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Susan Grant · William D. Grant · Brian E. Jones
Chiaki Kato · Lina Li

Novel archaeal phylotypes from an East African alkaline saltern

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Abstract DNA has been extracted on site from the brines of the final crystallizing pond of an alkaline saltern at Lake Magadi, Kenya. Amplification of 16S rRNA genes followed by cloning, sequencing, and phylogenetic analysis has revealed the presence of two distinct new archaeal lineages. The majority of cloned sequences showed greater than 95% identity to each other, but only 88%–90% similarity to any cultivated haloalkaliphilic Archaea, and form a distinct cluster within the known Haloarchaea. Two cloned genes showed close similarity to each other but only 76% similarity to any known archaeal sequence, and therefore represent a distinct phylotype only distantly related to the euryarchaeotal branch of the Archaea.

Key words Halophile · Haloalkaliphile · Phylogeny · Phylotype · 16S rDNA · Alkaline saltern · Archaea

Introduction

Soda lakes present stable, naturally occurring highly alkaline environments where pH values greater than 11.5 may be generated. They are characterized by the presence of high concentrations of carbonate or carbonate complexes as the major anions in solution and low levels of both Mg^{2+} and Ca^{2+}. The East African soda lakes of the Kenyan-Tanzanian Rift Valley provide a diversity of alkaline environments. The salinities of these lakes vary from about 5% total salts (w/v) in the more northerly lakes (Bogoria, Nakuru, Elmenteita, and Sonachi) to saturation in the south (Magadi and Natron), with roughly equal proportions of Na_2CO_3 and NaCl as the major salts (Jones et al. 1994). Nevertheless, because of the high ambient temperatures and high light intensities combined with a continuous supply of CO_2, these soda lakes are among the most productive natural environments in the world in terms of biomass. The less alkaline lakes are usually dominated by vast blooms of Cyanobacteria, while the hypersaline lakes such as Magadi on occasion support blooms of both cyanobacteria and alkaliphilic anoxygenic phototrophs belonging to the genera *Ectothiorhodospira* and *Halorhodospira* (Jones et al. 1998). The primary productivity of these phototrophic bacteria presumably drives all the biological processes in the soda lake environment.

The microbial diversity of soda lakes has been recently reviewed (Jones et al. 1998). Studies of nonphototrophic aerobic organotrophic isolates cultured from East African soda lakes has led to the characterization of a number of novel alkaliphilic and alkali-tolerant isolates, representing considerable phylogenetic diversity, particularly among the gram-positive bacteria (Duckworth et al. 1996). The anaerobic environment of these lakes has received less attention, with relatively few obligately alkaliphilic anaerobes having been isolated. Oligately anaerobic and alkaliphilic chemoorganotrophic isolates include separate and well-defined groups within the *Clostridium* spectrum (Owenson 1997).

Common salt is commercially harvested at Lake Magadi using a series of solar evaporation ponds. These salt-making ponds provide the most extreme alkaline environment at pH 12 and are colored bright red by blooms of haloalkaliphilic Archaea. Organisms cultured from these salterns have been assigned to a distinct physiological group of the *Halobacteriaceae* because they require not only high concentrations of NaCl but also high pH (between 8.5 and 11) and low Mg^{2+} (less than 10 mM) for growth. Microscopically, the isolates consist of rods and cocci, and were originally separated into two genera, *Natronobacterium* and

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S. Grant (✉) · W.D. Grant
Department of Microbiology and Immunology, University of Leicester, PO Box 138, Leicester LE1 9HN, UK
Tel. +44-116 252 2940; Fax +44-116 252 5030
e-mail: sg23@le.ac.uk

B.E. Jones
Genencor International BV, P.O. Box 642, 2600 AP Delft, The Netherlands

C. Kato · L. Li
The DEEPSTAR Group, Japan Marine Science and Technology Centre, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

Natronococcus (Tindall et al. 1984). However, recent phylogenetic analysis based on 16s rDNA sequences has indicated that members of the *Natronobacterium* group are diverse, and accordingly proposals for transfer of three of the species to other genera have been made (Kamekura et al. 1997). To date, only species closely related to the *Natronobacterium/Natronococcus* members of the alkaliphilic Haloarchaea have been cultured from these final crystallizing salterns, together with more rarely occurring nonarchaeal haloalkaliphilic bacilli, which form a distinct group in the *Bacillus* spectrum (Duckworth et al. 1996).

