Hlan), specific for members of the *Halanaerobiales* (appendix B). A comparative analysis of the secondary structure of the cloned 16S rDNA sequences from Lake Magadi and reference sequences of the halanaerobes revealed some helical regions that differed from helical regions of 16S rDNA of the other bacterial phyla (Rainey *et al.*, 1995b; Tourova *et al.*, 1995). Furthermore, some of the Lake Magadi clone sequence types showed significant differences in their higher order structures, i.e., length heterogeneity of a stem region, which distinguished them from all other reference sequences of the halanaerobes. Lake Magadi clone sequences B1 and B83, both affiliated with 16S rDNA sequences of the family *Halanaerobiaceae*, showed a striking shortened secondary structure in the hypervariable helix of positions 73 to 97 (*E. coli* 16S rRNA gene sequence numbering), where 14 and 4 nucleotides, respectively, were missing, in comparison to reference 16S rDNA sequences of the *Halanaerobiaceae* (appendix C). The length of this region was observed to be characteristic and could distinguish the two families from one another (Rainey *et al.*, 1995b; Tourova *et al.*, 1995): while all representatives of the family *Halobacteroidaceae* possessed a short stem in this region, all representatives of the family *Halanaerobiaceae* were observed to have a long stem. The second length heterogeneity of 16S rDNA secondary structure, which was found to be useful to differentiate the two subclusters of halanaerobes from each other, occurred in the helix covering *E. coli* 16S rRNA gene sequence positions 184 to 193, where all representatives of the *Halanaerobiaceae*, including Lake Magadi clone sequences B1 and B83 possessed a long stem, comprising a constant number of nucleotides, while Lake Magadi clone sequence B69 and most of the representatives of the *Halobacteroidaceae* (with the exception of *Acetohalobium arabaticum* and *Sporohalobacter lortetii*) possessed a short stem (appendix C). Notably, all three Lake Magadi clones showed the higher order structural idiosyncrasy in the penultimate helix (*E.coli* positions 1440 to 1460), where 16 nucleotides were missing in comparison with the secondary structure of *E. coli*, e.g. (appendix C).

The group of obligately anaerobic halophilic bacteria with fermentative metabolism and Gram-negative cell wall structure is currently attributed to a single order, *Halanaerobiales*, also known as the "halanaerobes", comprising ten genera with 24 species, whose names have been validly published. Based on comprehensive taxonomic studies combining phenotypic, chemotaxonomic, and genotypic approaches, the order *Halanaerobiales* was divided into two families, *Halanaerobiaceae* and *Halobacteroidaceae* (Rainey *et al.*, 1995b; Tourova *et al.*, 1995). The main phenotypic difference between the two families is that all representatives of the family *Halanaerobiaceae* are non-spore-forming, whereas most *Halobacteroidaceae* representatives are spore-forming (Zhilina *et al*., 1997a). The specific features of the primary and secondary structures of 16S rRNA of *Halanaerobiales* suggest that these organisms belong to a distinct evolutionary line within the phylogenetic division of Gram-positive bacteria (Bhupathiraju *et al*., 1994; Cayol *et al*., 1994; Rainey *et al.*, 1995b; Tourova *et al.*, 1995; Tsai *et al*., 1995). The most distinct structural difference between 16S rRNA sequences of the halanaerobes and all other known bacteria occurs in the penultimate helix (*E. coli* 16S rRNA gene sequence positions 1440 to 1460): the obvious change in this region is the loss of the apex helical element, and in almost all members of the halanaerobes 14 to 20 nucleotides are absent in this region. The abbreviated version of the local structure seems to be highly characteristic for the halanaerobes (Cayol *et al.*, 1994; Tourova *et al.*, 1995). Additionally, a signature region of 22 nucleotides (*E. coli* 16S rRNA gene sequence positions 821 to 842) had been identified, with the sequence GGATACTAGGTGTTGGRGGTTC, which differentiates the halophilic anaerobes from all other Gram-positive bacteria (Cayol *et al.*, 1994). The intracluster sequence similarities within the two families ranges from 85.6 to 99.7% for members of the *Halanaerobiaceae*, and from 84.7 to 98.5% 16S rDNA sequence similarity for members of the *Halobacteroidaceae.* The second family is separated from the first by similarity values that are 4-5% lower than the lowest intracluster similarity values.

Table 3.8: Bacterial strains, references and accession numbers of sequences used in the 16S rRNA(-gene) analysis including similarity calculations and generation of trees.

Order *Halanaerobiales*	**Strain**	**Accession no.**	**Reference**
Family *Halanaerobiaceae*			
Halanaerobium acetoethylicum	DSM 3532^T	X89071	(Rainey *et al.*, 1995b; Rengpipat *et al*., 1988)
Halanaerobium alcaliphilum	GSLS^T, DSM 8275^T	X81850	(Tsai *et al.*, 1995)
Halanaerobium congolense	SEBR 4224^T, DSM 11287^T	U76632	(Ravot *et al*., 1997)
Halanaerobium fermentans	R-9^T, JCM 10494^T	AB023308	(Kobayashi *et al*., 2000)
Halanaerobium kushneri	VS-751^T, ATCC 700103^T	U86446	(Bhupathiraju *et al*., 1999)
Halanaerobium lacusrosei	H 200^T, DSM 10165^T	L39787	(Cayol *et al*., 1995)
Halanaerobium praevalens	DSM 2228^T	AB022034	(Kobayashi *et al.*, 2000; Zeikus *et al*., 1983)
Halanaerobium saccharolyticum subsp. *saccharolyticum*	Z-7787^T, DSM 6643^T	X89069	(Rainey *et al.*, 1995b; Zhilina *et al*., 1992)
Halanaerobium salsuginis	VS-752^T, ATCC 51327^T	L22890	(Bhupathiraju *et al*., 1994)
Halocella cellulosilytica	Z-10151^T, DSM 7362^T	X89072	(Rainey *et al.*, 1995b; Simankova *et al*., 1993)
Halothermothrix orenii	H168^T, DSM 9562^T	L22016	(Cayol *et al.*, 1994)
Family *Halobacteroidaceae*			
Acetohalobium arabaticum	Z-7288^T, DSM 5501^T	X89077	(Rainey *et al.*, 1995b; Zhilina & Zavarzin, 1990)
Halanaerobacter chitinivorans	OGC229, DSM 9569	X89076	(Liaw & Mah, 1992; Rainey *et al.*, 1995b)
Halanaerobacter lacunarum	Z-7888^T, DSM 6640^T	X89075	(Rainey *et al.*, 1995b; Zhilina *et al*., 1991)
Halanaerobacter salinarius	SG 3903^T, DSM 12146^T	Y14212	(Mouné *et al*., 1999)
Halobacteroides elegans	Z-7287^T, DSM 6639^T	AJ238119	(Zhilina *et al.*, 1997a)
Halobacteroides halobius	MD-1^T, DSM 5150^T	X89074	(Oren *et al*., 1984; Rainey*, et al.*, 1995)]
Halonatronum saccharophilum	Z-7986^T, DSM 13868^T	AY014858	(Zhilina *et al.*, 2001a)
Natroniella acetigena	Z-7937^T, DSM 9952^T	X95817	(Zhilina *et al.*, 1996a)
Orenia marismortui	DY-1^T, DSM 5156^T	X89073	(Oren *et al*., 1987; Rainey *et al.*, 1995b)]
Orenia salinaria	SG 3902^T, ATCC 700911^T	Y18485	(Mouné *et al*., 2000)
Orenia sivashensis	Z-7191^T, DSM 12596^T	AF152595	(Zhilina *et al*., 1999)
Sporohalobacter lortetii	MD-2^T, DSM 3070^T	M59122	(Oren *et al.*, 1987)

Type strains are denoted by "^{T}".

3.2.4.3. Sequence types related to those of the *Proteobacteria*

3.2.4.3.1. Sequence types related to those of species of the *Alpha-Proteobacteria*

Lake Magadi cloned sequence types clustering within the alpha subdivision of the *Proteobacteria* were observed to be affiliated with three distinct lineages within this group and showed maximal similarity values ranging from 94.5 to 96.4% to reference 16S rRNA sequences of *Alpha-Proteobacteria* in the databases.

Methylobacterium

A single Lake Magadi clone sequence B65 showed relatively high similarity values, ranging from 95.3 to 96.4%, with reference 16S rDNA sequences of members of the genus *Methylobacterium*. More detailed analysis revealed that clone B65 was most closely affiliated (at least 96.0 % similarity) with members of subcluster II, as termed by Hirashi *et al.* (1995), within this genus, including the species *Methylobacterium mesophilicum* (JCM 2829^T) and *M. radiotolerans* (JCM 2831^T), as well as several *Methylobacterium* species, isolated from different environments and chlorine-resistant strains F18, F73, F05 and F15 (Hirashi *et al.*, 1995).

The genus *Methylobacterium* is a group of strictly aerobic, facultatively methylotrophic, Gram-negative, rod-shaped bacteria that are able to grow on C_1 compounds more reduced than carbon dioxide, as the sole carbon and energy sources (Green, 1992). Some species form pink to red colonies, because of the presence of carotenoids. They also produce bacteriochlorophyll *a* under aerobic conditions and some of them have been observed to contain a photochemical reaction center similar to the reaction center of purple phototrophic bacteria (Hirashi *et al.*, 1995). The genus *Methylobacterium* now consists of eleven species with *M. organophilum* as the type species. They are phenotypically and chemotaxonomically similar and phenotypic differences among the species are found in only a limited number of properties, such as carbon source utilisation. Phylogenetic analysis based on 16S rDNA sequences have shown that the genus *Methylobacterium* represents a line of descent in the alpha-2 subclass of the proteobacteria (Hirashi *et al.*, 1995).

Chelatococcus

Lake Magadi clone sequence B35 was most closely affiliated (94.9% similarity) with the 16S rDNA sequence of *Chelatococcus asaccharovorans* (strain TE2 = DSM 6462^T), belonging to the *Bejerinckia* group of *Rhizobiaceae* within the alpha branch of *Proteobacteria* (Auling *et al*., 1993).

Rhodobacter

Lake Magadi clone B135 displayed the highest 16S rDNA sequence relatedness (approximately 93.2 to 94.5%) with species of the *Rhodobacter/Rhodovolum/Rhodobaca* subgroup of the alpha *Proteobacteria,* and was specifically associated (94.5% similarity) with 16S rDNA sequences of *Rhodobacter sphaeroides* species (accession numbers X538543-X53855, D16424-D16425) (Dryden & Kaplan, 1990; Hiraishi & Ueda, 1994).

3.2.4.3.2. Sequence types related to those of species of the *Gamma-Proteobacteria*

Chromatiales, Ectothiorhodospiraceae

A total of three Lake Magadi clone sequences were affiliated with 16S rDNA reference sequences belonging to species of the family *Ectothiorhodospiraceae* and related taxa, by exhibiting maximal similarity values ranging from 92.3 to 95.9%.

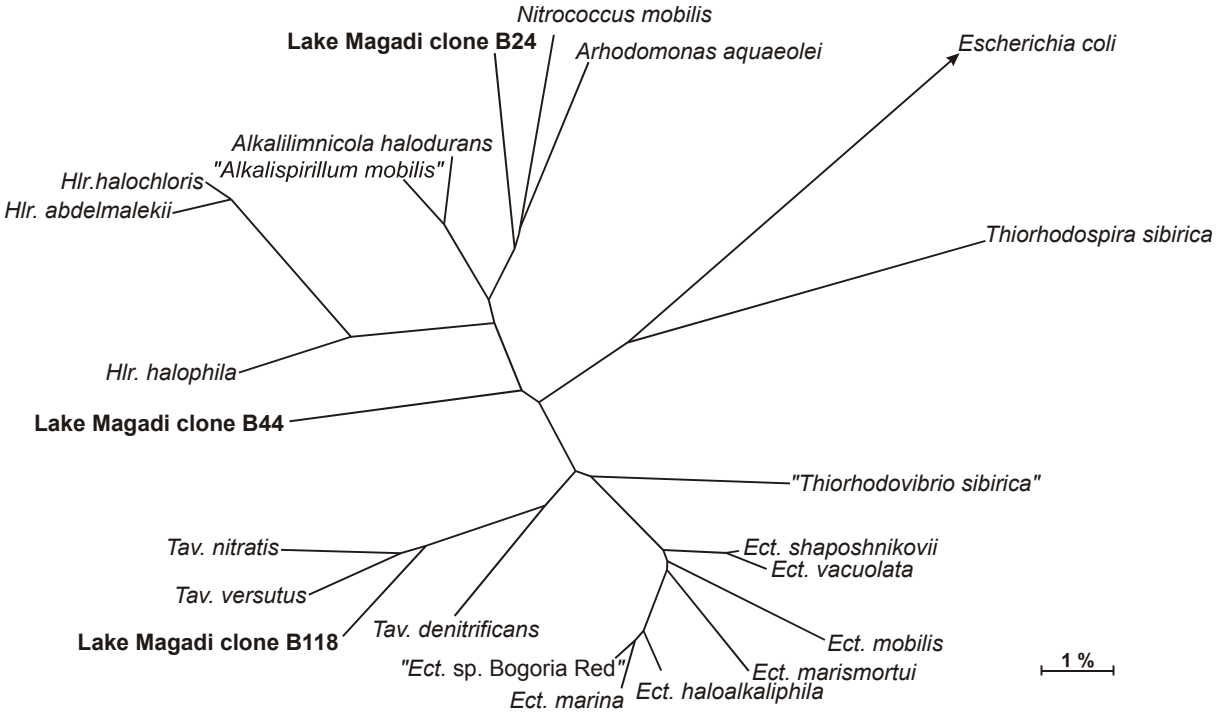

Fig. 3.11: Reconstructed phylogenetic tree showing the inferred relationships of Lake Magadi clones to members of the family *Ectothiorhodospiraceae* and relatives, based on their 16S rRNA gene sequences. The tree was constructed on the basis of 1131 unambiguously determined nucleotide positions, corresponding to positions 44 to 1387 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.*, 1978), that were common to all 16S rDNA sequences used in the analysis. The 16S rRNA gene sequence of *Escherichia coli* was used as an outgroup sequence. The scale bar represents one inferred nucleotide change per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.9. Abbreviations: *Ect.*, *Ectothiorhodospira*; *Hlr.*, *Halorhodospira*; *Tav.*, *Thioalkalivibrio*.

Clone sequence type B24 was most closely affiliated with two non-phototrophic species, including the species "*Alkalispirillum mobilis"* (94.4%) that have not been further characterised and, the alkaliphilic, halotolerant species *Alkalilimnicola halodurans*, isolated from sediments of Lake Natron, East Africa Rift Valley (Yakimov *et al.*, 2001). The Lake Magadi clone sequence B44 showed 91.6% similarity to B24 and was most closely affiliated (92.9%) with *Alkalilimnicola halodurans*. Additionally, both cloned sequences, B24 and B44, were observed to exhibit high simililarity values with *Arhodomonas aquaeolei* (94.0 and 91.2% 16S rDNA sequence similarity), a further non-phototrophic, obligately halophilic species within the family *Ectothiorhodospiraceae* (Adkins *et al.*, 1993).

Lake Magadi clone sequence B118 was most closely affiliated with 16S rDNA reference sequences of the recently published genus *Thioalkalivibrio*, including obligately alkaliphilic and obligately chemolithoautotrophic sulfur oxidising bacteria from soda lakes (Sorokin *et al.*, 2001). The highest value of sequence similarity was found with *Thioalkalivibrio nitratus* (95.9%). By comparison, similarity values calculated for clone sequence B118 and purple sulfur bacteria of the genera *Ectothiorhodospira* and *Halorhodospira* ranged from 89.9 to 92.1% and from 86.4 to 89.2%, respectively.

Within the *Gamma-Proteobacteria*, the *Ectothiorhodospiraceae* represents a group of haloalkaliphilic purple sulfur 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 & Süling, 1996; Imhoff *et al*., 1998;). In contrast to the *Chromatiaceae*, accumulating intracellular elemental sulfur, all strains of the family *Ectothiorhodospiraceae* produce external globules of elemental sulfur during photosynthetic sulfide oxidation (Imhoff, 1984). Isolates of *Ectothiorhodospiraceae* possess stacks of intracytoplasmatic membranes (Imhoff, 1989), and have been obtained from marine, hypersaline, and haloalkaline environments, and require or prefer saline and alkaline growth conditions (Imhoff & Süling, 1996). Within this family, two distinct genera, *Ectothiorhodospira* and *Halorhodospira*, have been described on the basis of phylogenetic evidence gathered from 16S rDNA sequence analysis and coherent physiological properties (Imhoff and Süling, 1996). Moderately halophilic species belong to the genus *Ectothiorhodospira*, while extreme halophilic species have been assigned to the genus *Halorhodospira*. More recently, the genus *Thiorhodospira* was described, which is currently represented by a single purple sulfur bacterium species *Thiorhodospira sibirica* (Bryantseva *et al.*, 1999b), and which clearly is affiliated on the

basis of its 16S rDNA sequence with the family *Ectothiorhodospiraceae* exhibiting sequence similarities to species of this genus in the range of 91.7 to 94.6%. Among the chemotrophic bacteria, *Arhodomonas aquaeolei* and *Nitrococcus mobilis* show a relationship on the basis of their 16S rDNAs to *Ectothiorhodospira* species (Adkins *et al.*, 1993; Teske *et al*., 1994).

Table 3.9: Bacterial strains, references and accession numbers of sequences used in the 16S rRNA(-gene) analysis including similarity calculations and generation of trees.

Species	**Strain**	**Accession no.**	**Reference**
Alkalilimnicola halodurans	DSM 13718^T	AJ404972	(Yakimov *et al.*, 2001)
"Alkalispirillum mobilis"	n.i.a.	AF114783	(Rijkenberg *et al*.,)
Arhodomonas aquaeolei	ATCC 49307^T	M26631	(Adkins *et al.*, 1993)
Ect. haloalkaliphila	ATCC 51935^T	X93479	(Imhoff & Süling, 1996)
Ect. marina	DSM 241^T	X93476	(Imhoff & Süling, 1996)
Ect. mobilis	DSM 237^T	X93481	(Imhoff & Süling, 1996)
Ect. mobilis (Basonym*: Ect. marismortui)*	DSM 4180	X93482	(Imhoff & Süling, 1996; Ventura *et al*., 2000)
Ect. shaposhnikovii	DSM 234^T	M59151	(Woese *et al*., 1985)
Ect. shaposhnikovii (Basonym*: Ect. vacuolata)*	DSM 2111	X93478	(Imhoff & Süling, 1996; Ventura *et al.*, 2000)
"Ect. sp. Bogoria Red*"*	n.i.a.	AF084511	(unpublished)
Hlr. abdelmalekii	DSM 2110^T	X93477	(Imhoff & Süling, 1996)
Hlr. halochloris	DSM 1059^T	M59152	(Woese *et al.*, 1985)
Hlr. halophila	DSM 244^T	M26630	(Woese *et al.*, 1985)
Nitrococcus mobilis	ATCC 25380^T	L35510	(Teske *et al.*, 1994)
Thioalkalivibrio denitrificans	ALJD^T	AF126545	(Sorokin *et al.*, 2001)
Thioalkalivibrio nitratis	ALJ12^T	AF126547	(Sorokin *et al.*, 2001)
Thioalkalivibrio versutus	AL2^T	AF126546	(Sorokin *et al.*, 2001)
Thioalkalivibrio sibirica	ATCC 700588^T	AJ006530	(Bryantseva *et al.*, 1999b)
"Thiorhodovibrio sibirica "	n.i.a.	AJ010297	unpublished

Species names that are not yet validly published were included, indicated by quotation marks. Type strains are denoted by "^{T}". Abbreviations: *Ect., Ectothiorhodospira*; *Hlr., Halorhodospira*. "n.i.a." means that no information was available.

Halomonadaceae

Lake Magadi clone sequence B19 was determined as a representative of eight cloned sequence types showing, at least, 96 % similarity to each other (400 nucleotides determined at the 3'-ends), and clustering within the radiation of reference 16S rDNA sequences of the family *Halomonadaceae*. The highest 16S rDNA sequence similarity (97.3%) for clone sequence B19 was found with the organism sequenced by Mellado *et al.* (1995), which bore the name *Halomonas salina*, although 16S rDNA analyses clearly indicate that it is not the authentic type strain *Halomonas salina* ATCC 49509, but a different, and probably new species within the genus *Halomonas* (Baumgarte *et al*., 2001;

Dobson & Franzmann, 1996). The next highest sequence similarity (96.3%) with an organisms whose name have been validly published, was observed with the 16S rDNA sequence from *Halomonas pacifica* (Baumann *et al*., 1983).

More detailed sequence analysis revealed that clone sequence B19 possessed the 15 signature nucleotides associated with the 16S rDNA sequences of the family *Halomonadaceae,* including a cytosine residue at position 486 (*E. coli* 16S rRNA gene sequence numbering), and, the 4 signature nucleotides that were observed to be common to members of the genus *Halomonas* (Dobson *et al*., 1993; Dobson & Franzmann, 1996). The 15 signature nucleotides common to members of the family *Halomonadaceae* are as follows (*E. coli* 16S rRNA gene sequence numbering, Brosius *et al.* 1978): a 6-bp stem at positions 76 to 93, A at position 484, C at position 486, G at position 640, A at position 660, A at position 668, A at position 669, U at position 737, U at position 738, U at position 745, U at position 776, U at position 1124, U at position 1297, C at position 1298, and A at position 1423. The four signature nucleotides common to members of the genus *Halomonas* are: C at position 1424, U at position 1439, A at position 1462 and C at position 1464.

Species belonging to the family *Halomonadaceae* (Dobson & Franzmann, 1996; Franzmann *et al*., 1988) are heterotrophic 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. Members of this family are slight or moderate halophiles or osmotolerant. They are aerobic, and some strains have the capacity for facultative anaerobic growth in the presence of nitrate. The major respiratory lipochinone is ubiquinone 9. There are also limited 16S rRNA signature characteristics available which are common to species belonging to the family *Halomonadaceae* (Dobson & Franzmann, 1996). Until recently, the family *Halomonadaceae* consisted of the four genera *Halomonas*, *Deleya*, *Halovibrio*, and *Chromohalobacter*. Lately, chemical studies and 16S rDNA sequence analysis on a wide number of isolates have shown that members of the genera *Halomonas*, *Deleya* and *Halovibrio*, as well as the species *Paracoccus halodenitrificans* and *Volcaniella eurihalina*, form a monophyletic group and share common chemotaxonomic and phenotypic characteristics (Dobson *et al.*, 1993; Franzmann & Tindall, 1990). Therefore the proposal was made to unit these genera and species in the single genus *Halomonas* (Dobson & Franzmann, 1996). Recently, it was proposed to include the genera *Alcanivorax* (Yakimov *et al*., 1998), *Carnimonas* (Garriga *et al*., 1998), *Chromohalobacter* (Ventosa *et al*., 1989b) and *Zymobacter* (Okamoto *et al*., 1993) in the family *Halomonadaceae* (Ludwig & Klenk, 2001).

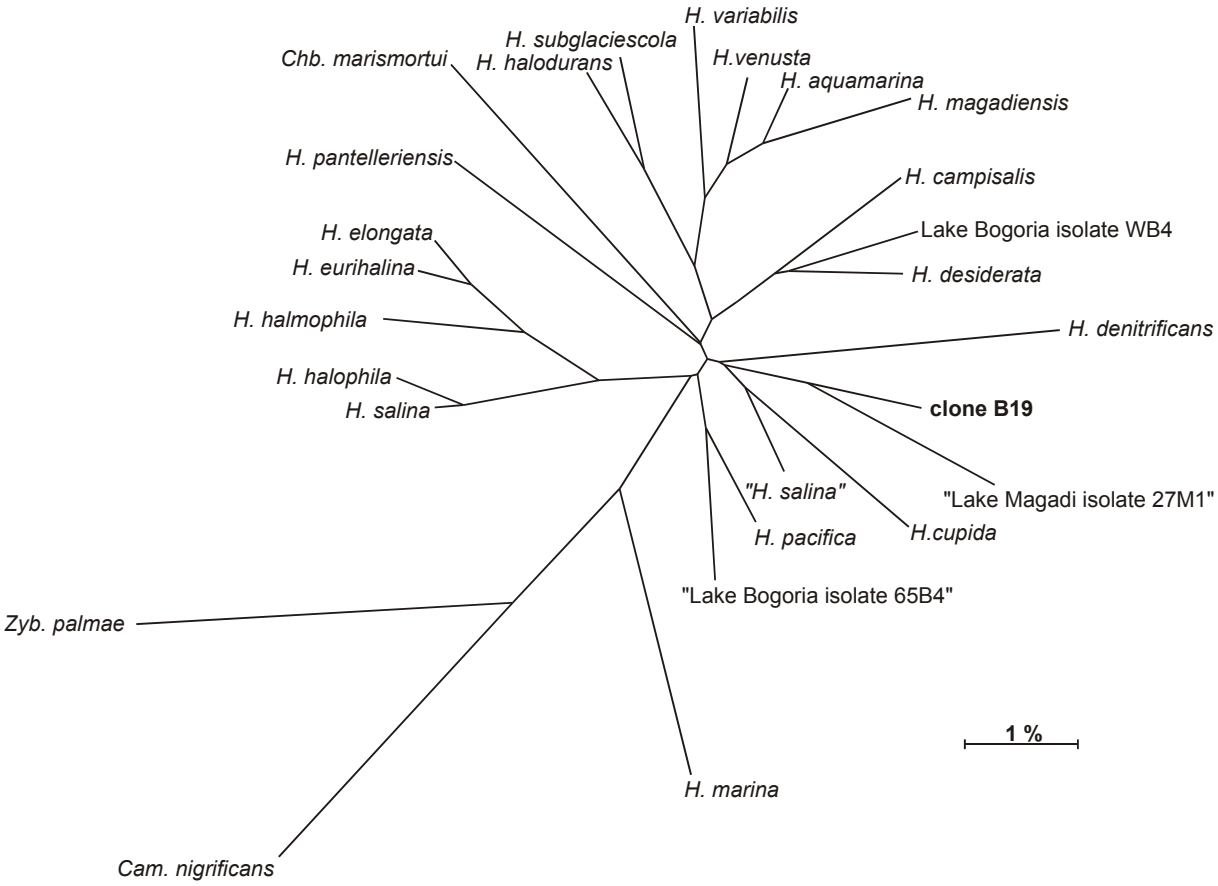

Fig. 3.12: Reconstructed unrooted tree showing the inferred phylogenetic relationship of Lake Magadi clone sequence B19 (printed in bold) to members of the family *Halomonadaceae* and relatives, based on their 16S rRNA gene sequences. The tree was constructed on the basis of 1016 unambiguously determined positions, corresponding to nucleotide positions 49 to 1387 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.* 1978), that were common to all 16S rDNA sequences used in the analysis. The scale bar represents one inferred nucleotide change per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.10. Abbreviations: *Cam.*, *Carnimonas*; *Chb.*, *Chromohalobacter*; *H.*, *Halomonas*.

