Chapter 4
The Hypersaline Lakes of Inner Mongolia: The MGAtech Project

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4.1 The MGAtech Project

After several years of informal collaboration based on a common interest in the microbial ecology of extreme environments, in the late 1990s, colleagues from five institutions ranging from academia to industry conceived the idea of an European Union–China project to investigate some of the many microbiologically largely unexplored thermal and saline sites in China. The partners in this ambitious venture were the University of Leicester in the UK, the University of Seville in Spain, the University of the Western Cape in South Africa, Beijing's Institute of Microbiology at the Chinese Academy of Sciences and industrial partner Genencor International in The Netherlands.

The concept of the project was that the Chinese environments were likely to harbor hitherto unaccessed microbial genetic resources that could be harnessed as sources of new and valuable products. Central to the rationale behind the bid for funding was the observation that while conventional culturing procedures were

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often quite successful in obtaining new organisms with their concomitant genetic resource, it is known that only a small proportion (0.01–10%) of the microbial population from any environment is cultivable, and as a consequence, the majority of microbial genes are inaccessible for study using culture techniques. Accordingly, the project was designed to be innovative in that it proposed the additional use of methods to efficiently access total microbial genomic diversity (both prokaryotic and eukaryotic) in these environments through the direct acquisition of the total metagenome by on site DNA and RNA extraction (hence the project acronym MGAtech-*Multigenome Access Technology for Industrial Catalysts*).

Three preparatory meetings in Beijing took place, and in October 2002, the five partners were successful in obtaining research funding of one million euros from the European Union under Framework V. Supporting funds were provided by agencies in China and South Africa plus the industrial partner. The University of Leicester coordinated the project in view of expedition skills developed over many years in African locations. This was the first time that European Scientists had been given access to Chinese extreme environments and established an important bridge-head in microbial biotechnology cooperation between the EU and China with all the concomitant scientific and political interactions.

The objectives of the project were:

1. To obtain material from a range of unique Chinese hypersaline and thermal sites.
2. Survey the microbial biodiversity in these environments. Establish and archive a collection of novel microbes.
3. Refine methods for the efficient isolation and recovery of microbial genes from these sites.
4. Identify and produce high added value new biomolecules derived from these sites and assess these new biomolecules for commercial exploitation.

Three sampling expeditions were conducted involving around ten personnel representing the five partners. The first expedition to the geothermal areas of the Yunnan Province (the Rehai area of the Tenchong region) was completed in March 2003. The second expedition was to the hypersaline lakes of Inner Mongolia in September 2003. The third expedition to hypersaline sites in Tibet took place in August 2004. Sites were extensively described, physical parameters measured and samples taken for later culture studies and water chemistry determinations. DNA and RNA extraction and stabilization on site was undertaken, modifying procedures previously developed on African expeditions.

The project ran from October 2002 to June 2006 and during this period more than 100 hydrolase-producing thermophiles were isolated from the Tenchong sites including examples of a new genus (Xue et al. 2006). The hypersaline sites of Inner Mongolia and Tibet produced nearly 400 isolates of halophilic and haloalkaliphilic bacteria and archaea including several new genera of each. Screening produced several enzymes appropriate for biotechnological exploitation. Access to the metagenome in Yunnan and Inner Mongolian samples showed great prokaryote biodiversity, indicating great potential for isolating novel genes from gene libraries. Eukaryote biodiversity was low but the proposed technology for isolating

functional eukaryote genes via cDNA generation from isolated RNA was proven to work well (Grant et al. 2006).

This chapter describes studies carried out on seven Inner Mongolian hypersaline lakes during the MGAtech project.

4.2 The Study Area

The Autonomous Region of Inner Mongolia in the northern part of the P.R. China is a large inland plateau. The area of study, around 600 km north of the Chinese capital, Beijing is located in the Xilin Gol League (Prefecture) in an area stretching roughly from the city of Xilinhot (43°57′N, 116°5′E) in the east to Erenhot (also called Erlian) (43°38′N, 111°58′E) on the border with Mongolia in the west. At an elevation of around 1,000 m the region experiences a typical temperate semi-arid continental climate with about 150–400 mm annual precipitation, mainly in the summer months, with mean maximum and minimum temperatures of 19°C and −22°C, respectively. Strong winds and high rates of evapotranspiration, often exceeding annual precipitation, are also characteristic of the region. The study area featured typical undulating steppe grassland at 1,250 m in the east descending to 900 m with [Gobi] desert scrub in the west. Two major fault lines, the Erenhot and Xilinhot faults, of tectonic (Paleozoic era) origin running SW to NE form shallow depressions in which salt lakes can form (Zheng 1991; Yu et al. 2001). The lake basins are often the sites of Quaternary precursor lakes with serial evaporite and sedimentary deposits reflecting climatic changes since the collision of the Indian and Laurasian tectonic plates. Since this is now a tectonically stable region (Zheng et al. 1993), it is aeolian activity and deflation of recent origin, not tectonic activity (in contrast to the soda lakes of the East African Rift Valley (Jones et al. 1994)) which has led to the development of the present large, shallow endorheic drainage basins in which one or more salt lakes have developed (Williams 1991). During the Holocene the climate became drier and lake waters evaporated and concentrated, creating conditions favorable for salt formation as evaporite deposits (Zheng 1991).

Many of the shallow lakes of Inner Mongolia are ephemeral or have variable morphometry. Few have been investigated limnologically and for many basic chemical data such as salinity and major ionic composition are lacking (Williams 1991). Such data that do exist (Zheng et al. 1992, 1993) suggest that many of the saline lakes along the Erenhot and Xilinhot faults are alkaline; $HCO_3^- + CO_3^{2-}$ type or Na_2SO_4 sub-type. An expedition was conducted in September 2003 (after the summer monsoon rains) to collect samples for microbiological and chemical analysis from seven lakes. It is worthy of note (since rainfall affects lake salinity) that Xilinhot weather records show that the summer of 2003 experienced 42% more rainfall than average and that the previous year had been unusually wet with almost four times the usual amounts. The lakes sampled were Ejinor, Bagaejinor, an unnamed lake west of Xilinhot on 101 Provincial Road now known to be called Bayannur (Google Maps), Shangmatala, Chahannor, Chagannor, and Erliannor.

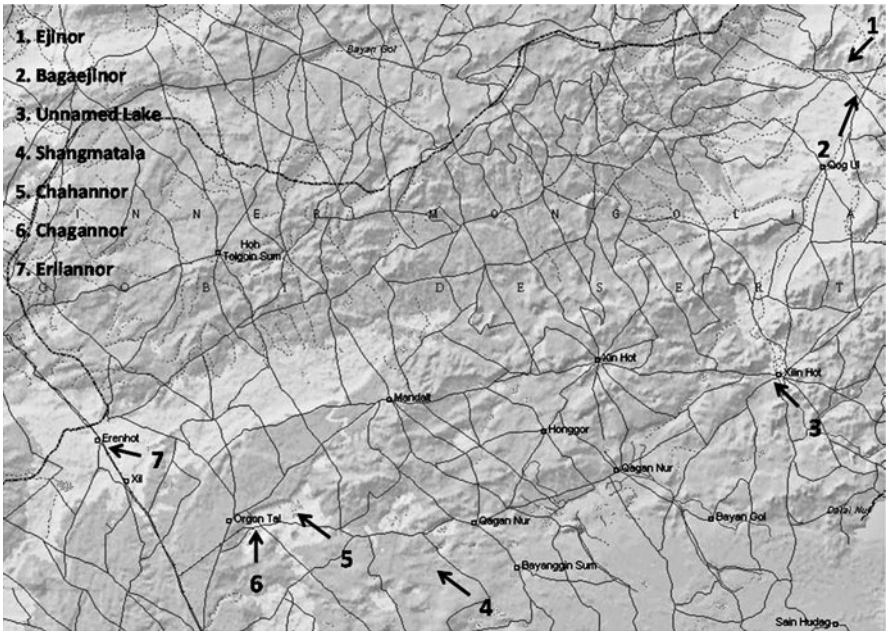

Fig. 4.1 Location of the seven hypersaline lakes studied from Inner Mongolia, northern area in P.R. China

Figure 4.1 shows the area and the location of the lakes. Samples consisted of water, sediment, salt crystals and microbial mass from filtered lake water. Secondary observations during sampling often indicated vigorous microbial activity and active mineral crystallization. Conditions during sampling were generally mild with air temperatures around 20°C, strong winds and occasional rain showers. Table 4.1 shows the features of the sampling sites in the seven lakes studied.

4.3 Description of the Lakes

Ejinor (EJ)

This is a sulfate-type salt lake with a surface area of 26 km^2. The lake is situated in the northern part of the Dabusugu tectonic basin (700 km^2) (Yu et al. 2001) and consists of an open body of water separated by a north-south causeway from a playa of salt (mainly mirabilite) sediment (15 km^2). On the north shore are a series of actively worked salterns. At the time of sampling the eastern shore consisted of a collection of dislocated evaporated pools and lagoons separated from the main body of water. The depth of brine varies with the seasons; 0.15–0.3 m in the rainy season, 0.05–0.1 m in the dry season. The lake is fed by direct precipitation (250–300 mm $year^{-1}$) and ground water. The average evaporation is 2,000 mm $year^{-1}$ (Yu et al. 2001).

Table 4.1 Features of the sampling sites from the seven lakes investigated

Sample	Coordinates	Description	T (°C)	pH	Salinity (%)	Conductivity (mS cm^{-1})
EJ1	45°14′4.52″N 116°32′4.77″E	Isolated pond	32	8.5	15.1	166
EJ2	–	Yellow colored lagoon	28	7.2	13.8	–
EJ3	45°14′7.94″N 116°32′8.52″E	Saltern of reddish water; orange flaky crystals on bottom	28	7.5	11.1	161
EJ4	–	Green scum, drainage channel	–	7.5	–	–
BJ1	45°08′5.27″N 116°36′1.67″E	Isolated pond	21	8.5	>30	147
XH1	43°55′3.55″N 115°36′7.57″E	Open water	26	9.0	–	–
XH2	43°55′1.85″N 115°37′5.20″E	Small lagoon	24	8.5	>30	185
XH3	43°55′1.70″N 115°37′.12″E	Drainage channel	21	9.5	–	287
SH1	43°12′7.51″N 114°01′3.61″E	West lagoon	20	8.5	16.7	480
SH2	43°12′7.51″N 114°01′3.61″E	East lagoon	22	8.5	–	502
CG1	43°16′1.31″N 112°55′6.36″E	South lagoon	17	10.5	18	213
CH1	43°21′5.83″N 113°08′1.93″E	Brown water with suspended silty clay	17	9.5	–	Very low
EN1	43°44′4.26″N 112°02′0.81″E	Saltern covered by white salt crust	17.9–17.2	7.5–8	–	44.9
EN2	–	Saltern covered by pink salt crust	18.4	7.5	–	–

–, No data available

Bagaejinor (BJ)

The lake lies in the SW of the Dabusu fault basin surrounded by grassland and has a surface area of 5 km^2. Zheng et al. (1992) classifies the lake as the sodium sulfate sub-type which is confirmed by our chemical analysis (Table 4.2). The lake had largely evaporated during the summer leaving a few small pools and lagoons in an extensive soft mud saline playa. The pools had a hard pink or brown crystalline base and comprised clear water surrounded by pink or brown flakes of salt crystals, probably mirabilite (Glauber's salt). There was much evidence of farm animal contamination close to the lake.