The development of molecular techniques that do not depend upon culture has revolutionized the approach to the analysis of prokaryote diversity. In particular, DNA extraction followed by the polymerase chain reaction (PCR) for the amplification of 16S rRNA genes, with or without appropriate cloning steps, has enabled the detection of groups that have yet to be brought into culture and demonstrated a high degree of sensitivity for those bacteria present in small numbers (Stahl 1997). Such methods have been applied to a number of marine, soil, thermal, and hypersaline environments (Amann et al. 1995; Benlloch et al. 1996), resulting in the description of as yet uncultivated groups of both Bacteria and Archaea. A new archaeal phylogenetic branch diverging deeply from the thermophilic or crenarchaeotal branch has been derived from cold deep ocean and Antarctic waters (Delong et al. 1994; Fuhrman et al. 1992), and sequences analyzed from a hot spring in Yellowstone National Park led the authors to propose that they might represent a previously uncharacterized lineage of Archaea, provisionally proposed as a third archaeal kingdom, the Korarchaeota (Barns et al. 1996).

This article reports the results of amplification and characterization of 16S rDNA from samples isolated on site from the final salt crystallizing pond at Lake Magadi in December 1996. A number of DNA samples from the East African Soda Lakes were obtained in this expedition, and shown to contain amplifiable DNA using primers specific for bacterial or archaeal 16S rDNA. This preliminary survey has indicated the existence of two novel archaeal groups, one phylogenetically related to but distinct from the known alkaliphilic halophiles, and the other showing little relatedness to any previously reported species.

Materials and methods

Sampling and DNA extraction procedure

Brine was sampled in a 250-ml stainless steel beaker mounted on the end of a flexible extendible 1-m pole. It was filtered on site through a sequence of sterile membrane filters (47 mm) in a 250-ml capacity polycarbonate filter unit (Sartorius) using a Nalgene hand pump, which produced a vacuum of 40–50 cmHg under field conditions. A glass fiber prefilter (GF/A, Whatman) was used first to remove suspended inorganic matter, which was discarded. The filtrate was then passed through an 8-μm cellulose nitrate filter (Sartorius) until the flow stopped. The collected filtrate then similarly was passed through a 3 μm cellulose nitrate filter, again until the flow stopped. (In the case of samples from lakes of less total salinity than the salterns of Lake Magadi, the process was repeated through a 0.22-μm cellulose acetate filter, but this was precluded by the viscosity of the brines in this instance).

The individual membrane filters were placed immediately into cold sterile buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA, 2 M NaCl) and kept on ice in a refrigerated cool box until they could be processed further, usually within 4 h of sampling. The material on the filters was dispersed by vortexing and the cells pelleted by centifugation. The DNA was extracted using a Pharmacia (Uppsala, Sweden) Rapid Prep Microgenomic Isolation kit, following the manufacturer's instructions. After isopropanol precipitation and centrifugation, the pelleted DNA was stored in the residual supernatant for transportation back to the UK, where the extraction was completed using standard procedures and the resulting DNA was dissolved in 50-μl sterile nanopure water for subsequent amplification by PCR.