Table 3.10: Bacterial strains belonging to the family *Halomonadaceae* and related taxa

Species	**Strain**	**Accession no.**	**Source**	**Reference**
Carnimonas nigrificans	CECT 4437^T	Y13299	Cured meat	(Garriga *et al.*, 1998a)
Chromohalobacter marismortui	ATCC 17056^T	X87219	Dead Sea	(Mellado *et al.*, 1995)
Halomonas aquamarina	DSM 30161^T	M93352	n.i.a.	(Dobson *et al.*, 1993)
Halomonas campisalis	ATCC 700597^T	AF054286	Sediment of Alkali Lake in Washington State, USA	(Mormile *et al.*, 1999)
Halomonas cupida	ACAM 343^T	L42615		(Dobson & Franzmann, 1996)
Halomonas desiderata	DSM 9502^T	X92417	Municipal sewage	(Berendes *et al.*, 1996)
Halomonas elongata	DSM 2581^T	M93355		(Dobson *et al.*, 1993)
Halomonas eurihalina	ATCC 49336^T	L42620	Hypersaline soil	(Dobson & Franzmann, 1996)
Halomonas halmophila	ATCC 19717^T	M59153	n.i.a.	(Woese)
Halomonas halodenitrificans	DSM 735^T	L04942	Wiltshire bacon curing brine	
Halomonas halodurans	DSM 5160^T	L42619	n.i.a.	(Dobson & Franzmann, 1996)
Halomonas halophila	DSM 4770^T	M93353	Hypersaline soil	(Dobson *et al.*, 1993)
Halomonas magadiensis	NCIMB 13595^T	X92150	Sediment, Lake Magadi	(Duckworth *et al.*, 2000)
Halomonas marina	DSM 4741^T	M93354	Seawater	(Dobson *et al.*, 1993)
Halomonas pacifica	ACAM 345^T	L42616	Seawater	(Dobson & Franzmann, 1996)
Halomonas pantelleriensis	DSM 9661^T	X93493	Sand of the Lake of Venere	(Romano *et al.*, 1996)
Halomonas salina	DSM 5928^T	AJ243448	Hypersaline soil	(Baumgarte *et al.*, 2001)
"Halomonas salina"	"false" ATCC 4950^T	X87217	unknown	(Mellado *et al.*, 1995)
Halomonas subglaciescola	ACAM 12^T	M93358	Water, organic lake	(Dobson*, et al.*, 1993)
Halomonas variabilis	DSM 3051^T	M93357	Hypersaline surface water	(Dobson*, et al.*, 1993)
Halomonas venusta	ACAM 346^T	L42618	Seawater	(Dobson & Franzmann, 1996)
"Lake Bogoria isolate WB4"	not deposited	X92145	Littoral mud/water, Lake Bogoria	(Duckworth *et al.*, 1996)
"Lake Bogoria isolate 65B4"	not deposited	X92142	Mud on shore line, Lake Bogoria	(Duckworth *et al.*, 1996)
"Lake Magadi isolate 27M1"	not deposited	X92137	Littoral mud/water, Lake Magadi	(Duckworth *et al.*, 1996)
Zymobacter palmae	IAM 14233^T	D14555	n.i.a.	(Okamoto *et al.*, 1993)

Accession numbers and references of EMBL/Genbank sequences used in the 16S rRNA(-gene) sequence analysis, including similarity calculations and generation of trees. Species names that are not yet validly published were included, indicated by quotation marks. Type strains are denoted by "^{T}". Abbreviation "n.i.a." means that no information was available.

3.2.4.3.3. Sequence types related to those of species of the *Delta-Proteobacteria*

A total of two Lake Magadi cloned sequence types, B20 and B47, were observed to cluster within the delta subgroup of the *Proteobacteria*. However, the 16S rDNA sequence of clone B20 revealed only a distant affiliation to reference sequences of the genus *Desulfuromusa* (Liesack & Finster, 1994), i.e., 82.8% sequence similarity to *Desulfuromusa kysingii* (DSM 7344-T), 82.3% to *Desulfuromusa bakii* (DSM 7345-T), and 82.4% to *Desulfuromusa succinoxidans* (DSM 8270-T). The partial 16S rDNA sequence of clone B47 was determined (480 nucleotides at the three-prime end of the gene) and was most closely affiliated with clone B20 (93.7% similarity) and *Desulfuromusa kysingii* (87.7%).

3.2.4.4. Sequence types related to those of species of the "CFB-group" (*Bacteroidetes*)

A single Lake Magadi cloned sequence type (B21) was distantly affiliated with members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum (Gherna & Woese, 1992; Paster *et al*., 1985). The highest 16S rDNA similarity values, ranging from 85.7 to 88.9%, were obtained with members of a deeply branching group within the CFB phylum, comprising *Chitinophaga pinensis* ACM 2043^T (87.2%), *Flexibacter filiformis* ATCC 29495^T (85.7%), *Flexibacter sancti* ATCC 23092^T (87.6), *Cytophaga arvensicola* IAM 12650^T (86.9%) and *Flavobacterium ferrugineum* ATCC 13524^T (88.9%) (Nakagawa & Yamasato, 1993 and 1996; Sly *et al*., 1999). Levels of intragroup sequence similarity of these species ranged from 88.5 to 95.3%.

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 & Klenk, 2001). More common labels, however, are *Cytophaga-Flavobacteria-Bacteroides* (acronym "CFB") or *Cytophaga-Flexibacter-Bacteroides* phylum, similar to the label *Cytophaga-Bacteroides* originally used by Woese (1987). As first described by Winogradsky, *Cytophaga*-like bacteria are unicellular, gliding, non-spore-forming Gram-negative rods, although shape can vary (Reichenbach, 1992). Colonies of many Cytophaga-like bacteria are pigmented due to flexirubin-type pigments found only in these bacteria and flavobacteria. *Cytophaga-Flavobacteria* are chemoorganotrophic and can degrade various biopolymers such as cellulose, chitin, and pectin (Reichenbach, 1992). *Cytophaga-Flavobacteria* are mainly aerobic, although some anaerobic strains are currently classified as *Cytophaga*

(Reichenbach, 1992). In contrast, members of the *Bacteroides* subgroup are all anaerobes and are found in the microflora of the human colon. 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).

3.2.5. Analysis of the "overall" diversity of bacterial 16S rDNA sequences

The extent of the diversity of the bacterial population of Lake Magadi sediment samples was estimated by analysis of a bacterial clone library, generated using undiluted template DNA during PCR amplification. Screening of the clone library included: (a) non-radioactive colony and dot blot hybridisations; (b) ARDRA-fingerprinting; and (c) partial or complete 16S rDNA sequence determination.

3.2.5.1. Non-radioactive colony and dot-blot hybridisations

The 16S rDNA clone library was screened initially for cyanobacterial sequence types, using a non-radioactive colony hybridisation approach to remove cloned sequence redundancy. The protocol entailed transfer of colonies of clones onto uncharged nylon-membranes, lysis of cells, followed by denaturation and immobilisation of cellular DNA, according to the protocol described above (see chapter 3.1.3.). Filters were successively hybridised with 5'-Biotin labelled oligonucleotide probes (designated CyanoK12 and Cyano-u) developed specifically for detection of cyanobacterial sequence types. Hybridisation with the general cyanobacterial probe, Cyano-u, and the more specific probe, CyanoK12, detected 34 clones, which produced positive signals in both hybridisation experiments, while a single clone (D67) hybridised exclusively with CyanoK12, but not with Cyano-u. Since partial sequence determination of five randomly chosen cyanobacterial clones revealed a close affiliation to Lake Magadi clone sequence B12 and sequences of the "Halothece" cluster (at least 98.0% similarity), only clone sequence D67 was included for subsequent sequence analysis. Finally, each filter was hybridised with the domain-specific probe EUB338 (Amann *et al*., 1990a; Daims *et al*., 1999) for estimating the amount of immobilised target DNA. The results of hybridisation experiments are summarised in Table 3.11. Examples of colony and dot blot hybridisation results are shown in Fig. 3.13.

Table 3.11: Screening of clone library D by hybridisation probing using oligonucleotide probes.

Probe	Specificity	Number of positive clones
Cyano-u	most *Cyanobacteria*	36
CyanoK12	"Halothece" cluster	37
Mor	clostridial clonal sequence types	11
Hlan	partly *Halanaerobium* group	10
Balc	partly *Bacillus* group VI	4
Total number of positive clones in hybridisation experiments		60
Total number of clones analysed		100

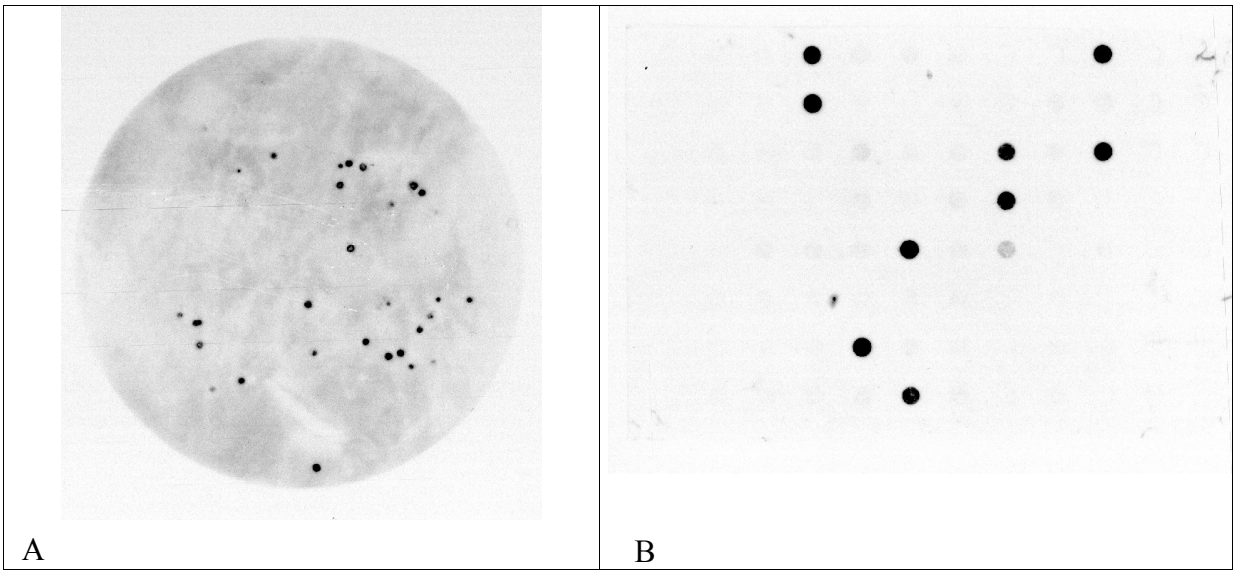

Fig. 3.13: **A)** Colony hybridisation detecting *E. coli* transformants with cyanobacterial 16S rDNA inserts. The colony blot was hybridised with a Biotin-labelled oligonucleotide probe (Cyano-u) specific for cyanobacteria and detected with chemiluminescense. **B)** Dot-blot hybridisation of plasmid DNA with the Biotin-labelled oligonucleotide probe (Hlan), specific for the *Halanaerobium* phyla.

3.2.5.2. ARDRA fingerprinting

All 63 non-cyanobacterial clonal sequence types were further screened and grouped on the basis of amplified rDNA restriction analysis (ARDRA), using *Taq*I, to obtain additional information about sequence diversity, and to systematically select clones for more detailed taxonomic studies. The 16S rDNA cloned inserts were reamplified, by PCR, using primers T3 and T7, which are complementary to sequence regions of the plasmid cloning vector. The use of these primers allowed PCR amplification with crude cell lysates, which can be performed in large numbers in a relatively short time. In the case of reamplification with 16S rDNA primers, background bands resulting from contaminating *E. coli* DNA were reported (DeLong *et al.*, 1993).

A total of 63 cloned sequences were separated into 34 different *Taq*I ARDRA patterns, which were designated alphabetically (Table 3.12). By far, the restriction pattern most frequently observed was ARDRA pattern type A, comprising twelve clones. The second most abundantly observed patterns were ARDRA types B and C, each comprising four clones. ARDRA type D was represented by three clones. Ten other ARDRA types (E - N), each represented by two clones were detected. The remaining 20 ARDRA types were represented by single cloned sequence types, cumulatively accounting for 20.0% of the SSU rDNA clone library. The phylogenetic affiliations of all detected ARDRA types are summarised in Table 3.13, together with the relative abundance of each ARDRA type.

Table 3.12: Screening of library D by ARDRA (amplified rDNA restriction analysis)

ARDRA-Type	Number of clones	Clones with identical ARDRA types (*were selected for further analysis)
A	13	**13**, **14**, 24, 35, 37, 38, 42, 55, 90, 91, *92*, 97, 104
B	4	**17**, *25*, 36, 102
C	4	*46*, 57, 64, 98
D	3	*3*, 56, 58
E	2	**9**, 63
F	2	**12**, 84
G	2	**15**, **82**
H	2	**16**, 21
I	2	23, 32
J	2	**31**, 81
K	2	**68**, 72
L	2	*70*, 89
M	2	**86**, 101
N	2	**87**, 88
ST[b]	20	**19**, 28 , 29 , **34**, *39*, *40*, *43*, *44*, **47**, **50**, **51**, **66**, **67**, **71**, *73*, *74*, *75*, *76*, 83, *100*
Σ=34[a]	Σ=63[b]	

Clones were selected for partial (printed in italics) and nearly complete (printed in bold) sequence analysis.
[a] Total number of different ARDRA types.
[b] Total number of clones analysed by ARDRA.

3.2.5.3. 16S rDNA sequence determination

The 16S rDNA sequences from representative clones of the different ARDRA groups were determined partially (at least 450 nucleotides at the five prime ends of the genes) or nearly completely (comprising approximately 1,500 nucleotides) and were compared with sequence types of clone library B and with sequences available from the DNA/RNA databases. Altogether, 20 cloned sequences of library D were determined completely and 15 partially. The results of nearly complete sequence analysis are summarised in Table 3.13.

Table 3.13: Nearest neighbours of Lake Magadi sediment SSU rDNA clones generated using undiluted DNA extract.

Taxonomic group	Clone	similarity (%)	Nearest neighbours	Accession no.	No. of clones
Cyanobacteria					
Chroococcales					34
	D5p	98.9	*Euhalothece sp.* MPI95AH10	AJ000709	
Firmicutes (Gram-positives with low G+C content of DNA)					**34**
Bacilli					
Bacillales					**5**
	D16	95.7	*Bacillus alcalophilus* DSM 485 ^{T}	X76436	2
	D87	96.0	*Bacillus alcalophilus* DSM 485 ^{T}	X76436	2
	D66	88.6	*Bacillus cohnii* DSM 6307 ^{T}	X76437	1
Clostridia					
Clostridiales					**18**
	D12p	87.3	*Thermobrachium celere* DSM 8682 ^{T}	X99238	2
	D97	87.6	isolate M12/2*	AJ271450	14
		86.5	*Thermobrachium celere* DSM 8682 ^{T}	X99238	
	D13	86.7	*Thermobrachium celere* DSM 8682 ^{T}	X99238	
	D14	85.7	*Thermobrachium celere* DSM 8682 ^{T}	X99238	
	D15	87.4	*Thermaerobacter marianensis* JCM 10246 ^{T}	AB011495	2
Thermoanaerobacteriales					**2**
	D68	90.9	isolate M12/2*	AJ271450	2
		86.4	*Thermoanaerobacter ethanolicus* ATCC 33223	L09164	
Halanaerobiales					**9**
	D46	94.2	*Natroniella acetigena* DSM 9952 ^{T}	X95817	4
	D47	85.3	*Orenia salinaria* ATCC 700911 ^{T}	Y18485	1
	D31	93.2	*Halocella cellulosilytica* DSM 7362 ^{T}	X89072	2
	D34	84.7	*Halocella cellulosilytica* DSM 7362 ^{T}	X89072	1
	D82	89.5	*Halocella cellulosilytica* DSM 7362 ^{T}	X89072	1
Proteobacteria					**7**
Alpha-Proteobacteria					
Rhodobacterales					**1**
	D50	99.0	*Rhodobaca bogoriensis* LBB1 ^{T}	AF248638	1

Taxonomic group	Clone	similarity (%)	Nearest neighbours	Accession no.	No. of clones
Gamma-Proteobacteria					
Chromatiales					**1**
	D39p	91.3	*Alkalilimnicola halodurans* DSM 13718 ^{T}	AJ404972	1
Oceanspirillales					**3**
	D3	96.1	*Halomonas desiderata* DSM 9502 ^{T}	X92417	3
Xanthomonadales					**1**
	D71	93.2	Fe(II)-oxidiser ES-1*	AF012541	1
		92.4	*Luteimonas memphitis* DSM 12574 ^{T}	AJ012228	
Delta-Proteobacteria					
Syntrophobacterales					**1**
	D73	80.0	*Desulfacinum hydrothermale* DSM 13146 ^{T}	AF170417	1
Bacteroidetes (CFB phylum)					
Sphingobacteria					
Sphingobacteriales					**1**
	D51	80.4	*Rhodothermus marinus* DSM 4252 ^{T}	X80994	1

Cloned 16S rRNA genes were sequenced completely (*E. coli* 16S rRNA gene sequence positions 27 to 1491). The closest matching sequences from environmental isolates or organisms whose names have been validly published were determined, using the FASTA analysis algorithm (Pearson, 1990) available from the EMBL (Stoesser *et al.* 2002). In some cases, indicated by "*", higher similarities were observed with environmental rDNA clones, or uncharacterised strains. Furthermore, the final number of clones is given, belonging to the same phylogenetic group based on 16S rDNA sequence determination, ARDRA or hybridisation probing.

3.2.5.3.1. Sequence types related to those of species of the *Bacilli*

The 16S rDNA cloned inserts of Lake Magadi clones D16 and D87, both giving positive signals in hybridisation experiments using probe "Balc", were sequenced completely, as representatives of two different ARDRA-types (H and N). The 16S rDNA sequences of clones D16 and D87 were closely related to each other (95.2% similarity) forming a single cluster, associated with sequences of the rRNA group 6 of *Bacillus* (Ash *et al.*, 1991; Nielsen *et al.*, 1994). The highest similarity values (95.7% for clone sequence D16, and 96.0% for D87) were observed with the sequence of *Bacillus alcalophilus* (DSM 485^T). A single sequence type (D66) showed relatively low sequence similarity values (84.4% to 88.6%) within the radiation of *Bacillus* and related taxa. The highest similarity value (88.6%) was obtained with the sequence of *Bacillus cohnii* (DSM 6307^T), a member of the *Bacillus* group 1. However, clone sequence D66 could not be assigned to any of the known *Bacillus* groups. This result suggests, that clone sequence D66, probably, represents a novel group of 16S rDNA sequence types related to *Bacillus*.

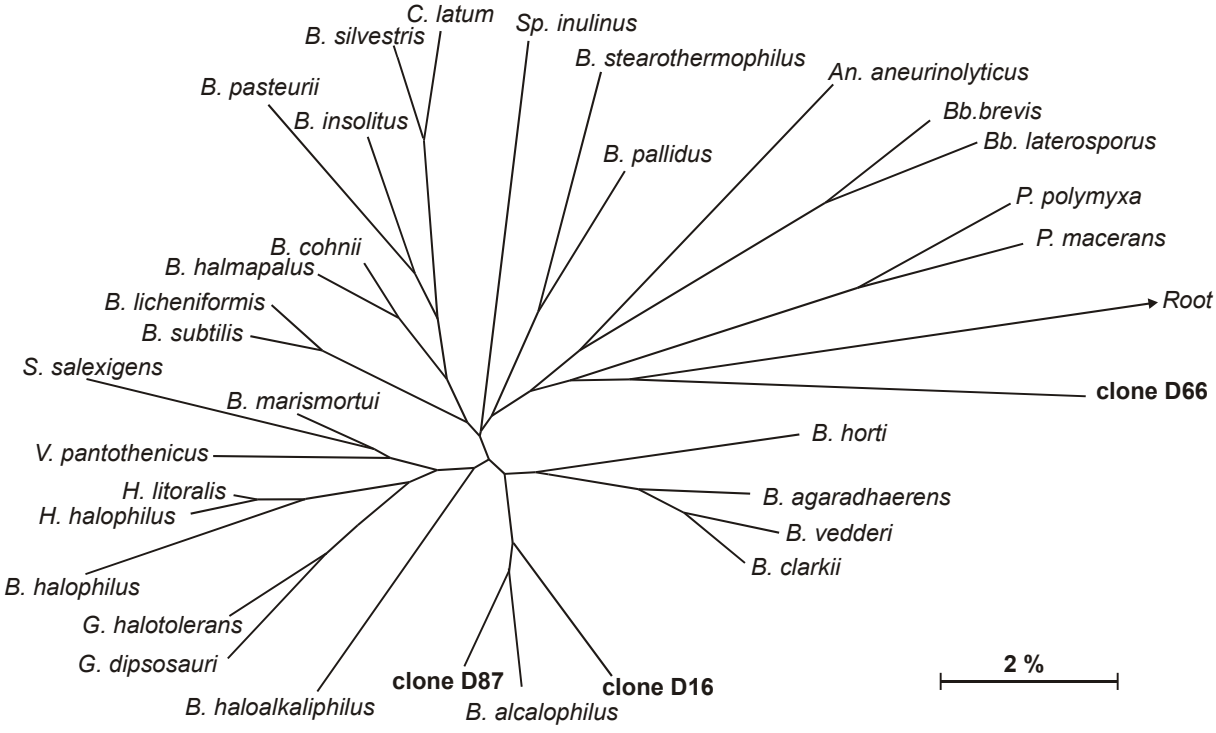

Fig. 3.14: Reconstructed tree showing the inferred phylogenetic relationships of Lake Magadi clone sequences of clone library D (generated using undiluted DNA extract for the PCR-amplification) to members of the genus *Bacillus* and some related organisms based on 16S rDNA sequence data. Abbreviations: *An., Aneurinibacillus; B., Bacillus; Bb., Brevibacillus; C., Caryophanon; G., Gracilibacillus; H., Halobacillus; P., Paenibacillus; S., Salibacillus; Sp., Sporolactobacillus; V., Virgibacillus;* The tree was rooted with *Alicyclobacillus acidocaldarius*. The scale bar represents two nucleotide substitution per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.5.

3.2.5.3.2. Sequence types related to those of species of the *Clostridia*

Clostridiales

Based upon the results of combined hybridisation probing, ARDRA-fingerprinting and 16S rDNA sequence determination, three different subgroups became apparent within the group of clostridial cloned sequence types of library D: a minute subgroup of Lake Magadi clone sequences, comprising D13, D14 and D97 and possessing a uniform ARDRA pattern (designated restriction type A), exhibited the highest similarity values to each other (91.8 to 92.4%), and to sequence type B3 (91.9 to 99.6%), whereas similarities with 16S rDNA sequences of other clostridial species were relatively low, ranging from maximal 85.7 to 86.5% for *Thermobrachium celere* DSM 8682^T and *Caloramator indicus*. The partial sequence of clone D12 (restriction type F) was determined (1035 nucleotides at the 5'-end), and showed the highest level of similarity (94.0%) with clone sequence D97. Notably, all of these clones (D12, D13, D14, and D97) did not hybridise with probe "Mor". The second, more abundantly represented subgroup, including ten cloned sequences (D24, D35, D37, D38, D42, D55, D90, D91, D92 and D104), also possessed the uniform ARDRA pattern (restriction type A), but gave positive signals in hybridisation experiments using "Mor". Lake Magadi clone sequence D92 was determined partially (almost 500 nucleotides at the 5'-end) as a representative of this group, and showed 98.6% similarity to clone sequence D14. Finally, a third ARDRA group (L) was detected, including clones D70 and D89. The cloned sequence D70 was determined partially at the 5'-end (approximately 500 nucleotides), and showed 98.9% similarity with clone B11. Both clone sequences (D70 and D89) gave positive signals with probe "Mor".

Frequently observed Lake Magadi cloned sequence types (e.g., D12, D13, D14 and D97) showed 88.6 to 94.0% similarity to each other, and were loosely affiliated (85.7 to 87.6% similarity) with reference 16S rDNA sequences of the genera *Caloramator* and *Thermobrachium,* which are peripherally affiliated to members of the Clostridia cluster I according to Collins *et al.* (1994). On the basis of ARDRA-fingerprinting, a total of 16 Lake Magadi cloned sequences (restriction type A), belonging to the "*Thermobrachium/Caloramator* group" were detected within clone library D.

Thermoanaerobacteriales

Lake Magadi clone D68 was sequenced as a representative of ARDRA type K, and showed the highest similarity values (89.7 to 90.0%) with 16S rDNA sequences of three haloalkaliphilic strains (designated strains M12/2, M14/4 and M16/4), isolated from Lake Magadi (Jones *et al.*, 1998), that have not been further characterised. The highest level of

sequence similarity (86.4%) of clone sequence D68 with an organism whose name is validly published was observed with *Thermoanaerobacter ethanolicus* (ATCC 33223), which is a member of cluster V as defined by Collins *et al.* (1994). Moreover, similarity values calculated for D68 and other clostridial Lake Magadi clone sequences indicated a remote relationship to clone sequence B66, by exhibiting 88.3% 16S rDNA sequence similarity.

A unique Lake Magadi clone sequence (D15) showed quite low similarity (78.6 to 85.0 % with other Lake Magadi clone sequences, clustering within the radiation of Clostridia, and did not hybridise with probe "Mor". The closest 16S rDNA sequences found in the databases were those of members of the genera *Thermaerobacter* (Takai *et al*., 1999), and *Moorella* (Collins *et al.*, 1994), the latter belonging to cluster VI within the *Bacillus-Clostridium* subphylum, according to Collins *et al.* (1994). The most similar rDNA sequence was that of the strictly aerobic, heterotrophic, and extremely thermophilic bacterium *Thermaerobacter marianensis* JCM 10246^T (87.4% similarity), isolated from the Mariana Trench Challenger Deep at a depth of 10897 m (Takai *et al.*, 1999). On the basis of its inferred phylogenetic placement, *Thermaerobacter marianensis* was the first described obligate aerobe among the Gram-positive, spore-forming, thermophilic, anaerobic bacteria with low G+C content, including the members of the genera *Clostridium*, *Caloramator*, *Desulfotomaculum*, *Thermoanaerobacter*, *Thermoanaero-bacterium*, *Anaerobranca*, *Thermosyntropha*, *Dictyoglomus* and *Moorella*. Lake Magadi clone sequence D15 was also remotely affiliated (86.1 to 86.9% similarity) with members of the genus *Moorella* (originally described as *Clostridium*), including the homoacetogenic, sporeforming, chemolithoautothrophic species *Moorella thermoautotrophica* JW/701/3 (Wiegel *et al*., 1981), *Moorella thermoacetica* LJD^T (Fontaine *et al*., 1942), and the glycerol utilising species *Moorella glycerini* JW/AS-Y6^T (Slobodkin *et al*., 1997).

Table 3.14: Levels of 16S rDNA sequence similarity for Lake Magadi clones, clustering within the radiation of Clostridia and relatives.

		% 16S rDNA sequence similarity											
group	clone	D12	D97	D13	D14	D68	D15	B3	B11	B66	B46	B45	B72
I	D12	100.0											
I	D97	94.0	100.0										
I	D13	90.9	91.8	100.0									
I	D14	88.6	90.0	92.4	100.0								
V	D68	83.7	85.1	83.6	83.0	100.0							
VI	D15	83.5	85.0	84.4	83.1	84.1	100.0						
I	B3	91.1	91.9	99.6	92.5	83.7	84.3	100.0					
I	B11	91.7	90.8	92.3	90.4	83.4	83.2	92.5	100.0				
VI	B66	85.0	85.1	84.5	82.7	88.3	84.2	84.5	84.4	100.0			
V	B46	87.4	88.7	87.0	85.2	84.5	83.3	87.1	87.6	83.7	100.0		
XI	B45	80.4	80.5	79.7	78.5	78.2	79.1	79.8	79.2	78.9	78.2	100.0	
XI	B72	80.4	81.2	79.7	78.6	78.6	78.6	79.2	78.6	78.5	74.4	95.1	100.0

Clones, labelled with "D", were generated using undiluted DNA-extract for PCR, while clones, named with "B", were generated using diluted DNA-extract. Roman numerals indicate closest affiliation of clones with clostridial clusters, as designated by Collins *et al*. (1994).