Table 4.2 Chemical composition of the seven lakes from Inner Mongolia, China

Lake	Year	Salinity (%)	pH	Na (M)	K (mM)	Ca (mM)	Mg (M)	Cl (M)	SO_4 (M)	HCO_3 (mM)	CO_3 (mM)	B_2O_3 (mM)	B (mM)	Li (mM)	Br (mM)	PO_4 (mM)	S (M)
Ejinor	1962	33.8	7.4	3.80	70.59		1.03	5.10	0.42		13.71	0.95					
	1983	33.3	7.5	4.35	31.07	5.09	0.69	5.17	0.32	6.87		1.81		0.61		0.008	
	2003 (EJ1)	15.1	8.5	2.71	38.26	10.85	0.68	3.87					2.83	0.56	16.2		0.36
	2003 (EJ3)	11.1	7.5	2.82	68.9	3.04	2.08	4.36		9.84	23.3		4.42	1.34	14.0		0.94
Bagaejinor	1962	26.9	8.2	3.77	103.0		0.32	4.15	0.23	21.68		1.67					
	1983	29.5	7.7	4.26	19.67	4.22	0.28	3.61	0.61	9.70	4.45	1.71		0.35		0.042	
	2003 (BJ1)	>30	8.5	5.32	33.2	0.77	0.35	4.61		7.40	3.30		4.25	0.33	8.05		1.07
Unnamed	2003 (XH2)	>30	8.5	5.06	53.1	3.1	0.08	5.4		13.9	1.7		2.87	0.23	7.78		0.33
Lake (Xilinhot)	2003 (XH3)		9.5	2.16	16.14	0.84	0.03	2.43					1.43	0.1	1.80		0.07
Shangmatala	1962	17.3	10.2	0.22	12.53	0.45	0.01	0.07	0.04	85.6	2.32	0.56					
	2003 (SH1)	16.7	8.5	5.38	150.0	2.62	0.26	4.69		7.4	13.0		13.0	1.93	5.18		0.81
Chagannor	1983	17.3	9.98	2.94	12.5		3.55	0.97	0.23	179.1	632.6	3.23				1.30	
	2003 (CG1)	18	10.5	2.89	14.0	0.12	0.001	1.08		360.0	410.0		3.81	0.014	1.25		0.43
Chahannor	1962	3.6	10.2	1.57	83.38			0.34	0.06	374.3	234.0	1.57					
	2003	Not recorded															
Erliannor	1962	20.3	8.2	2.80	61.38	17.09	0.27	2.91	0.26	12.3		0.67					
	1983	25.0	7.3	4.99	19.23	2.99	0.52	4.99	0.49	7.44		3.26		2.16		0.017	
	2003 (EN1)		8.2	4.2	39.0	0.12	0.86	5.33		4.1	8.3		5.24	3.3	9.57		0.48
	2003 (EN2)		7.5	3.23	61.66	4.61	1.64	5.35					10.57	6.4	15.3		0.53

Chemical composition in molarity. The elemental analysis was determined using Inductively Coupled Plasma Optical Emission Spectroscopy at the University of Leicester, UK. Carbonate and bicarbonate was determined by titration. The historical analyses are derived from Zheng et al. (1992)

Unnamed Lake West of Xilinhot (Bayannur) (XH)

No known descriptions of this lake exist; although it is close to the main 101 Provincial Road, about 40 km west of Xilinhot and it is clear from an abandoned soda factory and numerous lagoons and causeways, the former site of mineral extraction. An unusual feature of many of the lagoons was the active crystallization causing "iceberg-like" structures and a thick soda pavement. Also unusual in the colorless water was an orange colored brine shrimp. Since this alkaline water was close to the maximum pH for the survival of brine shrimp it is unclear if this is the same species of *Artemia* found in other salt lakes around the world. It may be more closely related to *Artemia monica*, a brine shrimp found in alkaline Mono Lake (California) than the more common *Artemia salina*.

Shangmatala (also known as Dermatala) (SH)

The lake is very isolated and lies in a shallow basin surrounded by low hills in a secondary depression of the Tenger fault basin at an elevation of 987 m. The surface area of lake is 2.5 km^2, with a 0.1–0.15 m depth of brine in the wet season. The lake is surrounded by grassland and vegetation almost up to the water's edge and there was much evidence of disturbance by grazing animals. The gravels and silty clays nearest the lake were soda soils covered by surface lichen. This layer was sampled. It was noted that an unpleasant odor was emitted, possibly dimethyl sulfide. Further disturbance by primitive mineral extraction was in evidence by piles of off-white "bricks," probably rock salt or Glauber's salt. The water was shallow – only a few cm deep and appeared pink due to the coloration of salt crystals. The surface of shore line mud was covered by rod-shaped crystals. The undisturbed lake sediments were stratified with a band of pink crystalline salt overlaying a green cyanobacterial layer (possibly *Microcoleus* sp.) above black anaerobic silt.

Chagannor (CG) (also known as Qagan Nur)

The lake is the site of the largest soda ash factory in China, the Xilingol Sunite Alkali Industry Company. The factory is served by a branch line of the Erenhot–Beijing railway and has several coal-fired kilns. A small town has grown up around the factory on the north side to accommodate factory workers. The lake consists of 21 km^2 of carbonate minerals mainly Pleistocene and Holocene series trona and/or natron deposits up to 30 meters deep (Zheng 1991). However, core samples reveal that this is a relict of a vast paleolake with nine different trona layers interspersed with muds reflecting the sedimentation cycles during the formation of a freshwater lake (20,000 years B.P.) and the evolution through wet and dry periods into an actively salt precipitating soda lake (Yu et al. 2001). Now there is little natural open water, only a series of peripheral [drainage] lagoons. The lake is situated in an arid area of scrubland and lies in the Erdaojing fault basin. Sampling took place on the south side well away from the industrial activity. Here the lake

water was greenish and the shoreline consisted of fine, soft, grey, viscous mud making conditions for sampling precarious.

Chahannor (CH)

The lake lies on the Erdaojing–Xilaxulun faultline in a vast basin of scrubland and dried mudflats with a typical pattern of surface cracking, surrounded by low hills and escarpments. Zheng et al. (1992) indicate that the area of salt sediment covers 15–20 km^2 but is covered with a 2 meter layer of sandy clay and "there is a silt clay deposit formed by recent freshening environment in the lake surface [sic]." It appears that the original concentrated alkaline lake which was recorded 10 years previously has been covered by thick aeolian sediment from the encroaching Gobi desert. The basin had partly filled with recent meteoric water and surface runoff to form a small, probably temporary, largely freshwater lake of brown water on a bed of impervious clay. Satellite imagery confirms this picture and the changing nature of the landscape.

Erliannor (EN) (also known as Erliandabusnoer)

The lake is located a few km NE of Erenhot, a large city on the border with Mongolia and the Beijing branch of the Trans-Siberian railway. The area of the lake is 8.75 km^2, with 0.1–0.3 m depth of brine which is recorded (Zheng et al. 1992) as being the magnesium sulfate sub-type. The lake is situated in the Erlian fault basin containing alluvial and lacustrine deposits of sand and silty clays with Glauber's salt and halite (Zheng et al. 1992). Zheng et al. (1993) comment on the high levels of Br^- (300 mg l^{-1}) in the lake. However, we found four times this amount (1,220 mg l^{-1}; sample EN2) with significantly higher levels of boron and lithium compared to the other lakes sampled (Table 4.2). The natural lake is largely unrecognizable due to the small-scale salt industry which has given rise to an extensive series of new and abandoned salterns. According to the local workers there is approximately 40 km^2 of underground soda, which is harvested in the winter months. In the summer, microbial blooms turn the salterns red (presumably due to haloarchaea) and salt (halite) is harvested. At the time of sampling (9 September 2003), all the salterns were white and the water was clear and colorless with a thermal gradient.

4.3.1 Chemical Composition of the Lakes

Data for the major ionic composition of the lakes derived from the present study are presented together with historical data derived from Zheng et al. (1992) in Table 4.2. The data are not entirely consistent but the variation may reflect seasonal and/or longer term climatic effects. Probably of greater influence is the degree of disturbance due to mineral extraction. Clearly the chemical composition will differ

depending upon whether the sample is from open water or from a crystallizer pond or saltern. The data indicates that these are all hypersaline lakes but suggest that only Chagannor can be considered to be a soda lake (pH > 9) with elevated $HCO_3^- + CO_3^{2-}$ and depleted Ca + Mg (Jones et al. 1994). The soda lake Chahannor no longer exists as a permanent surface feature and lies buried beneath windblown and alluvial sediment. Shangmatala may be evolving into an alkaline lake but the extensive disturbance of the natural lake into enclosed lagoons for mineral extraction make historical comparisons difficult. Our data confirm the lake as a source of Glauber's salt ($Na_2SO_4 \cdot 10H_2O$), halite (NaCl) and epsomite ($MgSO_4.7H_2O$) (Zheng et al. 1992). The data also confirm that Ejinor has the magnesium sulfate sub-type chemistry (Zheng et al. 1992) which concentrates in the crystallizer pond (EJ3) along with minor elements boron and lithium. A similar picture is seen at Erliannor which comprises almost entirely crystallizer ponds of various types where the co-concentration of Br^- is also significant.

4.4 Molecular Analysis of the Salt Lakes

An extensive study of the microbial populations in the water column of six salt lakes was done (at sampling points BJ1, CG1, EJ3, EN1, SH1, and XH2). Lake Chahannor was not included in this study due to the low salinity at the sampling time. Total genomic DNA was isolated from biomass collected at the sampling site by the GenomicPrep Cells and Tissue DNA Isolation Kit. The biomass (usually cells collected from brine filtration on site, filters being placed in ice-cold brine for transport to processing site) was pelleted in 1.5 ml eppendorf tubes by centrifugation at 13,000 rpm for 5 min and the DNA extracted according to manufacturer's protocols. These samples were then stable for at least 18 months at room temperature and were stored as such until further processing in the laboratory.

Molecular characterization involved cloning PCR amplified 16S rRNA archaeal and bacterial gene sequences from community DNA to construct 16S clone libraries. Unique clones were selected by patterns produced upon digestion with restriction endonucleases. Rarefaction curves were used to identify when enough clone sampling had taken place on each sample to guarantee extensive coverage of the diversity present. This statistical approach to determining overall biodiversity at these sites, together with the use of other biodiversity indices and richness estimators such as Chao1, Simpson's Index, the Shannon–Weaver Index and the Jaccard Index to compare sites is detailed in Pagaling et al. (2009).

4.4.1 Bacterial Diversity of the Salt Lakes

A total of 51% of the clone sequences were related to uncultured organisms. These sequences were found from a variety of sources including other salt lakes,

crystallizers, salt plains and marine environments. More surprisingly are the clone sequences related to clones detected in freshwater environments, hot springs and anoxic sediments (since samples were taken from the water column). Seven clone sequences are related to those detected in Lake Chaka in Tibet, which is an environment where some of the archaeal clones were affiliated with (Jiang et al. 2006). Six are related to sequences found in hypersaline saltern ponds in Salin-de-Giraud, France (Mouné et al. 2003). Five were related to those found in the Great Salt Plains of Oklahoma (Caton et al. 2004).

The remaining 49% of sequences were related to known species belonging to the *Bacteroidetes* (such as *Salinibacter* and *Salegentibacter*), *Firmicutes* (such as *Orenia salinaria*, *Halanaerobacter chitinovorans*), *Alphaproteobacteria* (such as *Roseovarius* sp., *Paracoccus* sp.), *and Gammaproteobacteria* (the Halomonads). There are also clone sequences related to chloroplast rRNAs from eukaryotes *Dunaliella salina* and *Chlamydomonas applanata*.

Phylogenetic trees were drawn containing clone sequences and existing 16S rRNA gene sequences from all major bacterial lines of descent known to have halophilic or halotolerant members (Grant 2004), as well as the sequences of the clones' nearest neighbors. The related numbered sequences shown in the trees are unpublished sequence data base entries. The root chosen for the bacteria was *Methanospirillum hungatei*. Resulting trees distribute the clone sequences from the salt lakes into nine monophyletic assemblages: *Bacteroidetes*, Chloroplasts (*Chlorophyceae*), *Cyanobacteria*, *Aquificae*, *Actinobacteria*, *Firmicutes*, *Alphaproteobacteria*, *Deltaproteobacteria,* and *Gammaproteobacteria*. Bootstrap values above 50% are shown and clone sequences are color coded according to the lake they were detected in. Many of these groups are well supported: Chloroplasts (97%), *Cyanobacteria* (68%), *Aquificae* (67%), and *Gammaproteobacteria* (67%). There are also additional well supported lineages (80–98%) that branch between these known groups, designated Salt Lake (SL) Clusters 1–3. Two additional SL Clusters (4 and 5) are near the *Aquificae* and the *Firmicutes*, both with low bootstrap values. The largest groups are the *Proteobacteria* with 30% of the clone sequences and the *Bacteroidetes* and *Firmicutes*, both with 20% of the clone sequences.