PCR amplification and cloning

The PCR protocol was modified from that of Embley (1991). The reaction mix included the following components: 3 mM $MgCl_2$; 0.2 mM (each) dGTP, dCTP, dATP, and dTTP (Promega, Madison, WI, USA); 20 pmol each of primers 27Fb (AGA GTT TGA TCC TGG CTC AG) and 1492R (ACG GHT ACC TTG TTA CGA CTT) for bacteria, based on DeLong (1992) and Duckworth et al. (1996); or 27Fa (TCY GGT TGA TCC TGS CGG) (McGenity et al. 1998) with 1492R for Archaea; or *Taq* DNA polymerase 1.25 units with a reaction buffer supplied by the manufacturer (Advanced Biotechnologies, Leatherhead, UK) in a total volume of 50 μl. The template DNA was 1 μl of the material extracted as described here, containing approximately 10–100 ng. The protocol consisted of a 2-min template melting step followed by 30 cycles of 30 s at 95°C, 40 s at 55°C, and 2 min at 72°C, with a final 10-min extension at 72°C.

The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Crawley, UK), and checked by agarose gel (1% w/v) electrophoresis (Sambrook et al. 1989). They were then cloned using the pCR-Script Amp SK(+) cloning kit (Stratagene, Cambridge, UK), following the manufacturer's protocol. White clones were checked for size of insert by the alkaline–SDS rapid colony disruption method (Sambrook et al. 1989).

Sequencing of the cloned PCR products

Selection of samples for sequencing on the Perkin Elmer/Applied Biosystems (Foster City, CA, USA) model 373S DNA sequencer was made on the basis of RFLP analysis with *Bam*HI + *Hin*dIII or *Rsa*1 + *Msp*1. Primers used for sequencing were M13(−20) and reverse; 533F (GTG CCA

GCM GCC GCG GTA A) (Robb et al. 1995); 958R (YCC GGC GTT GAM TCC AAT T) (DeLong 1992); 520R (GTA TTA CCG CGG CTG CTG) (Kato et al. 1997); and 1492R. In addition three new primers were synthesized by means of a model 392 DNA Synthesizer (Perkin Elmer/Applied Biosystems): 320F (CTA CGG GGC GCA GC); 800R (TGG CAC AGC GCA GGC); and 850F (GCT AAG CGG GCC GCC). This method permitted sequencing of about 1450 bases for each cloned 16S rDNA gene.

Analysis of DNA sequences

During analysis use was made of the GCG package (Wisconsin Package Version 9.1-UNIX, Genetics Computer Group [GCG], Madison, WI, USA), available at Leicester University, for FASTA searches and direct comparison of sequences. The Ribosomal Database Project was accessed via the Internet to download 16S rRNA secondary structure (Guttell 1994) and for the Check Chimera facility (Maidak et al. 1997). For further analysis the DNA sequences were compared with those in the EMBL and GenBank databases using the GENETYX-MAC/CD program (Version 34.0; Software, Tokyo, Japan). Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei 1987) with multiple alignments from the CLUSTAL W program (Thompson et al. 1994).

Accession numbers

The EMBL/GenBank 16S rDNA sequences used in the phylogenetic analysis are shown in Fig. 1. The 16S rDNA sequences determined in this study have been deposited in the DDBJ, EMBL, and Genbank databases, with accession numbers for the Magadi salt pond (MSP) clones 1, 8, 9, 11, 12, 14, 16, 17, 22, 23, and 41 as AB012049 to AB012059, respectively.

Results and discussion

The final salt pond at Lake Magadi yielded DNA samples that were amplifiable using the archaeal 16S rDNA primer, but not with the bacterial primer, which is unsurprising in view of the dominance of Archaea in these hypersaline environments. The inserts in a total of ten clones derived from this PCR product were sequenced fully and compared

