On the hunt for mesophilic Korarchaeota

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Introduction

In 1994 Barns et al. obtained two unusual archaeal clones from Obsidian Pool (Yellowstone) which did not fit into either the Euryarchaeota or the Crenarchaeota. After careful analysis of the data a new kingdom within the Archaea was proposed, the Korarchaeota (Barns et al., 1996). According to their data the Korarchaeota branch very near the root of the archaeal tree and the possibility exists that representatives of this ne kingdom may display novel biological properties of relevance to our understanding of ancient organisms. Although not in pure culture yet a Korarchaeote has been reported to be a prominent species in an anaerobic, thermophilic continuos culture (Burggraf et al., 1997). In 1999 (Takai and Sako) presented data on 3 additional clones which grouped with the clones described by Barns et al. (1996). Furthermore, another clone that did not fit into one of the three kingdoms was described. The new korarchaeal clones were isolated from a shallow water hot vent site and from an acidic hot spring. If the Korarchaeota indeed represent a new kingdom within the Archaea, one might expect a greater phylogenetic and physiological diversity than described so far. One could speculate that more than thermophilic and acidophilic members of this kingdom exist. However, so far nobody has looked for Korarchaeota in samples from mesophilic environments.

The scientific goal of this study is to expand the very limited phylogenetic diversity within the newly proposed kingdom of the Korarchaeota. The analysis of further clones from this lineage will help to clarify the position of the Korarchaeota within the phylogenetic tree of life and might extend the range of habitats in which this almost untouched group of Archaea can be found

Material and Methods

Genomic DNA was isolated from cyanobacterial mats from the Sippewissett salt marsh (Falmouth, Cape Cod) and from Guerrero Negro (Baja California, Mexico). Additionally, DNA from a hydrothermal vent site (Guaymas Basin, Mexico) was extracted. DNA was extracted from different depth within the core (5, 10, 15 cm.) which corresponds to in situ temperatures of 58, 90 and 100°C respectively.

DNA was extracted using a Soil DNA Kit™ (MO BIO, 12800-100-S). In case of the Guaymas sediments this method was not appropriate to isolate DNA and instead a modified phenol-chloroform-isopropylalcohol procedure was applied. To about 0.5 g (ww) of sample 895µl of lysis buffer, 62.5 µl SDS (20%) and 0.5 g beads (0.2 mm, Biospec) were added. The whole sample was then "bead beaten" at full speed for 15 s (which might have been somewhat short).

Then 42 µl Proteinase K (20 mg/ml) was added to the mixture and incubated at 55°C for one hour. This step can be done in a rotating hybridization oven.

The samples were divided into about 800 µl portions and then extracted with 100µl phenol-chloroform-isopropylalcohol (25:24:1) at 55°C for 10 min. After centrifugation (10 min., 14 krpm) the aqueous supernatant was carefully removed and the extraction step was repeated two more times. The aqueous phases were pooled and DNA was precipitated by adding 0.1 vol. NaAc (3M, pH 5.2) and 2 vol. ethanol (H_2O free, -20°C). The samples were kept overnight at -20°C and then spun down (14000 g, 20 min., 4°C). The pellet was washed with 200µl ice-cooled ethanol (70%) and spun down again (ca. 5 min.). The supernatant was withdrawn and the pellet was air dried and subsequently taken up in pure and sterile filtered water. In our case the DNA was dissolved in only 15µl of water.

PCR amplification

The oligonucleotide sequences used for Korarchaeal rDNA amplification were as follows (Brunk and Eis, 1998):

236F, 5′-GAG GCC CCA GGR TGG GAC CG-3′

626F, 5′-GTT AAA TCC GCC TGA AGA CA-3′

Either an universal- (U1391R) or an archaeal- (ARCH915R) specific sequence was used as the reverse primer. Arch21F and Eub8F were used as archaeal and eubacterial specific forward primers.