4.4.1.1 *Bacteroidetes*

This group is shown in Fig. 4.2, which has been magnified from the original tree (see Fig. 4.4), and therefore, does not show the root. A small group of sequences related to *Salinibacter* has formed their own well-supported clade (93%) within the *Bacteroidetes*. *Salinibacter* are typical inhabitants of salt lakes and salterns. One clone sequence from EJ3 (164) is closely related to halophilic eubacterium EHB-2 (99%). This clone was in fact the most frequently occurring in the 16S library for EJ3, with a clone frequency of 24. It was originally detected in salterns in Alicante, Spain, which had total salinities between 22.4% and 37% (w/v). Studies by Antón and colleagues (2000, 2002) recognised the existence of large numbers of these

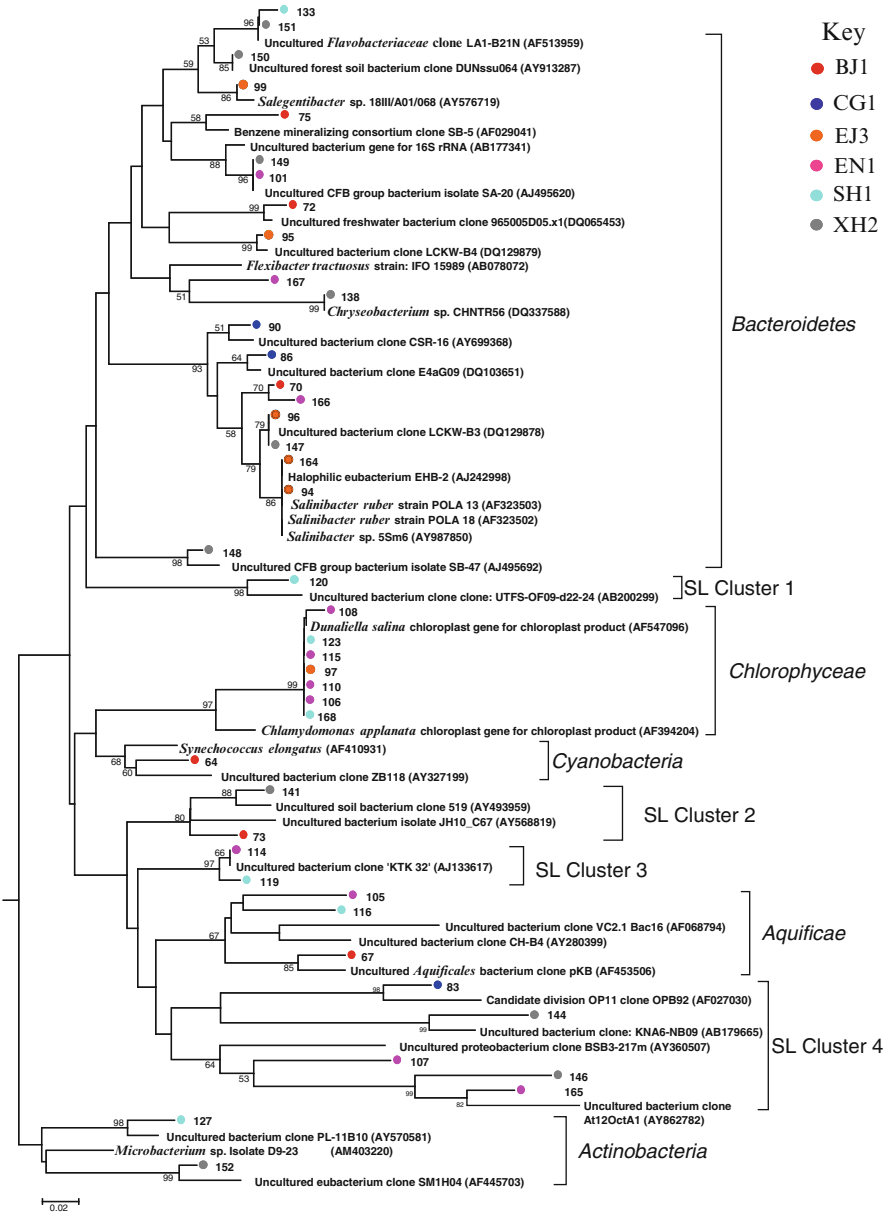

Fig. 4.2 Phylogenetic relationships showing the clone sequences with existing 16S rRNA gene sequences for *Bacteroidetes*, chloroplasts, *Cyanobacteria*, *Aquificae*, *Actinobacteria* and other uncultured isolates

Salinibacter types in hypersaline environments, making up the major bacterial constituent of these microbial ecosystems. One clone sequence from EN1 (101) and one from XH2 (149) are related to a bacterial isolate found in anoxic sediments underlying cyanobacterial mats in hypersaline saltern ponds in Salin-de-Giraud, France.

Apart from the *Salinibacter* clade, several other sequences are found within the *Bacteroidetes*. One clone sequence from SH1 (133) and XH2 (151) are related to *Flavobacteriaceae* clone LA1-B21N (both at 96%), detected in inland waters of remote Hawaiian islands, either from hypersaline Lake Laysan or from a brackish pond (Donachie et al. 2004). Several other sequences are related to unknown uncultured isolates from various environments. One clone sequence from BJ1 (75) is related to an uncultured bacterium clone: ODP1251B5.13 (93%) from deep marine sediments of the Pacific Ocean Margin (at the Peru and Cascadia Margins) that contain methane and methane hydrate. Phylogenetic analysis in that study similarly placed this clone with the *Bacteroidetes* (Inagaki et al. 2006).

4.4.1.2 Chloroplasts

Also illustrated in Fig. 4.2 are several sequences related to chloroplast genes from *Chlorophyceae*. It is likely that eukaryotes were present in the salt lakes, and the chloroplasts in them were lysed and DNA extracted along with the rest of the community DNA. The detection of these genes in the clone libraries has, therefore, given an insight into the eukaryotic diversity in the salt lakes. Eukaryotic algae, particularly *Dunaliella salina* are typical inhabitants of salt lakes, contributing to primary production, providing a source of organic compounds (Oren 1994). Several clone sequences from lakes EN1 (106, 108, 110 and 115) and SH1 (123 and 168) are related to chloroplasts in *Dunaliella salina* (97–99%). This is in fact the most frequently observed clone in the 16S libraries for both EN1 and SH1, with a clone frequency of 19 and 6, respectively. One clone sequence from EJ3 (97) is related to *Chlamydomonas applanata* (95%).

4.4.1.3 *Cyanobacteria*

Only one clone sequence from BJ1 (64) was detected, appearing in the same clade as *Synechococcus elongatus* (see Fig. 4.2) an oxygenic photosynthetic cyanobacterium also contributing to primary production in saline environments. This sequence is related to clone ZB118, showing 95% similarity. This clone was detected in a slightly saline sulfide-rich spring. Phylogenetic analysis in that study also placed clone ZB118 with the *Cyanobacteria*, with *Oscillatoria terebriformis* as its nearest neighbor, a known mat-building organism.

4.4.1.4 *Aquificae*

It is surprising to find sequences related to *Aquificales*, since representatives of this group are not halophilic or halotolerant (Grant 2004), although they have been found in deep sea hydrothermal vent systems (Reysenbach et al. 2000) so are presumably tolerant to marine conditions. Nevertheless, there appears to be one clone sequence from BJ1 (67) that is related to an uncultured *Aquificales* clone pKB (94%), which comes from a near-neutral thermal Spring in Kamchatka, Russia's largest volcanic belt flanked by the Pacific Ocean and the Sea of Okhotsk. Moreover, other sequences appear in the same clade as this *Aquificales* clone. A clone sequence from EN1 (105) is distantly related to an uncultured bacterium clone VC2.1 Bac16 (88%). This clone comes from a growth chamber deployed in a Mid-Atlantic Ridge hydrothermal vent. Another clone sequence from SH1 (116) is related to a sequence that came from a *Paralvinella sulfincola* tube-worm and the adjacent substratum on an active deep-sea vent chimney (Page et al. 2004).

4.4.1.5 *Actinobacteria*

Actinobacteria are high% G+C Gram-positives that are readily found in saline environments (Grant 2004). However, only two clone sequences were detected that appear in the same clade as *Microbacterium* sp. isolate D9–23 (Fig. 4.2), which was found in a marine aquaculture biofilter. One clone sequence from SH1 (127) is distantly related to clone PL-11B10 (91%), which was detected in the waters of a low temperature biodegraded oil reservoir. Clone sequence 152 from XH2 is distantly related to eubacterium clone SM1H04 (91%), which was detected in Angel Terrace at the Mammoth Hot Springs in Yellowstone National Park, Wyoming, USA.

4.4.1.6 *Firmicutes*

The *Firmicutes* are low% G+C Gram-positives, also readily isolated from saline environments (Grant 2004) and was in fact one of the largest groups detected in five of the six salt lakes. This group consists of several haloanaerobes: *Halanaerobacter*, *Halanaerobium*, *Orenia* and "*Natronoanaerobium*," which can be seen in Fig. 4.3. This has also been magnified from the original tree, and therefore, does not show the root. Several clone sequences from SH1 (124, 125 and 134) and one from XH2 (143) are related to *Halanaerobacter lacunaris* (96–98%). This isolate was found in the silt of the hypersaline Lake Chokrak on the Kerch Peninsula (Zhilina et al. 1992). One clone sequence from XH2 (136) was related to *Halanaerobacter chitinovorans* (98%). This isolate was found in sediment in a solar saltern in Chula Vista in Southern California (Liaw and Mah 1992). One sequence from CG1 (77) is distantly related to *Halanaerobiaceae* bacterium SLAS-1 (90%) isolated from Searles Lake, which is an alkaline salt-saturated brine,

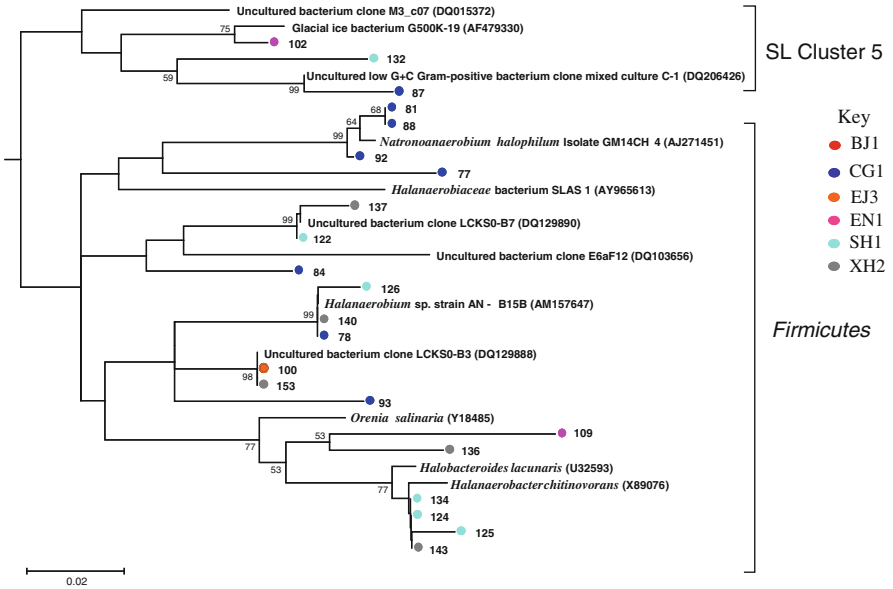

Fig. 4.3 Phylogenetic relationships showing the clone sequences with existing 16S rRNA gene sequences for *Firmicutes* and other uncultured isolates

rich in arsenic (Oremland et al. 2005). One clone sequence from CG1 (78), one from SH1 (126) and one from XH2 (140) are closely related to *Halanaerobium* sp. AN-B15B (97–98%), originally isolated from the oxic-anoxic transition of a deep sea halocline (Daffonchio et al. 2006). This clone was also one of the most dominant in the library for SH1, with a clone frequency of 6. EN1 (109) is distantly related to *Orenia salinaria* (88%). This fermentative bacterium was isolated from anoxic sediment that was rich in sulfides in Salin-de-Giraud salterns, France (Mouné et al. 2000). Three clone sequences from CG1 (81, 88, and 92) are related to "*Natronoanaerobium halophilum*" isolate G-M14CH-4 (93, 95, and 96%, respectively), which was detected in soda lakes (Jones et al. 1998).

The remaining clone sequences are related to uncultured isolates. One clone sequence from EJ3 (100), one from SH1 (122), and two from XH2 (137, 153) are related to two clones found in Lake Chaka in Tibet. One sequence from CG1 (93) is distantly related to uncultured clone E6aF12 (94%), which was detected in a hypersaline endoevaporitic microbial mat from a saltern in Israel. Phylogenetic analysis similarly placed this clone with the *Firmicutes*, with *Halocella cellulolytica* as its nearest neighbor (Sørensen et al. 2005).

4.4.1.7 *Proteobacteria*

This group comprises the largest detected from all six salt lakes. Sequences from three divisions of *Proteobacteria* were found as seen in Fig. 4.4 (4% belong to the *Alphaproteobacteria*, 6% to the *Deltaproteobacteria* and 20% to the *Gammaproteobacteria*).