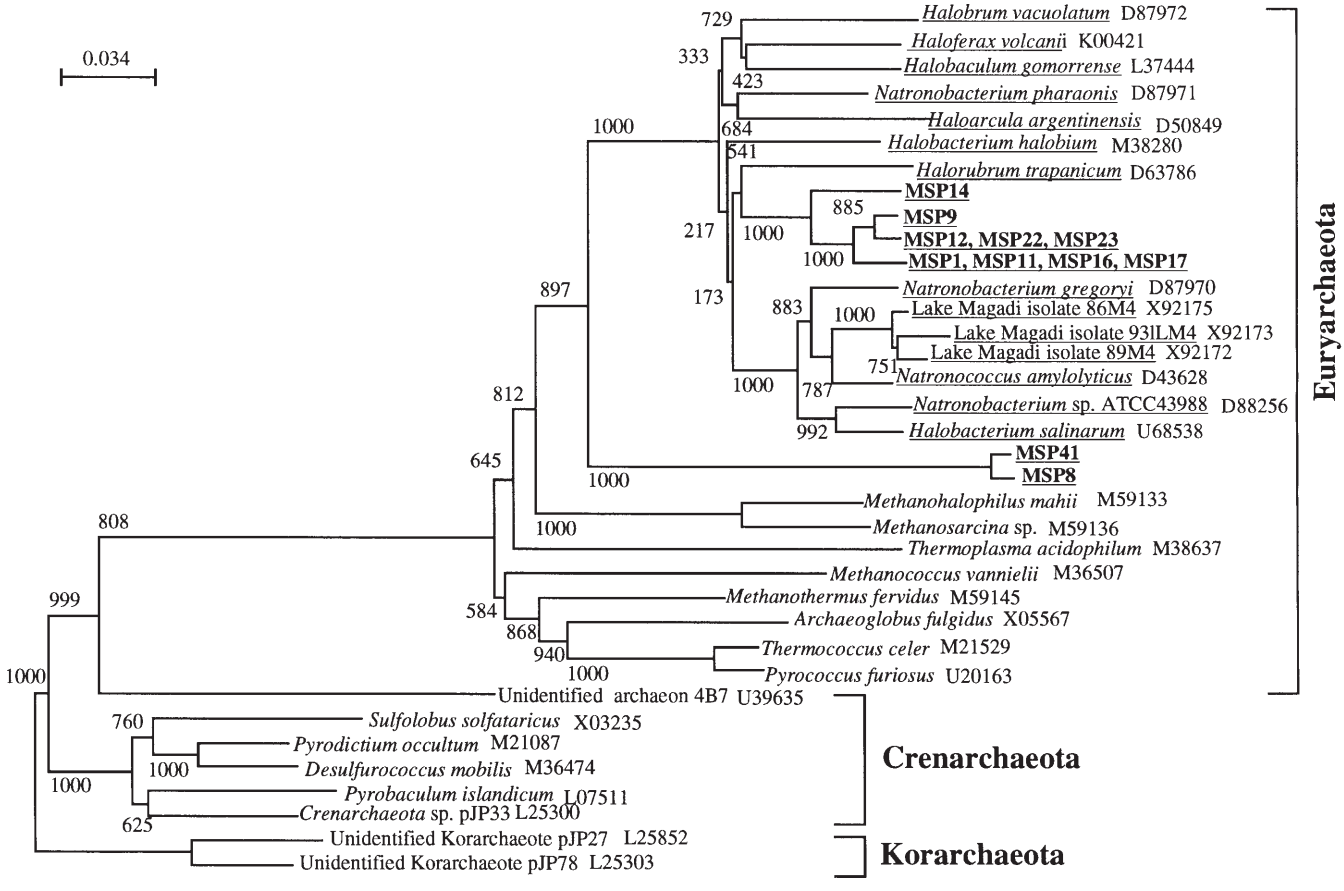

Fig. 1. Unrooted phylogenetic tree showing the relationship between the 16S rDNA gene sequences of the MSP clones and those of other Archaea, using the neighbor-joining method of Saitou and Nei (1987). The scale represents the average number of nucleotide substitutions per site. Bootstrap values (1000 replicates) are shown at the nodes. Haloarchaeal sequences are underlined. *a*, "*Halorubrum trapanicum*" D63786 (JCM 8979) is probably a *Halococcus* sp. (Kamekura et al. 1997)