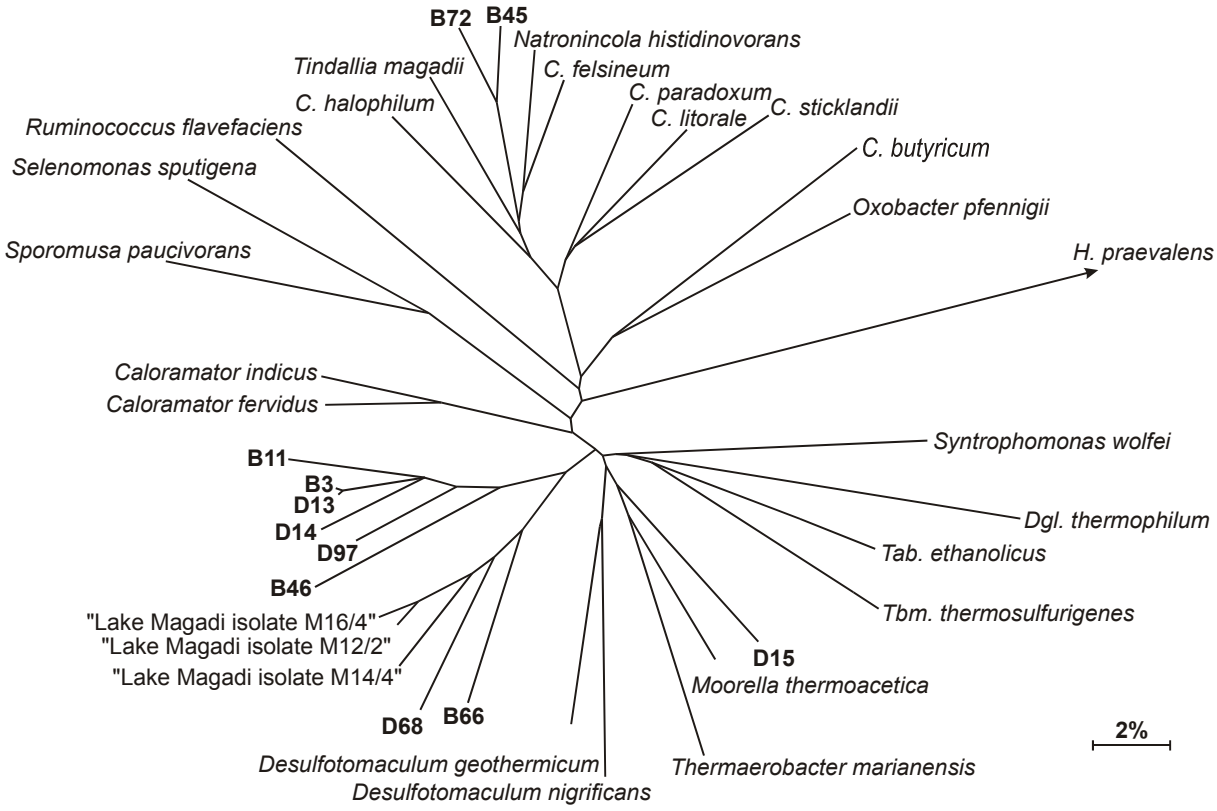

Fig. 3.15: Reconstructed tree showing the inferred phylogenetic relationships of Lake Magadi clones (printed in bold) to members of the clostridia assemblage, based on their 16S rRNA gene sequences. The tree was constructed on the basis of 1214 unambiguously determined positions, corresponding to positions 60 to 1431 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.* 1978), that were common to all 16S rDNA sequences used in the analysis. The 16S rRNA gene of *Halanaerobium praevalens* (AB022034) was used as an outgroup sequence. The scale bar represents two inferred nucleotide changes per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.7. Abbreviations: *C.*, *Clostridium*; *Dgl.*, *Dictyoglomus*; *H.*, *Halanaerobium*; *Tab.*, *Thermoanaerobacter*; *Tbm.*, *Thermoanaerobacterium*.

Halanaerobiales

The Lake Magadi clone sequences clustering within the halanaerobes were observed to be affiliated with two distinct lineages, and showed maximal similarity values ranging from 84.7 to 94.2% to reference 16S rRNA sequences of the order *Halanaerobiales* in the databases. Frequently observed Lake Magadi cloned sequence types (D9, D40, D43, D46, D47, D57, D63, D64, D74 and D98), belonging to six different ARDRA groups, were affiliated with members of the family *Halobacteroidaceae.* Lake Magadi clone sequences D46 and D47 were determined completely as representatives of this group, and exhibited 86.8% similarity to each other. All other cloned sequences of this group were observed to show, at least, 97% simililarity at their 5'-ends (approximately 500 nucleotides) to D46 or D47. Clone sequence D46 possessed a substantial degree of relatedness (94.2%) to the 16S rDNA sequence of the haloalkaliphilic, chemoorganotrophic, homoacetogenic Lake

Magadi isolate *Natroniella acetigena* (Zhilina *et al.*, 1996a). Relatively low similarity values (at most 85.3%) were observed for Lake Magadi clone D47 and the 16S rDNA sequence of the halophilic fermentative species *Orenia salinaria*, isolated from salt ponds in the salterns of Salin-de-Giraud, Camargue, France (Mouné *et al.*, 2000). The second frequently observed cloned sequences (D31, D34, D81, D82, D86 and D101), belonging to four different ARDRA-types, were affiliated with members of the family *Halanaerobiaceae*. Lake Magadi clone sequences D31, D34 and D82 were determined completely as representatives of this group, and exhibited the highest values of 16S rDNA sequence similarity (93.2, 84.7, and 89.5%, respectively) with the halophilic, cellulolytic species *Halocella cellulosilytica*, isolated from sediment of Lake Sivash, Crimea (Simankova *et al.*, 1993). Intra-group similarities of D31, D34 and D82 were in the range of 86.2 to 92.2%.

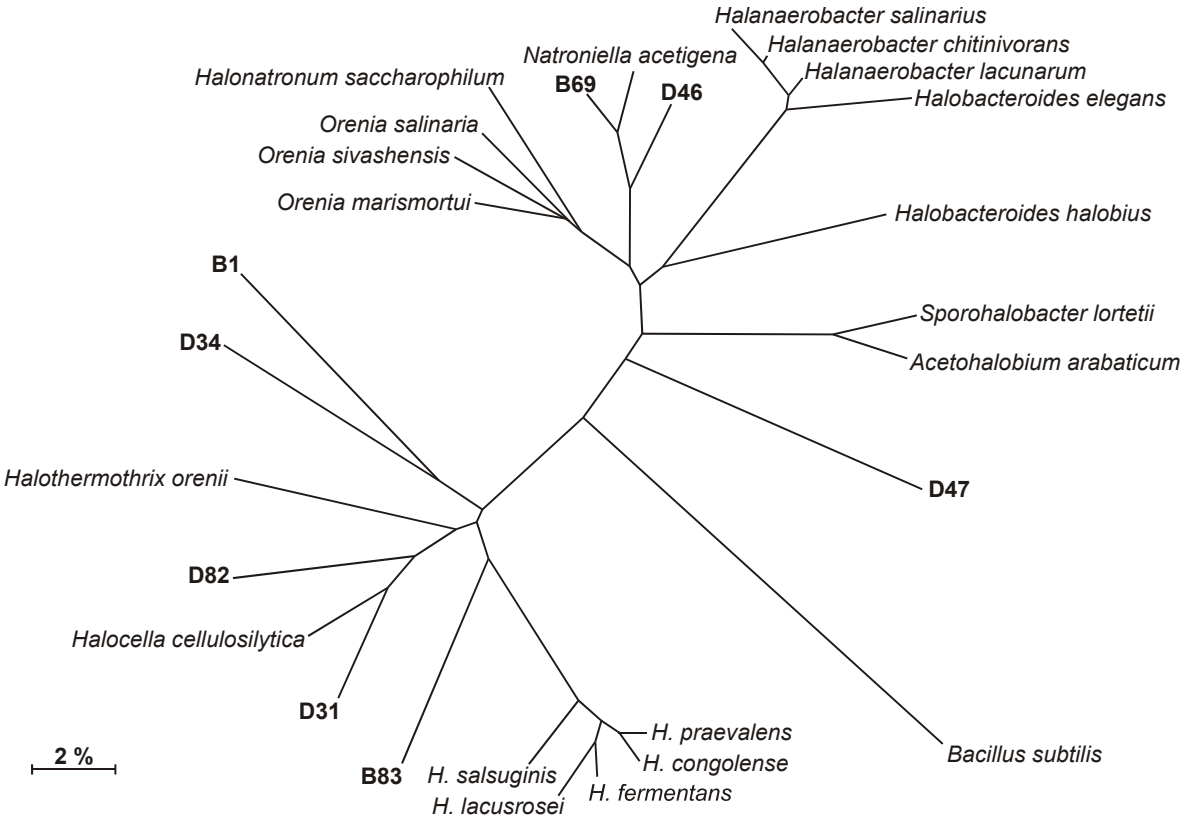

Fig. 3.16: Reconstructed tree showing the inferred phylogenetic relationships of Lake Magadi clone sequences (printed in bold) to members of the order *Halanaerobiales*, based on their 16S rRNA gene sequences. The tree was constructed on the basis of 1253 unambiguously determined positions, corresponding to positions 45 to 1368 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.* 1978), that were common to all 16S rDNA sequences used in the analysis. The 16S rRNA gene of *Bacillus subtilis* (X60646) was used as an outgroup sequence. The scale bar represents two inferred nucleotide changes per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.8. Abbreviation: *H.*, *Halanaerobium*.

Peculiarities of cloned 16S rDNA sequences

All Lake Magadi clone sequences of the "halanaerobes", with the exception of clone D34, showed positive signals in hybridisation experiments using the oligonucleotide probe Hlan2, specific for members of the *Halanaerobiales*.

Lake Magadi clone sequence D86, which was most closely related with members of the family *Halanaerobiaceae*, showed a striking shortened secondary structure (14 nucleotides missing) in the hypervariable helix of positions 73 to 97 (*E. coli*), which was also found in clone B1. All other Lake Magadi clone sequences possessed the same helix length in this region, previously observed to be characteristic to distinguish the two families *Halanaerobiaceae* (long stem) and *Halobacteroidaceae* (short stem) from each other (Rainey *et al.*, 1995b; Tourova *et al.*, 1999). A further significant secondary structure distinguishing the two families from each other was observed in the helix of positions 184 to 193 (*E. coli*) where all reference 16S rDNA sequences and Lake Magadi clone sequences belonging to the *Halanaerobiaceae* showed a long stem, identical in terms of numbers of nucleotides in each helix and in each loop, while most of the representatives of the *Halobacteroidaceae* and Lake Magadi clones (D9, D46 and D47) possessed a shortened stem of variable length. In the penultimate helix (*E. coli* positions 1440 to 1460), which is generally abbreviated in most 16S rDNA sequences of the halanaerobes, Lake Magadi clone sequence D47 (most closely related to the *Halobacteroidaceae*), and D34 and D82 (both affiliated with the *Halanaerobiaceae*) showed a distinctive long helix structure.

3.2.5.3.3. Sequence types related to those of species of the *Alpha-Proteobacteria*

A single Lake Magadi clone sequence (D50) was observed to be affiliated with members of the alpha-3 subgroup of the Proteobacteria by exhibiting 99.0% sequence identity with a recently described alkaliphilic purple nonsulfur bacterium, *Rhodobaca bogoriensis* LBB1^{T}, isolated from water/sediment samples from Lake Bogoria, Kenya (Milford *et al.*, 2000). Phylogenetic analysis as determined by ribosomal RNA gene sequence analysis placed *R. bogoriensis* within the *Rhodobacter/Rhodovolum* clade of the *Alpha-Proteobacteria*.

3.2.5.3.4. Sequence types related to those of species of the *Gamma-Proteobacteria*

Chromatiales, Ectothiorhodospiraceae

A single Lake Magadi clone sequence (D39) of this library was affiliated with 16S rDNA reference sequences and clones belonging to the family *Ectothiorhodospiraceae* (order *Chromatiales*) and showed the highest similarity value (98.5%) to Lake Magadi clone sequence B44 at 475 nucleotides at their five-prime ends. The highest similarity value (91.3%) for sequence type D39 and an organism whose name has been validly published was observed for the alkaliphilic, halotolerant species *Alkalilimnicola halodurans*, isolated from sediments of Lake Natron, East Africa Rift Valley (Yakimov *et al.*, 2001).

Oceanspirillales, Halomonadaceae

Three cloned sequences of this library (D3, D17, and D44) were observed to be affiliated with reference 16S rDNA sequences of the family *Halomonadaceae* (order *Oceanspirillales*): clone sequence D3 was determined completely and showed the highest 16S rDNA similarity value (96.1%) with the alkaliphilic, halotolerant and denitrifying species *Halomonas desiderata*, isolated from a municipal sewage works (Berendes *et al.*, 1996). The sequences of clones D3 and B19 (clone library B) showed a similarity value of 94.8% to each other. More detailed sequence analysis for clone D3 revealed, that the 16S rDNA insert possessed the 15 signature characteristics associated with 16S rDNA sequences of the family *Halomonadaceae,* including a distinctive cytosine residue at position 486 (*E. coli* 16S rRNA gene sequence numbering), and four signature characteristics that were observed to be common to members of the genus *Halomonas* (compare chapter 3.2.4.3.2., *Halomonadaceae*).

The 16S rDNA sequences of Lake Magadi clones D17 and D44 were determinded partially (appoximately 500 nucleotides at the five-prime ends of the genes), and showed 97.1% similarity to each other, and 95.3 to 98.8% similarity with clone sequence B19.

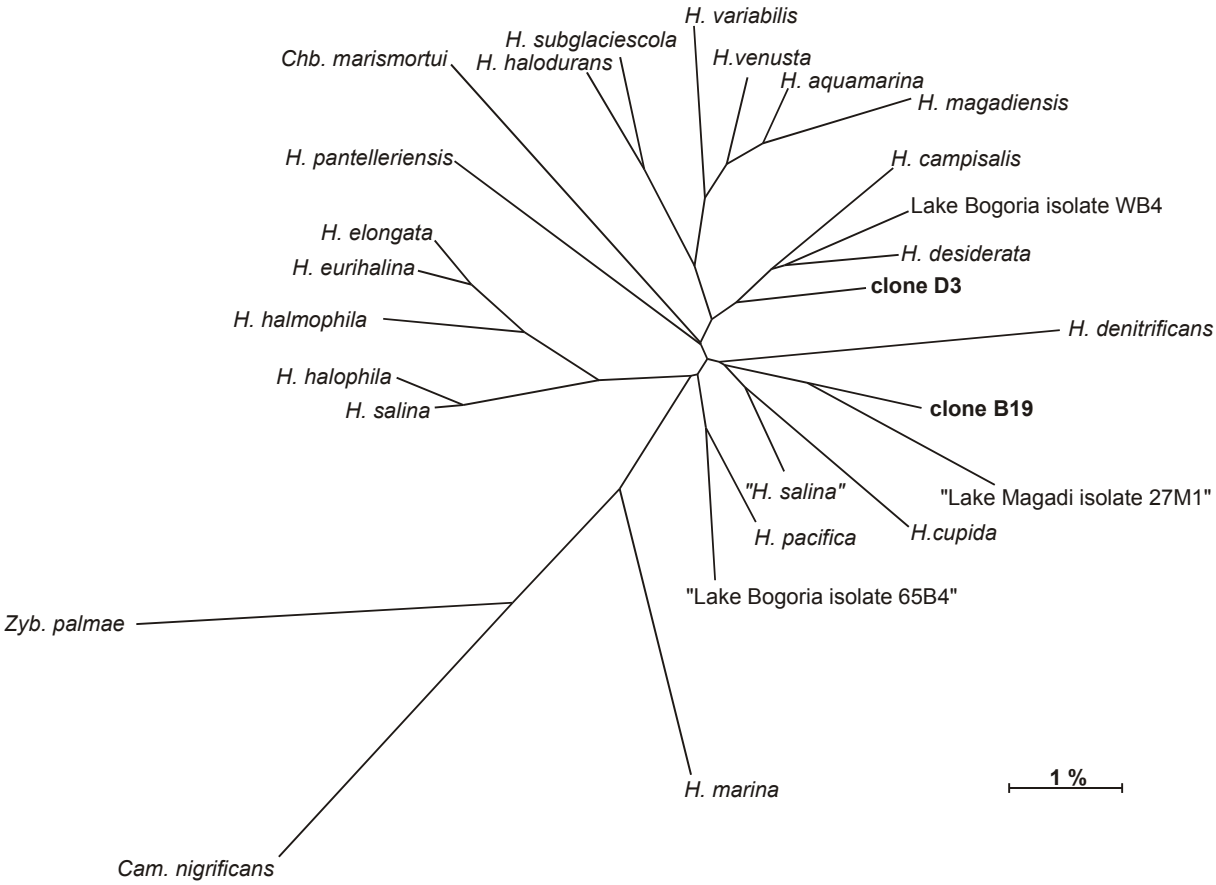

Fig. 3.17: Reconstructed unrooted tree showing the inferred phylogenetic relationships of Lake Magadi clone sequences (printed in bold) to members of the family *Halomonadaceae* and relatives, based on their 16S rRNA gene sequences. The tree was constructed on the basis of 1016 unambiguously determined positions, corresponding to positions 49 to 1387 (*Escherichia coli* 16 S rRNA gene numbering, Brosius *et al.* 1978), that were common to all 16S rDNA sequences used in the analysis. The scale bar represents one inferred nucleotide change per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.10. Abbreviations: *Cam.*, *Carnimonas*; *Chb.*, *Chromohalobacter*; *H.*, *Halomonas; Zyb.*, *Zymobacter.*

Xanthomonas group

A single Lake Magadi clone sequence (D71) was observed to be remotely affiliated with members of the family *Xanthomonadaceae* within the gamma subdivision of Proteobacteria. The 16S rDNA sequence of clone D71 had a maximal similarity of 93.0% to a group of highly similar (99.2-99.4%) sequences, including two cloned sequences, PVB3 and PVB4, obtained from a microbial mat at a deep-sea hydrothermal vent system at the Loihi Seamount near Hawaii (Moyer *et al.*, 1995), one sequence of the Fe-oxidising lithotrophic isolate ES-1 (Emerson & Moyer, 1997), and one sequence from soil bacterium N4-7 for which no further information was available. The highest

similarity value (92.4%) for clone sequence D71 and an organism whose name has been validly published was observed for *Luteimonas memphitis*, isolated from experimental biofilters supplied with the waste gas of an animal-rendering plant (Finkmann *et al*., 2000).

The family *Xanthomonadaceae* (order *Xanthomonadales* according to the taxonomic outline in *Bergey's Manual of Systematic Bacteriology* (Ludwig & Klenk, 2001), is phylogenetically placed in the gamma-subclass of Proteobacteria, currently comprising the genera *Frateuria* (Swings *et al*., 1980), *Luteimonas* (Finkmann *et al.*, 2000), *Lysobacter* (Christensen & Cook, 1978) *Nevskia*, *Pseudoxanthomonas* (Finkmann *et al.*, 2000), *Rhodanobacter* (Nalin *et al*., 1999), *Schineria* (Tóth *et al*., 2001), *Stenotrophomonas* (Hauben *et al*., 1999; Palleroni & Bradbury, 1993), *Xanthomonas* (Dowson, 1939; Hauben *et al*., 1997; Vauterin *et al*., 1995), and *Xylella* (Wells *et al*., 1987).

The "Xanthomonas group" became of increasing interest since several strains with fundamentally different phenetic characteristics compared with the known plant- and human-pathogenic genera were isolated from different and sometimes extreme environments and were assigned to this branch based on their 16S rDNA sequences (Finkmann *et al.*, 2000). Among these, chemolithoautotrophic Fe(II)-oxidising strains were isolated from groundwater (Emerson & Moyer, 1997), an alkaliphilic *Stenotrophomonas*-like strain was isolated from Lake Natron, Kenya, (Duckworth *et al.*, 1996), and *Stenotrophomonas maltophila*-like strains were isolated from the gut and faeces of the arthropod *Folsomia candida* (Hoffmann *et al*., 1998). Additionally, the analysis of a bacterial community from a microbial mat at an active, deep-sea hydrothermal vent system by using 16S rRNA gene analysis, indicated the presence of *Xanthomonas*-related strains in this microbial community (Moyer *et al.*, 1995). Recently, several N_2O-producing *Xanthomonas*-like strains were isolated from ammonia supplied biofilters forming two new lines within the *Xanthomonas* branch of the Proteobacteria and leading to the proposal of the two new genera *Luteimonas* and *Pseudoxanthomonas* (Finkmann *et al.*, 2000).

3.2.5.3.5. Sequence types related to those of species of the *Delta-Proteobacteria*

A single Lake Magadi clone sequence (D73) was observed to cluster within the delta subdivision of the Proteobacteria. The 16S rDNA sequence of clone D73 was determined partially (1028 nucleotides, including *E.coli* positions 28-510 and 948-1491) and showed the closest affiliation (92.5% similarity) with clone sequence B20. However, similarity values calculated for clone sequence D73 and 16S rDNA sequences of organisms of the

delta subgroup of Proteobacteria were relatively low, ranging from approximately 72.0 to 80.0%. The highest level of similarity (80.0%) was observed with the 16S rDNA sequence of the thermophilic sulfate-reducing bacterium *Desulfacinum hydrothermale* DSM 13146^T isolated from geothermally heated sediments near Milos Island, Greece (Sievert & Kuever, 2000).

3.2.5.3.6. Sequence types related to those of species of the *Bacteroidetes* (CFB phylum)

A single Lake Magadi clone sequence (D51) showed a remote affiliation with members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum. The highest level of 16S rDNA sequence similarity (80.4%) was found with the thermophilic and slightly halophilic species *Rhodothermus marinus* DSM 4252^T, isolated from shallow marine hot springs of the coast of Iceland (Alfredsson *et al*., 1988). On the basis of 16S rRNA gene sequence analysis, *R. marinus* is considered to represent a deep lineage within the *Cytophaga-Flavobacterium-Bacteroides* phylum (Andrésson & Fridjónsson, 1994). Organisms belonging to the species *Rhodothermus marinus* are widely distributed in saline hot springs with neutral pH and were isolated from marine hot springs at a beach on the island of S. Miguel in the Azores, from a saline hot spring near the Bay of Naples et Campi Flegri, Italy, and from marine hydrothermal vents in Japan (Moreira *et al*., 1996; Nunes *et al*., 1992a; Sako *et al*., 1996; Silva *et al*., 2000). Comparative sequence analysis for clone sequences D51 and B21 (library B) revealed only a distant relationship (75.3% similarity) of these two representatives of the CFB phylum.

3.2.5.3.7. Chimeric sequences

The detection of possible PCR chimeric clones was restricted to the bacterial library, generated using undiluted DNA extract during PCR amplification, and showing higher diversity in 16S rDNA sequence types as the second bacterial library generated from diluted template DNA. This observation is in accordance with the hypothesis that higher frequencies of chimera formation should be expected when DNA with high complexity is used for PCR (Wang & Wang, 1996). The sequence analysis of Lake Magadi 16S rDNA clones generated using undiluted DNA extract during PCR amplification, revealed two chimeric clone sequences from a total of 21 that were analysed by sequence determination. A 16S rDNA clone was classified as chimeric if its 5'-terminal sequence comprising approximately 450 nucleotides and the almost complete sequence were placed into different species clusters. As nearly full-length 16S rDNA sequences were obtained,

the phylogenetic placement of two Lake Magadi clones changed dramatically, implying possible chimeras. Searching for chimeric junctions was done using the CHECK_CHIMERA program of the RDP (Maidak *et al.*, 2001) and by manual inspection of predicted secondary structures and analysis of nucleotide signatures.

Clone sequence D19 was observed to be a hybrid between a *Methylobacterium* sequence and a clostridial relative. The junction of the chimera was found to be in the semiconserved helix 12 (Gutell, 1993) between *E. coli* 16S rRNA gene sequence positions 240 and 290. The only abnormal base pairing detected in the helical regions formed by the two phylogenetically distinct fragments of the chimera was a single-base-pair mismatch at *E. coli* positions 242 and 284. Lake Magadi clone sequence type D67 was an evident hybrid between a clostridial and a cyanobacterial sequence. This clone attracted attention since it led to distinct results in hybridisation experiments: positive signals were obtained in hybridisation reactions using the specific cyanobacterial probe (CyanoK12), targeting a variable region between *E. coli* 16S rRNA gene sequence positions 647 and 664, while no signals were revealed in hybridisations using the general cyanobacterial probe, targeting a highly conserved region close to the 5'-prime end of the 16S rDNA molecule (*E.coli* positions 363 to 380). The junction was found to be in a highly conserved region between *E. coli* 16S rRNA gene sequence positions 510 and 540 (helix 19), where a single mismatch in base pairing was detected.

Table 3.15: Similarity analysis demonstrating that different domains of a chimeric sequence have different inferred phylogenetic affiliations.

Clone	Phylogenetic affiliation	Nucleotides	similarity (%)	Nearest neighbour	Accession no.
D19					
full	Gram+ lowGC	1-1455	86.1	*Thermobrachium celere* DSM 8682^T	X99238
5'-end	α-Proteobacteria	1-220	99.0	*Methylobacterium sp.* PC30.39	Z23158
3'-end	Gram+ lowGC	221-1189	87.5	*Thermobrachium celere* DSM 8682^T	X99238
D67					
full	Cyanobacteria	1-1446	93.5	*Cyanothece sp.* PCC 7418	AJ000708
5'-end	Gram+ lowGC	1-480	83.9	*Caloramator indicus* IndiB4	X75788
3'-end	Cyanobacteria	481-1446	97.4	*Euhalothece sp.* MPI 95AH10	AJ000709

The closest matching sequence from a cultivated species was determined using the FASTA analysis algorithm (Pearson, 1990) available from the EMBL (Stoesser *et al.*, 2002).

Formation of chimeric molecules

A potential hazard associated with PCR amplification of target DNA of mixed sequences is the creation of recombinant ("shuffle-gene") or chimeric products that consist of mixtures of parts of different sequences. This phenomenon has been widely observed and is not restricted to 16S rDNA amplification from complex communities (i.e., Choi *et al*., 1994; Kopczynski *et al*., 1994; Meyerhans *et al*., 1990; Paabo *et al*., 1992; Shuldiner *et al*., 1989; Von Wintzigerode *et al.*, 1997; Wang & Wang, 1996 and 1997). A chimeric molecule is generated when a fragment of one gene competes with specific primers during the annealing step of PCR and anneals with a homologous template to prime the next cycle of DNA synthesis. Investigation of the contributions of several PCR parameters to the formation of chimeric molecules revealed that prematurely terminated DNA strands, especially in later cycles of PCR, are the main cause of chimera formation (Wang & Wang, 1996 and 1997).