DNA was amplified on a Eppendorf Mastercycler Gradient using AmpliTaq Gold™ polymerase (Perkin Elmer) and the following reaction mixture:

10x Ampli Taq Buffer II	2.5	µl
25 mM MgCl2	2.5	µl
dNTP's	1	µl
Primer forward	1	µl
Primer reverse	1	µl
1% BSA	1.25	µl
dH2O	10.55	µl
Ampli Taq Polymerase	0.2	µl
Template DNA	5	µl
Total volume	25	µl

The quality (size) of PCR products was routinely checked by agarose-gel electrophoresis (1.25 % SeaKem w/v in 0.5x TBE).
PCR products were then cloned using the Invitrogen TOPO TA cloning kit, according to the descriptions of the kit. The transformed E. coli TOP10 cells were grown overnight on LB-Amp plates (50 µg/ml ampicillin, x-gal). Only white colonies were picked for a PCR of the insert.

RFLP digest (Restriction Fragment Length Polymorphism)
In order to determine the diversity of the obtained PCR products of inserts an aliquot of the product was digested by means of restriction enzymes.

PCR product	10	µl
d H_2O	8	µl
10x Buffer2	2	µl
Hind PI	0.1	µl
MspI	0.05	µl

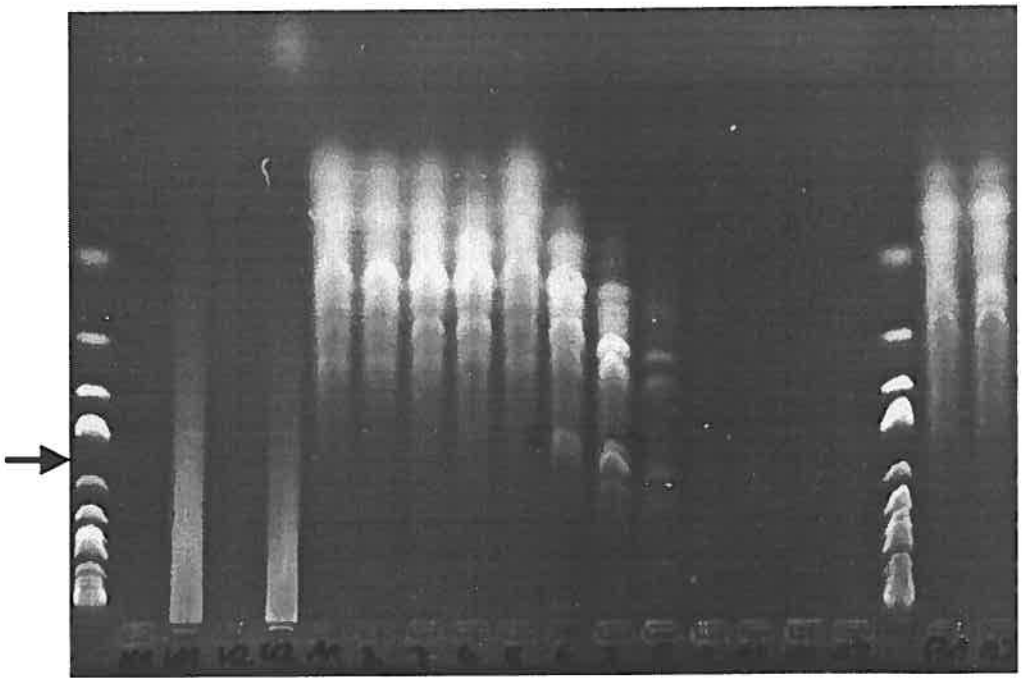
The whole assay was incubated at room temperature for 1-3 hours (or overnight).
Products were subsequently separated by gel electrophoresis (2% MetaPhor, 70 V). Only clones that exhibited a different RFLP pattern were sent for sequencing.
Some of the sequences were determined in house on a PE ABI 310 capillary sequencer.
The phylogenetic analysis of sequences was done with ARB (TU Munich) and BLAST

searches were done on the web site of the Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast).