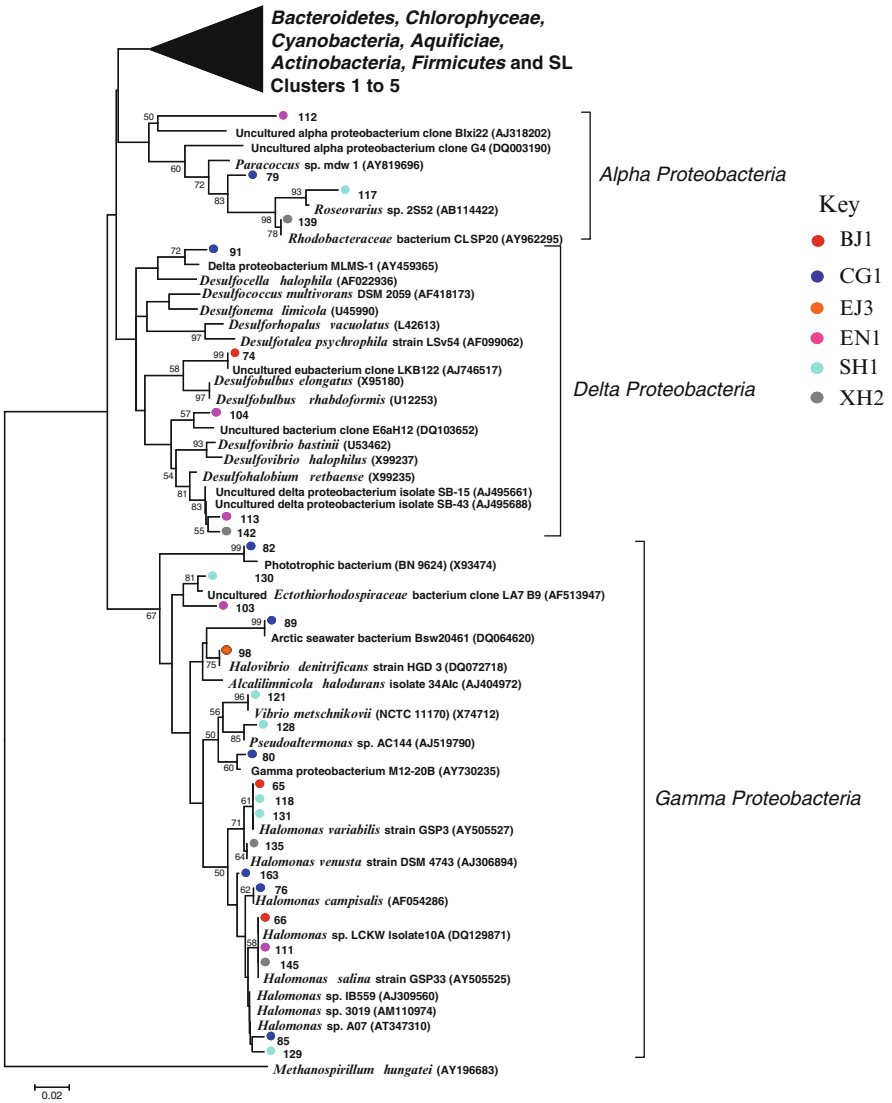

Fig. 4.4 Phylogenetic relationships showing the clone sequences with existing 16S rRNA gene sequences for *Proteobacteria* and other uncultured isolates

4.4.1.8 *Alphaproteobacteria*

A small proportion of sequences were affiliated with this group. One clone sequence from XH2 (139) is closely related to a *Rhodobacteriaceae* clone (98%), which is from a solar saltern. One clone sequence from SH1 (117) is related to *Roseovarius* sp. 2S5-2 (95%) (Fuse et al. 2003).

4.4.1.9 *Deltaproteobacteria*

Five clone sequences were found in this group of sulfate reducers. One clone sequence from CG1 (91) is distantly related to a *Deltaproteobacteria* clone MLMS-1 (92%), which was isolated from anoxic bottom water of Mono Lake, and is known to reduce arsenate using sulfide as the electron donor (Hoeft et al. 2004). One sequence from EN1 (113) and a sequence from XH2 (142) are related to uncultured isolates SB-15 and SB-43, respectively (95% and 98%, respectively). These isolates were found in anoxic sediments underlying cyanobacterial mats in Salin-de Giraud salterns in France. Phylogenetic analysis in that study also placed these clones with the *Deltaproteobacteria* near the sulfate reducers; *Desulfohalobium retbaense* their nearest neighbor (Mouné et al. 2003). Another clone sequence from EN1 (104) is distantly related to an uncultured clone E6aH12 (88%), which was detected in a hypersaline endoevaporitic microbial mat in Israel placed with the *Deltaproteobacteria*, near *Desulfocella halophila* (Sørensen et al. 2005).

4.4.1.10 *Gammaproteobacteria*

This is the largest group of *Proteobacteria* detected in the salt lakes. The majority of these sequences are Halomonads, but there are also two sequences related to Halovibrios, one to *Pseudomonas* and one to an *Ectothiorhodospiraceae* clone which are all typical of hypersaline environments, particularly the halomonads (Grant 2004).

Clone sequences from BJ1 (65) and SH1 (118 and 131) are related to *Halomonas variabilis* strain GSP 3 (96–98%). In addition, one clone sequence from BJ1 (66) and EN1 (111) are related to *Halomonas salina* strain GSP33 (99% and 97%, respectively). These isolates were found in the Great Salt Plains in Oklahoma, USA. One clone sequence from CG1 (76) is related to *Halomonas campisalis* (97%). This isolate was found in the sediments of the Salt Plain of Alkali Lake in Washington, USA (Mormile et al. 1999). One sequence from XH2 (135) is closely related to *Halomonas venusta* strain DSM 4743 (97%). This clone is the most frequently occurring clone in the library for XH2, with a clone frequency of 9.

Several clone sequences are related to other *Halomonas* species. One clone sequence from CG1 (163) is closely related to *Halomonas* sp. A-07 (99%), which was detected in soda lakes in Tanzania. Another clone sequence from CG1 (85) is closely related to *Halomonas* sp. 3019 (97%), which was detected in deep sea sediment in the East Pacific. One clone sequence from SH1 (129) is related to *Halomonas* sp. IB-559, showing 95% identity. One clone sequence from XH2 (145) is closely related to *Halomonas* sp. LCKW-Isolate10 (99%), which is another isolate found in Lake Chaka. Phylogenetic analysis in that study placed this clone with the *Gammaproteobacteria*, with *Halomonas ventosae* as its nearest neighbor (Jiang et al. 2006). Another clone sequence

from EJ3 (98) is closely related to *Halovibrio denitrificans* strain HGD 3 (98%). One clone sequence from SH1 (130) is related to an uncultured *Ectothiorhodospiraceae* bacterium clone LA7-B9. *Ectothiorhodospiraceae* are halophilic anoxygenic phototrophs. A clone sequence from CG1 (82) is related to Phototrophic bacterium (BN 9624) (97%). This clone was shown to be related to *Ectothiorhodospira* species (Imhoff and Süling 1996). One sequence clone from EN1 (103) is related to *Alcalilimnicola halodurans* isolate (94%) which was found in sediments from Lake Natron in the African Rift Valley (Yakimov et al. 2001). One clone sequence from CG1 (80) is related to gammaproteobacterium M12-20B (95%) detected in Mono Lake.

4.4.1.11 Novel Lineages

These novel lineages have been called Salt Lake (SL) Clusters 1–5 (Figs. 4.2 and 4.3). SL Cluster 1 branches near to the *Bacteroidetes*. It consists of just one clone sequence from SH1 (120), which is distantly related to uncultured bacterium clone: UTFS-OF09-d22-24 (92%). This clone was detected in activated sludge.

SL Clusters 2 and 3 branch between the *Cyanobacteria* and the *Aquificae*. SL Cluster 2 consists of one sequence from BJ1 (73), which is distantly related to an uncultured bacterium isolate JH10_C67 (90%). This clone was detected in the intertidal flat of Ganghwa Island. Another sequence from XH2 (141) is distantly related to uncultured soil bacterium clone 519 (91%), which was detected in soil aggregates.

SL Cluster 3 consists of one sequence from EN1 (114) and one from SH1 (119), which are both related to an uncultured bacterium "KTK 32" (91% and 97%, respectively). This clone is from highly saline brine sediments of Kebrit Deep in the Northern Red Sea. Phylogenetic analysis in that study similarly placed this clone in a separate clade that did not belong to any of the known genus in the domain Bacteria, but was positioned between the orders *Thermotoga* and *Aquificae* (Eder et al. 1999).

SL Cluster 4 branches from the *Aquificae*, consisting of several clone sequences. One sequence from CG1 (83) is distantly related to an uncultured candidate division OP11 bacterium FL11H04 (88%). This clone was detected in a hot spring called Obsidian Pool in Yellowstone National Park, Wyoming, USA. The group, designated OP11, was positioned near the *Deinococci*. This study also demonstrated the presence of this division in a range of environments including Carolina Bay sediment, Amazonian soil and Australian deep subsurface water, thereby demonstrating the ubiquitous nature of this novel lineage in the environment (Hugenholtz et al. 1998). One clone sequence from EN1 (165) and one from XH2 (146) are related to an uncultured bacterium clone At12OctA1 (95% and 89%, respectively). This clone was detected in Salar de Atacama in Northern Chile (Demergasso et al. 2004).

SL Cluster 5 branches near the *Firmicutes*. One clone sequence from EN1 (102) is closely related to a glacial ice bacterium G500K-19, showing 98% sequence similarity (Christner 2002). One clone sequence from SH1 (132) is related to an uncultured bacterium clone M3_c07 (94%), which was detected in the gut of obese

mice (Ley et al. 2005). One clone sequence from CG1 (87) is closely related to an uncultured low G+C Gram-positive bacterium clone mixed culture C-1 (98%). This clone was also detected in Mono Lake. Phylogenetic analysis in that study positioned this clone as distantly related to *Bacillus selenitireducens*, which belongs to the *Firmicutes* (Hollibaugh et al. 2006).

4.4.2 Archaeal Diversity of the Salt Lakes

The vast majority (81%) of the clone sequences obtained for the archaeal 16S rRNA gene libraries from the salt lakes were related to uncultured organisms. The related sequences were found in other salt lakes, salterns or crystallizer ponds, saline soils, saline waters of Antarctica and one sequence found in Permo-triassic rock (Walsh et al. 2005). Fifteen of the clone sequences are related to those found in crystallizer ponds in Australia (Burns et al. 2004) and a further fifteen related to those found in saline soils in British Columbia, Canada. Many of the sequences found in the British Columbian soils were closely related to those found in Permo-triassic rock. Nine clone sequences are related to those found in Lake Zabuye in Tibet (Fan et al. 2004).

The remaining 19% of clone sequences are related to known species. Three sequences are related to *Halorubrum saccharovorum*; two are related to *Halorubrum tibetense*; two are related to *Halorubrum vacuolatum*; one sequence is related to *Halorubrum xingjiangense*; and one sequence is related to *Halorubrum terrestre*. Four other sequences are related to unspecified *Halorubrum* type species. One sequence is related to *Halosimplex carlsbadense* and one sequence is related to a *Haloarcula* species.

Phylogenetic trees were drawn containing clone sequences and existing 16S rRNA gene sequences from all major archaeal lines of descent known to have halophilic or halotolerant members, as well as unpublished sequences of the clones' nearest neighbors deposited in sequence data bases. The root chosen for the archaea was *Escherichia coli*. This phylogenetic analysis shows the distribution of sequences into eight monophyletic assemblages within the order *Halobacteriales*. Many branch within the previously mentioned groups *Halorubrum*, *Halosimplex* and *Haloarcula*. Additional sequences branch with *Halobacterium*, *Halobaculum*, *Halogeometricum*, *Halomicrobium,* and the alkaliphilic group *Natronomonas*. However, there are additional lineages that form between these nodes, designated Salt Lake (SL) Clusters 1–3. This is summarized in Figs. 4.5–4.7; only bootstrap values above 50% are shown and all clone sequences are color coded according to the lake that they were detected in. All known genera within the order *Halobacteriales* are represented in the tree; however, none of the sequences in the SL Clusters affiliate closely with any of them. There appears to be good bootstrap support for the branches *Halorubrum* (82%) and *Natronomonas* (100%). All the SL Clusters are also well supported (98–100%), with the latter forming an outer group to the *Halobacteriales* (see later).

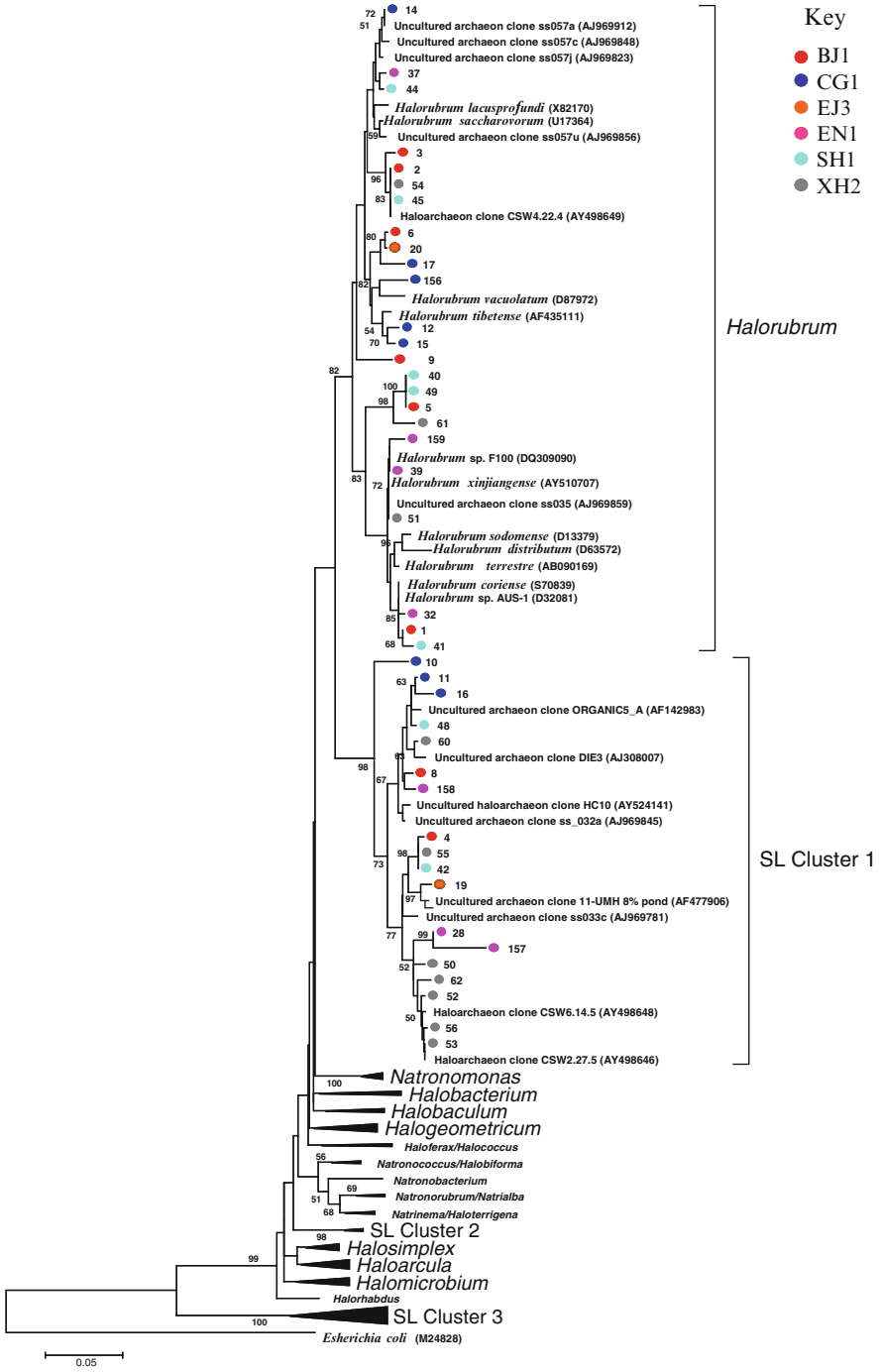

Fig. 4.5 Phylogenetic relationships showing the clone sequences with existing 16S rRNA gene sequences for *Halorubrum* and other uncultured isolates