The frequency of occurrence of chimeric sequences in clone libraries has been reported, from independent studies, more than once to range from 4 to 20% (Barns *et al.*, 1994; Choi *et al.*, 1994; Kopczynski *et al.*, 1994; Robison-Cox *et al*., 1995). However, by using oligonucleotide probes to detect exchanges of fragments between parental DNA molecules, Wang and colleagues (1996) were able to detect chimeras formed during co-amplification of nearly identical sequences. They observed frequencies of 12.9 and 30% between sequences of 82 and 99.7% similarity, respectively, and concluded that previous studies (Kopczynski *et al.*, 1994; Liesack *et al*., 1991; Meyerhans *et al.*, 1990) had underestimated the actual frequencies of recombination events, as they had not considered recombinations between identical molecules, which would generate undetectable chimeric sequence types.

Detection of chimeric SSU rRNA genes is difficult but of importance since they could otherwise lead to erroneous reporting of "novel" lines of evolutionary descent, giving a false impression of biodiversity within microbial communities, and reducing the quality of centralised sequence data bases. Detection of chimeric 16S rRNA (-gene) sequences is possible by observing base pair mismatches in secondary structures, although this method is not fail-safe, given that some chimeras do not exhibit such abnormalities and conservation in helices between mixed populations of organisms can be high enough to produce normal SSU rRNA secondary structure (Kopczynski *et al.*, 1994). Chimeras can also be detected by demonstrating that separate domains of an unknown 16S rRNA (-gene) sequence are identical to two ore more different known sequences (Kopczynski *et al.*, 1994; Liesack *et al.*, 1991). However, natural habitats have been shown to contain mainly uncultivated microorganisms, whose 16S rRNA (-gene) sequences are only now being determined (Robison-Cox *et al.*, 1995). The chance that

the databases will contain the sequences that are identical to specific domains of chimeric sequences and, thus, useful for the detection of chimeras, depends on the degree to which the species of the habitat have been characterised by sequence analysis. Thus, methods based on demonstrating that different domains of a query sequence have different inferred phylogenetic affiliations have been developed to detect chimeras (Barns *et al.*, 1994; Fuhrman *et al.*, 1993; Komatsoulis & Waterman, 1997; Kopczynski *et al.*, 1994; Maidak *et al.*, 2001; Robison-Cox *et al.*, 1995). This difference can be demonstrated by comparing trees derived from different sequence domains or by pairwise similarity analysis of the domains. Meanwhile, computer software programs are the main tools for identification of chimeric sequences, and there are three currently available nearest-neighbour methods: (a) the CHECK_CHIMERA method of Larsen *et al.* (Maidak *et al.*, 2001); (b) the aligned similarity method of Robison-Cox *et al.* (1995); and (c) the so-called chimeric alignment method (mglobal CHI) of Komatsoulis & Waterman (1997). Robison-Cox and coauthors carried out a mathematical evaluation of the CHECK_CHIMERA program, which is currently the most popular of the methods, and found that the confidence in detection of chimeras by this method decreased from 95 to 50% as the estimated similarity between template DNA parental sequences increased from 82 to 96%. In conclusion, one should be aware that this service may be unable to detect all chimeras, since the ability to detect all chimeric 16S rRNAs decreases as the parental sequences which contribute to chimera formation become more similar. The presence of chimeric sequences in the databases may further reduce the usefulness of a program that depends on comparison of an input sequence with all sequences in the databases.

3.2.6. Analysis of the archaeal 16S rDNA clone library

The diversity of archaeal 16S rDNA sequence types from Lake Magadi sediment samples was assessed by analysis of a single archaeal clone library, generated using undiluted template DNA, during PCR amplification, and primers specific for *Archaea*. The screening of the clone library included ARDRA-fingerprinting and partial or complete 16S rDNA sequence determination.

3.2.6.1. ARDRA fingerprinting

A total of 96 archaeal clones were grouped on the basis of amplified rDNA restriction analysis (ARDRA), using the tetrameric restriction enzyme *Taq*I, to obtain an overview of the diversity of archaeal sequence types and to select clones for more detailed taxonomic studies. The 16S rDNA inserts of archaeal clones were reamplified by PCR using the primers 16F23A, specific exclusively for *Archaea* and 16R1492, specific for both, *Archaea* and *Bacteria*. The use of these primers for reamplification of 16S rDNA inserts allowed the comparison of results from ARDRA analyses of archaeal clones and haloalkaliphilic isolates. The 96 archaeal clone sequences were clustered into 41 different ARDRA types, indicated by Roman numerals and corresponding to the nomenclature of ARDRA groups obtained for the haloalkaliphilic soda lake isolates (compare Table 3.20, Table 3.21 and Table 3.16). Additionally, various haloalkaliphilic reference strains of the family *Halobacteriaceae* were included in the restriction analysis (compare Table 3.21). In some cases (i.e., restriction types I, III, Vb, VI and VIII), the ARDRA profiles of archaeal clone sequences were identical with those obtained for certain reference strains (i.e., *Natronomonas pharaonis, Halorubrum vacuolatum, Natronobacterium gregoryi,* Wadi Natrun isolate W3B, *Natronococcus amylolyticus*) included in the analysis. The most frequently encountered ARDRA type (defined IXa) was represented by 15 clones. The second abundantly represented ARDRA group (Vb) comprising 13 clones, possessed identical patterns with *Natronobacterium gregoryi*. A third commonly encountered ARDRA group (Xa) included nine clones. A considerable number of ARDRA types, comprising 24 different patterns (defined ST, single-type), occurred only once in the archaeal clone library. The affiliations of archaeal cloned sequence types to the corresponding ARDRA groups are summarised in Table 3.17.

Table 3.16: Screening of library A by ARDRA (amplified rDNA restriction analysis)

ARDRA-Type	**Number of clones**	**Clones with identical ARDRA types**
I	1	90, *Natronomonas pharaonis*
III	4	**10**, **38**, 55, 82, *Halorubrum vacuolatum*
Vb	13	**2**, *8*, *15*, *17*, *22*, *24*, *32*, *37*, *41*, **50**, 59, 65, 69, *Natronobacterium gregoryi*
VI	3	**43**, 77, 93, Wadi Natrun isolate W3B
VIII	1	**33**, *Natronococcus amylolyticus*
IXa	15	*5*, *13*, **26**, *39*, *40*, *46*, *47*, 54, 70, 71 73, 74, 95, 96, 97
IXb	3	**14**, **42**, 58
Xa	9	**9**, *12*, *25*, *60*, 62, 72, 76, 83, 87
Xb	2	*27*, **35**
XI	2	**86**, 98
XII	2	**4**, **31**
XIII	2	**49**, *84*
XIV	3	**44**, 61, 88
XV	3	**3**, *7*, **30**
XVI	5	**6**, *16*, *48*, 79, 80
XVII	2	**23**, **34**
XIX	2	*68*, *78*
ST[a]	24	*1*, **11**, **18**, **20**, **21**, **28**, **29**, **36**, **51**, **52**, *53*, **56**, *57*, *63*, 64, 66, **67**, **75**, **81**, *85*, **89**, *91*, *92*, 94
Σ=41[a]	Σ=96[b]	

Clones were selected for partial (printed in italics) and nearly complete (printed in bold) sequence analyses.
[a] Total number of different ARDRA types.
[b] Total number of clones analysed by ARDRA.

3.2.6.2. 16S rDNA sequence determination

All of the ARDRA types that occurred more than once in the library, as well as many of the unique ARDRA types, were sequenced partially (at least, 450 nucleotides at the five prime ends of the genes) and compared to each other. If partial sequences showed less than 97.0 % similarity to any other sequence of this library, they were selected for more detailed sequence analysis (comprising approximately 1,490 nucleotides). In total, 35 Lake Magadi clone sequence types were determinded completely, and 31 partially. The results of nearly complete 16S rDNA sequence analysis are summarised in Table 3.17 and Fig. 3.18. For the most part (including 93 of a total number of 96 clones), sequences were observed to be affiliated with members of the extremely halophilic archaea, the halobacteria, assembled in the family *Halobacteriaceae,* order *Halobacteriales* (Grant & Larsen, 1989). A minority of three clones was found to show a remote affiliation to members of the *Methanococcaceae*.

Table 3.17: Nearest neighbours of Lake Magadi sediment SSU rDNA cloned sequence types

Group	Clone	ARDRA type	Similarity (%)	Nearest neighbours	Accession no.	No. of clones
Euryarchaeota						
Halobacteria						
"Natro group"						
	A2	Vb	97.9	**O2C**	this study	13
			95.7	*Natronobacterium gregoryi* NCIMB 2189^T	D87970	
	A50	Vb	97.7	**O2C**	this study	
			95.5	*Natronobacterium gregoryi* NCIMB 2189^T	D87970	
	A43	VI	99.2	**W3B**	this study	3
			94.4	*Natronococcus amylolyticus* Ah-36^T	D43628	
	A33	VIII	99.4	*Natronococcus amylolyticus* Ah-36^T	D43628	1
	A44	XIV	93.0	**W3B, W1YE, O2C, O2B**	this study	3
	A3	XV	96.7	**W3B**	this study	3
			94.9	*Haloterrigena turkmenica* VKM B-1734^T	AB004878	
			94.9	Little Lake Magadi isolate 93dLM4*	X92170	
			93.7	*Natronococcus amylolyticus* Ah-36^T	D43628	
	A30	XV	94.0	**W1YE, O2C, W3B**	this study	
			93.2	*Haloterrigena thermotolerans* PR5^T	AF115478	
	A6	XVI	94.6	**O2B**	this study	5
			94.0	*Haloterrigena thermotolerans* PR5^T	AF115478	
	A23	XVII	94.9	**W3B**	this study	2
			94.0	*Natronococcus amylolyticus* Ah-36^T	D43628	
	A34	XVII	94.8	**W1YE, W3B, O2B, O2C**	this study	
			94.0	*Natrinema versiforme* XF10^T	AB023426	
	A56	ST	95.0	**W3B**	this study	
			95.0	*Natrinema versiforme* XF10^T	AB023426	
	A29	ST	95.3	**O2B**	this study	
			94.6	*Haloterrigena turkmenica* VKM B-1734^T	AB004878	
	A81	ST	97.2	**W1YE**	this study	
			93.4	*"Natronococcus xinjiangense"*	AF251285	
	A28	ST	93.5	**W3B, W1YE, O2C, O2B**	this study	
			93.4	*Natronococcus amylolyticus* Ah-36^T	D43628	
	A36	ST	92.8	**W3B**	this study	
			92.8	*Natronococcus amylolyticus* Ah-36^T	D43628	
	A67	ST	93.7	**W3B**	this study	
			92.3	*Haloterrigena thermotolerans* PR5^T	AF115478	

Group	Clone	ARDRA type	Similarity (%)	Nearest neighbours	Accession no.	No. of clones
"*Halorhabdus* group"						
	A9	Xa	91.5	*Halorhabdus utahensis* AX-2^T	AF071880	9
	A18	ST	91.7	*Halorhabdus utahensis* AX-2^T	AF071880	
	A86	XI	91.5	*Halorhabdus utahensis* AX-2^T	AF071880	2
	A11	ST	91.5	*Halorhabdus utahensis* AX-2^T	AF071880	
	A4	XII	90.7	*Halorhabdus utahensis* AX-2^T	AF071880	2
	A31	XII	91.7	*Halorhabdus utahensis* AX-2^T	AF071880	
"*Halorubrum* group"						
	A10	III	98.3	*Halorubrum vacuolatum* JCM 9060^T	D87972	4
	A38	III	98.4	*Halorubrum vacuolatum* JCM 9060^T	D87972	
"*Natronomonas* group"						
	A52	ST	95.8	**OS2**	this study	
			95.8	*Natronomonas pharaonis* JCM 8858^T	D87971	
"MSP clones"						
	A26	IXa	99.0	Magadi salt pond (MSP) clone 23*	AB012058	15
			89.7	*Natronomonas pharaonis* JCM 8858^T	D87971	
	A14	IXb	99.6	Magadi salt pond (MSP) clone 16*	AB012055	3
			89.4	*Haloterrigena thermotolerans* PR5^T	AF115478	
	A42	IXb	95.4	Magadi salt pond (MSP) clone 9*	AB012051	
			90.8	*Natrinema pellirubrum* NCIMB 786^T	AJ002947	
	A21	ST	92.9	Magadi salt pond (MSP) clone 9*	AB012051	
			90.8	*Natrinema pellirubrum* NCIMB 786^T	AJ002947	
	A35	Xb	93.4	Magadi salt pond (MSP) clone 9*	AB012051	2
			89.9	*Natronomonas pharaonis* JCM 8858^T	D87971	
	A51	ST	91.4	Magadi salt pond (MSP) clone 9*	AB012051	
			90.4	*Haloterrigena thermotolerans* PR5^T	AF115478	
New lineages within the halobacteria						
	A49	XIII	88.3	*Halorhabdus utahensis* AX-2^T	AF071880	2
	A75	ST	90.0	**W3B**	this study	
			89.5	*Natronorubrum tibetense* AS 1.2123^T	AB005656	
	A89	ST	90.0	*Haloferax mediterranei* ATCC 33500^T	D11107	
Ungrouped sequence types						
	A20	ST	79.0	*Methanococcus infernus* ME^T	AF025822	

Cloned 16S rRNA genes were sequenced (*E. coli* 16S rRNA gene sequence positions 23 to 1491). The most similar sequences from environmental isolates and organisms whose names have been validly published were determined using the FASTA search available in the EBI. In some cases, indicated by "*", higher similarities were observed with environmental rDNA clones or, printed in bold, with haloalkaliphilic isolates from Owens Lake (California) or the lakes of Wadi Natrun (Egypt). The total number of clones belonging to the same ARDRA group is indicated.

Halobacteria

Cloned 16S rDNA sequences were observed to be affiliated with four major lines of described halobacteria, i.e., the genera *Natronomonas* (Kamekura *et al.*, 1997), *Halorubrum* (McGenity & Grant, 1995), *Halorhabdus* (Wainø *et al.*, 2000) and the relatively diffuse cluster of halobacteria including *Natronobacterium gregoryi* and related organisms, which was temporarily named the "Natro group" (McGenity *et al.*, 1998). A further group of cloned sequences was found to be affiliated with a recently described, new lineage of clonal sequence types within the known *Halobacteriaceae* (Grant *et al.*, 1999).

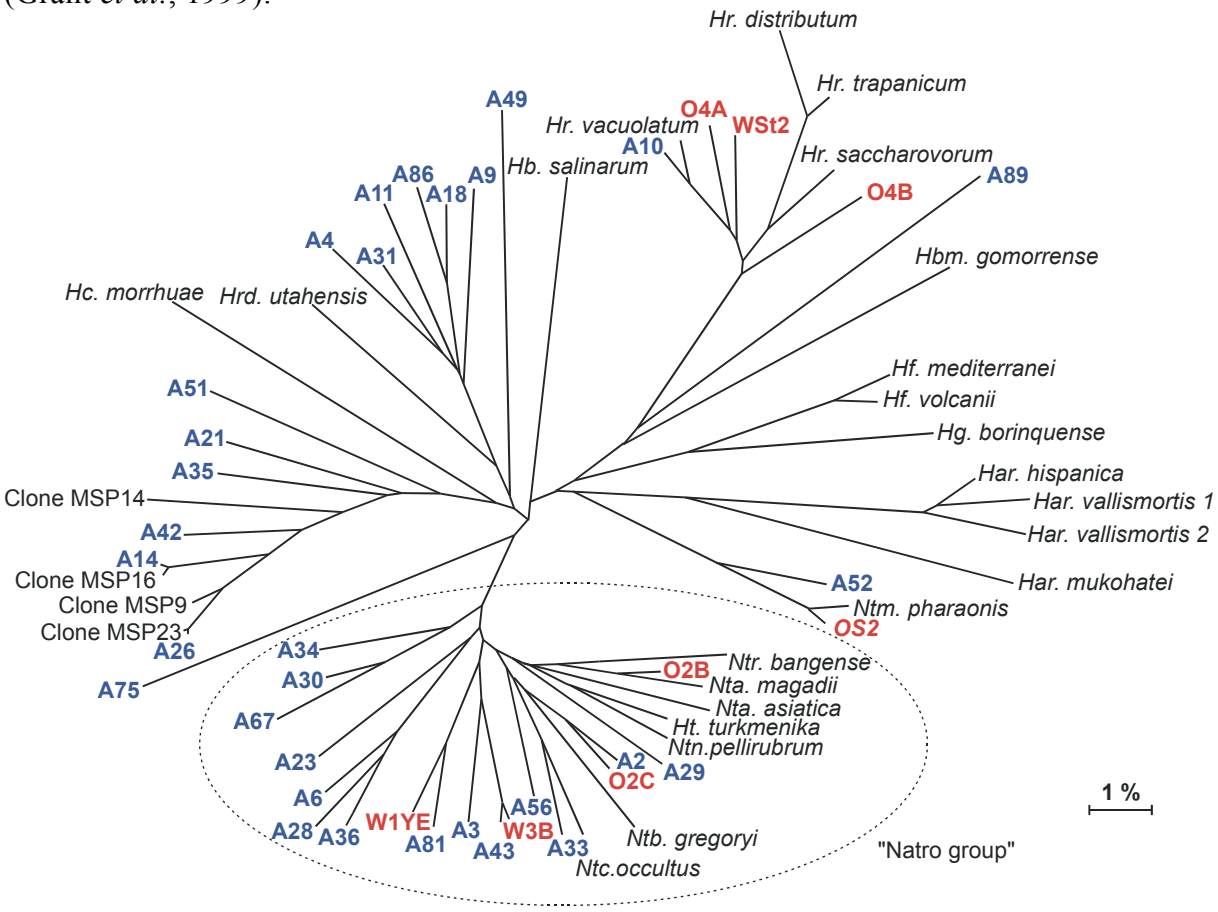

Fig. 3.18: Reconstructed unrooted tree on the basis of 16S rDNA sequence data, indicating the inferred phylogenetic positions of Lake Magadi clones (printed in blue) within the radiation of halobacteria, including haloalkaliphilic soda lake isolates (printed in red) from Owens lake, California, and Wadi Natrun, Egypt. The tree was constructed on the basis of 1318 unambiguously determined positions, corresponding to positions 24 to 1474 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.* 1978), that were common to all 16S rDNA sequences used in the analysis. The scale bar represents one inferred nucleotide change per 100 nucleotides. Accession numbers of 16S rDNA sequences are indicated in Table 3.24. Abbreviations: *Har.*, *Haloarcula*; *Hb.*, *Halobacterium*; *Hbm.*, *Halobaculum*; *Hc.*, *Halococcus*; *Hf.*, *Haloferax*;. *Hg.*, *Halogeometricum; Hr.*, *Halorubrum*; *Hrd.*, *Halorhabdus; Ht.*, *Haloterrigena*; *Nta.*, *Natrialba*; *Ntb.*, *Natronobacterium*; *Ntc.*, *Natronococcus*; *Ntm.*, *Natronomonas*; *Ntn.*, *Natrinema; Ntr.*, *Natronorubrum.*

“Natro group”

The most frequently encountered group of Lake Magadi clone sequences (comprising 43 sequences types, separated into 19 different ARDRA groups) was observed to be affiliated on the basis of their 16S rDNA sequences with known members of the so called “Natro group”, an assemblage of mainly haloalkaliphilic species of the genera *Natronobacterium*, *Natronococcus*, *Natrialba*, *Natronorubrum*, *Natrinema*, and *Haloterrigena*. The inferred positions of cloned sequence types clustering within the radiation of the “Natro group” are shown in detail in Fig. 3.19. Reference species of this cluster were observed to be more than 89.5% similar in their 16S rDNA sequences. All Lake Magadi clone sequences of this group exhibited similarity values to each other in the range of 91.2 to 97.0% and similarities to reference sequences of this group in the range of 92.3 to 99.4%. Some clonal sequence types, i.e., A2, A43 and A81 exhibited relatively high sequence similarities (97.9 to 99.2%) to haloalkaliphilic organisms isolated from Wadi Natrun and Owens Lake, i.e., O2C, W3B and W1YE (see chapter 3.3.1.), wherein isolate W3B and clone A43 possessed the same ARDRA patterns (group VI). An abundantly represented group of archaeal sequence types, comprising 13 clones and clustering near the “Natro group” of halobacteria, was found to be most closely affiliated to the haloalkaliphilic Owens Lake isolate O2C (97.7-97.9%), and to the 16S rDNA sequence of the alkaliphilic organism *Natronobacterium gregoryi* (95.5-95.7% similarities), isolated from the solar salt pans at Lake Magadi (Tindall *et al.*, 1980). Notably, all of these clones demonstrated identical ARDRA patterns (group Vb) with that of *Natronobacterium gregoryi* and, at least, very similar patterns with isolate O2C (belonging to ARDRA type Va).

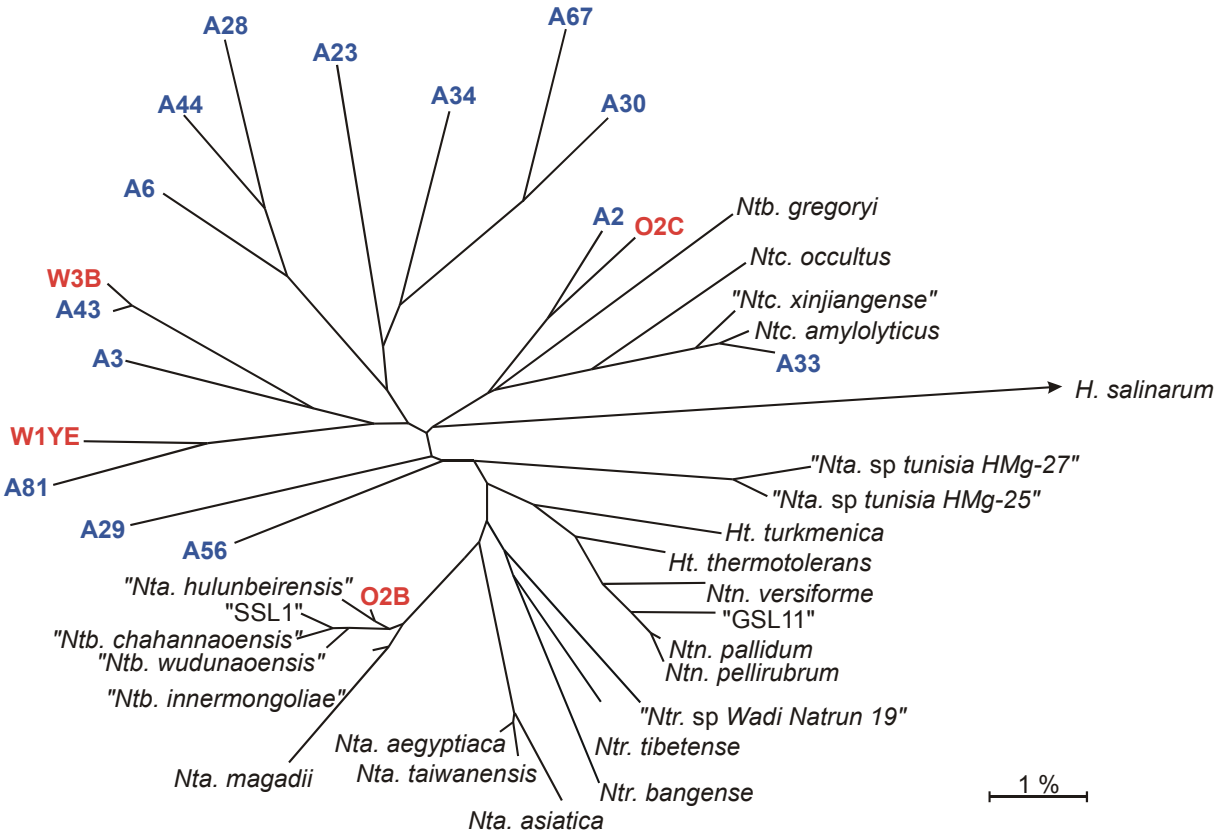

Fig. 3.19: Reconstructed tree on the basis of 16S rDNA sequence data, indicating the inferred phylogenetic positions of Lake Magadi clones (printed in blue) within the **"Natro group"** of halobacteria, including haloalkaliphilic soda lake isolates (printed in red) from Owens Lake, California, and Wadi Natrun, Egypt. The tree was constructed on the basis of 1333 unambiguously determined positions, corresponding to positions 25 to 1474 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.* 1978), that were common to all 16S rDNA sequences used in the analysis. The sequence of *Halobacterium salinarum* DSM 3754^T was used as an outgroup. The scale bar represents one inferred nucleotide change per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.24. Species names that are not yet validly published are indicated by quotation marks. Abbreviations: *H.*, *Halobacterium*; *Ht.*, *Haloterrigena*; *Nta.*, *Natrialba*; *Ntb.*, *Natronobacterium*; *Ntc.*, *Natronococcus*; *Ntn.*, *Natrinema*; *Ntr.*, *Natronorubrum.*

"*Halorhabdus* group"

A second abundantly represented group of Lake Magadi clone sequences (comprising 15 sequence types, separated into 5 different ARDRA types) demonstrated relatively high 16S rDNA similarities to each other (93.5 to 96.8%), forming a single cluster that grouped within known lineages of the halobacteria. Clonal sequence types belonging to this cluster were observed to be most closely affiliated (90.6 to 91.6% 16S rDNA sequence similarities) with the recently described, extremely halophilic species *Halorhabdus utahensis*, isolated from sediment of the hypersaline Great Salt Lake, Utah, USA, and presently representing the sole known member of the genus *Halorhabdus* (Wainø *et al.*, 2000). On the basis of 16S rDNA sequence comparisons, the unique

sequence type A49 showed a remote relationship (87.6 to 89.2%) with other clonal sequences of this library and with *Halorhabdus utahensis* (87.7% similarity).

"*Halorubrum* group"

Four Lake Magadi clones were observed to be affiliated on the basis of their 16S rDNA sequences with members of the genus *Halorubrum* (McGenity & Grant, 1995). Cloned sequence types A10 and A38 were determined nearly completely as representatives of the uniform ARDRA pattern (III), which was observed to be common to all clones within this group, as well as *Halorubrum vacuolatum* and the haloalkaliphilic Owens Lake isolate O4A. The highest similarity values for clones A10 and A38, were observed to each other (99.4%) and to the 16S rDNA sequence of *Halorubrum vacuolatum* (approximately 98%), isolated from Lake Magadi (Mwatha & Grant, 1993).

"*Natronomonas* group"

A single Lake Magadi clone (A52) was most closely affiliated (95.8% similarity) with the 16S rDNA sequence of the alkaliphilic organism *Natronomonas pharaonis*, originally isolated from the alkaline brine of eutrophic desert lakes of Wadi Natrun, Egypt (Soliman & Trüper, 1982), and later from Lake Magadi, as strain Sp1 (Tindall *et al.*, 1984). The same level of sequence similarity (95.8%) was obtained for clone sequence A52 and the Owens Lake isolate OS2, clustering nearby *Natronomonas pharaonis*.