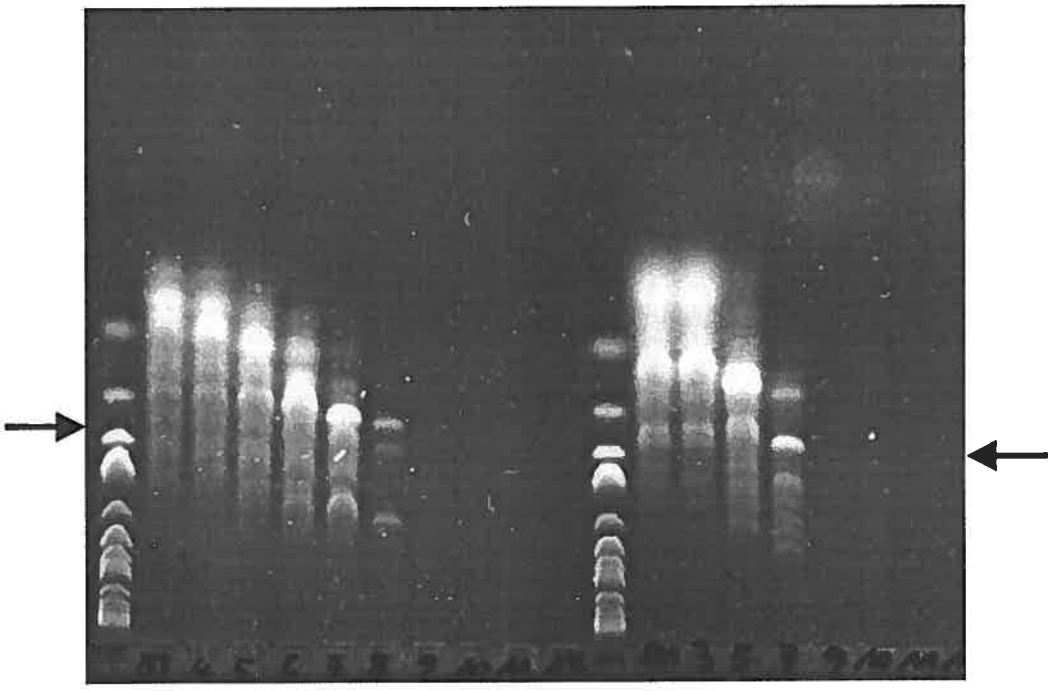
Results and Discussion

Bacterial mats: DNA was successfully isolated from the Sippewissett bacterial mat and the cynobacterial mat from Guerero Negro (Baja California, Mexico) using a soil DNA extraction kit. Without further purification but a 1/10 dilution step this DNA could be used for PCR. In order to determine the optimal annealing temperature and primer combinations for Korarchaeota 16S rDNA amplification a gradient PCR (52-72°C) with Baja cyanobacterial mat DNA and different sets of primer combinations was done (Fig. 1). Although the primers bound more specifically at higher temperatures (t_{opt} = 64-66°C) there were still several other bands present which indicates unspecific binding of the primers to genomic DNA. Nevertheless, the appearance of PCR products of approximately the right size class may indicate the presence of Korarchaeota 16S rDNA. Attempts to purify the PCR products on a low melt agarose gel were partially successful but the cloning of the DNA bands that were cut out from the gel failed. Only very few colonies of transformed E. coli grew on LB-AMP-X-gal plates and most of them were blue. This indicates (I) that the transformation itself was not very efficient and (II) that almost all of the transformants contained a plasmid without insert. Eventually this was due to loss of A-tags at the ends of the PCR product which greatly diminishes the ligation efficiency.

Hydrothermal sediment: In contrast to the cyanobacterial mat samples the extraction of DNA from deep-sea vent sediments failed with the MO BIO Kit. A more elaborate procedure that involved phenol-chloroform-isoamylalcohol extraction steps was used to obtain DNA from these sediments. Even with this method, initially no DNA was detected in the extract by means of gel electrophoresis. Only after concentrating the extract about 15 times the DNA was concentrated enough for PCR. The low extraction efficiency could either be due to the high content of hydrocarbons present at that particular site or due to very low bacterial biomass. Evidence for the latter is given in Fig. 2 and 3. The photomicrographs of DAPI stained sediment samples show very little bacterial cells. FISH (fluorescence in situ hybridization) with an Archaea specific oligonucleotide probe indicate again the presence of only a few cells and most of the probe seem to bind unspecifically to inorganic sediment particles.