with the DNA databases. Figure 1 shows the results of phylogenetic analysis for these ten 16S rDNA sequences and representative members of the Archaea, including named haloalkaliphilic species and uncharacterized isolates from the saltern site. Nine of the sequences (MSP1, 9, 11, 12, 14, 17, 22, and 23) are closely related to each other, forming a single cluster of three groups within the known halobacteria. Sequences within this cluster had 95%–99.7% identity, but showed only a maximum of 88%–90% identity to any 16S rDNA sequence in the databases, including the soda lake isolates 93dLM4, 82M4, 89M4, 93lLM4, 98NT4, and 86M4 reported by Duckworth et al. (1996).

The closest match was generally to *Natronobacterium* (now proposed as *Natronomonas*) *pharaonis* (Kamekura et al. 1997), with identities to species such as *Natronococcus occultus, Natrobacterium* (now *Natrialba*) *magadii*, or *Natronobacterium* (now *Halorubrum*) *vacuolatum*, species that are cultivable from this environment, showing a similar relatedness of only 87%–89%. This result suggests that the organisms concerned probably represent a novel group of Archaea, probably at the generic level. Although quantitative estimates of relative numbers have not been carried out for this study, these results, together with partial sequence of a further ten clones, indicate that this group must be a major contibutor to the archaeal population in these salterns.

Table 1 shows signature sequence analysis of the region 610–620 (*Escherichia coli* numbering) for the MSP clones and selected halophilic and haloalkaliphilic archaeal 16S rDNA sequences from a total of 68 included in the analysis. The sequences for the MSP clones of the main cluster are identical, but differ from all others by at least two bases. Analysis of the sequences from nucleotide positions 151–200 (174–218 by *E. coli* numbering) suggested by Benlloch et al. (1996) as differentiating at genus level is shown in Table 2. When MSP sequences are compared with those of cultivable haloalkaliphilic Archaea, the sequences for four closely related strains of the *Natronococcus occultus* group (Duckworth et al. 1996) are identical, but differ from that of *Natronococcus amylolyticus.* Similarly, sequences in this region for *Natrialba magadii* and the related strain 98NT4 are identical. All other sequences differ in all three areas. With relatively few strains available for analysis, and the taxonomic relationships between them still not fully resolved, sequence signatures in this region cannot be designated as genus specific. However, when applied to the MSP clones, it differentiates into four groups which correlate with the results of the phylogenetic analysis of the full 16SrDNA sequence, although placing MSP9 with MSP1, 11, 16, and 17.

The tenth sequence, MSP8, showed even less affiliation to other halobacteria. A FASTA search using the GCG package revealed only a maximum 76% identity with known Archaea. It possesses 43/49 of the homologous and 11/12 nonhomologous signature features defining the archaeal domain as described by Winker and Woese (1991). Because this sequence MSP8 was unique, it was decided to screen for related clones by PCR amplification using a specific primer that was designed particularly to distinguish it from the major cluster revealed in this study. This primer, 286F (AGC TGT CAG TCG TGT TCT G), when used with 1492R gave a product of approximately 1200 bp when amplifying directly from a colony or plasmid preparation of MSP8, but gave no product with members of the main cluster group such as MSP9 or MSP12. Screening of further clones derived from the 8-μm filter sample of the final salt pond by an independent PCR amplification reaction was therefore carried out. The majority of clones failed to amplify with primers 286F and 1492R (although positive with the control primers 27Fa and 1492R). Of 45 clones

Table 1. 16S rDNA signature sequences of MSP clones and halophilic and haloalkaliphilic Archaea