"MSP clones"

Another frequently encountered group of Lake Magadi cloned sequence types (comprising 23 sequences, separated into 5 different ARDRA types) was found to be closely affiliated with other cloned sequence types of uncultured organisms, forming a distinct lineage of 16S rDNA sequences within the known *Halobacteriaceae* (Grant *et al.*, 1999). These reference clone sequences were also derived from Lake Magadi samples, specifically from the final crystallising pond of an alkaline saltern and were, therefore, called "Magadi salt pond (MSP) clones". In this study, nearly complete 16S rDNAs (comprising approximately 1,400 nucleotides) of representative clones, exhibited 91.1 to 95.6% similarity to each other, and 91.4 to 99.6% similarity to several Magadi salt pond clones, whereas similarities calculated with any 16S rDNA sequence from cultivated organisms in the databases showed maximal similarity values of 89.7 to 90.8%. The closest affiliations were generally obtained to those of *Natronomonas pharaonis* (Kamekura *et al.*, 1997; Soliman & Trüper, 1982), *Haloterrigena thermotolerans* (Montalvo-Rodríguez *et al.*, 2000) or to *Natrinema pellirubrum* (McGenity *et al.*, 1998).

New lineages within the *Halobacteriaceae*

A few Lake Magadi cloned sequence types (4 sequences, separated into 3 different ARDRA types) were observed to be the most deeply branching cloned sequence types within the *Halobacteriaceae* and exhibited only slight affiliations (at most 90.0% similarity) to 16S rDNA sequences of known species within the halobacteria: Lake Magadi clone sequence A75 represented a unique sequence type, distantly related with members of the "Natro group" (87.2 to 89.0% similarity). A further unique sequence type (A89) was observed to show maximal sequence similarities with species of the genera *Haloferax* (88.2 to 88.6%), *Halorubrum* (86.6 to 88.1%) and *Halobaculum* (87.0%). Lake Magadi clone sequence A49 was determined completely as a representative of two clones (ARDRA type XIII), and exhibited a remote affiliation (87.6 to 89.2% similarity) with sequences of the *Halorhabdus* group.

Ungrouped sequence types within the *Euryarchaeota*

Three Lake Magadi cloned sequences (A20, A57 and A63) were observed to represent new sequence types within the *Euryarchaeota*, but outside the halobacteria cluster. These clones possessed 93.7 to 95.2% 16S rDNA sequence similarities to each other, but only a maximum of 79.0% to any other known species within the *Archaea*. The highest similarity value was obtained for clone A20 and *Methanococcus infernus*.

Estimation of the resolving capacity of the *Taq*I-ARDRA approach

The resolving capacity of the *Taq*I amplified rDNA restriction analysis (ARDRA) for the analysis of archaeal cloned sequence types was estimated to correspond with 95 to 97% 16S rDNA sequence similarities, thus, allowing a differentiation between sequences belonging, presumably (at least) to different genera. The majority of the seventeen ARDRA types, occurring more than once in the archaeal clone library, comprised sequences with intragroup similarities in the range of 95.0 to 99.8% (Table 3.18). The ARDRA types XV and XVII were observed to exhibit minimal intragroup similarity values of 92.0 and 93.6%. Two cloned sequence types (A44 and A36) were almost identical (99.8% similarity), but possessed distinct ARDRA patterns (XIV and ST, respectively), differing in a single band, that occurred only in A44, but not in A36.

Table 3.18: Correlation between screening of library A by ARDRA (amplified rDNA restriction analysis), and partial/complete 16S rDNA sequence analysis.

Group	ARDRA -Type	Similarity	Number of clones	Clones with identical ARDRA types
"Natro group"				
	Vb	98.5-99.8	13	**2**, *8*, *15*, *17*, *22*, *24*, *32*, *37*, *41*, **50**, 59, 65, 69, *Natronobacterium gregoryi*
	VI	99.2	3	**43**, 77, 93, Wadi Natrun isolate W3B
	VIII	99.4	1	**33**, *Natronococcus amylolyticus*
	XIV		3	**44**, 61, 88
	XV	92.0-99.8	3	**3**, *7*, **30**
	XVI	99.3-99.5	5	**6**, *16*, *48*, 79, 80
	XVII	93.6	2	**23**, **34**
	XIX	99.6	2	*68*, *78*
	ST	92.8-97.2	11	*1*, **28**, **29**, **36**, *53*, **56**, **67**, **81**, *85*, *91, 92*
			Σ=43	
"*Halorhabdus* group"				
	Xa	97.1-99.5	9	**9**, *12*, *25*, *60*, 62, 72, 76, 83, 87
	XI		2	**86**, 98
	XII	96.0	2	**4**, **31**
	ST	95.0	2	**11**, **18**
			Σ=15	
"*Halorubrum* group"				
	III	99.4	4	**10**, **38**, 55, 82, *Halorubrum vacuolatum*
			Σ=4	
"*Natronomonas* group"				
	I		1	90, *Natronomonas pharaonis*
	ST		1	**52**
			Σ=2	
"Magadi salt pond clones"				
	IXa	98.7-99.8	15	*5*, *13*, **26**, *39*, *40*, *46*, *47*, 54, 70, 71 73, 74, 95, 96, 97
	IXb	95.0	3	**14**, **42**, 58
	Xb	98.2	2	*27*, **35**
	ST	93.3	2	**21**, **51**
			Σ=22	
New lineages within the *Halobacteriaceae*				
	XIII	99.5	2	**49**, *84*
	ST	86.6	2	**75**, **89**
			Σ=4	
Ungrouped *Euryarchaeota*				
	ST	93.7-95.2	3	**20**, *57*, *63*
			Σ=3	

The ARDRA groups were arranged according to the affiliations of the corresponding cloned sequences analysed by partial (printed in italics) or complete (printed in bold) 16S rDNA sequence determination. ST = single cloned sequence types, i.e., that occurred only once in the clone library.

3.2.7. Statistical approaches to estimating 16S rDNA sequence diversity

The description of microbial communities by analysing clone-libraries usually leads to the question of how well a given sample (the clones analysed) reflects the community's "true" diversity (Hughes *et al.* 2001).

There are several statistical approaches for describing the microbial diversity, such as,

1. diversity indices;
2. estimated species richness;
3. compared sample diversity (rarefaction analysis);
4. different "new" diversity statistics (e.g., extrapolation from accumulation curves, parametric and non-parametric estimators).

Most of these statistical approaches were derived from the field of plant and animal ecology and evaluated for their applicability in describing microbial communities, as well. While the above mentioned approaches 1 to 3 can be used for the description of a sampled community, parametric and nonparametric estimators (4) try to estimate the "black box" of the total community. Recently, Hughes *et al.* (2001) compared the utility of various statistical approaches for assessing the diversity of microbial communities. Methods from macroorganismal ecology were reviewed regarding the success of these diversity estimators for the description of microbial communities for which the true diversity is unknown. The findings of this comprehensive work can be summarised as follows:

1. Evaluating microbial diversity with statistical approaches available for macroorganisms seems feasible but further work using larger data sets is needed to investigate the general applicability of these approaches for microbial diversity studies.
2. The sample size is a key-parameter for the estimation. The larger the sample size, the higher the fit of the estimated richness. For some habitats the minimum sample size required to detect richness differences of only tens of species was estimated by the authors with "only" 200-1000 clones.
3. The diversity comparison requires a clear definition of OTU (in the field of molecular biology different authors using different cut-offs of percent genetic similarity for the definition of OTUs)

4. Also the abundance of amplified genes in PCR's may not reflect the relative abundance of template DNA because of differences in primer binding and elongation efficiency.
5. The calculation of rarefaction curves may be a valuable way to compare the relative diversity of communities.

The screening process using ARDRA fingerprinting was tested by statistical analysis to evaluate whether the total diversity in clone libraries A and D was covered by screening 96 and 100 clones, respectively. Generally, species diversity can be considered to be composed of two components, i.e., species richness (the number of species in a community) and species evenness (the distribution of levels of abundance among the species). Both species richness and relative abundance indices are based on the correct identification to the species level, which is seldom possible in bacteriology (Watve & Gangal, 1996). Therefore, instead of attempting species identification, most researchers subject the characterisation data to cluster analysis and categorise groups of isolates or clones that lie close to each other. In order to categorise the groups, it is necessary to define a cutoff level of similarity above which isolates/clones one of a given group will belong. This cutoff point is arbitrary and may appear to be without justification (Watve & Gangal, 1996). Throughout this chapter, the term "species richness" is used to mean the richness of different OTUs (operational taxonomic units), wherein an OTU was defined as a group of cloned 16S rDNA sequence types exhibiting identical *Taq*I-ARDRA patterns.

The following graph shows a plot of the number of OTUs observed versus the number of clones found in the samples investigated.

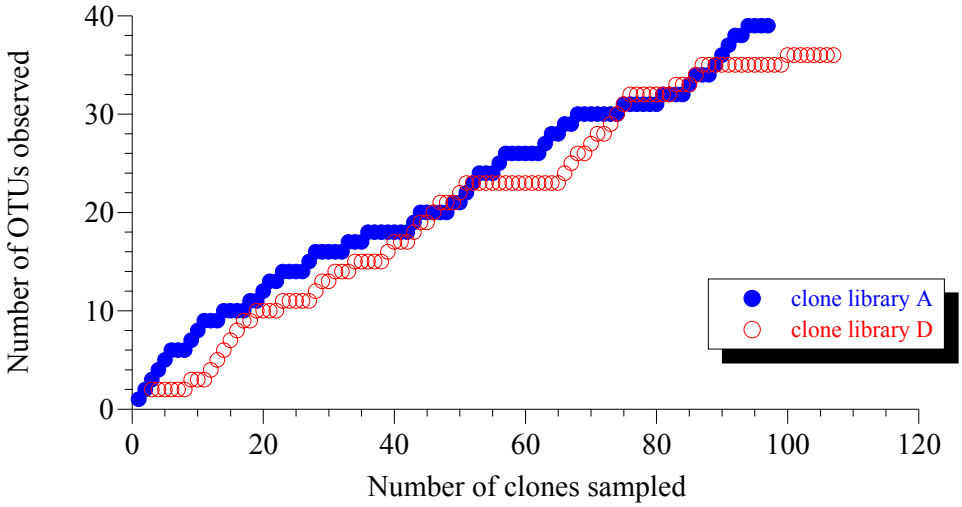

Fig. 3.20: Cumulative difference of observed OTUs, randomly ordered, versus the number of clones sampled for the clone-libraries A and D.

Both curves demonstrate an increase in the number of OTUs detected with an increase in the number of clones sampled. Assuming that the molecular biological approach chosen for the preparation and determination of any clone library leads, for an infinitely high number of sampled clones, to a total maximum number of different OTUs in the environmental sample, the curves shown in Fig. 3.20 should follow a saturation function reaching its maximum (asymptote) at infinite effort. The estimators (asymptote = total "species richness" expected) can be estimated, e.g., by using the Michaelis-Menten equation (Raaijmakers, 1987) or a negative exponential function (Soberón & Llorente, 1993).

A curve fit of the raw data plotted in Fig. 3.20, using the following negative exponential function (equ. 3.1), models the process of observing new ARDRA types as sampling effort increases and estimates the richness (of OTUs) expected for the clone libraries A and D.

$$y = a + b \cdot e^{-c \cdot x} \qquad \text{(equ. 3.1)}$$

with:

x: number of clones sampled

y: number of different OTUs observed

a: estimated richness (asymptote)

b and c: coefficients defining the specific shape of the curve

The parameter estimation was carried out by performing a non-linear regression of the raw data of the clone libraries A and D, with equ. 3.1, using the software tool SPSS 10.0. For lim x $\rightarrow \infty$, the term $b \cdot e^{-c \cdot x}$ derives versus the value of zero. Thus the asymptote (estimated richness) is represented by the coefficient a. The results (raw data in comparison to the regression curves) are shown in Fig. 3.21.

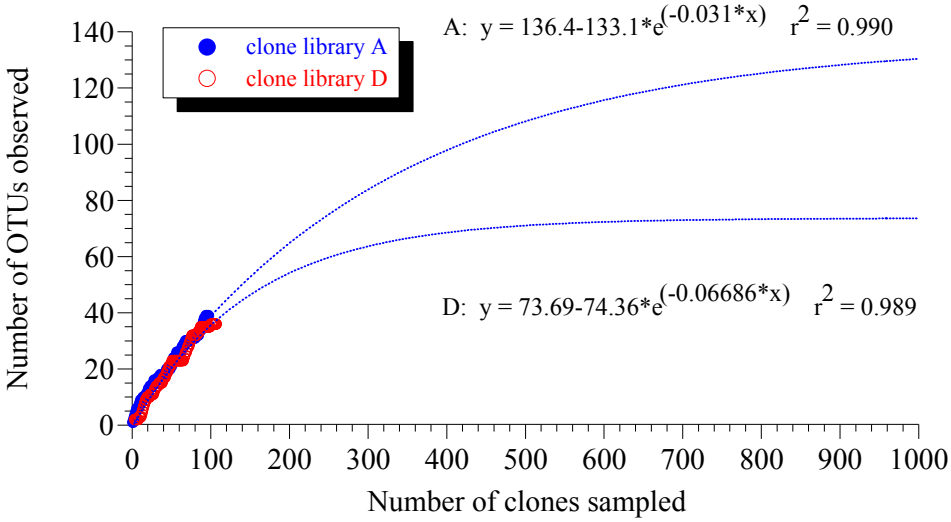

Fig. 3.21: Cumulative difference of OTUs observed versus the number of clones for the clone-libraries A and D and non-linear regression of the raw-data using a negative exponential function.

The non-linear regression of the raw-data, using equ. 3.1, estimates a richness of approximately 136 OTUs for clone library A and 74 OTUs for clone library D. The analytical effort to reach the saturation area of the curves can be estimated to be in the order of approximately 400 clones (clone library A) and 1000 clones (clone library D). These numbers have to be sampled to reliably obtain the "whole" richness of OTUs (detectable with the chosen approach) within the environmental sample.

Although both curve fits show quite good correlation coefficients of $r^2 = 0.99$ (clone library A) and $r^2 = 0.989$ (clone library D), respectively, the raw-data measured are still within the linear area of the curves. Therefore, these estimates should not be used to determine the absolute richness expected, but can provide a figure about the effort that would be necessary to detect more OTUs, as well as an order of OTUs that could be detected in the environmental sample, using the chosen method. Slight changes within the

raw data could result in different curve fits leading to different values for the estimated richness.

Another way to compare how well communities have been sampled is to plot their rank-abundance curves, wherein the abundance (number of clones per OTU) is plotted versus the richness (number of different OTUs). The OTUs are ordered from the most to the least abundant on the x-axis, and the abundance of each type observed is plotted on the y-axis. The rank-abundance plot for the clone libraries A and D is shown in Fig. 3.22. In clone library A, 5 OTUs were represented by 4-16 clones and 26 OTUs by unique clones. Clone library D comprised two abundant OTUs with 37 and 12 clones, respectively, 13 OTUs with 2-4 clones and 20 OTUs with single types.

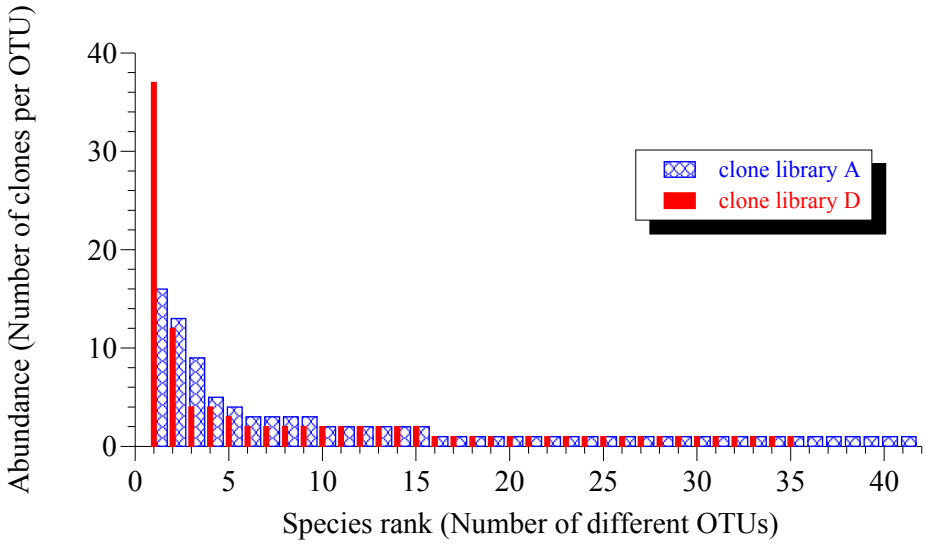

Fig. 3.22: Rank-abundance plots for the clone libraries A and D

Another approach to describe microbial communities is the rarefaction method (Dunbar *et al.*, 1999). Rarefaction compares observed richness among sites, treatments, or habitats that have been unequally sampled. A rarefied curve results from average randomisations of the observed accumulation curve (Heck *et al.*, 1975). The variance around the repeated randomisations allows one to compare the observed richness among samples, but it is distinct from a measure of confidence about the actual richness in the communities.

The raw-data of the clone libraries A and D were used to perform a rarefaction analysis using the software Analytic Rarefaction 1.3 (Holland, 2001). The results are shown in Fig. 3.23.

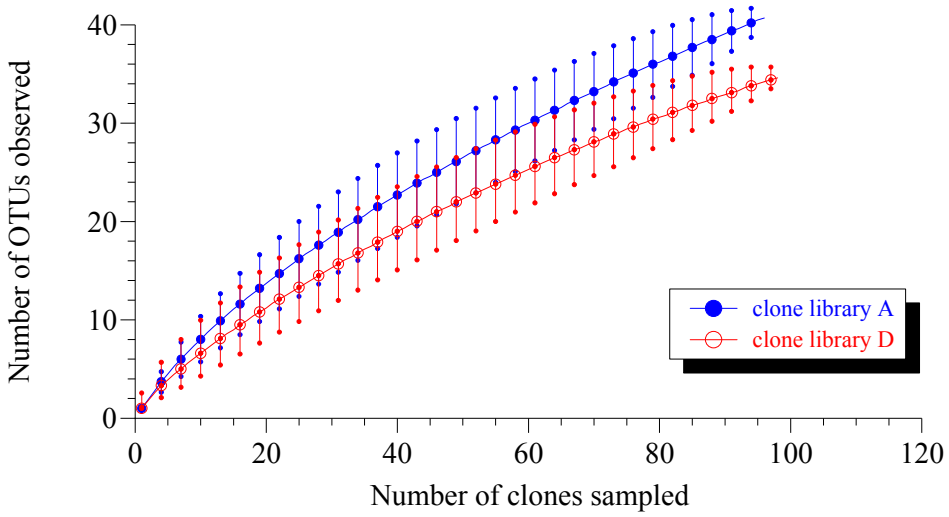

Fig. 3.23: Rarefaction analysis of observed OTU richness in the clone-libraries A and D. The error bars give the 95% confidence intervals.

Fig. 3.23 demonstrates that more OTUs were observed in clone-library A than in D. At the highest shared sample size (96 clones), 41 (40.7) OTUs were observed in clone library A versus 34 (34.2) OTUs in clone library D. However, rarefaction curves do not address the precision of the observed species richness. Thus, although the rarefaction curves suggest that the clone library A is more diverse than the clone library D, one cannot address the statistical significance of this evidence with rarefaction curves.

3.3. Cultivation-dependent analysis of microbial diversity

3.3.1. Analysis of *Archaea* isolates derived from other haloalkaline environments

In this study, various haloalkaliphilic isolates of halobacteria, derived from different highly saline and alkaline habitats, such as the Wadi Natrun, Egypt, and Owens Lake, California, were analysed using a molecular approach, including *Taq*I-ARDRA (amplified rDNA restriction analysis) and sequence analysis of 16S rRNA genes.

3.3.1.1. Halobacteria

The term "halobacteria" refers to the extremely halophilic Archaea, members of the family *Halobacteriaceae* (order *Halobacteriales*) (Grant & Larsen, 1989). The most striking feature of the halobacteria is their absolute requirement for high concentrations of NaCl. Although some strains may grow at salt concentrations as low as 1.5 M, most of the strains grow best at concentrations of 3.5-4.5 M and grow well in saturated NaCl (5.2 M). Halobacteria are among the most halophilic organisms known and comprise the dominant microbial population when hypersaline waters approach saturation (Rodríguez-Valera *et al.*, 1981), frequently imparting a red or pink coloration to the brines. The organisms accumulate mainly KCl, up to 5 M, in order to compensate for the high salt concentrations in the environment, and may be growth limited by the amount of KCl in media (Grant *et al*., 2001). The majority of halobacteria examined to date have retinal-based pigments capable of the light-mediated translocation of ions across the cell membrane (Grant *et al.*, 2001). Bacteriorhodopsin, as a light-driven, outward-directed proton pump, and halorhodopsin, as an inward-directed chloride pump, became models for energy conversion leading to the conservation of light energy in a form directly usable by the cell (Oren, 1998). It is probable that all halobacteria possess halorhodopsin and the photoreceptor pigments, but not all possess bacteriorhodopsin (Grant *et al.*, 2001). Because halobacteria can also grow in the dark, the systems are, presumably, not essential. Colonies of most strains are various shades of red due to the presence of C_{50} carotenoids (bacterioruberins) that impart red or pink coloration to mass developments in the natural environment. The importance of this reddening in promoting rapid precipitation of sea salt has also been documented. It is now known that the carotenoid pigments of halobacteria trap solar radiation, increasing the ambient temperature and evaporation rates in salterns (Jones *et al.* 1981). The characteristic red bacterioruberins, possessed by most naturally occurring isolates, seem to play a protective role against photooxidative damage caused by the strong sunlight where these organisms are found (Wu *et al*., 1983). Colorless strains are rarely reported, but have been found, for example, in the genus *Natrialba*, e.g., strain 172P1, isolated from beach sand in Japan

(Kamekura *et al*., 1992), *Natrialba aegyptiaca*, isolated from salt soil in Egypt (Hezayen *et al*., 2001) and *Natrialba taiwanensis* (classified initially as *N. asiatica* strain B1T), isolated from salts produced in solar salterns in Taiwan (Hezayen *et al.*, 2001; Kamekura & Dyall-Smith, 1995).

The halobacteria can be distinguished from other extremely halophilic procaryotes by their archaeal characteristics, particularly the possession of ether-linked phosphoglycerides. The lipids of all halobacteria examined to date contain phytanyl ether analogues of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. Many strains also contain glycerol sulfate. One or more glycolipids and sulfated glycolipids are also present in most strains including a sulfated tetraglycosyl diether, triglycosyl diethers and diglycosyl diethers. All halobacteria have diphytanyl (C_{20}–C_{20}) glycerol core lipids, although some strains have additional phytanyl-sesterterpanyl (C_{20}–C_{25}) glycerol ether core lipids (Kamekura and Dyall-Smith, 1995). Isopreniod quinones are of the menanquinone type (MK8 and $MK8H_2$), not the ubiquinone type (Collins *et al*., 1981).

Strains of aerobic, extremely halophilic members of the *Archaea* have been isolated from various hypersaline environments, such as hypersaline lakes (e.g., Oren *et al*., 1995; Xin *et al*., 2000), soda lakes (e.g., Soliman & Trüper, 1982; Tindall *et al*., 1984), solar salterns (e.g. Montalvo-Rodríguez *et al*., 1998; Nuttall & Dyall-Smith, 1993), saline soils (e.g., Zvyagintseva & Tarasov, 1987), beach sands (Kamekura & Dyall-Smith, 1995), salt mines (e.g., Denner *et al*., 1994; Norton *et al*., 1993) or fish and hides (Klebahn, 1919; Petter, 1931).

Taxonomy of halobacteria

Before the 1970s, halobacterial taxonomy was mainly based on standard biochemical tests and morphology (Gibbons, 1974). At the end of the 1970s 16S rRNA-DNA hybridisation studies demonstrated that the halobacteria should be classified into nine clades of two groups (Ross & Grant, 1985). The polar lipid compositions had proven particularly useful in the classification of halobacteria (Ross & Grant, 1985; Torreblanca *et al*., 1986). Polar lipid analysis and 16S rRNA-DNA hybridisation studies not only produced groupings of six genera of halobacteria but also induced a further reclassification of some uncertain halobacterial species (Grant & Larsen, 1989). Many halobacteria have been described since, which, together with the reassessment of existing taxa using genotypic methods, has led to the taxonomy of the group being in a state of flux (McGenity *et al*., 1998). However, the use of genotypic parameters alone to describe taxa was considered to be not very accurate, and the combination of genotypic and phenotypic features, known as polyphasic taxonomy, is now promoted for the delineation of taxa (Oren *et al*., 1997). At present, the following fourteen generic names have been

validly published within the family *Halobacteriaceae*: *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Halorubrum*, *Natrialba*, *Halobaculum*, *Halogeometricum*, *Haloterrigena*, *Natrinema*, *Natronomonas*, *Natronobacterium*, *Natronococcus*, and *Natronorubrum* (Grant *et al.*, 2001; Kamekura *et al*., 1997; McGenity *et al.*, 1998; Montalvo-Rodríguez *et al.*, 1998; Oren *et al.*, 1997; Ventosa *et al*., 1999).

Halobacteria constitute a monophyletic group with the most distantly related species having 83.2% 16S rRNA gene sequence similarity, indicating the genotypic diversity of the halobacteria. The methanogens are the closest relatives (Olsen *et al*., 1994) but still have less than 80% 16S rRNA gene sequence similarity to halobacteria. Probably, halobacterial diversity extends beyond the fourteen formally described genera when uncultivated halobacteria are considered. For example, Benlloch *et al*. (1995) described two 16S rRNA clones from a crystallizer pond, forming a distinct phylogenetic group of sequence types, and Munson *et al.* (1997) obtained several distinct halobacterial cloned sequences from salt marshes. A common feature of all members of the genus *Haloarcula* is the presence of at least two heterogeneous copies of the 16S rRNA gene, differing in sequence similarity by up to 5% (Dennis *et al*., 1998). This property has been reported in the species *Haloarcula marismortui* A and B (94.5% similarity), "*Haloarcula sinaiiensis*" major and minor (97.3%), two genes for *Haloarcula vallismortis* (96.2%) and *Haloarcula hispanica* (Kamekura, 1998). Recently, the expression of two 5% different 16S rRNA genes in individual cells of *Haloarcula marismortui* was shown by fluorescence in situ hybridisation (Amann *et al.* 2000).

Alkaliphilic halobacteria

The alkaliphilic members of the *Halobacteriaceae* form a distinct physiological group, as they require high NaCl concentrations, high pH (between 8.5 and 11) and low Mg^{2+} concentrations (less than 10 mM) for growth. They have been isolated from a variety of alkaline, hypersaline lakes and soils (see Table 3.19). Microscopically, the initial isolates consisted of rods and cocci and were accordingly separated into the two genera, *Natronobacterium* and *Natronococcus* (Tindall *et al.*, 1984). All haloalkaliphilic archaea characterised to date have C_{20}–C_{25} diether core lipids of phosphatidyl glycerol (PG) and phosphatidyl glycerol phosphate (PGP), together with minor amounts of unidentified phospholipids (Morth & Tindall, 1985; Tindall, 1985; Xu *et al.*, 1999). On the other hand, DNA-DNA hybridisation experiments have indicated that they share relatively little genomic DNA similarity (31-38% binding only) (Mwatha & Grant, 1993). Studies using 16S rRNA(-gene) sequences indicated that the alkaliphilic halobacteria, originally classified in the genera *Natronobacterium* and *Natronococcus*, are genotypically more diverse than one would have predicted (Kamekura, 1998; Kamekura *et al.*, 1997). Re-

examination of the data of Morth & Tindall (1985) and Tindall (1985) suggest a significant correlation between the 16S rRNA and chemotaxonomic data. Duckworth *et al.* (1996) have isolated a variety of alkaliphilic Archaea from hypersaline soda lakes Natron, Little Lake Magadi and Magadi, all of which were, on the basis of 16S rDNA sequence comparisons, relatively closely associated with species of the genera *Natronococcus*, *Natrialba* or *Natrinema*. More recently, Grant *et al*. (1999) described several 16S rDNA cloned sequence types from the brines of the final crystallising pond of an alkaline saltern at Lake Magadi, Kenya. These cloned sequences showed only 88-90% similarity to any cultivated haloalkaliphilic Archaea.