K236F / U1391R 66°C



K624F / U1391R 66°C K236F / ARCH915R 64°C

Fig. 1: PCR products of a temperature gradient (52-72°C) amplification with different primer sets specific for Korarchaeota. Sample: Cyanobacterial mat Baja California (Mexico).

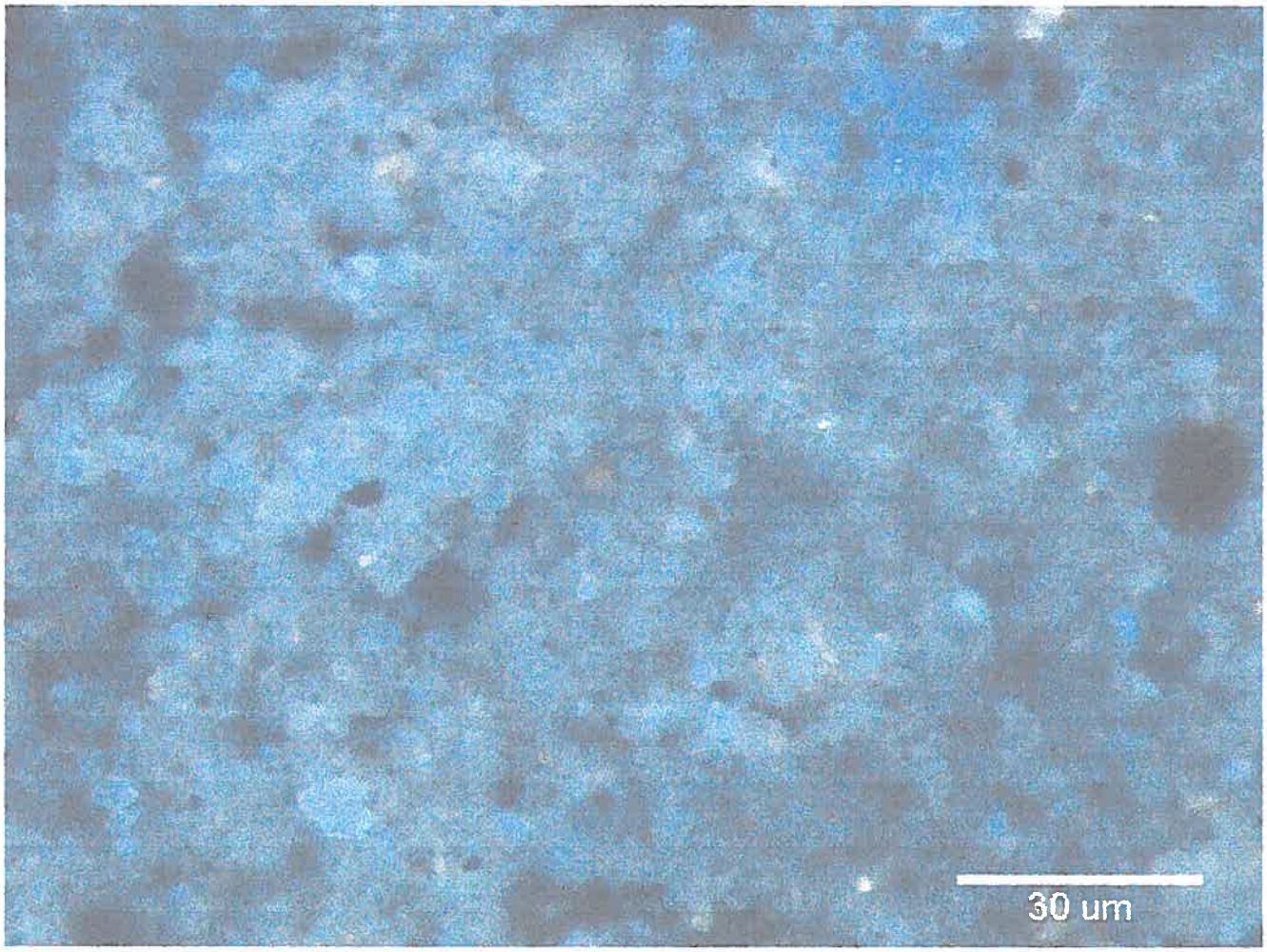
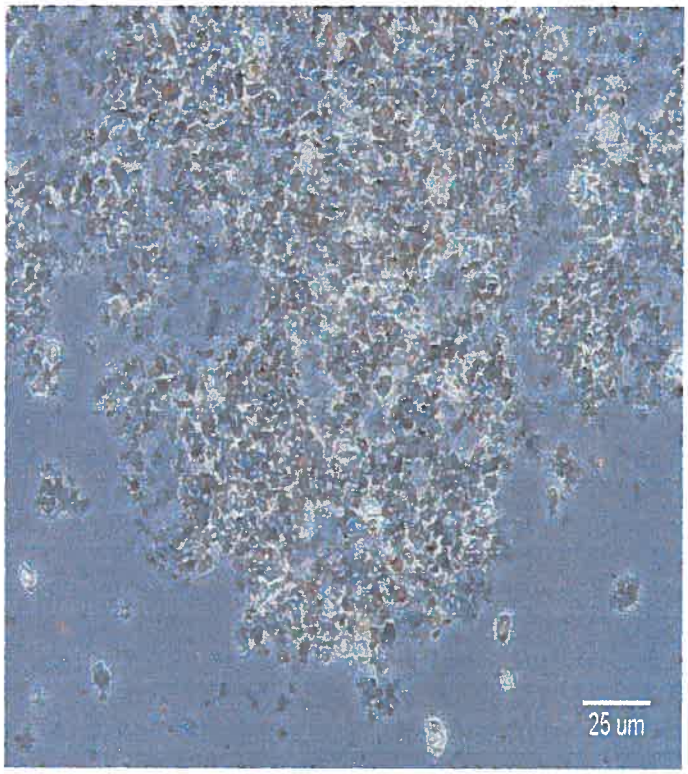