Haloarchaea	
Haloferax species (4)	TCC GCC AGC TC
Haloarcula species (8)	TCG ACC AGC TC
Haloarcula species (1)	TCG ACG CGC TC
'Natrinema' species (3)	TCC GCG CGC TT
Halorubrum species (7)	TCC ACC TGC TC
Halorubrum species (2)	TCC ACC CGC TC
Haloalkaliphilic Archaea	
Natronococcus occultus group (4)	TCT CCC CGC CT
Strains SSL1 and 98NT4	TCT CTC CGC TT
Natronococcus amylolyticus	TCC GCA CGC TT
Strains 93dLM4 and 82M4	TCC GCG CGC TC
Natrialba magadii	TCT CTT CGC TT
Natronobacterium gregoryi	TCT CCT CGC TC
Natronomonas pharaonis	TCC ACT CGC TT
Halorubrum vacuolatum	TTC ACC CGC TC
MSP clones	
MSP1, 9, 11, 12, 14, 16, 17, 22, and 23	TCT GCA CGC CC
MSP8 and 41	TCC ACG CGC TC

Based on the region 610–620 (*E. coli* numbering)
The numbers in brackets refer to the number of species in the genus or group that have this identical sequence

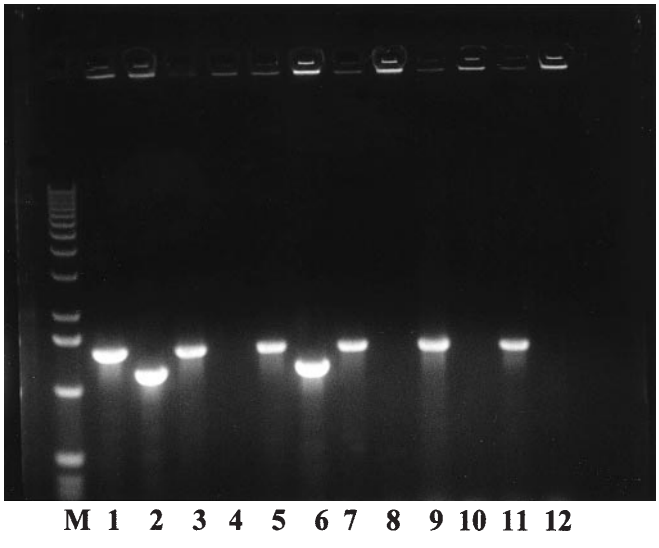

Fig. 2. Gel electrophoresis of PCR products from MSP clones amplified with the archaeal primer 27Fa (*odd-numbered lanes*) or the MSP8-specific primer 286F (*even-numbered* lanes), together with reverse primer 1492R (see text for details). *Lane M*, 1-Kb ladder molecular markers; *lanes 1*, *2*, MSP8 control; *lanes 3*, *4*, MSP40; *lanes 5, 6*, MSP41; *lanes 7*, *8*, MSP42; *lanes 9*, *10*, MSP43; *lanes 11*, *12*, MSP44

Table 2. Alignment of 16S rDNA in the region 174–218 for haloalkaliphilic archaea and the MSP clones