Table 3.19: Representatives of alkaliphilic species of halobacteria, sources of isolation, and references.

Species/strain	**Strain**	**Source**	**Reference**
Natrialba magadii	NCIMB 2190^T	Lake Magadi, Kenya	(Tindall *et al.*, 1984)
Natrialba sp. strain SSL1	ATCC43988^T	Sambhar Salt Lake, India	(Upasani & Desai, 1990)
Natrialba sp.	strain 98NT4	Lake Natron, Kenya	(Duckworth *et al.*, 1996)
Natronobacterium gregoryi	NCIMB 2189^T	Lake Magadi saltern, Kenya	(Tindall *et al.*, 1984)
Natronococcus amylolyticus	JCM 9655^T	Lake Magadi, Kenya	(Kanai *et al.*, 1995)
Natronococcus occultus	NCIMB 2192^T	Lake Magadi, Kenya	(Tindall *et al.*, 1984)
Natronococcus sp.	strain 86M4	Lake Magadi, Kenya	(Duckworth *et al.*, 1996)
Natronococcus sp.	strain 89M4	Lake Magadi, Kenya	(Duckworth *et al.*, 1996)
Natronococcus sp.	strain 931LM4	Little Lake Magadi, Kenya	(Duckworth *et al.*, 1996)
Natronomonas pharaonis	JCM 8858^T	Soda lake, Wadi Natrun, Egypt	(Soliman & Trüper, 1982)
Natronorubrum bangense	A33^T	Soda lake, Tibet	(Xu *et al.*, 1999)
Natronorubrum tibetense	GA33^T	Soda lake, Tibet	(Xu *et al.*, 1999)
Halorubrum vacuolatum	JCM 9060^T	Lake Magadi, Kenya	(Mwatha & Grant, 1993)

Type strains are denoted by "^{T}".

3.3.1.2. ARDRA fingerprinting

The diversity of haloalkaliphilic soda lake isolates was estimated using a PCR 16S rDNA restriction analysis (ARDRA) with the tetrameric restriction enzyme *Taq*I. The 39 halobacterial isolates were clustered into eight different groups of identical ARDRA profiles (see Table 3.20), indicated by Roman numerals. Some of these ARDRA groups showed quite similar restriction patterns, and were, therefore, marked by a small letter following the Roman numeral (see Table 3.22). Additionally, various haloalkaliphilic reference strains of the family *Halobacteriaceae* were included in the amplified rDNA

restriction analysis (see Table 3.21). In some cases (i.e., restriction types I, IIa and III), the ARDRA profiles of certain reference strains corresponded exactly with those obtained for the haloalkaliphilic soda lake isolates or were, at least, similar to each other (patterns Va and Vb). The most frequently encountered ARDRA type (defined IIa) was observed for the reference strain *Natrialba magadii* and for a total of twelve haloalkaliphilic isolates, including isolates from Owens Lake and from the Wadi Natrun. The second abundantly represented group of identical ARDRA patterns (type I) comprised ten haloalkaliphilic isolates from both soda lake habitats, as well as the reference organism *Natronomonas pharaonis*. A third group of isolates, comprising four strains from Owens Lake, possessed identical ARDRA patterns with *Halorubrum vacuolatum*. However, the remaining six ARDRA types represented "new" patterns differing from those of reference halobacteria included in the analysis. Notably, these ARDRA groups comprised exclusively isolates of one of the two soda lake habitats studied.

Table 3.20: Arrangement of haloalkaliphilic isolates in restriction type groups (indicated by Roman numerals) based on amplified ribosomal restriction analysis (ARDRA) using *Taq*I.

Isolate	Restriction Type	Source	Reference strains with same ARDRA pattern
OS2	**I**	Owens Lake, California	***Natronomonas pharaonis***
O3A	**I**	Owens Lake, California	
O1Bh	**I**	Owens Lake, California	
O1C	**I**	Owens Lake, California	
O3C	**I**	Owens Lake, California	
O6G	**I**	Owens Lake, California	
O3G	**I**	Owens Lake, California	
W2A	**I**	Wadi Natrun, Egypt	
W3A	**I**	Wadi Natrun, Egypt	
W1C	**I**	Wadi Natrun, Egypt	
O1A	**IIa**	Owens Lake, California	***Natrialba magadii***
O2B	**IIa**	Owens Lake, California	
O5B	**IIa**	Owens Lake, California	
O1YE	**IIa**	Owens Lake, California	
OSt1	**IIa**	Owens Lake, California	
W1A	**IIa**	Wadi Natrun, Egypt	
W4A	**IIa**	Wadi Natrun, Egypt	
W2B	**IIa**	Wadi Natrun, Egypt	
W2C	**IIa**	Wadi Natrun, Egypt	
W2G	**IIa**	Wadi Natrun, Egypt	
W3YE	**IIa**	Wadi Natrun, Egypt	
WSt1	**IIa**	Wadi Natrun, Egypt	
W1YE	**IIb**	Wadi Natrun, Egypt	
W1G	**IIb**	Wadi Natrun, Egypt	
O4A	**III**	Owens Lake, California	***Halorubrum vacuolatum***
O1G	**III**	Owens Lake, California	
OS1	**III**	Owens Lake, California	
O4G	**III**	Owens Lake, California	
WSt2	**IVa**	Wadi Natrun, Egypt	
W2YE	**IVa**	Wadi Natrun, Egypt	
W1B	**IVa**	Wadi Natrun, Egypt	
W4B	**IVa**	Wadi Natrun, Egypt	
O4B	**IVb**	Owens Lake, California	
O5G	**IVb**	Owens Lake, California	
O2C	**Va**	Owens Lake, California	
O4C	**Va**	Owens Lake, California	
O5C	**Va**	Owens Lake, California	
O6C	**Va**	Owens Lake, California	
W3B	**VI**	Wadi Natrun, Egypt	

Table 3.21: Reference strains of haloalkaliphilic organisms used in the *Taq*I ARDRA analysis and the affiliations to restriction type groupings (indicated by Roman numerals).

Reference species	**Strain**	**Restr. type**	**Source**	**Reference**
Natronomonas pharaonis	HP^T (DSM 2160^T)	**I**	Saline, Lake Magadi, Kenya	(Soliman & Trüper, 1982)
Natronomonas pharaonis	SP1 (DSM 3395)	**I**	Saline, Lake Magadi, Kenya	(Tindall *et al.*, 1984)
Natronococcus occultus	DSM 3396^T	**VII**	Lake Magadi, Kenya	(Tindall *et al.*, 1984)
Natronococcus occultus	SP4^T	**VII**	Lake Magadi, Kenya	(Tindall *et al.*, 1984)
Natronococcus amylolyticus	DSM 10524^T	**VIII**	Lake Magadi, Kenya	(Kanai *et al.*, 1995)
Natronobacterium gregoryi	SP2^T (DSM 3393^T)	**Vb**		(Tindall *et al.*, 1984)
Natrialba magadii	MS3^T	**IIa**	Lake Magadi, Kenya	(Tindall *et al.*, 1984)
Halorubrum vacuolatum	DSM 8800^T	**III**	Lake Magadi, Kenya	(Mwatha & Grant, 1993)

Table 3.22: Restriction types (indicated by Roman numerals) and corresponding lengths of fragments (number of nucleotides) experimentally determinded by *Taq*I amplified restriction analysis (ARDRA) of haloalkaliphilic isolates and reference strains.

	ARDRA-Types										
	I	**IIa**	**IIb**	**III**	**IVa**	**IVb**	**Va**	**Vb**	**VI**	**VII**	**VIII**
Length	711	932	933	715	715	677	932	750	1190	773	750
	679	463	455	462	256	256	750	462	159	256	256
	48	48	48	217	217	217	462	182	48	158	186
				48	158	158	182	48		48	159
					48	48	48				48
∑	1438	1443	1436	1442	1394	1395	2374	1442	1397	1235	1399

3.3.1.3. 16S rDNA sequence determination

Partial 16S rDNA sequences from one to six randomly chosen representatives of each restriction type group were determined (at least 450 nucleotides at the five prime ends of the genes) or nearly completely (approximately 1,490 nucleotide positions), and were compared with sequences available from the DNA databases. The results of almost complete sequence analysis are summarised in Table 3.23 and Fig. 3.24. Sequences were observed to cluster within three major lineages of existing halobacteria, i.e., the genera *Natronomonas* (Kamekura *et al.*, 1997) and *Halorubrum* (McGenity & Grant, 1995), as well as the relatively diffuse cluster of halobacteria including *Natronobacterium gregoryi* and related organisms, which was temporarily called the "Natro group" (McGenity *et al.*, 1998). Notably, all sequences analysed possessed relatively high similarity values (93.1% to 98.8%) to 16S rDNA sequences from known, cultivated halobacteria.

Table 3.23: Nearest neighbours of haloalkaliphilic isolates.

Group	Isolate	Restriction type	similarity (%)	Nearest neighbours	Accession no.
Natronomonas					
	OS2	I	98.8	*Natronomonas pharaonis* JCM 8858^T	D87971
Halorubrum					
	O4A	III	95.8	*Halorubrum vacuolatum* JCM 9060^T	D87972
	O4B	IVb	94.9	*Halorubrum saccharovorum* JCM 8865^T	U17364
	WSt2	IVa	96.2	*Halorubrum saccharovorum* JCM 8865^T	U17364
"Natro group"					
	O2B	IIa	99.3	*Natrialba hulunbeirensis* AS1.1986^T	AF262026
	O2C	Va	94.3	*Natronobacterium gregoryi* NCIMB 2189^T	D87970
	W1YE	IIb	93.7	*Natrinema versiforme* XF10^T	AB023426
	W3B	VI	93.1	*Natronococcus amylolyticus* Ah-36^T	D43628

Almost complete 16S rRNA genes (*E. coli* 16S rRNA gene sequence positions 24 to 1,491) from representatives of each restriction group were sequenced and compared with those available from the DNA databases. The closest sequences from environmental isolates and organisms whose names have been validly published were determined. Type strains are denoted by "^{T}".

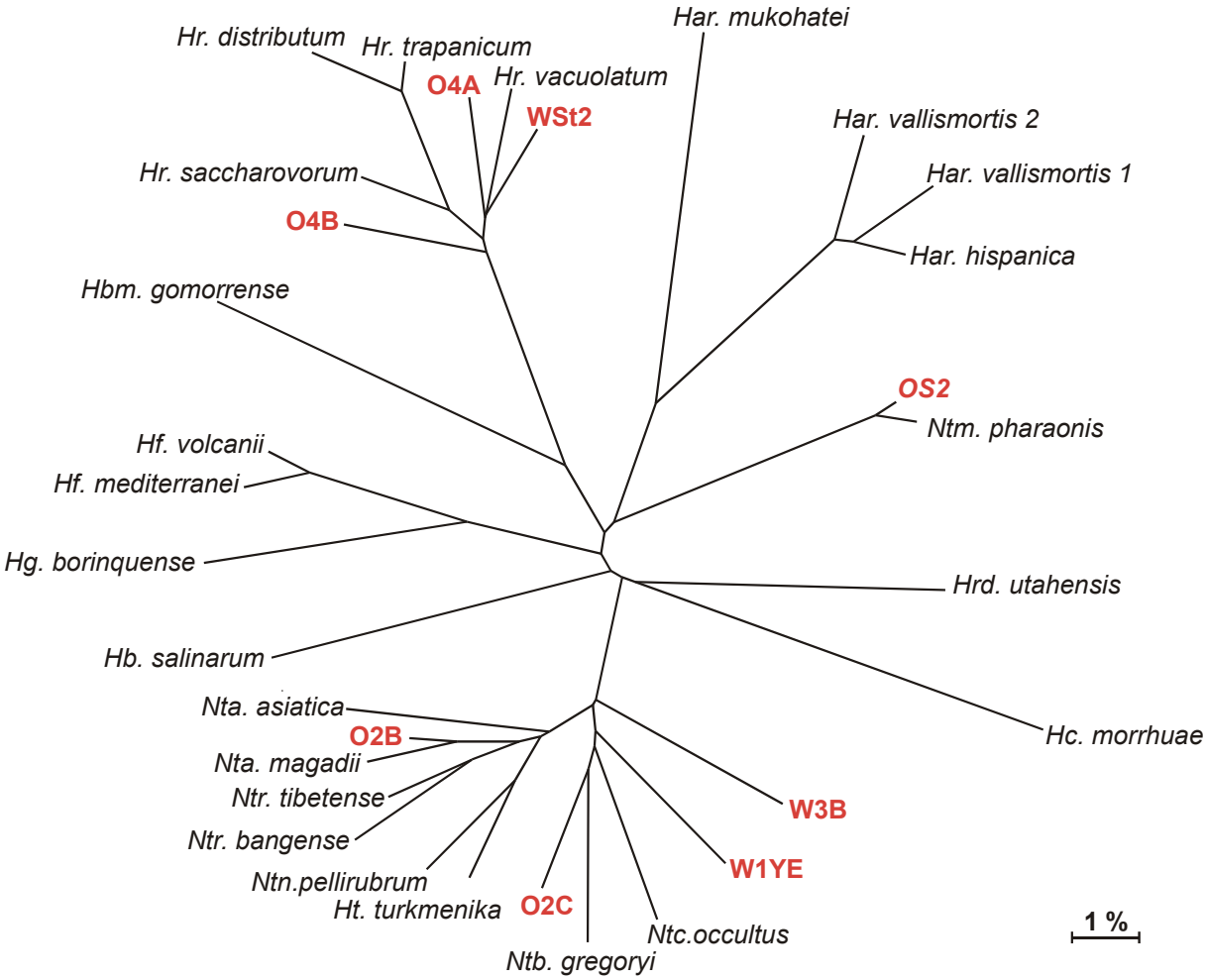

Fig. 3.24: Reconstructed unrooted tree based on 16S rDNA sequence data, indicating the inferred phylogenetic positions of haloalkaliphilic soda lake isolates (printed in red) from Owens Lake, California, and Wadi Natrun, Egypt, within the radiation of the *Halobacteriaceae*. The tree was constructed from 1318 unambiguously determined nucleotide positions, corresponding to positions 24 to 1474 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.* 1978), that were common to all 16S rDNA sequences used in the analysis. The scale bar represents one inferred nucleotide change per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.24. Abbreviations: *Har.*, *Haloarcula*; *Hb.*, *Halobacterium*; *Hbm.*, *Halobaculum*; *Hc.*, *Halococcus*; *Hf.*, *Haloferax*; *Hg., Halogeometricum;*. *Hr.*, *Halorubrum*; *Hrd., Halorhabdus;* *Ht.*, *Haloterrigena*; *Nta.*, *Natrialba*; *Ntb.*, *Natronobacterium*; *Ntc.*, *Natronococcus*; *Ntm.*, *Natronomonas*; *Ntn.*, *Natrinema*; *Ntr.*, *Natronorubrum.*

Table 3.24: Bacterial strains, references and accession numbers of sequences used in the 16S rRNA(-gene) analysis including similarity calculations and generation of trees.

Species	**Strain**	**Accession no.**	**Source**	**Reference**
Haloarcula hispanica	ATCC 33960^T	U68541	Saltern, Alicante, Spain	(Arahal *et al.*, 1996)
Haloarcula mukohatei	JCM 9738^T	D50850	Salt flat, Salinas Grandes Argentina	(Ihara *et al.*, 1997)
Haloarcula vallismortis no.1	ATCC 29715^T	U17593	Salt flat, Death Valley, USA	(Kamekura & Dyall-Smith, 1995)
Haloarcula vallismortis no.2	ATCC 29715^T	D50851	Salt flat, Death Valley, USA	(Ihara *et al.*, 1997)
Halobaculum gomorrense	DSM 9297^T	L37444	Dead Sea	(Oren *et al.*, 1995)
Halobacterium salinarum	NRC 34001	K02971	Salted buffalo hide	(Hui & Dennis, 1985)
Halobacterium salinarum	DSM 3754^T	this study	Salted cow hide	this study
Halococcus morrhuae	ATCC 17082^T	X00662	Dead Sea	(Leffers & Garrett, 1984)
Haloferax mediterranei	ATCC 33500^T	D11107	Saltern, Alicante, Spain	(Kamekura & Seno, 1992)
Haloferax volcanii	ATCC 29605^T	K00421	Dead Sea	(Gupta *et al.*, 1983)
Halogeometricum borinquense	ATCC 700274^T	AF002984	Solar salterns, Cabo Rojo, Puerto Rico	(Montalvo-Rodríguez *et al.*, 1998)
Halorhabdus utahensis	AX-2^T	AF071880	Great salt lake, UT, USA	(Wainø *et al.*, 2000)
Halorubrum distributum	JCM 9100^T	D63572	Sulphate saline soil, Turkmenia	(Kamekura & Dyall-Smith, 1995)
Halorubrum trapanicum	NCIMB 13488	this study		
Halorubrum saccharovorum	JCM 8865^T	U17364	Saltern, San Francisco, USA	(Kamekura & Dyall-Smith, 1995)
Halorubrum vacuolatum	JCM 9060^T	D87972	Lake Magadi, Kenya	(Kamekura *et al.*, 1997)
Haloterrigena turkmenica	VKM B-1734^T	AB004878	Sulfate saline soil, Turkmen, Turkmenistan	(Ventosa *et al.*, 1999)
Haloterrigena thermotolerans	PR5^T	AF115478	Solar salterns, Cabo Rojo, Puerto Rico	(Montalvo-Rodríguez *et al.*, 2000)
Natrialba aegyptiaca	DSM 13077^T	AF251941	Salt soil, Aswan, Egypt	(Hezayen *et al.*, 2001)
Natrialba asiatica	JCM 9576^T	D14123	Beach sands, Japan	(Kamekura & Dyall-Smith, 1995)
Natronobacterium chahannaoensis	AS 1.1988 ^{T}	AJ004806	Chahannao soda lake, China	(Xu *et al.*, 2001)
Natrialba hulunbeirensis	AS1.1986 ^{T}	AF262026	Soda lake, Hulunbeir, China	(Xu *et al.*, 2001)
Natrialba magadii	NCMB 2190^T	X72495	Lake Magadi, Kenya	(Lodwick *et al.*, 1991)
Natrialba sp. strain SSL1	ATCC 43988^T	D88256	Sambhar Salt Lake, India	(Kamekura *et al.*, 1997)
Natrialba sp. strain 98NT4	not deposited	X92174	Lake Natron. Kenya	(Duckworth *et al.*, 1996)
Natrialba taiwanensis	JCM 9577^T	D14124	Solar salts	(Hezayen *et al.*, 2001)

Species	Strain	Accession no.	Source	Reference
Natrinema pallidum	NCIMB 777^T	AJ002949	Salted cod	(McGenity *et al.*, 1998)
Natrinema pellirubrum	NCIMB 786^T	AJ002947	Salted hide	(McGenity *et al.*, 1998)
Natrinema versiforme	XF10^T	AB023426	Aibi salt lake, China	(Xin *et al.*, 2000)
Natrinema sp. GSL11	GSL11	D14126	Great Salt Lake, UT, USA	(Kamekura & Dyall-Smith, 1995)
Natronobacterium gregoryi	NCIMB 2189^T	D87970	Lake Magadi saltern, Kenya	(Kamekura *et al.*, 1997)
Natronococcus amylolyticus	Ah-36^T	D43628	Lake Magadi, Kenya	(Kanai *et al.*, 1995)
Natronococcus occultus	NCIMB 2192^T	Z28378	Lake Magadi, Kenya	(McGenity & Grant, 1993)
Natronomonas pharaonis	JCM 8858^T	D87971	Soda lake, Wadi Natrun, Egypt	(Kamekura *et al.*, 1997)
Natronorubrum bangense	AS 1.1984^T	Y14028	Soda lake, Tibet	(Xu *et al.*, 1999)
Natronorubrum tibetense	AS 1.2123^T	AB005656	Soda lake, Tibet	(Xu *et al.*, 1999)
Other				
Natrialba sp. tunisia	HMg-25	AB049461		(unpublished)
Natrialba sp. tunisia	HMg-27	AB049462		(unpublished)
"Natronococcus xinjiangense"	not deposited	AF251285	Aiding Lake, Xinjian	(unpublished)
"Natronobacterium innermongoliae"	AS 1.1985 (HAM-2)	AF009601	Hamatai soda lake, China	(Tian *et al.*, 1997)
"Natronobacterium wudunaoensis"	Y21	AJ001376	Hypersaline ponds, China	(unpublished)
"Natronorubrum sp. Wadi Natrun19"		AB046926	Wadi Natrun	(unpublished)
clone MSP9		AB012051	Magadi salt pond	(Grant *et al.*, 1999)
clone MSP14		AB012054	Magadi salt pond	(Grant *et al.*, 1999)
clone MSP16		AB012055	Magadi salt pond	(Grant *et al.*, 1999)
clone MSP23		AB012058	Magadi salt pond	(Grant *et al.*, 1999)

Some species names were included, indicated by quotation marks, that are not yet validly published. Type strains of species are denoted by "^{T}".

Natronomonas

One frequently observed group, comprising ten haloalkaliphilic isolates from soda lakes, was affiliated with reference sequences of the genus *Natronomonas*. The nearly complete 16S rDNA sequence of the haloalkaliphilic isolate OS2 from Owens Lake was determined as a representative of this ARDRA group (defined I). Comparative sequence analysis showed that OS2 was affiliated (98.8% similarity) with the alkaliphilic species *Natronomonas pharaonis*, originally isolated from the alkaline brines of eutrophic desert lakes of Wadi Natrun, Egypt (Soliman & Trüper, 1982) and later from Kenyan soda lake (Magadi) as strain SP1 (Tindall *et al.*, 1984). On the basis of their 16S rDNA sequences, *Natronomonas pharaonis* and isolate OS2 are distantly related to the other taxa of the family *Halobacteriaceae*, exhibiting similarity values less than 90%.

Halorubrum

The second abundantly represented group, comprising ten haloalkaliphilic soda lake isolates, was found to be affiliated on the basis of their 16S rDNA sequences with members of the genus *Halorubrum* (McGenity & Grant, 1995). The isolates O4A, O4B and WSt2 were sequenced nearly completely as representatives of three different ARDRA patterns (III, VIb and IVa, respectively). The Owens Lake isolate O4A was most similar to the 16S rDNA sequence of *Halorubrum vacuolatum* (95.8%), isolated from Lake Magadi (Mwatha & Grant, 1993) and possessing the same ARDRA pattern (III). A further isolate from Owens Lake (O4B) and the Wadi Natrun isolate WSt2 were most closely affiliated (94.9% and 96.2 % 16S rDNA sequence similarities) with the sequence of the organism *Halorubrum saccharovorum,* isolated from marine salterns (Tomlinson & Hochstein, 1976).

On the basis of 16S rDNA sequence comparisons, the genus *Halorubrum* was found to be quite diverse, with three main clusters, containing firstly *Halorubrum saccharovorum* and *H. lacusprofundi* (98.3% 16S rDNA sequence similarity), secondly, *H. sodomense*, *H. trapanicum*, *H. coriense* and *H. distributum* (>97.7% 16S rDNA sequence similarity), and thirdly, *H. vacuolatum*, the most deeply branching species (Grant *et al.*, 2001). All known species, except *H. vacuolatum,* are neutrophilic and *H. vacuolatum* is presently the only representative of the genus with gas vacuoles. The polar lipids of the six neutrophilic species are C_{20}-C_{20} derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, and sulfated mannosyl-glucosyl-glycerol diether (Kamekura & Dyall-Smith, 1995; Ross & Grant, 1985). *Halorubrum vacuolatum*, however, has both C_{20}-C_{20} and C_{20}-C_{25} derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, and two unidentified phospholipids but has no glycolipid (Mwatha & Grant, 1993), although the authors give no information on the relative composition. *Halorubrum* species have been isolated from hypersaline environments varying widely in chemical and physical properties, e.g., salterns, salt mines, Antarctic salt lakes, the Dead Sea, Lake Magadi and Solonchak soil.

"Natro group"

The most frequently encountered group of haloalkaliphilic soda lake isolates, including O2B, O2C, W1YE, and W3B (restriction types IIa, Va, IIb, and VI, respectively) as representatives of a total of 19 isolates, was affiliated on the basis of their 16S rDNA sequences with organisms of a rather diffuse assemblage of halobacteria temporary called the "Natro group" (McGenity *et al.*, 1998), comprising the genera *Natronobacterium*, *Natronococcus*, *Natrialba*, *Natronorubrum*, *Natrinema*, and *Haloterrigena* (Fig. 3.25).

Reference species of this cluster were observed to be more than 89.5% similar in their 16S rDNA sequences. Most of the halobacteria of this cluster are alkaliphiles, but all presently known members of the genera *Natrinema* (McGenity *et al.*, 1998) and *Haloterrigena* (Ventosa *et al.*, 1999) are neutrophilic, although, some *Natrinema* strains are able to grow slowly at pH 8.6. The genera *Haloterrigena* and *Natrinema* were created independently from each other to accommodate a number of strains formerly classified in other genera and a few new isolates. Since some strains have been assigned to both genera, and may thus overlap, additional studies will be required to reassess the taxonomic status of the two genera. The genus *Natrialba* is the first genus within this cluster which is comprised of both neutrophilic and alkaliphilic strains.

The haloalkaliphilic isolate O2B from Owens Lake, California, showed the highest 16S rDNA similarity values with organisms of the genus *Natrialba*, including the haloalkaliphilic species *Natrialba hulunbeirensis* (99.3%) and *Natrialba chahannaoensis* (98.6%), both isolated from soda lakes in the Inner Mongolia Autonomous Region, China (Xu *et al.*, 2001). Additionally, high similarity values (98.6 to 99.1%) were obtained for isolate O2B and a number of haloalkaliphilic strains, whose names have not yet been validly published, including *"Natronobacterium innermongoliae"*, *"Natronobacterium wudunaoensis"* and *Natrialba* sp. strain SSL1, originally isolated from an alkaline saline brine from Sambhar Salt Lake, India (Upasani & Desai, 1990). These strains might belong to the genus *Natrialba* as judged from the 16S rDNA tree reconstruction (see Fig. 3.25). Isolate O2B possessed the same ARDRA pattern (IIa) as *Natrialba magadii*, and the 16S rDNA sequences exhibited 97.1% similarity to each other. Also, O2B showed a remote affiliation to other members of the genus *Natrialba* including the neutrophilic species *Natrialba aegyptiaca* (95.8) and *Natrialba taiwanensis* (95.4%) (Hezayen *et al.*, 2001), wherein *Natrialba taiwanensis*, isolated by Kamekura & Dyall-Smith (1995) and classified initially as *Natrialba asiatica* strain B1T, is able to grow at alkaline pH.

The 16S rDNA sequence of the Owens Lake isolate O2C (ARDRA group Va) was most similar to that of the alkaliphilic species *Natronobacterium gregoryi* (95.1%). Until now, members of the genus *Natronobacterium* have been isolated only from the solar salt pans at Lake Magadi (Tindall *et al.*, 1980 and 1984) and there are no reports that members of the genus occur in other alkaline, highly saline environments. The two Wadi Natrun isolates W3B and W1YE (ARDRA groups VI and IIb, respectively) were on the basis of 16S rDNA analysis most closely affiliated with members of the genus *Natronococcus*, including the Lake Magadi isolates *Natronococcus occultus* and *Natronococcus amylolyticus* (Kanai *et al.*, 1995; Tindall *et al.*, 1984) and the Aiding Lake isolate "*Natronococcus xinjiangense"*, by exhibiting similarity values ranging from

92.2 to 93.2%. The isolates W3B and W1YE showed 93.7% 16S rDNA sequence similarity to each other.