4.4.2.1 *Halorubrum*

The 34% of clone sequences branch with representatives of *Halorubrum*, therefore, comprising the largest group detected in all six salt lakes. This group is shown in more detail in Fig. 4.5. *Halorubrum* includes extreme halophiles requiring between 1.5 and 5.2 M NaCl for growth and are strict aerobes (Grant et al. 2001). One clone sequence from BJ1 (5) and two from SH1 (40 and 49) are related to *Halorubrum saccharovorum* (all at 94%). The gene of this isolate is in fact closely related to the dominating clone found in the 16S library for SH1, showing a clone frequency of 15. The isolate was first isolated by Tomlinson and Hochstein (1976) from a mixture of mud and brine from a saltern in the southern section of San Francisco Bay. This isolate is a strict aerobe, requiring 1.5–5.2 M NaCl for growth, with an optimum concentration of 3.5–4.5 M. It also requires 0.005 M Mg^{2+}. It can grow between 30°C and 56°C, growing optimally at 50°C (Grant et al. 2001). Two clone sequences from CG1 (12 and 15) are closely related to *Halorubrum tibetense* (96% and 97%, respectively). Moreover, the gene of this isolate is closely related to the second most frequent clone in the library for lake CG1, with a clone frequency of 11. This is a haloalkaliphilic archaeon isolated from Lake Zabuye on the Tibetan Plateau, which is an alkaline chloride–sulfate salt lake with a pH of 9.4 (Fan et al. 2004). Two more clone sequences from CG1 (17 and 156) are related to *Halorubrum vacuolatum* (95% and 96%, respectively). This is also an obligate haloalkaliphilic archaeon requiring an optimum pH of 9.5 for growth (Grant et al. 2001). It was first isolated from Lake Magadi in Kenya, an alkaline soda lake with a pH of 11 (Mwatha and Grant 1993; Grant et al. 1998). One clone sequence from XH2 (51) is closely related to *Halorubrum xinjiangense* (99%). This was isolated from Xiao-Er-Kule Lake in Xinjiang, China and grows optimally at 3.1–3.4 M NaCl and pH 7.0–7.5 (Feng et al. 2004). Another clone sequence from XH2 (61) is related to *Halorubrum terrestre* (93%). Isolated from saline soils, *Halorubrum terrestre* grows optimally at 25% (w/v) NaCl and pH 7.5 (Ventosa et al. 2004). One clone sequence from EN1 (159) is closely related to *Halorubrum* sp. F100 (98%). This was found in saline lakes in Turkey. Clone sequences from BJ1 (1), EN2 (32) and SH1 (41) are closely related to *Halorubrum* AUS-1, which was isolated from a nameless clay pan in Western Australia. This isolate grows on medium containing 25% (w/v) NaCl at pH 7.4 (Mukohata et al. 1988).

The remaining clone sequences are related to uncultured isolates that cluster with *Halorubrum*. Four clone sequences from CG1, EN1 and SH1 (14, 37, 39 and 44) are related to uncultured archaeon clones ss057_a, ss057j, ss057u and ss035. These were sequences detected in saline soils in British Columbia, Canada at distances between 0 and 300 cm from the salt spring source exhibiting 7% (w/v) NaCl, and pH 7–10. The phylogenetic tree in that study similarly placed clones ss057_a, ss057j, ss057u and ss035 with *Halorubrum* (Walsh et al. 2005). Five sequences from BJ1, EJ3, SH1 and XH2 (2, 3, 20, 45 and 54) are related to haloarchaeon clone CSW4.22.4 (96–99%). This is an isolate from crystallizer ponds of Corio Bay in Victoria, Australia, which exhibited a total salt concentration

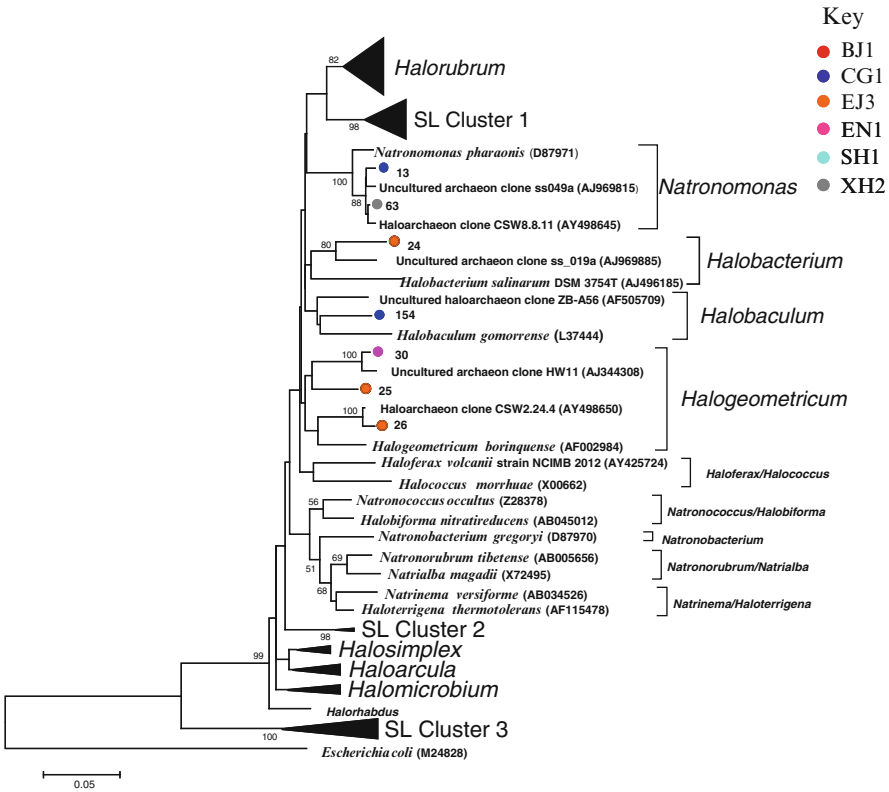

Fig. 4.6 Phylogenetic relationships showing the clone sequences with existing 16S rRNA gene sequences for *Natronomonas*, *Halobacterium*, *Halobaculum* and *Halogeometricum*

of 33% (w/v) and a pH of 8.1 (Burns et al. 2004). This clone is also the most abundant in the 16S library for lake BJ1, with a clone frequency of 22.

4.4.2.2 *Natronomonas*

This group of alkaliphiles grow at 2–5.2 M NaCl and pH 7–10 (Grant et al. 2001). Only two clone sequences from the libraries are found in this group. One sequence from CG1 (13) is closely related to an uncultured clone ss049a (97%), which is another clone detected in saline soils in British Columbia as previously described. Phylogenetic analysis in that study has shown this clone to be distantly related to *Natronomonas pharaonis* (Walsh et al. 2005). One clone sequence from XH2 (63) is closely related to an uncultured clone CSW8.8.11 (97%) from Australian crystallizer ponds as previously described. Although both are related to uncultured archaea, they are distributed within the same clade as *Natronomonas pharaonis* (Fig. 4.6). In fact, both are distantly related to *Natronomonas pharaonis*, showing 95% and 94% similarity, respectively.

4.4.2.3 *Halobacterium*

Members of this group are extremely halophilic, requiring 3.0–5.2 M NaCl, but grow optimally at 3.5–4.5 M NaCl. They also grow at temperatures between 35 and 50°C, with a pH range of 5.5–8.5 (Grant et al. 2001). Only one clone sequence from EJ3 (24) clusters within the same clade as *Halobacterium* (Fig. 4.6). It is related to an uncultured clone ss_019a, found in saline soils in British Columbia as previously described (92%). It is also distantly related to *Halobacterium salinarum* showing 87% similarity.

4.4.2.4 *Halobaculum*

These are aerobic extreme halophiles, which grow optimally in 1–2.5 M NaCl and 0.6–1 M $MgCl_2$ (Grant et al. 2001). One clone sequence from CG1 (154) is related to an uncultured haloarchaeon clone ZB-A56 (95%), which is from Lake Zabuye (as previously described). These sequences are distributed within the same clade as *Halobaculum gomorrense* (see Fig. 4.6). Sequence 154 is distantly related to *Halobaculum gomorrense*, showing 88% similarity.

4.4.2.5 *Halogeometricum*

These aerobes grow optimally at 3.5–4 M NaCl and 0.04–0.08 $MgCl_2$, and so are also extremely halophilic (Grant et al. 2001). This genus currently has two species being *Halogeometricum borinquense* the type species, which is distantly related to *Haloferax* (Montalvo-Rodríguez et al. 1998). Three clone sequences appear in the *Halogeometricum* clade (Fig. 4.6). Clone sequence 26 from EJ3 is related to clone CSW2.24.4, a sequence found in an Australian crystallizer pond as previously described. Phylogenetic analysis in that study similarly placed this clone near to, but distantly related to *Halogeometricum borinquense* (Burns et al. 2004). One clone sequence from EN1 (30) is closely related to uncultured archaeon clone HW11 (97%), which is a sequence found in Permo-Triassic rock salt. Clone sequence 25 showed a high localized similarity to *Halosimplex* but clearly full length homology places this sequence near *Halogeometricum*. Sequences 25, 26, and 30 form separate branches within the *Halogeometricum* clade with low bootstrap values, suggesting that they are only distantly related. In fact, they only show 88–89% similarity to *Halogeometricum borinquense* (AF002984).

4.4.2.6 *Haloarcula*

The genus *Haloarcula* includes a group of extremely halophilic archaea, requiring 2.0–5.2 M NaCl for growth. They are aerobic or facultative anaerobes

(Grant et al. 2001). They are shown in Fig. 4.7. One clone sequence from BJ1 (7) is related to an uncultured clone Sec16SC8 (95%), which was a sequence found in the crystallizers of an Adriatic solar saltern. This had a water activity of 0.759 at 30°C (the water activity for a saturated NaCl solution at 30°C is 0.769) and was at pH 8.0. The phylogenetic tree in that study also showed clone Sec16SC8 affiliated with *Haloarcula* (Pašić et al. 2005). One clone sequence from XH2 (58) is related to a *Haloarcula* sp. (94%). Finally, another clone sequence from EN2 (31) is related to *Haloarcula* sp. NCIMB 720 (92%).