Natronococcus amylolyticus	CGTGC	ATA	CCGCTCTC	ATGCTGGAAGTTGCAGAGA	GCTC	GAAATGCCCCGG
Nc. occ.[a] 89M4, 93ILM4, 86M4	TCCCC	ATA	CCGCTCGA	CGCCTGGAA-CTGC-GCGA	GCCC	GAAACGCTCCGG
Natronobacterium gregoryi	CCTGC	ATA	CGGCTCTC	ATGCTGGAAGTGCA-GAGA	GCTC	GAAACGCTCCGG
Natrialba magadii; Str. 98NT4	CTGGA	ATA	CGACTCTC	ATCCTGGAG-TGGA-GAGA	GTCC	GAAATGCTCCGG
Natrialba sp. str. SSL1	CCGGA	ATA	GGGCTCTC	ATCTTGGAG-TGGA-GAGA	GCCT	GAAACGCTCCGG
Strain 93dLM4	CCGGA	ATA	GGAGTCCC	AGGCTGGAA-CTGCCGGGA	CTCA	GAAATGCTCCGG
Strain 82M4	CCGTG	ATA	CGATTCTC	AGCCTGGAA-GTGGCGAGA	ATCC	GAAACGCTCCGG
Halorubrum vacuolatum	AGTGC	ATT	CCACACTC	AGCCTGGAA-TGGC-GAGT	GTGA	CAAACGCTCCGG
Natronomonas pharaonis	TCCGG	ATA	CCGATTTC	ATCCTGGAATTGGA-GAAT	GTGC	GAAATGCTCCGG
Clones MSP1, 9, 11, 16, 17	TCTGA	ATA	AGACTCAC	ATCTTGGAG-TAGA-GTGA	GTCT	GAAATGCTACGG
Clones MSP12, 22, 23	TCTGA	ATA	GGGCTTGC	ATCTTGGAG-TAGA-GCAA	GTCC	GAAATGCTTCGG
Clone MSP14	ACCGG	ACA	ACGGTCTC	ACGTTGGAG-TGCA-GAGA	TCGT	GAAACGCTCCGG
Clones MSP8,41	GCCGG	ATA	AGAGAGGG	ATACTGGAA-AGTT-CCCT	CTCA	GAAATGCTACGG

Numbering is based on that for *E. coli*
The three regions highlighted by brackets are those suggested by Benlloch et al. (1996) as differentiating at genus level
[a] *Nc. occ.*, *Natronococcus occultus*

Table 3. Interdomain and kingdom signatures for clones MSP8 and 41

	Bacteria	Archaea				
Base position(s)[a]		Euryarchaeota	Crenarchaeota	Korarchaeota	Lake Magadi Sequence 8 and 41	
					Bases	Signature
27:556	G:C	G:C	C:G	Eury	G:C	Eury
28:555	R:Y	G:Y	C:G	Eury	G:C	Eury
30:553	Y:R	Y:R	G:C	A:U (Euk)	C:G	Eury
34:550	C:G	U:G	C:G	Cren	U:G	Eury
289:311	G:C	C:G	G:C	Eury	**A:U**	?
501:544	Y:R (C:G)	R:Y (G:C)	C:G	Eury	G:C	Eury
503:542	Y:R or A:U	C:G	G:C	Cren	**G:C**	**Cren**
504:541	C:G	Y:R	G:Y	Eury	U:A	Eury
513:538	Y:R or A:U	C:G	U:A	Cren	**G:C**	?
518	C	C	U	Eury/G	C	Eury
658:747	N:N	Y:R (C:G)	G:C	Cren	C:G	Eury
692	U	U	C	Eury	C or U	Cren / Eury
965	Y or A	Y	G	Eury	U	Eury
1074:1083	G:U	A:C	G:U	Cren	**G:U**	**Cren**
1244:1293	N:N	Y:R	R:Y	Eury	C:G	Eury
1252	Y or A	U	C	Cren	**A**	**Bact**
1335	N	C	G	ND	**G**	**Cren**
1408	A	A	G	ND	A	Eury

Eury, Euryarchaeota; Cren, Crenarchaeota; Bact, Bacteria
The signatures are based on those of Winker and Woese (1991) and Barns et al. (1994). Those in MSP8 and 41 that do not conform to the euryarchaeotal sequences are shown in bold
[a] Positions based on *E. coli* numbering

screened, 1 clone, MSP41, gave a PCR product with specific primer, as shown in Fig. 2. The 16S rDNA insert in this clone was accordingly sequenced for comparison with MSP8. It showed 98.7% identity to MSP8, and a corresponding close phylogenetic relatedness. As shown in Fig. 1, both sequences position on the fringes of the known halobacteria, showing little relationship to the cultivable alkaliphilic halophiles, and placing MSP8 and 41 as members of a deeply branching group of the Euryarchaeota.