Fig. 2: Photomicrograph of a DAPI stained sediment sample from the Guaymas Basin (Mexico). The in situ temperature of this sediment layer was about 100°C.



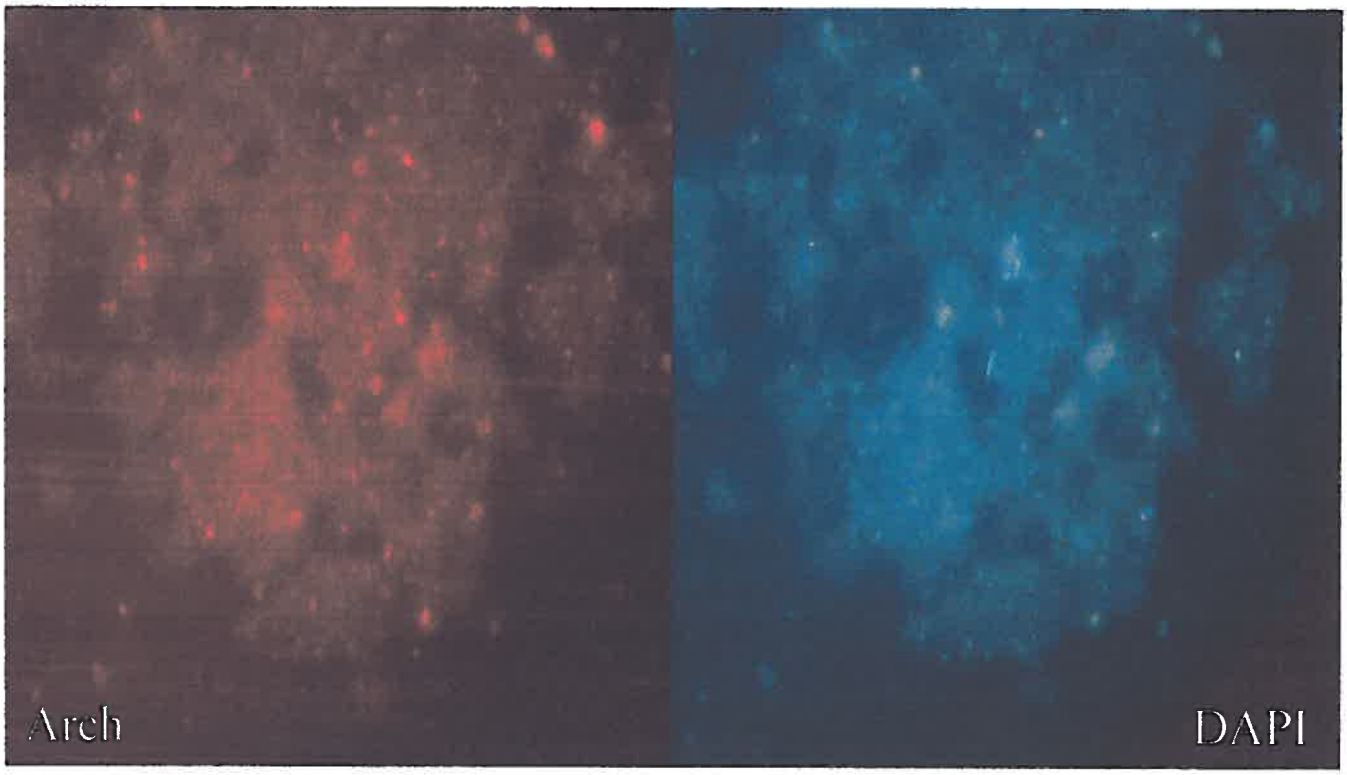


Fig. 3: Photomicrographs of a Guaymas vent sediment sample. Top: phase contrast. Bottom left: FISH with rhodamine labeled Archaea specific oligonucleotide probe. Bottom right: DAPI stained sample. Scale bar is valid for all three pictures.

Eventually, a longer bead beating step (> 15s) at the beginning of the extraction procedure may have increased the DNA recovery.
However, even the little DNA we had obtained was enough for some PCR reactions. Instead of starting directly with Korarchaeota specific primers this time the first amplification of the DNA was done with Eubacteria and Archae specific primers (Fig. 4). The eventually obtained archaeal PCR product would then be screened for Kor-sequences using the specific primers and amplification conditions described above.
Surprisingly, neither in the Guaymas nor in the cyanobacterial mat samples from Guerrero Negro were archaeal PCR products of the expected size observed (Fig. 4.). The only specific amplification products were obtained with eubacterial primers in Guaymas sediments. However, the in situ temperature of these sediment samples was between 58 and 100°C at 15 cm depth, which is above the temperature range reported for Eubacteria. Thermophilic Eubacteria are often gram negative and show optimal growth between 40 and about 80 °C. So far only members of the genus Thermotoga and Aquifex are known to thrive at temperatures above 90°C, but not at 100°C. The so called hyperthermophillic microorganisms growing at 100°C and above are exclusively Archaea.
A possible explanation for the lack of archaeal sequences could be that the beat beating step of only 15 s in the DNA extraction procedure preferentially destroyed eubacterial cells and left the more resistant Archaea unaffected. This, however, is an assumption and not based on real data. On the other side, eubacterial contaminants (introduced during sampling, sample treatment, DNA extraction...) could lead to an eubacterial signal although there were no Eubacteria present in the original sample, or there was even no DNA at all. In order to clarify these questions the PCR products were cloned in E. coli. After picking about 80 colonies 13 different (indicated by RFLP) clones were isolated and sent for sequencing. Unfortunately, only three of them could be sequenced. A BLAST search with three of the obtained sequences revealed *Thermus* and *Actinothermus* but also *Nitrosococcus*, *Caulobacter* and *Propionigenium* to be closest relatives. However the similarity between the clone sequence and the database hint was usually rather low (about 91%). It has to be stressed that the obtained sequences were short (about 150 nt) and of a rather poor quality, which makes a further interpretation of the data difficult. However, the fact that often thermophilic Eubacteria appeared in the BLAST search suggests that the obtained clones were indeed members of the original bacterial community and not simply contaminants.