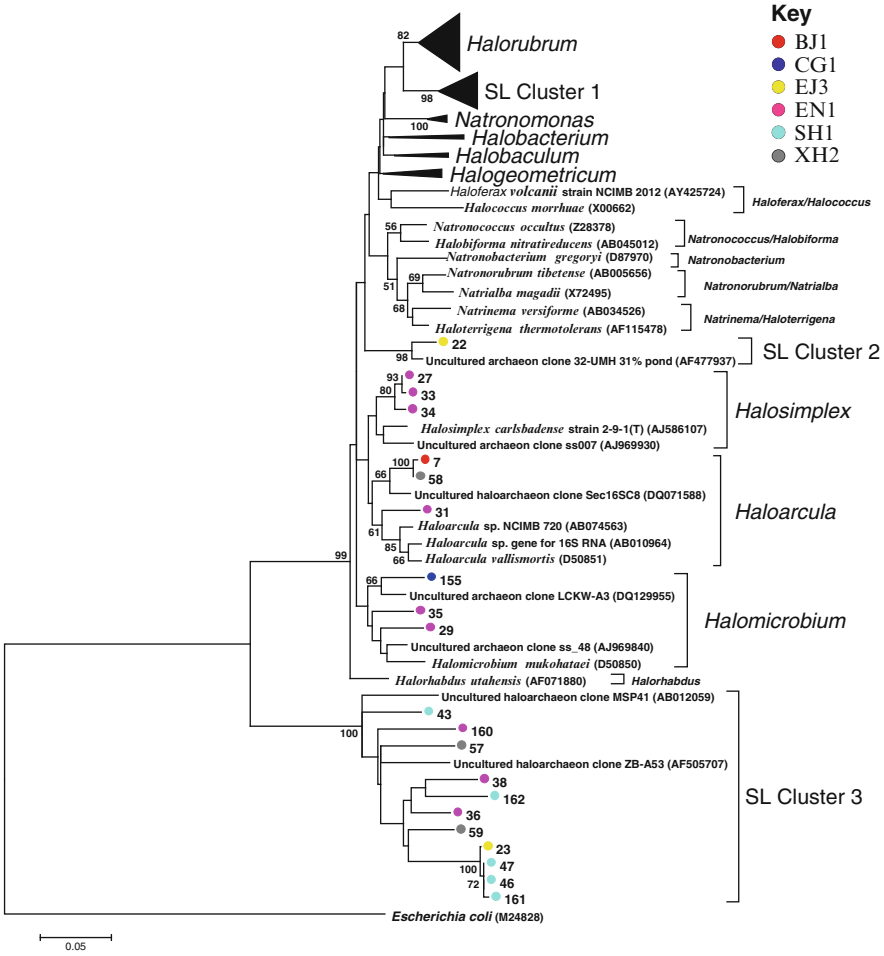

Fig. 4.7 Phylogenetic relationships showing the clone sequences with existing 16S rRNA gene sequences for *Halosimplex*, *Haloarcula*, *Halomicrobium,* and other uncultured isolates

4.4.2.7 *Halosimplex*

The genus *Halosimplex* is a deep branch distantly related to *Haloarcula* (Fig. 4.7). They are unique in that they have three different genes encoding their 16S rRNA. The strain *Halosimplex carlsbadense* was isolated from a Permian halite deposit in south eastern Mexico. It requires a defined medium for growth containing glycerol and acetate or pyruvate (Vreeland et al. 2002). Three clone sequences from EN2 (27, 33, 34) branch with the *Halosimplex* clade. Although related to an uncultured archaeon ss007, which was detected in saline soils in British Columbia (93–94%), they all show 93% similarity to *Halosimplex carlsbadense* strain 2-9-1.

4.4.2.8 *Halomicrobium*

This genus currently contains two species, *Halomicrobium mukohataei* being the type species, which was isolated from salt flats in Argentina and somewhat related to *Haloarcula*. However, analysis of its morphology, polar lipid composition and 16S rRNA gene sequence showed that it was significantly different from *Haloarcula* and was proposed as a new genus within the *Halobacteriales*. It is extremely halophilic aerobe and a facultative anaerobe in the presence of nitrate (Oren et al. 2002). Three clone sequences appear in the same clade as *Halomicrobium mukohataei* (Fig. 4.7). One clone sequence from CG1 (155) is related to an uncultured clone LCKW-A3 (93%) from Lake Chaka. This athalassohaline lake possesses a total salinity of 32.5% (w/v) with a water temperature between −8 to 4.2°C in the winter and 6 to 20°C in the summer. It also had a pH of 7.4 (Jiang et al. 2006). Two clone sequences from EN2 (29 and 35) are related to an uncultured clone ss_48 from saline soils in Canada as previously described (93% and 94%). That study also placed clone ss_48 near the *Haloarcula-Halomicrobium* branch (Walsh et al. 2005).

4.4.2.9 Novel Lineages

These novel lineages have been called Salt Lake (SL) Clusters 1–3 as shown in Figs. 4.5 and 4.7. Branching near the *Halorubrum* group, SL Cluster 1 is the second largest group comprising 27% of the clone sequences. Several clone sequences from XH2 (50, 52, 53, 56, and 62) and one from EN1 (28) are again closely related to uncultured clones from Australian crystallizer ponds as described. Haloarchaeon clone 6.14.5 is the most frequently observed clone in the library for Lake EN1, with a clone frequency of 13. Haloarchaeon clone 2.27.5 is also the dominating clone in the library for XH2, with a clone frequency of 11. Clone sequences from BJ1 (4), CG1 (16), EN2 (158), SH1 (42) and XH2 (55) are related to saline soils in Canada. Clone sequences from BJ1 (8) and CG1 (10) are related to uncultured clones ORGANIC4_A and ORGANIC5_A (95% and 94%, respectively). These sequences

were found in anoxic sediment in the Vestfold Hills in Eastern Antarctica. The lakes that were sampled in this basin were at temperatures between −1.8 and 3°C, with salinities between 14 and 45 g/kg (Bowman et al. 2000). Clone ORGANIC5_A is also the most frequently occurring clone in the 16S library for Lake CG1, with a clone frequency of 12. One clone sequence from XH2 (60) is related to an uncultured archaeon DIE3 (92%). This sequence was detected in a hypersaline pond from the bottom of a slag heap of a former potassium mine in Germany. This pond had a pH of 5.6 with a total salinity above 32% (w/v). As expected, this pond had a very high potassium concentration. Phylogenetic analysis in that study also placed this clone in an unknown cluster that formed an outer group to *Halorubrum* (Ochsenreiter et al. 2002). One clone sequence from CG1 (11) and one sequence from SH1 (48) are related to a haloarchaeon clone HC10 (95% and 94%, respectively). This clone was detected in a solar saltern in San Diego, USA (Bidle et al. 2005). One clone sequence from EJ3 (19) is related to an uncultured clone 11-UHM 8% pond (97%). This was detected in a multipond solar saltern "Bras del Port" located in Alicante, Spain, showing 8% (w/v) total salinity (Benlloch et al. 2002).

SL Cluster 2 is a small group branching near the group of known haloarchaea. It consists of just one clone sequence from EJ3 (22), which is related (93%) to an uncultured archaeon from a solar saltern in Alicante, Spain, showing 31% (w/v) total salinity (Benlloch et al. 2002).

SL Cluster 3 appears to form its own separate lineage to the order *Halobacteriales*. Several sequences from EJ3 (23), EN2 (38), SH1 (46, 47, 161 and 162) and XH2 (57and 59) are related (88–91%) to sequences detected in Zabuye Lake. Two sequences from EN2 (36 and 160) and one from SH1 (43) are related to an uncultured haloarchaeon clone MSP41. This clone was detected in salt crystallizing pond at Lake Magadi, Kenya. Similarly, phylogenetic analysis in that study positioned this clone in a novel lineage on the periphery of known haloarchaea. It was suggested that this clone was a member of a deeply branching group of the *Euryarchaeota* (Grant et al. 1999).

4.5 Biogeography

The data set just described was subject to a rigorous statistical analysis (Pagaling et al. 2009). The archaeal community was found to consist of closely related lineages whereas the bacterial community showed much more diversity. This analysis was also used to determine important factors correlating with biotic composition. This showed that temperature, Na^+ and Mg^{2+} ions, and pH were the factors that drive microbial community composition in these salt lakes. In general, the closer the chemical composition of the lakes, the closer the biotic composition. Geographic distance was not a statistically significant driver but there was a positive correlation between lake proximity and biotic composition. In other words, with regard to micro-organism biogeography in closely spaced saline systems, "the milieu selects."

4.6 Haloarchaeal Viruses

Strains with 16S rRNA gene sequences 98% identical to *Halorubrum saccharovorum* were isolated from Lake Bagaejinor in Inner Mongolia. Two lytic viruses infecting these were isolated from the lake water. The host has since been designated as *Halorubrum kocurii* (Gutiérrez et al. 2008b). The BJ1 genome sequence has been determined and has very low sequence identity to any previously described virus (Pagaling et al. 2007). BJ1 has an icosahedral head and tail morphology and most likely a linear double stranded DNA genome exhibiting terminal redundancy. Its genome sequence has 42,271 base pairs with a G+C content of ~65 mol%. The genome of BJ1 is predicted to encode 70 ORFs, including one for a tRNA. Fifty of the seventy ORFs had no identity to data base entries; twenty showed sequence identity matches to archaeal viruses and to haloarchaea. ORFs possibly coding for an origin of replication complex, integrase, helicase, and structural capsid proteins were identified. Evidence for viral integration was obtained. The second virus BJ2 has been partially sequenced; 44 contigs containing a total of 97,602 base pairs with a G+C content of 51 mol%. It has no discernible sequence identity to the BJ1 virus. This virus is more fully described by Pagaling (2007).

4.7 Culture-Dependent Study of the Salt Lakes

In addition to the cultivation-independent approach described previously, culture-dependent microbiological techniques were employed to investigate the microbial community and abundance from salt lakes. Water, soil and sediment samples were collected from the athalassohaline Lakes Bagaejinor, Chagannor, Chahannor, Ejinor, Erlianor, Shangmatala, and Xilinhot (see Sect. 4.2 and 4.3 of this chapter).

The number of microorganisms in the samples collected from these environments was determined. MH medium (Ventosa et al. 1982) with different NaCl concentrations (0.5, 10, 20, and 25%) was used to determine the viable cells. The number of colony forming units (cfu) per milliliter of water ranged from 1×10^2 to 1.2×10^4 cfu/ml in MH medium with 10% NaCl and from 1.9×10^3 to 2.6×10^4 cfu/ml in the same medium with 25% NaCl. With respect to the soil/sediment samples, these values were in the range 1×10^3 to 1.4×10^6 cfu/g in media with 10% NaCl, and from 1×10^2 to 3.8×10^4 cfu/g in media with 25% NaCl. On the other hand, the determination of the total and viable cell counts in the samples collected was carried out by epifluorescence by using the Live-Dead Cell Staining kit. The total counts ranged from 6.6×10^6 to 1.3×10^7cells/ml, while the number of viable cells ranged from 1.3×10^6 to 8.4×10^6 cells/ml. The higher numbers of cells determined by direct counts are normal in comparison with the colony counts since in the last procedure only those microorganisms able to grow in culture media are detected (Castillo 2008; Carrasco 2009).

Direct plating on different media containing similar salts to those found in the salt lakes, but present in different concentration, was used to allow maximum recovery of halophilic bacterial and archaeal microorganisms from these hypersaline environments. The isolates were selected by their colony morphology, salt response and later by their 16S rRNA gene sequences (Castillo 2008; Carrasco 2009).

4.7.1 Bacterial Diversity of the Salt Lakes

A total of 179 moderately halophilic bacterial strains were isolated; however, no extremely halophilic bacteria were isolated from any of the salt lakes studied. The 16S rRNA sequence of the isolates was determined by PCR amplification of the 16S rRNA gene by using the forward primer 16F27 and the reverse primer 16R1488. The 16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig et al. 2004).

The phylogenetic analysis determined that all the bacterial isolates were closely related to members of *Firmicutes* (representing 70%) and *Gammaproteobacteria* (30%). These results contrast with those obtained in other studies on saline environments, in which the larger proportion of isolates are Gram-negative bacteria (Yeon et al. 2005; Jiang et al. 2006). Within the *Firmicutes*, most isolates were closely related to endospore-forming bacteria belonging or closely related to the genera *Halobacillus* (40 strains), *Alkalibacillus* (27), *Bacillus* (21), *Oceanobacillus* (7), *Gracilibacillus* (6), *Thalassobacillus* (3), *Filobacillus* (2), *Halalkalibacillus* (2), *Virgibacillus* (2), and *Salimicrobium* (2), whereas only 10% of isolates were closely related to Gram-positive non-endospore-forming cocci: *Marinococcus* (12 strains), and *Staphylococcus* (1). The second largest group cultivated from the salt lakes was the *Gammaproteobacteria*, *Halomonas* being the most prominent genus, with 40 isolates, followed by *Halovibrio* (4), *Chromohalobacter* (3), *Pseudomonas* (3), *Alkalispirillum* (2), *Salicola* (1) and *Stenotrophomonas* (1) (Carrasco 2009). The abundance of endospore-forming microorganisms in the salt lakes studied may be due to the ability of the endospores to be transported and their remarkable capacity for resistance and dormancy, which might allow them to survive in these habitats subjected to substantial seasonal changes (Claus and Berkeley 1986).

Novel Taxa

Some of the isolates were distantly related to any previously known bacterial species and thus, a polyphasic taxonomic study, based on phenotypic, genotypic and phylogenetic characteristics, was performed in order to describe them and to determine their correct taxonomic position.

4.7.1.1 Study of Strains CG12 and CG13 (Described as *Aquisalimonas asiatica* gen. nov., sp. nov.)

The proposal of the creation of the novel genus *Aquisalimonas* was based on two strains, designated CG 12 and CG 13, which were isolated from water samples collected from Lake Chagannor (Márquez et al. 2007). This genus belongs to the *Gammaproteobacteria*, and constitutes a new phyletic sublineage within the *Alkalispirillum-Alkalilimnicola* group of the family *Ectothiorhodospiraceae*. The genus *Aquisalimonas* was defined as Gram-negative, motile, non-endospore forming, moderately halophilic and alkali-tolerant rods, occurring singly, in pairs or long chains, catalase and oxidase positive, and able to reduce nitrate to nitrite. Cellular fatty acids mainly consist of $C_{18:1}\omega7c$, $C_{16:0}$, and $C_{12:0}$. *Aquisalimonas asiatica* is the type species of the genus and strain CG12 is the type strain. The G+C content of its genomic DNA is 63.6 mol%. The polar lipids detected in this strain are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, phosphoglycolipid and six different unidentified phospholipids (Márquez et al. 2007).