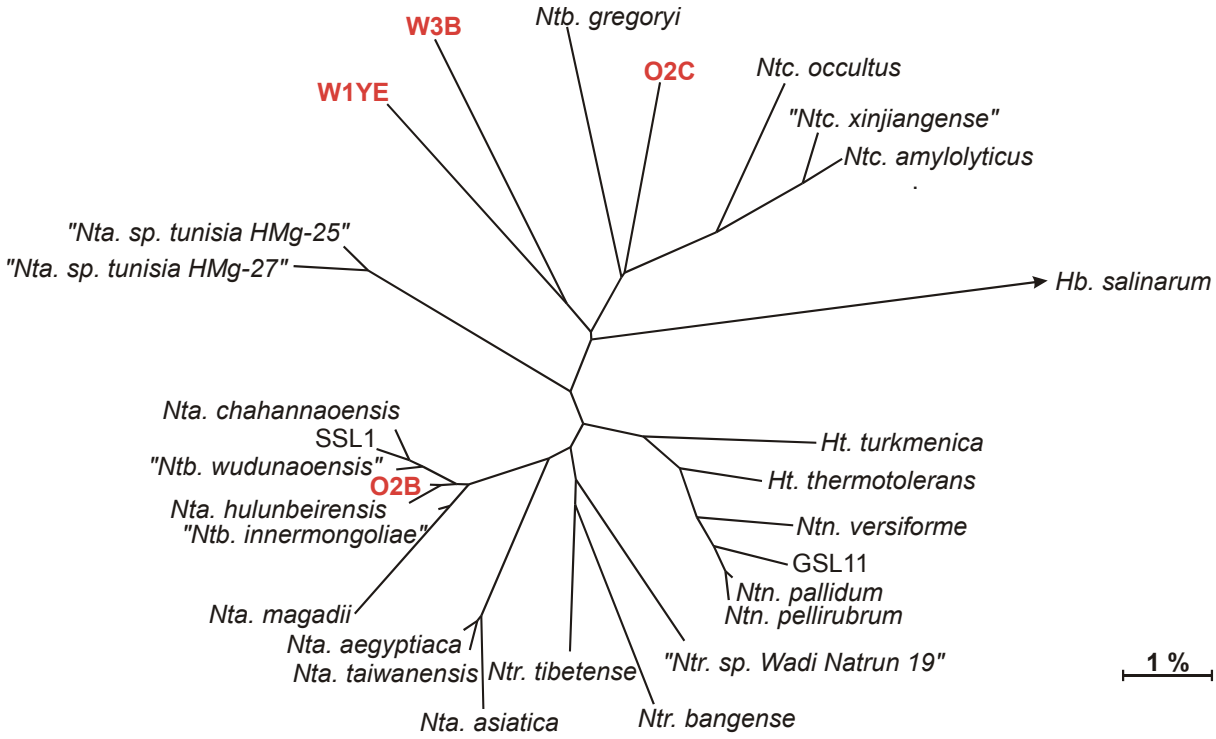

Fig. 3.25: Reconstructed tree, based on 16S rDNA sequence data, indicating the inferred phylogenetic positions of haloalkaliphilic soda lake isolates (printed in red) from Owens lake, California, and Wadi Natrun, Egypt, within the **"Natro group"** of halobacteria. The tree was constructed on the basis of 1333 unambiguously determined positions, corresponding to positions 24 to 1474 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.* 1978), that were common to all 16S rDNA sequences used in the analysis. The sequence of *Halobacterium salinarum* DSM 3754^{T} was used as an outgroup. The scale bar represents one inferred nucleotide change per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.24. Species names that are not yet validly published are indicated by quotation marks. Abbreviations: *Hb.*, *Halobacterium*; *Ht.*, *Haloterrigena*; *Nta.*, *Natrialba*; *Ntb.*, *Natronobacterium*; *Ntc.*, *Natronococcus*; *Ntn.*, *Natrinema*; *Ntr.*, *Natronorubrum.*

3.3.2. Analysis of aerobic sporeformers isolated from soda lake samples

A set of fourteen aerobic and sporeforming strains, isolated from sediment samples of the two Kenyan soda lakes, Lake Magadi and Lake Natron, were analysed on the basis of ARDRA (amplified rDNA restriction analysis) and subsequent sequence determination and comparison of their 16S rRNA genes. Almost complete 16S rRNA genes (*E. coli* 16S rRNA gene sequence positions 28 to 1491) were amplified using template DNA generated according to the rapid protocol for preparation of genomic DNA from single colonies (see chapter 2.2.2.). At the outset of the analysis the *Taq*I ARDRA approach was applied as a screening method to obtain indicative 16S rDNA sequence information, that were used to estimate the diversity of the fourteen isolates, and to select strains for more detailed 16S rDNA studies.

3.3.2.1. ARDRA fingerprinting

The ARDRA profiles of 16S rDNAs (digested with *Taq*I) of the strains isolated from sediments of Lake Magadi and Lake Natron formed five groups with distinctive patterns (Table 3.25 and Fig. 3.26).

Table 3.25: Aerobic sporeforming isolates AS1 to AS15 and original strain designations.

Isolate	Orig. Code	Restriction Type	Source
AS 1	**2A1**	**A**	Lake Natron, site 2
AS 5	**1.2-2-1**	**A**	Lake Natron, site 1
AS 9	**1AN6-1**	**A**	Lake Natron, site 1
AS 12	**1AN7**	**A**	Lake Natron, site 1
AS 2	**2AN13**	**B**	Lake Natron, site 2
AS 3	**1.2-1-1**	**C**	Lake Natron, site 1
AS 7	**2.53**	**C**	Lake Natron, site 2
AS 8	**2.91**	**C**	Lake Natron, site 2
AS 14	**9.31-1.**	**C**	Lake Magadi, site 9
AS 15	**2.92-1**	**C**	Lake Natron, site 2
AS 4	**1-2-2**	**D**	Lake Natron, site 1
AS 10	**9.41**	**D**	Lake Magadi, site 9
AS 11	**2.92-2**	**D**	Lake Natron, site 2
AS 13	**9.1-1-2**	**E**	Lake Magadi, site 9

Isolates analysed by *Taq*I ARDRA and the affiliations to restriction type groupings (indicated by capital letters A to E).

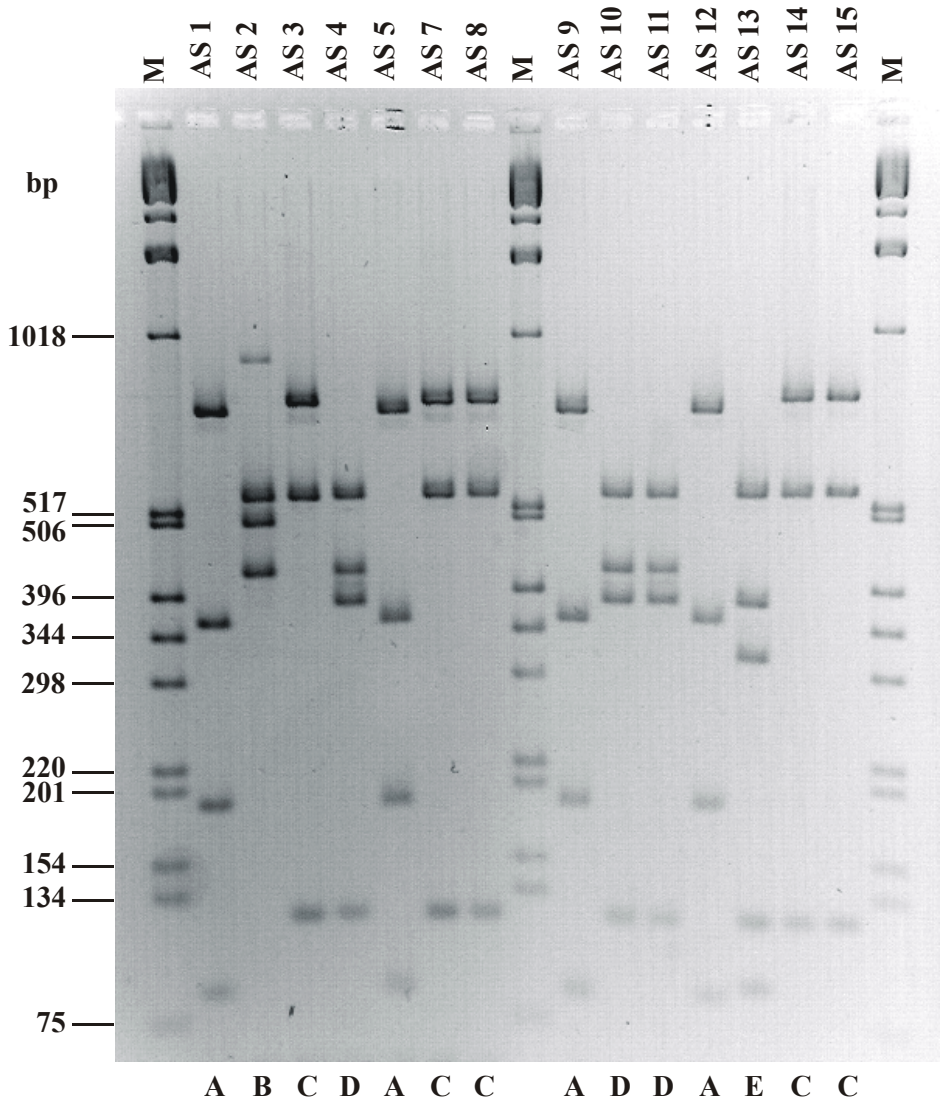

Fig. 3.26: Photograph of ethidium bromide stained agarose gel of *Taq*I ARDRA patterns from aerobic sporeforming isolates (defined AS1 to AS15). Lanes: M, molecular weight DNA markers with molecular sizes indicated on the left in base pairs; all other lanes are PCR amplified 16 rDNAs digested with *Taq*I. Capital letters below each lane correspond to ARDRA pattern designations as used in Table 3.25.

3.3.2.2. 16S rDNA sequence determination

Subsequent to the first screening of the fourteen soda lake isolates by ARDRA fingerprinting, partial 16S rDNA sequences from one to three randomly chosen representatives of each restriction type were determined (at least 450 nucleotides at the five prime ends of the genes) or nearly completely (comprising approximately 1,490 nucleotides) and were compared with sequences available from the DNA databases. The results of sequence analysis are summarised in Table 3.26. Sequences were observed to cluster within two major groups of the established lineages of bacteria: mainly the low G+C Gram-positive bacteria (*Bacillus* and relatives) and the gamma subdivision of the

Proteobacteria. Notably, all sequences analysed showed relatively high similarities (98.9% to 99.6%) to 16S rDNA sequences from cultivated and characterised organisms, with the exception of isolate AS2 exhibiting maximal 95% sequence similarity to any known species.

One frequently encountered group of 16S rDNA sequence types was affiliated with sequences of organisms in the rRNA group 6 of *Bacillus* (Ash *et al.*, 1993; Nielsen *et al.*, 1994) and comprised isolates AS3, AS14 and AS15, all of them belonging to the ARDRA type C. These sequences showed 100% identity to each other and were most closely related (99% similarity) to the sequence of the obligate alkaliphilic and extremely halotolerant (up to 17% NaCl) soil isolate *Bacillus pseudofirmus* (Nielsen *et al.*, 1995).

Table 3.26: Nearest neighbours of aerobic sporeforming soda lake isolates.

Isolate	ARDRA type	Length of seq. (bp)	similarity (%)	Nearest neighbour	Accession no.
AS 1	**A**	481	99.0	*Halomonas desiterata* DSM 9502^T	X92417
AS 5	**A**	460	98.9	"	"
AS 12	**A**	472	98.9	"	"
AS 2	**B**	1,490	95.0	*Gracilibacillus halotolerans* DSM 11805^T	AF036922
AS 3	**C**	456	99.3	*Bacillus pseudofirmus* DSM 8715^T	X76439
AS 14	**C**	488	99.2	"	"
AS 15	**C**	469	99.1	"	"
AS 4	**D**	511	99.4	*Bacillus agaradhaerens* DSM 8721^T	X76445
AS 10	**D**	496	99.6	"	"
AS 11	**D**	485	99.4	"	"
AS 13	**E**	1,492	99.3	*Bacillus halodurans* ATCC 27557^T	AB021187

The sequences of 16S rRNA genes from representatives of each *Taq*I ARDRA group (defined A to E) were determined almost completely or partially (at the five prime ends of the genes) and were compared with those available from the DNA databases.

Additionally, Lake Magadi isolate AS13 (the sole representative of ARDRA type E) was affiliated with group 6 of *Bacillus*, and showed 99.3% sequence similarity to the obligate alkaliphilic soil isolate *Bacillus halodurans* (Nielsen *et al.*, 1995), but merely 91.5% to members of the ARDRA group C. The second abundantly represented group of 16S rDNA sequences clustering within *Bacillus* consisted of isolates AS4, AS10 and

AS11, all belonging to restriction group D. These sequences were closely related to each other (approximately 99% similarity) and were most closely associated (at least 99.0%) with sequences of organisms forming a distinct group of obligately alkaliphilic *Bacillus* strains, including the soil isolates *B. agaradhaerens*, *B. clakii* (Nielsen *et al.*, 1994 and 1995), as well as *B. vedderi* (Agnew *et al.*, 1995) isolated from bauxite waste.

The 16S rDNA sequence of isolate AS2, possessing the unique ARDRA type B, was observed to be loosely associated with sequences of the genus *Gracilibacillus* (Wainø *et al.*, 1999). The highest affiliation (95.0% sequence similarity) was observed with the extremely halotolerant species *Gracilibacillus halotolerans* isolated from Great Salt Lake, Utah (Wainø *et al.*, 1999).

The third commonly encountered group of sequence types comprised isolates AS1, AS5 and AS12, all members of restriction group A. These 16S rDNA sequences were closely affiliated with the *Halomonas* group of the gamma subdivision of Proteobacteria. The highest similarity values (of approximately 99%) were observed with the sequence of the alkaliphilic, halotolerant organisms *Halomonas desiderata* (Berendes *et al.*, 1996).

Peculiarities of 16S rDNA sequences

In this study, all partially or nearly completely determined 16S rDNA sequences of isolates belonging to the genus *Bacillus* and relatives were observed to exhibit low levels of ambiguous nucleotides at approximately 0.1 to 0.2% of the determined nucleotide positions. Most of the ambiguous nucleotides were located within certain helical regions of the RNA secondary structure or single-stranded loop regions and occurred at phylogenetically hypervariable positions (see Table 3.27). Surprisingly, the ambiguous positions were not complementary, i.e., these positions did not affect both components of a nucleotide base pair. Each 16S rRNA gene sequence in this study was confirmed by determining contiguous overlapping sequences of PCR-DNA, produced by two separate PCR reactions. All sequences, for the most part, have been determined in both the forward and reverse directions. Therefore, sequencing errors can be excluded, to a large extent, as an explanation for the occurrence of ambiguous nucleotides. Moreover, they may presumably represent interoperon variation within single strains.

Table 3.27: Occurrence of ambiguous nucleotide positions.

Isolate	ARDRA type	Length of seq. (bp)	ambiguous nucleotide	correspondin g nucleotide	*E. coli* positions	RNA secondary structure
AS 2	**B**	1,490	**R**	**T**	75:95	helix E
			M	**G**	186:191	helix N
AS 3	**C**	456	**K**	**T**	93:75	helix D
AS 14	**C**	488	**Y**	**G**	186:191	helix N
AS 15	**C**	469				
AS 4	**D**	511	**R**	**C**	93:75	helix E
AS 10	**D**	496				
AS 11	**D**	485				
AS 13	**E**	1,492	**Y**	**-**	845	loop
			R	**A**	1442:1460	helix E

Occurrence of ambiguous nucleotide positions within 16S rDNA sequences of aerobic sporeforming soda lake isolates, clustering within the radiation of *Bacillus* and relatives. Additionally, the corresponding nucleotides of base pairs within a certain helical region of the RNA secondary structure are included. *Escherichia coli* positions according to Brosius *et al*. 1978.

3.3.3. Analysis of a unicellular cyanobacterium isolated from Lake Magadi

A unicellular unicyanobacterial, non-axenic culture was analysed by molecular characterisation, including a non-radioactive colony hybridisation approach and 16S rDNA sequence analysis. The cyanobacterial culture (Z-9405) was isolated from samples of water with mass development of phytoplankton collected in the littoral waters of Lake Magadi at the end of the rainy season of 1993 (Dubinin *et al.*, 1995). Morphologically, the cyanobacterial culture was assigned to *Synechococcus elongatus* according to the traditional classification. The optimal parameters for growth of the moderate halophilic strain Z-9405 were determined as 13.0% NaCl, 2.5% Na_2CO_3, pH 10.1, 2293 mg-equivalent/l alkalinity, and 17.4% salinity (Dubinin *et al.*, 1995).

The increasing interest toward this group of halophilic aerobic oxygenic phototrophic microorganisms is motivated by both, their substantial role in the primary productivity of the hypersaline habitats and the perspective of their biotechnological exploitation. The production of specific metabolites, such as new exopolysaccharides of industrial interest, could be a promising field for application of halotolerant cyanobacterial species (de Philippis *et al*., 1998; Sudo *et al*., 1998).

Traditional techniques for the identification and systematics of cyanobacteria have relied primarily on observed morphological characteristics. However, the cell morphology of a species may change depending on environmental conditions (e.g., Dubinin *et al.*, 1995; Evans *et al*., 1976). Photosynthetic pigment content, lipid composition, differentiated cell structures and other characters may also be subject to change because of variable expression of cyanobacterial gene products in culture (Kenyon *et al*., 1972). The availability of nucleic acid sequence data from cyanobacteria is today forming the basis for new identification techniques such as restriction fragment length polymorphism (Lyra *et al*., 1997; Neilan *et al*., 1997b), PCR (Nübel *et al*., 1997; Rudi *et al*., 1997), denaturing gradient gel electrophoresis (Ferris *et al*., 1996; Nübel *et al.*, 1997), and *in situ* hybridisation (Schönhuber *et al.*, 1999). Other molecular approaches that have been described to identify certain groups of cyanobacteria include the analysis of genes encoding phycocyanin (Neilan *et al*., 1995) or nitrogenase (Ben-Porath & Zehr, 1994) and the detection of a repetitive DNA sequence in toxin-producing heterocystous cyanobacteria (Rouhiainen *et al*., 1995).

The purification of cyanobacteria still can be a difficult and time-consuming procedure and often they are cultivated more easily when accompanied by heterotrophic bacteria (Castenholz & Waterbury, 1989). Therefore, some strains growing as unicyanobacterial but non-axenic cultures have been included in the bacteriological classification system (Castenholz & Waterbury, 1989). The molecular analysis of such non-axenic unicyanobacterial cultures poses some difficulties, since they constitute

mixed bacterial cultures. Several different approaches have been described to enable the analysis of 16S rRNA from non-axenic cyanobacterial cultures. These include special isolation techniques such as antibiotic treatment to suppress heterotrophic bacteria or physical cleaning of cyanobacteria by micromanipulation (Garcia-Pichel *et al.*, 1996). Furthermore, molecular approaches were applied to separate cyanobacterial from other bacterial 16S rRNA genes including selective PCR (Nübel *et al.*, 1997), or molecular cloning combined with screening for inserts of interest (Nelissen *et al.*, 1994). The PCR procedure enables the selective retrieval of cyanobacterial 16S rRNA gene fragments that can be sequenced directly. A drawback of this method is that the amplification products comprise only 700 nucleotides, i.e., approximately 50% of the 16S rRNA gene. In contrast to that, molecular cloning seems to be more time-consuming, although this method leads to more sequence information. Therefore, the almost complete 16S rRNA gene of the unicyanobacterial culture Z-9405 from Lake Magadi was analysed using a molecular cloning approach combined with a powerful screening method based on non-radioactive colony hybridisation with an oligonucleotide probe.

3.3.3.1. Generation of a 16S rDNA clone library

Cells were harvested from 0.5 ml of hypersaline viscous culture by centrifugation. DNA was extracted according to the standard protocol for preparation of genomic DNA from microbial cultures (see chapter 2.2.1.). The crude DNA extract with a concentration of approximately 200 ng/µl was gel-purified (see chapter 2.2.3.) and 5 µl of a 1+24 dilution (in TE-buffer) were used as template for amplification of 16S rRNA genes by PCR in a 100 µl reaction volume. Nearly complete 16S rRNA genes were amplified by using conventional primers 16F27 and 16R1492, targeting highly conserved regions of the 16S rRNA genes and containing the appropriate tails for subsequent cloning. Since PCR yields a heterogeneous mixture of amplification products from a non-axenic culture, different DNA molecules were gel-purified and separated by cloning in *E. coli*, using the regenerated pDIRECT vector. The transformation of 0.5 ml competent cells resulted in roughly 350 white clones, while no blue or faint blue clones occurred.

3.3.3.2. Screening of transformants by using colony hybridisation

The 16S rDNA clone library was screened for plasmid inserts of interest, using a non-radioactive colony hybridisation approach: colony blotting, including transfer of colonies onto uncharged nylon-membranes, lysis of cells, followed by denaturation and immobilisation of cellular DNA, performed according to the protocol described above. A filter comprising approximately 50 blotted colonies was hybridised with a Biotin-labelled oligonucleotide probe (Cyano-u) specific for cyanobacteria. Hybridisation with Cyano-u

combined with chemiluminescence detection revealed 16 positive and 32 negative clones (see Fig. 3.27).

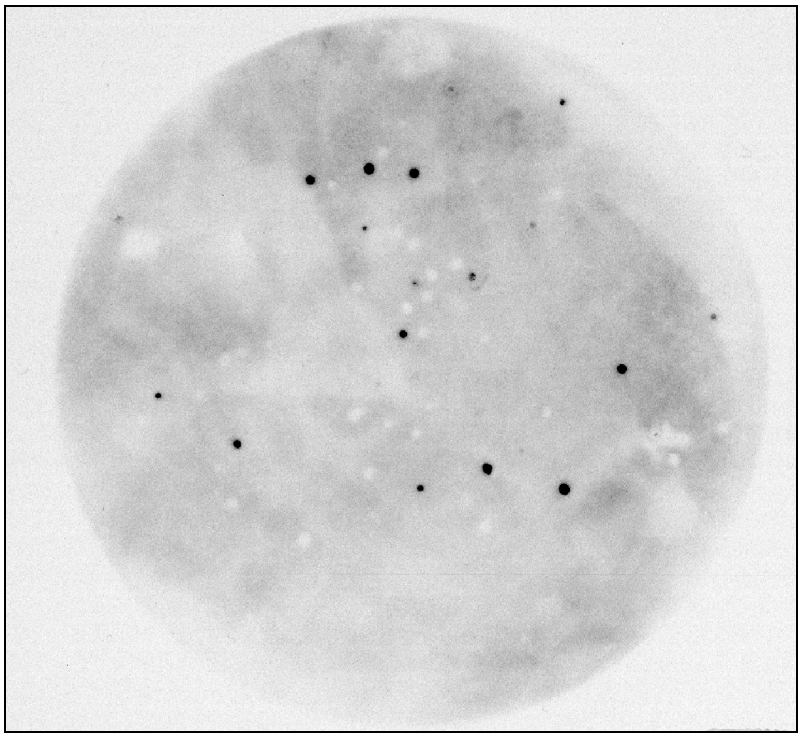

Fig. 3.27: Colony hybridisation detecting *E. coli* transformants with cyanobacterial 16S rDNA inserts. The colony blot was hybridised with a Biotin-labelled oligonucleotide probe (Cyano-u) specific for cyanobacteria and detected with chemiluminescence.

3.3.3.3. Analysis of 16S rRNA gene sequences

Partial 16S rDNA sequences from five randomly selected positive clones were determined using sequencing primer 16SR518. Since these partial sequences possessed 100% identity to each other, a single 16S rDNA insert was sequenced completely as a representative. The nearly complete 16S rRNA gene sequence (comprising 1,475 nucleotides) of the unicellular cyanobacterial strain Z-9405 was found to be most closely associated with reference sequences of cyanobacteria in the "Euhalothece" group (Garcia-Pichel *et al.*, 1996). The maximal similarity value (98.6%) was obtained for strain MPI 95AH10, an extremely halotolerant unicellular cyanobacterium, isolated from benthic gypsum crusts in solar evaporation ponds in Eilat, Israel. Notably, strain Z-9405 was almost identical (99.8% similarity) to the cloned sequence type B12 (clone library B), obtained from Lake Magadi sediment samples. Fig. 3.28 shows the estimated position of strain Z-9405 within the radiation of cyanobacteria, on the basis of 16S rDNA sequence analyses.

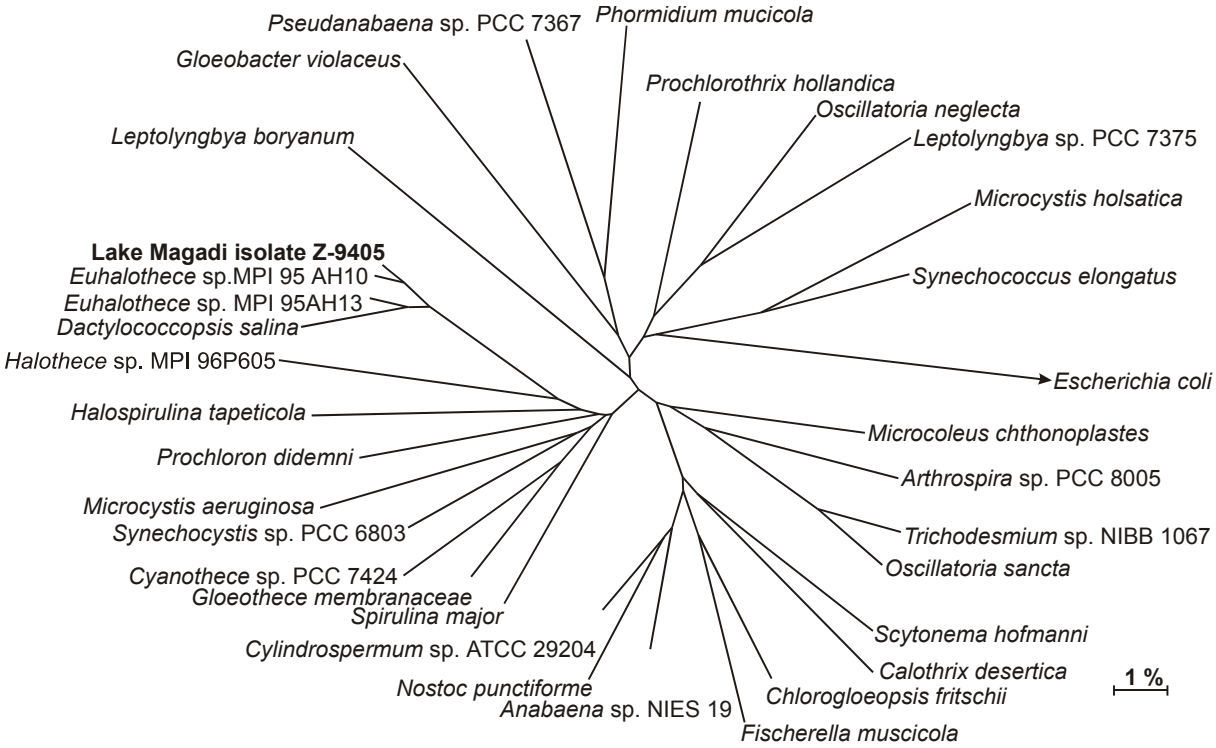

Fig. 3.28: Reconstructed tree, indicating the inferred phylogenetic position of Lake Magadi isolate Z-9405 within representative species of the Cyanobacteria, based on their 16S rRNA gene sequences. The tree was constructed on the basis of 1323 unambiguously determinded nucleotide positions, corresponding to positions 38 to 1455 (*Escherichia coli* 16S rRNA gene sequence numbering, Brosius *et al.* 1978), that were common to all of the 33 almost complete 16S rDNA sequences used in the analysis. The 16S rRNA gene from *Escherichia coli* was used as an outgroup sequence. The scale bar represents one inferred nucleotide change per 100 nucleotides. Accession numbers of 16S rDNA sequences included in the tree are indicated in Table 3.4.