MSP41 shared all the signature features described earlier for MSP8. Both possess the simpler structure of the helix located in position 198–220 (*E. coli* numbering), which distinguishes most euryarchaeotes from the crenarchaeotes (Winker and Woese 1991). Analysis of domain and kingdom signatures (Winker and Woese 1991; Barns et al. 1994) for clones 8 and 41, shown in Table 3, reveals a majority of euryarchaeotal signature features (11/18), consistent with the phylogenentic placement, and only 3 crenarchaeotal, 1 bacterial, and 2 features uncharacteristic of any domain. One of these signature bases differs in the two clones, leading to uncertain assignment. There is no indication of affiliation to the Korarcheaota.

The lack of relatedness of the new phylotype represented by clones MSP8 and 41 to other Archaea raises the

Table 4. Sequence signatures of MSP8 and 41 compared with those defining the three main methanogen groups and the extreme halophiles

Position(s)[a] of base or pair	*Methanomicrobiales*	*Methanobacteriales*	*Methanococcales*	Extreme halophiles	MSP8 and 41
321:332	U:A	C:G	A:G	U:A	U:A
369:392	U:A	C:G	C:G	U:A	**U:G**
370:391	U:A	C:G	C:G	U:A	**C:G**
371:390	A:U	G:C	G:C	A:U	A:U
438:496	G:G	U:A	U:A	U:A	U:A
740	C	U	U	G	G
741	A	G	G	A	A
825:875	G:C	A:U	A:U	G:C	G:C
871	G	U	U	G	**U**
1069	U	C	C	C	C
1076:1081	U:A	C:G	C:G	C:G	C:G
1084	A	G	G	G	G
1122:1151	C:G	U:A	A:U	U:A	**A:U**
1409:1491	A:U	C:G	C:G	A:U	A:U
1482	A	G	G	A	A

Sequence signatures positions for the methanogens and extreme halophiles are those defined by Rouvière et al. (1992). Those in MSP8 and 41 that do not conform to the signatures for the extreme halophiles are shown in bold

[a] Positions based on *E. coli* numbering

question of physiology. The affiliation with the euryarcheotal branch suggests the possibility that the organisms concerned could be methanogens. Alkaliphilic and alkalitolerant methanogens have been isolated from soda lakes, including Lake Magadi (Kevbrin et al. 1997). The obligate alkaliphiles such as *Methanohalophilus* (*Methanosalsus*) *zhilinaeae* fall within the *Methanomicrobiales*, and are characterized by the utilization of one-carbon substrates such as methanol rather than H_2/CO_2. However, available 16S rDNA sequence for organisms of this type show less than 72% similarity to that of clones MSP8 and 41. In addition, the latter lack the distinctive composition in the region 266–273, TACCTACT, which distinguishes the methanosarcina group of the *Methanomicrobiales* (Rouvière et al. 1992), instead conforming to the dominant archaeal sequence with GCTCACCA.

Signature sequences of the type shown in Table 3 for the methanogens as a whole are generally almost identical with those of the extremely halophilic archaea (Burggraf et al. 1991). Interestingly, the single exception, the presence in methanogens of a cytosine residue rather than uridine at nucleotide position 1159 (*E. coli* numbering), is also found in clones MSP8 and 41. 16S rDNA sequence signatures defining the three major groups of methanogens, the *Methanomicrobiales, Methanobacteriales*, and *Methanococcales*, and of the extreme halophiles (Rouvière et al. 1992) are compared with those of MSP8 and 41 in Table 4. Three of the signatures in this novel phylotype are those found in methanogens (*Methanobacteriales* or *Methanococcales*), and only one is distinctly that of the extreme halophiles. However, because the majority are shared between one or more of the methanogen groups and the halophiles, no firm conclusions can be drawn from this analysis. Clearly the possibility that this phylotype represents a new methanogenic branch would need to be addressed in any future attempts to culture the organisms concerned.

These results further indicate the extent of biodiversity still undetermined in these extreme environments.

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