4.7.1.2 Study of Strain CH9d (Described as *Salsuginibacillus kocurii* gen. nov., sp. nov.)

Strain CH9d was isolated from a sediment sample of Lake Chahannor. When phylogenetic analysis was carried out, it was observed that its 16S rRNA gene sequence was very distant from those of other known taxa. *Thalassobacillus devorans* was its closest neighbor with a similarity value of only 91%, followed by other members of the genera *Bacillus, Halobacillus* and *Marinococcus*. The phylogenetic results, together with the phenotypic and chemotaxonomic differences found between the isolate and its phylogenetically related relatives led to the proposal of the creation of a novel genus, *Salsuginibacillus,* with the single species *Salsuginibacillus kocurii* (Carrasco et al. 2007b). Strain CH9d is a Gram-positive rod that forms non-pigmented colonies when grown at 37°C on alkaline, saline medium and produces ellipsoidal endospores located in central or subterminal position in swollen sporangia. However, *Thalassobacillus devorans* produces endospores located centrally without swelling of the sporangia (García et al. 2005). Conversely, species of the genus *Marinococcus* are non-spore-forming cocci characterized by their orange-pigmented colonies (Hao et al. 1984; Li et al. 2005). Strain CH9d is moderately halophilic and alkali-tolerant, whereas *Bacillus agaradhaerens* (its nearest *Bacillus* species) is a halotolerant and strictly alkaliphilic bacterium (Nielsen et al. 1995). In addition, strain CH9d differs from *T. devorans* and *B. agaradhaerens* in its inability to hydrolyze gelatin. In contrast to species of the genus *Halobacillus,* strain CH9d is negative for oxidase and is able to reduce nitrate to nitrite (Spring et al. 1996). On the other hand, the DNA base composition of strain CH9d (44.7 mol%) is similar to those of the previously

described *Halobacillus* species (40–45 mol%) (Spring et al. 1996; Amoozegar et al. 2003; Yoon et al. 2003, 2004, 2005; Liu et al. 2005) but different to those described for *T. devorans* (42.4 mol%) (García et al. 2005), *B. agaradhaerens* (39.3–39.4 mol %) (Nielsen et al. 1995), and *Marinococcus* species previously described (46.4–48.5 mol%) (Hao et al. 1984; Li et al. 2005). The peptidoglycan type of the strain CH9d is A1γ based on *meso*-diaminopimelic acid, the isoprenoid quinones are MK-7 (88%) and MK-6 (12%), and its major fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{17:0}$ and iso-$C_{15:0}$. With respect to the polar lipid composition, strain CH9d contains diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and two phospholipids of unknown structure (Carrasco et al. 2007b).

4.7.1.3 Study of Strain SH4s (Described as *Aquisalibacillus elongatus* gen. nov., sp. nov.)

The genus *Aquisalibacillus* with the single species *Aquisalibacillus elongatus* was proposed on the basis of the features of strain SH4s, isolated from water of the saline Lake Shangmatala. This strain is phylogenetically related to the genera *Filobacillus*, *Piscibacillus* and *Tenuibacillus,* within the family *Bacillaceae*. The 16S rRNA gene sequence similarity values between strain SH4s and the type strains of these three genera ranged from 95.4 to 95.9%. These values are lower than those found between the genera *Tenuibacillus* and *Piscibacillus* and the genus *Filobacillus*, their most closely phylogenetically related taxon (97 and 96.9%, respectively). Strain SH4s shares some phenotypic features with these three genera but, in contrast to *Piscibacillus* and *Tenuibacillus*, it is negative for Gram-staining and oxidase activity. Besides, the isolate clearly differs from members of the three genera in the motility (cells of strain SH4s are non-motile), ability to reduce nitrate to nitrite, DNA base composition (45.9 mol%), fatty acids profile (with iso-$C_{16:0}$ and iso-$C_{15:0}$ as the major components) and, overall, the peculiar peptidoglycan composition present in its cell wall (type A4β, based on L-Orn-D-Asp) (Márquez et al. 2008).

4.7.1.4 Study of Strain EN8d (Described as *Sediminibacillus halophilus* gen. nov., sp. nov.)

Strain EN8d was isolated from a sediment sample from Erliannor salt lake. This strain is most closely related phylogenetically to the genera *Thalassobacillus* (93.6% 16S rRNA gene sequence similarity) and *Halobacillus* (95–96%), although constitutes a separate line of descent within the radiation of Gram-positive rods. Cells of strain EN8d are Gram-positive, rod-shaped that occur singly, in pairs or in short chains. This strain is motile, moderately halophilic, facultatively anaerobic, oxidase and catalase positive, and is able to reduce nitrate to nitrite. Its genomic DNA G+C content is 47.5 mol%, it has a cell-wall peptidoglycan type A1γ with *meso*-diaminopimelic acid, MK-7 as the predominant menaquinone and

anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ as the major fatty acids. The polar lipids include diphosphatidylglycerol, phosphatidylglycerol, and a glycolipid. The novel genus *Sediminibacillus,* and the novel species *S. halophilus* was proposed for this strain (Carrasco et al. 2008).

4.7.1.5 Study of Strains XH-62, XH-63 and EJ-15 (Described as *Gracilibacillus orientalis* sp. nov.)

Three strains (designated XH-62, XH-63 and EJ-15) were phylogenetically related to species of the genus *Gracilibacillus*, *Gracilibacillus dipsosauri* their nearest phylogenetic neighbor (with 16S rRNA sequence similarities ranging between 95.4 and 95.8%). Strains XH-62 and XH-63 were isolated from water samples from the lake near Xilinhot (Lake Bayannur), whereas strain EJ-15 was isolated from sediments collected from Lake Ejinor. The three strains are Gram-positive, moderately halophilic rods that grow optimally at 10% NaCl. They are oxidase negative, catalase positive and strictly aerobic. They produce spherical, terminal and deforming endospores as well as colonies with a cream pigmentation. DNA G+C content of the three strains is in the range 36.1–37.1 mol%. They have *meso*-diaminopimelic acid in the cell wall peptidoglycan. The polar lipid pattern of strain XH-63, selected as representative strain of the isolates, consists of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and a phospholipid and two amino phospholipids of unknown structure. Besides, strain XH-63 possess menaquinone of the MK-7 type and a fatty acid composition very similar to those described for other species of the genus *Gracilibacillus* (Wainø et al. 1999). All the features of the three new isolates clearly indicated that they represent a new species for which the new name *Gracilibacillus orientalis* sp. nov. was proposed, with strain XH-63 as the type strain (Carrasco et al. 2006).

4.7.1.6 Study of Strain CG-15 (Described as *Bacillus chagannorensis* sp. nov.)

Strain CG-15 was isolated from a water sample collected from Lake Chagannor. The phylogenetic analysis showed that this strain belongs to the genus *Bacillus,* and forms a phyletic group with *Bacillus saliphilus* (96.0% 16S rRNA gene sequence similarity), *Bacillus agaradhaerens* (94.0%) and *Bacillus clarkii* (93.5%). Strain CG-15 is a Gram-positive, endospore-forming motile rod, facultative anaerobe that grows over a wide range (3–20%) of salt concentrations, with optimal growth at 7% salts and thus, it can be considered as a moderately halophilic microorganism. Besides, it is an alkalitolerant bacterium that grows at pH 5.8–11.0 (optimal at pH 8.5) and 6–40°C (optimal at 37°C). The DNA G+C content of this isolate is 53.8 mol%; this value is within the range for *Bacillus* but is higher than those of *Bacillus saliphilus* (48.4 mol%) (Romano et al. 2005), *Bacillus agaradhaerens* (39.2 mol%) (Nielsen et al. 1995) and *Bacillus clarkii* (42.2 mol%) (Nielsen et al. 1995).

Strain CG-15 contains a cell wall type based on *meso*-diaminopimelic acid and MK-7 as the major menaquinone. With respect to the cellular fatty acids composition, anteiso-$C_{15:0}$, iso-$C_{15:0}$ and anteiso-$C_{17:0}$, are its major components. The polar lipids detected are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and three different phospholipids of unknown structure. These chemotaxonomic characteristics are typical of those found in members of the genus *Bacillus* previously described (Priest et al. 1988; Heyrman et al. 2004, 2005; Wieser et al. 2005; Lim et al. 2006a, 2006b). However, strain CG-15 could be distinguish from other phylogenetically related *Bacillus* species in some features, such as cell morphology, range and optimal salt concentration for growth and optimal pH for growth as well as the genomic DNA G+C content or fatty acids composition. Therefore, on the basis of these polyphasic taxonomic data, the name *Bacillus chagannorensis* sp. nov. was proposed for the novel isolate (Carrasco et al. 2007a).

4.7.1.7 Study of Strain XH-22 (Described as *Virgibacillus salinus* sp. nov.)

A strain, related to the genus *Virgibacillus,* that was also studied in detail is strain XH-22. This isolate exhibited 16S rRNA gene sequence similarity values of 97.6 and 97.5%, with respect to *V. carmonensis* and *V. necropolis,* and values comprised between 96.9 and 94.9% with respect to the other *Virgibacillus* species. The values of DNA–DNA hybridization between strain XH-22 and the type strains of *V. carmonensis* and *V. necropolis* were 32% and 28%, respectively. These values are clearly lower than 70% cutoff generally accepted for species delineation and support the placement of strain XH-22 as a genotypically distinct species within the genus *Virgibacillus* (Wayne et al. 1987). Strain XH-22 is a Gram-positive, motile rod, with optimal growth at 10% total salts, pH 7.5 and 37°C. It has *meso*-diaminopimelic acid in the cell wall peptidoglycan and anteiso-$C_{15:0}$, $C_{16:0,}$ and iso-$C_{14:0}$ as the major fatty acids. As other members of the genus *Virgibacillus* previously described, MK-7 is its predominant menaquinone*;* however, strain XH-22 has in addition to this component, MK-6, MK-5 and an unidentified respiratory quinone in minor amounts, whereas *Virgibacillus carmonensis* and *Virgibacillus necropolis* have MK-6 and MK-8 as minor compounds (Heyrman et al. 2003). With respect to the polar lipid composition, strain XH-22 contains diphosphatidylglycerol, phosphatidylglycerol, a glycolipid and two different phospholipids of unknown structure. The presence of diphosphatidylglycerol and phosphatidylglycerol as major polar lipids are common characteristics of *Virgibacillus* species (Heyrman et al. 2003; Wang et al. 2008) but the presence of a glycolipid had not been reported previously in any species of this genus. Strain XH-22 can be clearly distinguished from *V. carmonensis*, its nearest phylogenetic neighbor, by differences in the oxidase reaction, colony pigmentation, optimum temperature for growth and acid production from some substrates. Therefore, on the basis of the data obtained, strain XH-22 belongs to the genus *Virgibacillus,* but constitutes a novel species, for which the name *Virgibacillus salinus* sp. nov. was proposed (Carrasco et al. 2009).

4.7.2 Archaeal Diversity of the Salt Lakes

On the basis of their growth at different salt concentrations, cell morphology and colony pigmentation and some biochemical tests, 268 isolates, representing extremely halophilic archaea, were obtained. We did not isolate any haloarchaea from Lake Chahannor, probably because the salinity of this lake was too low and haloarchaea were not present. The 16S rRNA gene sequences of extremely halophilic archaea isolated were amplified by PCR using forward primer ARCHF and reverse primer ARCHR (López-García et al. 2001; Arahal et al. 1996). The sequences obtained were analyzed and aligned using the ARB software package (Ludwig et al. 2004). The result of the comparison of these sequences with those from previously described haloarchaeal species obtained from the databases indicate that most of the archaea isolated belong to the genera *Halorubrum* (68), *Haloarcula* (60), and *Haloterrigena* (51). The other halophilic archaea are related to haloarchaea belonging to the following genera: *Natrinema* (27), *Haloferax* (19), *Natronococcus* (17), *Natronobacterium* (9), *Halomicrobium* (7), *Natrialba* (6), *Halobacterium* (2), and *Natronolimnobius* (2). The hypersaline environments studied show a great haloarchaeal diversity, since representatives from at least 11 different genera belonging to the family *Halobacteriaceae* were isolated. Besides, the higher number of isolates belong to well known genera, that according to previous studies are frequently isolated from hypersaline habitats, such as the genera *Halorubrum*, *Haloarcula* or *Haloferax* (Torreblanca et al. 1986; Grant and Ross 1986; Gutierrez et al. 1989; McGenity and Grant 1995; Oren and Ventosa 1996; Ochsenreiter et al. 2002). However, a large number of isolates were related to the genera *Haloterrigena* (51) or *Natrinema* (27), which are considered as not so widely represented in hypersaline environments. On the contrary, only two strains were related to the genus *Halobacterium,* which according to previous studies is very abundant in other hypersaline habitats (McGenity et al. 1998; Ventosa et al. 1999).