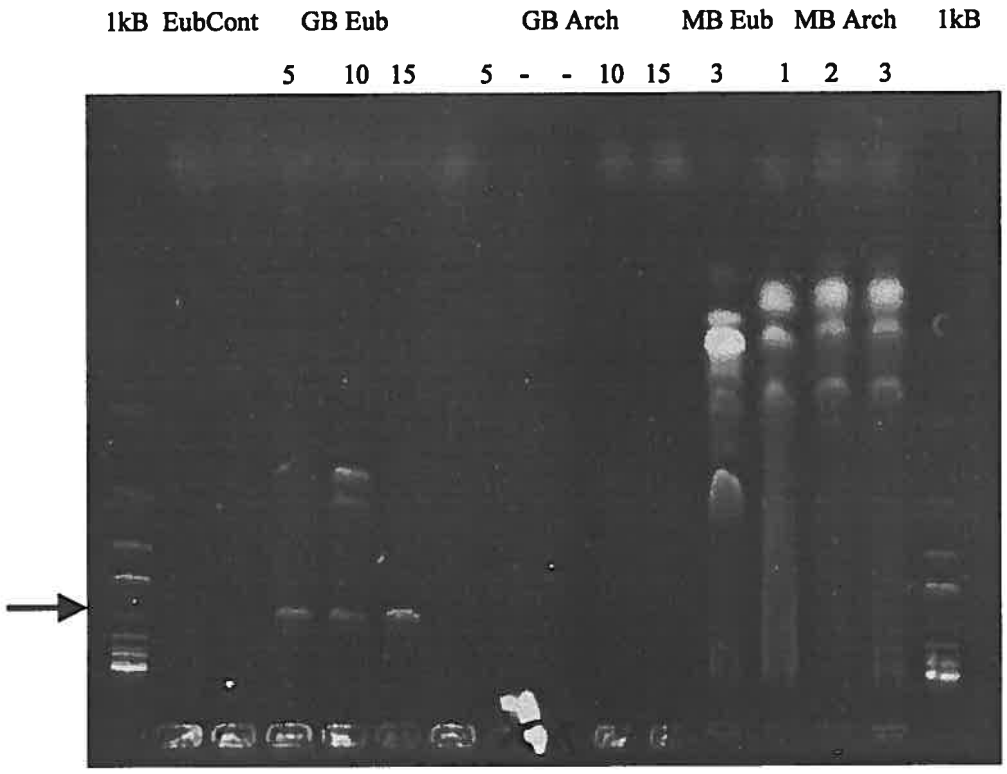


Fig. 4: PCR products of Guaymas (GB) sediments and Baja mat (BM) with Eubacterial and Archaeal primers. Numbers indicate sample depth in cores (in cm).

Conclusions

No mesophilic and thermophilic korarchaeal PCR products were obtained using the different Kor-specific forward primers. The cell and DNA content in sediment from the Guaymas hot vent site was extremely low. This was indicated by the low DNA yield and confirmed by DAPI staining and FISH. DAPI as well as FISH bound rather unspecifically to sediment particles than to microorganisms. Despite *in situ* temperatures of up to 100°C a variety of 13 different eubacterial clones was obtained. A BLAST search with three of the obtained sequences revealed Thermus and Actinothermus but also Nitrosococcus, Caulobacter and Propionigenium to be closest relatives.

References

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