4. Summary and concluding remarks

A molecular, cultivation-independent, approach has been utilised for an analysis of the procaryotic diversity and community structure in sediment samples from Lake Magadi, an African soda lake in the Rift Valley, Kenya. Ecosystems such as soda lakes represent unusual natural environments about which very little is known of the bacterial and archaeal diversity and ecology. The extreme conditions, i.e., pH values above 11 combined with total salinities to saturation (30% w/v or greater), as well as marked temperature extremes, provide a selection for microorganisms whose ecological activities and biotechnological potential are not well known or understood.

Cultivation-independent analysis

The molecular approach for the analysis of procaryotes inhabiting soda lakes allows for the detection of a broad taxonomic spectrum of individuals within a complex microbial community without the requirement for the cultivation and isolation of the organisms. The molecular analysis was accomplished through the general scheme: a) extraction of total DNA from sediment samples; b) PCR-amplification using primers specific for *Bacteria* or *Archaea* to target 16S rRNA genes (rDNA); c) ligation-independent cloning of the PCR-products; d) screening of the clone libraries using hybridisation probing, ARDRA (Amplified Ribosomal DNA Restriction Analysis) fingerprinting and partial sequencing of the cloned 16S rDNA; e) sequence analysis of entire 16S rDNA.

Several new protocols were developed and optimised to support the analyses. A ligation independent cloning (LIC) vector was developed for the directional cloning of PCR products without restriction enzyme digestion or ligation reactions. Additionally, a protocol for colony hybridisation was established, allowing for the application of non-radioactive hybridisation experiments, using single-end-labelled oligonucleotide probes for rapid screening of 16S rDNA clone libraries.

The analysis of a bacterial, PCR-amplified 16S rDNA clone library generated from serially-diluted template DNA extracted from Lake Magadi sediment samples, allowed the "identification" of the microorganisms expected to be predominant within the environmental sample. Sequence analysis revealed the detection of 16S rDNA sequence types clustering, predominantly, with the taxa of cyanobacteria, particularly the "*Euhalothece"*, as well as other taxa, including Gram-positive bacteria with low G+C-content (*Firmicutes*), i.e., *Bacilli* and *Clostridia*, members of the *Halanaerobiales* and *Thermoanaerobacteriales*, as well as the alpha-, gamma-, and delta-subclasses of the

Proteobacteria and the "*Cytophaga-Flavobacter-Bacteroides*" (CFB) phylum. Approximately 55% of the sequence types found in this clone library were highly similar (greater than 95% sequence similarity) to database entries, while 18% showed only slight relationships (90-95% sequence similarity), and approximately 27% of the sequences showed less than 90% similarity to other known sequences. Based upon the results of the initial screening, specific oligonucleotide probes were generated to target sequences detected in high frequency in the clone libraries. Hybridisation experiments combined with ARDRA analysis enabled a rapid screening of bacterial clone libraries, detecting cloned sequence redundancy, and allowing the focus to be directed at the analysis of sequences of special interest, e.g., new sequence types.

Screening of cloned sequence types of the archaeal 16S rDNA clone library, using ARDRA fingerprinting and partial sequence analysis, detected different sequence types clustering within the *Euryarchaeota*, mainly, with organisms of the family *Halobacteriaceae* (order *Halobacteriales*, class *Halobacteria*), indicating a diversity of new species and genera, presumably, belonging to the family *Halobacteriaceae*. A minority of three cloned sequence types was observed to possess a remote affiliation to members of the *Methanococci*.

Cultivation-dependent analysis

A unicellular, unicyanobacterial, non-axenic culture, representing a predominant species isolated from Lake Magadi was analysed by molecular characterisation, including non-radioactive colony hybridisation and 16S rDNA sequence analysis. The almost complete 16S rDNA sequence of the unicellular cyanobacterial strain Z-9405 was observed to be closely affiliated (98.6% similarity) with members of the *"Euhalothece"* group, encompassing unicellular, extremely halotolerant cyanobacteria. Furthermore, strain Z-9405 was almost identical (99.8% similarity) to the cloned sequence type B12, obtained from Lake Magadi sediment samples.

Another fourteen aerobic sporeforming strains, isolated from sediment samples of Lake Magadi and Lake Natron, Kenya, were analysed on the basis of ARDRA and sequence analysis. Sequences were detected clustering within two major groups of established lines of bacteria: mainly the group of Gram-positive bacteria with low G+C content (*Firmicutes*) and the gamma subdivision of the Proteobacteria. All sequences analysed showed relatively high similarities (98.9% to 99.6%) to 16S rDNA sequences from

cultivated and characterised organisms, with the exception of isolate AS2, exhibiting less than 95% sequence similarity to any known species.

Additionally, 39 haloalkaliphilic archaeal isolates of the halobacteria, derived from different saline and alkaline habitats, such as the Wadi Natrun (Egypt) and Owens Lake (California) were analysed by molecular, genotypic characterisation, including *Taq*I-ARDRA and sequence analysis of 16S rRNA genes. These organisms were observed to be affiliated with three major lines of existing *Halobacteria*, i.e., the genera *Natronomonas* and *Halorubrum*, as well as the rather diffuse cluster of halobacteria including *Natronobacterium gregoryi* and relatives, exhibiting 16S rDNA sequence similarity values in the range of 93.1 to 98.8%.

Conclusions

The strategy of total DNA extraction, PCR-amplification, screening of clone libraries and sequence determination of cloned 16S rRNA genes enabled the detection and recognition of here-to-fore unknown archaeal and bacterial sequence types from sediment samples of the extreme environment of Lake Magadi and provided new insights into the procaryotic composition of the soda lake environment. These data could serve as starting-points for the development of new cultivation techniques for as yet uncultivated microorganisms and unrecognised species.

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Abbreviations

ARDRA	amplified rDNA restriction analysis
bp	Base pair
CTAB	Cetyltrimethyl ammonium bromide
DNA	Desoxyribonucleic acid
dNTP	2'-desoxynucleoside-5'-triphosphate
EDTA	Ethylenediamine-tetraacetic acid
EMBL	European Molecular Biology Laboratory
IPTG	Isopropyl-β-thiogalactoside
kb	Kilo base
OPD	Oligonucleotide Probe Database
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
rDNA	Ribosomal desoxyribonucleic acid
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SLS	N-laurylsarcosine
SSU	Small subunit
St	Single type
Tris	Tris-(hydroxymethyl-)aminomethane
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

Culture Collections

ACAM	Australian Collection of Antarctic Microorganisms, Cooperative Research Centre for Antarctic and Southern Ocean Environment, University of Tasmania, GPO Box 252C, Hobart. Tas. 7001, Australia.
ACM	Australian Collection of Microorganisms, Department of Microbiology and Parasitology, The University of Queensland, Brisbane. Qld. 4072, Australia.
AS	Academia Sinica, China General Microbiological Culture Collection Centre, Zhong-guancun, Beijing 100080, China

ATCC	American Type Culture Collection, Manassas, VA, USA
CECT	Colección Espagñola de Cultivos Tipo, Universitat de Valencia, Edeficio de Investigación, Campus de Burjasot, 46100 Burjasot (Valencia), Spain.
DSM	=DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany
IAM	Institute of Applied Microbiology, University of Tokyo Yayoi, Bunko-Ku, Tokyo, Japan.
JCM	Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198, Japan.
LMG	= BCCM/LMG: Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Rijksuniversiteit, Ledeganckstraat 35, B-9000, Gent, Belgium.
NCDO	National Collection of Dairy Organisms. See: NCFB.
NCFB	National Collection of Food Bacteria (previously named NCDO). Transferred from the IFR (Institute of Food Research) Reading to National Collections of Industrial, Food and Marine Bacteria, 23 Machar Drive, Aberdeen, AB24 3RY, Scotland.
NCIM	National Collection of Industrial Microorganisms, National Chemical Laboratory, Poona 8, Maharashtra, India.
NCIMB	National Collection of Industrial and Marine Bacteria, National Collections of Industrial, Food and Marine Bacteria, 23 Machar Drive, Aberdeen, AB24 3RY, Scotland.
NCMB	National Collection of Marine Bacteria, Torry Research Station Aberdeen, Scotland, United Kingdom. See NCMB.
NRC	= NRCC: National Research Council of Canada Culture Collection, 100 Sussex Drive, Ottawa, Ontario K1A OR6, Canada.
PCC	Pasteur Culture Collection of Cyanobacteria, Unité de Physiologie Microbienne, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France.
VKM	All-Russian Collection of Microorganisms, Russian Academy of Sciences, Institute of Biochemistry and Physiology of Microorganisms, 142292 Pushchino, Moscow Region, Russia.

Appendix

A. PCR and Sequencing

PCR

Standard protocol for PCR assay.

compound	volumes per reaction	final concentration
10x PCR buffer [with: Tris/HCl (100 mM), $MgCl_2$ (15 mM), KCl (500 mM) Gelatine (0.01%, w/v)]	10 µl	1x
dNTPs (each 1.25 mM)	10 µl	800 µM
forward primer (12 µM)	2.5 µl	0.3 µM
reverse primer (12 µM)	2.5 µl	0.3 µM
template DNA	variable	500-900 ng
Taq-polymerase (5U/µl)	0.5 µl	2.5 U
H_2O	ad 100 µl	

Sequencing reaction

Standard sequencing reaction for AmpliTaq,FS BigDyeTerminator kit: (ABI Prism protocol 10/97):

reagent	quantity
DNA-template	
PCR-product (1.6 kb)	0.1-0.5 µg
plasmid-DNA	0.5-1.0 µg
Primer	10 pmol
Premix	4 µL
H_2O	ad 20 µl

Oligonucleotide primers for PCR and sequencing

Name	Sequence[a] (5'---3')	T_m [°C]	Annealing [°C]	Specifity
PCR primers				
16F27	AGAGTTTGATCMTGGCTCAG	59	60	most bacteria
16F23A	TCYGGTTGATCCTGCC	51	55	archaea, incl. korarchaeota
16R1492	TACGGYTACCTTGTTACGACTT	63	55/60	bacteria, archaea, incl. korarchaeota
PCR primers for cloning with pDirect-vector				
CT16F27	ctcgctcgcccaAGAGTTTGATCMTGGCTCAG	59	60	most bacteria
CT16F23A	ctcgctcgcccaTCYGGTTGATCCTGCC	51	55	archaea, incl. korarchaeota
CT16R1492	ctggttcggcccaTACGGYTACCTTGTTACGACTT	63	55/60	bacteria, archaea, incl. korarchaeota
Sequencing primers				
16F357	ACTCCTACGGGAGGCAGCAG	66	60	most bacteria
16F530	TTCGTGCCAGCAGCCGCGG	66	60	most bacteria, eukaryotes, archaea
16F945	GGGCCCGCACAAGCGGTGG	68	60	most bacteria
16F1103	TGTTGGGTTAAGTCCCGCAAC	64	60	most bacteria
16R518	CGTATTACCGCGGCTGCTGG	66	60	most bacteria
16R1087	CTCGTTGCGGGACTTAACCC	64	60	most bacteria
A16F340	GGGGCGCAGCAGGCGCG	64	60	archaea, incl. korarchaeota
A16F764	GGATTAGATACCCGGGTAGTC	64	60	archaea, incl. korarchaeota
A16F1017	TGAGAGGWGGTGCATGGCC	64	60	archaea, incl. korarchaeota
A16R514	CCGCGCCGGCTGGCACC	64	60	archaea, incl. korarchaeota
A16R1067	TCTCGCTCGTTGCCTGACTT	62	60	archaea, incl. korarchaeota
Plasmid primers				
T3	GCAATTAACCCTCACTAAAGGG	64	60	plasmid
T7	CGCGTAATACGACTCACTATA	58	60	plasmid

M=C:A, Y=C:T, all 1:1

B. Oligonucleotide probes

Oligonucleotide probes, sequences and target sites.

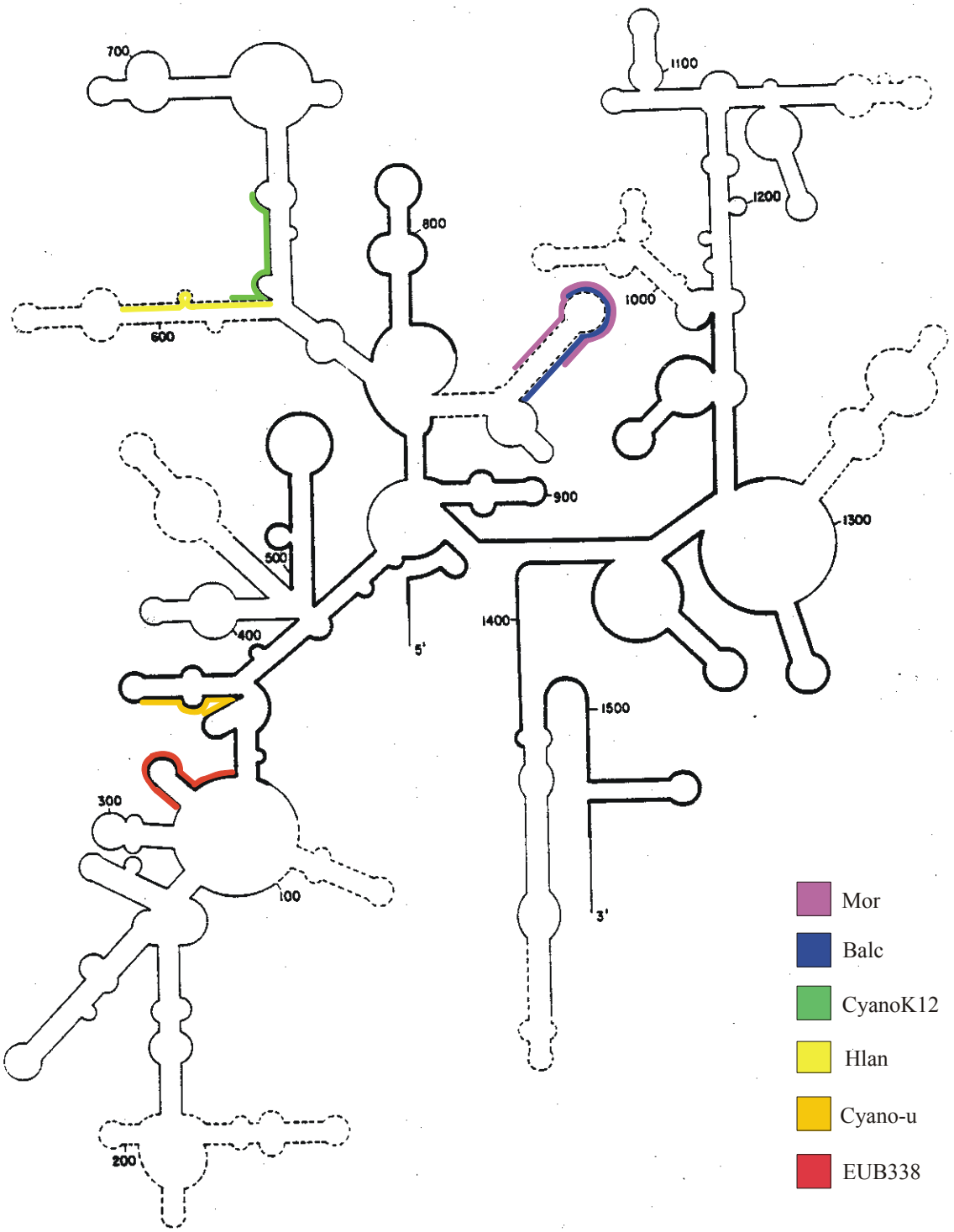
Probe	Specificity	Probe sequence 5' – 3'	*E.coli* positions[1)]
Cyano-u	most *Cyanobacteria*	CGCCCATTGCGGAAAATT	363-380
CyanoK12	"Halothece" cluster	CTGTCGCCCTCTAGCGCA	647-664
Mor	clostridial clonal sequence types	CCCACCAGTTTCAATGGC	633-650
Hlan	partly *Halanaerobium* group	TCGAACCYCCAACACCTA	827-844
Balc	partly *Bacillus* group VI	GGCACTAAGGGCATCGAA	841-857
EUB338[2)]	*Bacteria*	GCTGCCTCCCGTAGGAGT	338-355

Y=C:T (1:1)

Conditions for hybridisation and washing.

	Hybridisation		Washing	
Probe	**Formamide**	**Temperature**	**NaCl**	**Temperature**
Cyano-u	30%	50°C	100 mM	53°C
CyanoK12	30%	50°C	100 mM	53°C
Mor	45%	50°C	50 mM	53°C
Hlan	30%	50°C	100 mM	53°C
Balc	30%	50°C	100 mM	53°C
EUB338[2)]	25%	45°C	200 mM	48°

[1)] Brosius *et al.*, 1978
[2)] Amann *et al.*, 1990a



16S rRNA secondary structure model, indicating target regions (coloured) of the oligonucleotide probes used in hybridisation experiments. Highly conserved regions of the 16S rRNA molecule are indicated by fat black lines, less conserved regions by thin lines, and hypervariable regions by dotted lines.

C. Peculiarities of 16S rDNA sequences within the *Halanaerobiales*

Secondary structure I: *E.coli* positions 73 to 97

```
  PAIRING                          Eeeeeee .    . eeeeeeE
  E.coli                  ---------GAAGAAG--CUUG--CUUCUUU-----------------

Haloanaerobiaceae (long stem)
  clone D31               --CCTCAACAGATTCC--TTCG--GGATGACGATGAGAG---------
  clone D82               --CCTCAACAGATTCC--TTCG--GGATGAAGATGAGA----------
  clone D34               -CCATCGGTTTAGACC--TTCG--GGTCTTACCGGTGA----------
  clone D86p              ---------GCTTACC--TTCG--GGTAAG------------------
  clone B1                ---------GCTTACC--TTCG--GGTAAGT-----------------
  clone B83               ----CCCGGCGGAGCC--TTCG--GGTGAAGCCGGA------------
  Han.fermentans          --ccctgacagatacc--ttcg--ggttgaagacagga----------
  Han.kushneri            --cctcgacagaaacc--ttcg--ggttgacgacgaga----------
  Han.congolense          --cctcgacagaaacc--ttcg--ggttgaagacgaga----------
  Han.praevalens          --cctcgactgatacc--ttcg--ggttgaagacgaga----------
  Han.saccharolyticum     --cctcgactgaaacc--ttcg--ggttgacgacgaga----------
  Han.alcaliphilum        --cctcgactgaaacc--ttcg--ggttgatgacgaga----------
  Han.acetoethylicum      --cctcgactgaaacc--ttcg--ggttgacgacgaga----------
  Han.salsuginis          --CCCUGACAGAUACC--UUCG--GGUUGAAGACAGGA----------
  Han.lacusrosei          --cctcgactgatacc--ttcg--ggttgacgacgaga----------
  Hcl.cellulolytica       ---ccccgactgaacc--ttcg--ggatgacgacggga----------
  Htt.orenii              --ccccgactgaatcc--ttcg--ggatgacgacggga----------

Halobacteroidaceae (short stem)
  clone B69               -----------CTACC--TTCG--GGTAG-------------------
  clone D47               -----------TCACC--TTCG--GGTGA-------------------
  clone D46          -    -----------CTACC--TTCG--GGTAG-------------------
  clone D40p              -----------CTACC--TTCG--GGTAG-------------------
  clone D74p              -----------TCACC--TTCG--GGTGA-------------------
  clone D43p              -----------CTACC--TTCG--GGTAG-------------------

  Hbac.halobius           -----------ttacc--ttcg--ggtaa-------------------
  O.marismortui           -----------ttacc--ttcg--ggtaa-------------------
  O.salinaria             -----------ttacc--ttcg--ggtaa-------------------
  O.sivashensis           -----------ctacy--yycg--ggtag-------------------
  Hbac.elegans            -----------ctac---ttcg--ggtag-------------------
  Hla.salinarius          -----------ctacc--ttcg--ggtag-------------------
  Hla.chitinovorans       -----------ctacc--ttcg--ggtag-------------------
  Hla.lacunaris           -----------ctacc--ttcg--ggtag-------------------
  Na.acetigena            -----------ctacc--ttcg--ggtag-------------------
  Ach.arabaticum          ----------ctgctc--ttt---gagcag------------------
  Shb.lortetii            -----------CUGcc--uUCG--GGUAG-------------------
```

```
Secondary structure II: E. coli positions 184 to 193

  PAIRING                          NNn...........    ...........nNN
  E.COLI                 -----------------GUC---GCAA---GAC---------------
```

Haloanaerobiaceae ***(long stem)***

```
  clone D31               ---------CTGATTAGATA---GCAT---TATCAAATCAG---------
  clone D82               ---------CTGATTAGATA---GCAT---TATCAAATCAG---------
  clone D34               ---------CTAATCGGAAA---GCAT---TTTTTGATTAG---------
  clone D86p              ---------CTGGCTGGTGG---TTAT---CCTCCGGTCAG---------

  clone B1                ---------CTGGCTGGTGG---TTAT---CCTCCGGTCAG---------
  clone B83               ---------CTGACAGATAG---GCAT---CTATCAGTCAG---------
  Han.fermentans          ---------ctgagagtgtg---gcat---cacacaatcag---------
  Han.kushneri            ---------ctgagagtgtg---gcat---cacacaatcag---------
  Han.congolense          ---------ctgagagtgtg---gcat---cacacaatcag---------
  Han.praevalens          ---------ctgagagtgtg---gcat---cacacaatcag---------
  Han.saccharolyticum     ---------ctgagagtgtg---gcat---cacacaatcag---------
  Han.alcaliphilum        ---------ctgagagtgtg---gcat---cacacaatcag---------
  Han.acetoethylicum      ---------ctgagagtgtg---gcat---cacacaatcag---------
  Han.salsuginis          ---------CUGAGAGAGUG---GCAU---CACUCAAUCAG---------
  Han.lacusrosei          ---------ctgagagtgtg---gcat---cacacaatcag---------
  Hcl.cellulolytica       ---------cttaacatgta---gcat---tacaagttaag---------
  Htt.orenii              ---------ctgtcggatga---gcat---tcaactgacag---------

Halobacteroidaceae : (variable length)
  clone B69               ----------------CTCC---TTTTT----GGAG--------------
  clone D9p               ------------------------------------------------
  clone D47               ------------------------------------------------
  clone D46               ----------------CTCCT---TTT----AGGAG--------------
  clone D40p              ------------------------------------------------
  clone D74p              ------------------------------------------------
  clone D43p              ------------------------------------------------

  Hbac.halbius             ----------------ctcc----ttt---ggag----------------
  O.marismortui            -----------------ctc---ttac---gag-----------------
  O.salinaria              ----------------ctcc---ttt----ggag----------------
  O.sivashensis            ----------------ccct--ctttt---agag----------------
  Hbac.elegans             ------------------------ag------------------------
  Hla.salinarius           ------------------------aa------------------------
  Hla.chitinovorans        ------------------------aa------------------------
  Hla.lacunaris            ------------------------ag------------------------
  Na.acettgena             ----------------ctcc---tttc---ggag----------------
  Ach.arabaticum           ---------atgctgcctgg---ataa---ccaggctgcat---------
  Shb.lortetii             ---------AUACUUUCUGC---ACAA---GCAgAUUGUAU---------
```

Secondary structure III: E. coli positions 1440 to 1460

```
 PAIRING                 DD.D E.EEE  .FF....    ....FF EEEE-DDDDD.D   CCCCC
 E.COLI                  GG-UAG-CUUAA-CC----UUCG----GGAGGGC-GCUUA-CCACUUUGU
Halobacteroidaceae clones

Haloanaerobiaceae
 clone B1                AC-C---------------TGCG------------GGTGC-CGAAGGTGT
 clone B83               AC-C---------------TGAG------------GGTGC-CAAAGGTGT
 clone D31               AT-T---------------TGCG------------AATGC-CGAAGGTGT
 clone D82               GG-TGG-GCCAA-CCC---TTTA--GGGGAGGCA-GCCGT-CGAAGGTGG
 clone D34               GG-TGG-GCCAA-CCC---TTTA--GGGGGGGCA-GCCGT-CGAAGGTGG

 Han.fermentans          at-c---------------tgag------------gatgc-c
 Han.kushneri            at-c---------------tgcg------------gatgc-caaaggtgn
 Han.congolense          at-c---------------tgag------------gatgc-caaaggtgt
 Han.praevalens          at-c---------------tgcg------------gatgc-c
 Han.saccharolyticum     at-c---------------tgcg------------gatgc-caaaggtgt
 Han.alcaliphilum        nn-n---------------nnnn------------gatgc-caaaggtgt
 Han.acetoethylicum      at-c---------------tgcg------------gatgc-caaaggtgt
 Han.salsuginis          AU-C---------------UGCG------------GAUGC-CAAAGGUGU
 Han.lacusrosei          at-c---------------tgcg------------gatgc-caaaggtgt
 Hcl.cellulolytica       at-t---------------tgcg------------aatgc-caaaggtgt
 Htt.oreniii             ac-c---------------tgcg------------ggtgc-cgaaggtgt


Halobacteroidaceae
 clone B69               GT-C---------------TAAG------------GACGC-CGAAGGTGT
 clone D9p
 clone D47               GG-TGG-GCCAA-CCC---GTAA---GGGAGGCA-GCCGT-CGAAGGTGG
 clone D46               AT-C---------------TATAT-----------GATGC-CGAAGGTGT

 Hbac.halobius           ac-t-gacctaa-ctt---ttag------aagga-agtgc-cgaaggtgt
 O.marismortui           at-cgg-cccaa-c-----ttagt----gaggga-gatgc-cgaaggtgt
 O.salinaria
 O.sivashensis           at-c-g-gc-------tctttatatc-----ttt-ggtgc-cgaag
 Hbac.elegans            at-t-c-gc----------aata--------gcg-antgc-cgaaggtgg
 Hla.salinarius
 Hla.chitinovorans       at-t-t-gc----------gata--------gca-agtgc-cgaaggtgt
 Hla.lacunaris           at-t-c-gc----------aata--------gcg-agtgc-cgaaggtgt
 Na.acetigena            gt-t---------------taag------------gacgc-cgaaggtgt
 Ach.arabaticum          gt-t---------------tacg------------ggcgc-cgaaggtgt
 Shb.lortetii            gc-c---------------UACG------------GGCGC-CGAAGGUgu
```

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