4.7.2.1 Novel Taxa

Several new isolates showed a low phylogenetic relationship with the previously described haloarchaeal species and they have been studied in more detail in order to determine their taxonomic position.

4.7.2.2 Study of Strain EJ-46 (Described as *Halovivax asiaticus* gen. nov., sp. nov.)

Strain EJ-46 was isolated from sediment of the saline lake Ejinor. This organism is neutrophilic and requires at least 15% NaCl for growth. $MgCl_2$ is not required. Optimal growth is in media containing 20% NaCl at pH 7.0–7.5. Polar lipids

analysis revealed the presence of phosphatidylglycerol and phosphatidylglycero-methylphosphate derived from both $C_{20}C_{20}$ and $C_{20}C_{25}$ glycerol diethers. Four glycolipids were detected, one of which may be novel. The 16S rRNA gene analysis revealed that strain EJ-46 is a member of the phylogenetic group defined by the family *Halobacteriaceae*, but there was a low degree of similarity to other members of this family. The highest 16S rRNA similarity values (94.9–94.8%) were obtained with the haloalkaliphilic species *Natronococcus occultus* and *Natronococcus amylolyticus,* respectively. The phenotypic, genotypic and phylogenetic analysis of strain EJ-46 showed that it should be classified as a new genus and species within the haloarchaea, for which the name *Halovivax asiaticus* gen. nov., sp. nov. was proposed (Castillo et al. 2006a).

4.7.2.3 Study of Strain XH-70 (Described as *Halovivax ruber* sp. nov.)

The pleomorphic, extremely halophilic archaeon strain XH-70 was isolated from the saline lake near Xilinhot. The strain required at least 2.5 M NaCl and 5 mM Mg^{2+} for growth. The 16S rRNA gene sequence analysis indicated that this strain belongs to the family *Halobacteriaceae*, showing 99.5% similarity with *Halovivax asiaticus,* and 94.7% and 94.6% similarity with *Natronococcus amylolyticus* and *Natronococcus occultus,* respectively. Polar lipid analysis supported the placement of strain XH-70 in the genus *Halovivax*. DNA–DNA hybridization studies (32% with *H. asiaticus*), as well as biochemical and physiological characterization, allowed strain XH-70 to be differentiated from *Halovivax asiaticus*. A novel species, *Halovivax ruber* sp. nov., was proposed to accommodate this strain (Castillo et al. 2007a).

4.7.2.4 Study of Strain XH-48 (Described as *Halostagnicola larseni* gen. nov., sp. nov.)

This organism is pleomorphic and exhibits optimal growth at 20% NaCl. The G+C content of its DNA is 61 mol%. TLC of polar lipids showed that strain XH-48 contained phosphatidylglycerol and phosphatidylglyceromethylphosphate derived from both $C_{20}C_{20}$ and $C_{20}C_{25}$ glycerol diethers and two unidentified glycolipids. The 16S rRNA gene phylogenetic analysis showed the position of strain XH-48 as a new distinct branch in the *Natrialba* clade, related to *Natrialba aegypticaca* (94.5%) and *Natrialba asiatica* (93.3%). Phenotypic characteristics of these two species of the genus *Natrialba* are very different from those of strain XH-48. The phenotypic, polar lipid profile and phylogenetic data based on the 16S rRNA sequence comparison support the placement of strain XH-48 in a new genus and species within the haloarchaea, for which was proposed the name *Halostagnicola larsenii* gen. nov., sp. nov. (Castillo et al. 2006b).

4.7.2.5 Study of Strain SH-6 (Described as *Halopiger xanaduensis* gen. nov., sp. nov.)

Strain SH-6 was isolated from a sediment sample from the Shangmatala salt lake. This strain was capable of growing over a wide range of NaCl concentrations, ranging from 15 to 30% NaCl. It grew optimally in the presence of 25% NaCl, as has been shown for most extremely halophilic archaea. $MgCl_2$ was not required for growth. Strain SH-6 was non-motile and pleomorphic, although long rod-shaped cells were most common. TLC of polar lipids showed that strain SH-6 contained phosphatidylglycerol and phosphatidylglyceromethylphosphate derived from both $C_{20}C_{20}$ and $C_{20}C_{25}$ glycerol diethers. The glycolipid S2-DGD-1 was also detected. The DNA G+C content of strain SH-6 is 63.1 mol%. Comparison of its 16S rRNA gene sequence with those of members of the family *Halobacteriaceae* revealed that strain SH-6 was distantly related to the other haloarchaeal genera investigated. *Natronolimnobius innermongolicus* and *Natronolimnobius baerhuensis* were shown to be the closest relatives of strain SH-6, having a 16S rRNA sequence similarity value of 94.6%. However, the similarity values were almost identical for several haloarchaea representing various genera, e.g., *Natrialba aegypticaca* (94.5%), *Natronorubrum tibetense* (93.5%) and *Natronococcus occultus* (94.1%). The phenotypic, polar lipid composition and phylogenetic data based on the 16S rRNA gene sequence comparison clearly supported the placement of strain SH-6 in a new genus and species within the haloarchaea, for which was proposed the new name *Halopiger xanaduensis* gen. nov., sp. nov. (Gutiérrez et al. 2006).

4.7.2.6 Study of Strains EJ-52, EJ-32, BG-1, EN-2 and SH-4 (Described as Four Species of the Genus *Halorubrum: Hrr. orientale* sp. nov., *Hrr. ejinorense* sp. nov., *Hrr. kocurii* sp. nov. and *Hrr. aquaticum* sp. nov.)

Five novel halophilic archaeal strains (designated EJ-52, EJ-32, BG-1, EN-2 and SH-4) were isolated from the saline lakes Ejinor, Bagaejinor, Erliannor and Shangmatala. Phylogenetic analysis based on the 16S rRNA gene sequence comparison, polar lipid composition and phenotypic characteristics indicate that these isolates belong to the genus *Halorubrum*. All strains require at least 2.5 M NaCl but $MgCl_2$ is not required. Strain EJ-52 was motile, pleomorphic and red-pigmented. Analysis of its 16S rRNA gene sequence showed that the isolate was closely related to *Halorubrum saccharovorum* (96.1% sequence similarity), *Halorubrum lacusprofundi* (95.9%), *Halorubrum tibetense* (95.2%), *Halorubrum alcaliphilum* (95.2%) and *Halorubrum vacuolatum* (95.1%). The polar lipids of strain EJ-52 were $C_{20}C_{20}$ derivatives of phosphatidylglycerol phosphate and phosphatidylglycerol phosphate methyl ester and a sulfated diglycosyl diether. On the basis of the data obtained in this study, strain EJ-52 represents a novel species, for which the name *Halorubrum orientale* sp. nov. was proposed (Castillo et al. 2006c).

On the basis of 16S rRNA gene sequence similarities, strain EJ-32 was shown to be phylogenetically related to *Halorubrum coriense* (97.9%), *Halorubrum trapanicum* (97.9%), *Halorubrum sodomense* (97.8%), *Halorubrum tebenquichense* (97.8%), *Halorubrum xinjiangense* (97.6%) and *Halorubrum terrestre* (97.4%). Strain EJ-32 was found to be non-motile and Gram-negative. The G+C content of its DNA is 64.0 mol%. Values for DNA–DNA hybridization with respect to phylogenetically related *Halorubrum* species were $\leq$ 49%, indicating that strain EJ-32 represents a novel species of the genus *Halorubrum*, for which the name *Halorubrum ejinorense* sp. nov. was proposed (Castillo et al. 2007b).

Strain BG-1 grows over a pH range from 6.0 to 9.0 with an optimum at pH 7.5. Hypotonic treatment caused cell lysis. Analysis of its 16S rRNA gene sequence positioned this isolate within the genus *Halorubrum* in the family *Halobacteriaceae*. Strain BG-1 was most closely related to *Halorubrum aidingense* (98.8% sequence similarity), *Halorubrum saccharovorum* (98.6%), *Halorubrum lacusprofundi* (98.6%), and *Halorubrum lipolyticum* (98.4%). However, DNA–DNA hybridization values between strain BG-1 and the most closely related members of the genus *Halorubrum* were below 40%. The polar lipid composition revealed the presence of mannosyl-2-sulfate-(1–4)-glycosyl-archaeol (S-DGA-3), the main glycolipid of neutrophilic species of the genus *Halorubrum*. The G+C content of its genomic DNA was 69.4 mol%. Comparison of the phenotypic characteristics between strain BG-1 and *Halorubrum* species supported the conclusion that BG-1 is a novel species within this genus, for which the name *Halorubrum kocurii* sp. nov. was proposed (Gutiérrez et al. 2008b).

Cells of strains EN-2 and SH-4 were motile rods and strictly aerobic. On the basis of 16S rRNA gene sequence analysis, these strains were closely related to *Halorubrum cibi* (97.9 and 98.0% similarity, respectively), *Halorubrum tibetense* (97.6 and 97.3%), *Halorubrum alkaliphilum* (96.8 and 97.1%), *Halorubrum luteum* (96.8 and 97.0%) and *Halorubrum lipolyticum* (96.8 and 97.0%). DNA–DNA hybridization experiments showed that strains EN-2 and SH-4 had relatedness higher than 70% but were not related to these species, with levels of DNA–DNA hybridization equal to or below 45%. Polar lipid analysis revealed that strains EN-2 and SH-4 contained phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester; sulfated diglycosyl diether and several unidentified glycolipids. It was concluded that both strains represent a novel species of the genus *Halorubrum*, for which the name *Halorubrum aquaticum* sp. nov. was proposed (Gutierrez et al. 2011).

4.7.2.7 Study of Strain EJ-57 (Described as *Natrinema ejinorense* sp. nov.)

A Gram-negative, non-motile, neutrophilic, pleomorphic and extremely halophilic archaeon, strain EJ-57, was isolated from the salt lake Ejinor. Strain EJ-57 is able to grow at 25–50°C and requires at least 1.8 M NaCl for growth, with an optimum at 3.4 M NaCl, and grows over a pH range from 6.0 to 8.5, with an optimum at pH 7.0. Hypotonic treatment caused cell lysis. Analysis of its almost complete 16S rRNA

gene sequence positioned the isolate within the genus *Natrinema*, in the family *Halobacteriaceae*. Strain EJ-57 was most closely related to *Natrinema versiforme* (96.2% sequence similarity), *Natrinema pallidum* NCIMB (95.9%), *Natrinema altunense* (95.8%) and *Natrinema pellirubrum* (95.5%). However, DNA–DNA hybridization experiments showed that this strain was not related to these species, with DNA relatedness equal or lower than 39%. The major polar lipids of the isolate were $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and a disulfated glycolipid S2-DGA-1. Comparative analysis of the phenotypic characteristics between strain EJ-57 and *Natrinema* species supported the conclusion that EJ-57 is a novel species within this genus, for which the name *Natrinema ejinorense* sp. nov. was proposed (Castillo et al. 2006d).

4.7.2.8 Study of Strains XH-65 (Described as *Haloterrigena salina* sp. nov.)

The extremely halophilic strain XH-65, isolated from the unnamed salt lake near Xilinhot was subjected to a polyphasic taxonomic characterization. This strain is neutrophilic, non-motile and grows optimally at 3.4 M NaCl, at pH 7.5 and at 37°C. Magnesium is not required for growth. On the basis of 16S rRNA gene sequence analysis, strain XH-65 was shown to belong to the genus *Haloterrigena* and was related to *Haloterrigena turkmenica* (98.1% sequence similarity) and other *Haloterrigena* species ($\leq$96.9%). DNA–DNA hybridization revealed 37% relatedness between strain XH-65 and *Haloterrigena turkmenica*. The polar lipid analysis revealed the presence of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and mannose-2,6-disulfate (1 $\rightarrow$ 2)-glucose glycerol diether (S_2-DGD). The results of the DNA–DNA hybridization and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain XH-65 from the *Haloterrigena* species with validly published names. Strain XH-65 represents a novel species, for which the name *Haloterrigena salina* sp. nov. was proposed (Gutiérrez et al. 2008a).

4.7.2.9 Study of Strains CG-6 and CG-4 (Described as *Natronorubrum sediminis* sp. nov.)

Two novel haloalkaliphilic archaea, strains CG-6 and CG-4, were isolated from the sediment of Lake Chagannor. Cells of the two strains were pleomorphic, non-motile and strictly aerobic. They required at least 2.5 M NaCl for growth with an optimum at 3.4 M NaCl, and grew at a pH range from 8.0 to 11.0, with an optimum at pH 9.0. Hypotonic treatment with less than 1.5 M NaCl caused cell lysis. They had similar polar lipid composition, possessing the $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of phosphatidylglycerol phosphate and phosphatidylglycerol phosphate methyl ester. No glycolipids were detected. Comparison of 16S rRNA sequences and morphological features placed them in the genus *Natronorubrum*. Their 16S rRNA sequence similarities with the recognized species of the genus *Natronorubrum* were 96.2–93.8%. Detailed phenotypic characterization and

DNA–DNA hybridization studies revealed that the two strains belong to a new species in the genus *Natronorubrum*, for which the name *Natronorubrum sediminis* sp. nov. was proposed (Gutiérrez et al. 2010).

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