0014-4754/93/490503-11$1.50 + 0.20

Partial sequence of the gene for a serine protease from a halophilic archaeum *Haloferax mediterranei* R4, and nucleotide sequences of 16S rRNA encoding genes from several halophilic archaea

M. Kamekura and Y. Seno

Noda Institute for Scientific Research, 399 Noda, Noda-shi, Chiba-ken 278 (Japan)

Abstract. A part of the gene coding for a halophilic serine protease from a halophilic archaeum *Haloferax mediterranei* R4 was amplified by PCR and its 672 nucleotide sequence was determined. Tentative translation to the amino acid sequence suggested that the enzyme was quite similar to halolysin produced by another halophilic archaeum strain 172P1. Nucleotide sequences of 16S rRNA encoding genes from 9 halophilic archaea were determined. Alignment of 19 sequences known so far showed that there are more than 20 positions carrying bases or deletions specific for each halobacterial genus: *Halobacterium*, *Haloarcula*, *Haloferax*, and *Halococcus*.
Key words. Halophilic; archaea; serine protease; *Haloferax mediterranei*; 16S rRNA; nucleotide sequence; *Halobacterium*; *Haloarcula*; *Halococcus*; *Natronobacterium*.

Introduction

Most of the enzymes from halophilic archaea (extreme halophiles) require high concentrations of salt for their activities and stabilities and become inactive below salt concentrations of about 2 M. A thorough analysis of the interaction of *Haloarcula marismortui* malate dehydrogenase with salts demonstrated that the enzyme protein binds unusual quantities of salt and water[23]. The mechanism of halophilicity of the halophilic enzymes has not been elucidated at the level of the amino acid sequence. Enzymes suitable for manipulation by protein engineering techniques should be relatively small in size, should have their genes cloned, and should have homologs whose three dimensional structures have been determined at high resolution. Dihydrofolate reductase from *Haloferax volcanii*[24] and glutamate dehydrogenase from *Halobacterium salinarium*[1] are excellent subjects for comparative studies of the relationship between structure and enzyme activity-stability.

The authors have previously reported purification and characterization of a serine protease, halolysin, from an unidentified halophilic archaeum strain 172P1[6,9]. Halolysin showed highest activity at 3–4 M NaCl and was stable when kept at 5 °C in the presence of 4 M NaCl. Though it showed high homology (approximately 43%) to thermitase[20], a protease from *Thermoactinomyces vulgaris*, which is not halophilic, the identification of another halophilic serine protease with greater homology would be of help in designing strategies for engineering halolysins. An extra-cellular protease produced by another halophilic archaeum *Haloferax mediterranei*[5–7] may be a candidate. In this article we report the partial sequence of the gene encoding the serine protease from *Hf. mediterranei* R4.

We have also determined nucleotide sequences of the genes encoding 16S rRNAs from several halophilic archaea, to explore the taxonomic position of the halolysin producer, strain 172P1.

Materials and methods

Bacterial strains

Haloferax mediterranei R4 ATCC 33500, *Halobacterium* sp.Y12, and *Hb. trapanicum* NCMB 767 were obtained from Dr. F. Rodriguez-Valera of the University of Alicante, Spain; strain L-11 from Dr. F. J. Post of Utah State University, USA; strain B1T from Dr. K. Tojo of Nihon University, Japan; other strains were obtained from ATCC.

Production and purification of the serine protease

Protease activities were measured as described previously[5] with azocasein or synthetic oligopeptides as substrates.

Preliminary experiments showed that the highest production of the protease from *Hf. mediterranei* R4 ATCC 33500 was obtained in a chemically defined synthetic medium of the following composition (g/l): sucrose 10, sodium glutamate monohydrate 20, $MgSO_4 \cdot 7H_2O$ 20, KCl 4, KH_2PO_4 0.3, and NaCl 160, pH 6.4. The strain was precultured in 1 l of the medium in a 5-liter Erlenmeyer flask and inoculated into a 30-liter jar fermentor containing 20 l of the medium. After cultivation at 37 °C for 70 h with an aeration of 20 l/min and agitation of 240 rpm, cells were harvested by centrifugation and the supernatant was concentrated to 5 l at 5 °C, using an ultrafiltration module with a mol.wt cut-off of 3000 (AsahiKasei Industries Ltd., Tokyo, Japan). The concentrate was centrifuged to get rid of insoluble materials and solid ammonium sulfate was added to a concentration of 150 g per liter. The enzyme solution was loaded onto a column of Butyl-Toyopearl 650C (Tosoh MFG

Co. Ltd., Japan, 3 × 20 cm) which had been equilibrated with 10 mM Tris-hydrochloride, pH 7.6 containing 25% NaCl and 15% ammonium sulfate. After washing the column with the same buffer, the protease was eluted with 10 mM Tris–25% NaCl–5% ammonium sulfate. The eluate was concentrated, dialysed at 5 °C against 10 mM Tris–25% NaCl, pH 7.6, and subjected to gel permeation chromatography using Toyo-Pearl HW-50Fine (Tosoh, MFG Co. Ltd). Active fractions were pooled, concentrated and used as a protease preparation.

A preliminary report of the purification of the enzyme has been given in the proceedings of a FEMS-NATO Advanced Research Workshop on General and Applied Aspects of Halophilic Microorganisms, held September 17–22, 1989 in Alicante, Spain[7].

Polymerase chain reaction for amplification of the protease gene

For the isolation of a part of the gene for R4 protease, a probe was prepared by PCR. The sense and antisense primers were synthesized by the phosphoramide method. The sense primer had a base sequence 5′-CAGTACGCTCCACAGAAGGTCAAC (amino acid sequence Gln-Tyr-Ala-Pro-Gln-Lys-Val-Asn). The antisense primer had a sequence 3′-AGGCCGTGCAGCTACCGC (amino acid sequence Ser-Gly-Thr-Ser-Met-Ala). PCR was performed with Gene Amp PCR system 9600 (Perkin Elmer Cetus) in 100 µl of 10 mM Tris-hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM each deoxynucleotide triphosphate, 20 µM sense and antisense primers, 0.1 µg chromosomal DNA and 2 units of AmpliTaq DNA polymerase. After 25 cycles of amplification (30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C), products were detected by electrophoresis through a 1% agarose gel. To give blunt ends, the fragment was treated with T4 DNA polymerase, then ligated to *Sma*I-cut plasmid pUC119, and transformed into *E. coli* JM109. White colonies on TY-agar plates (containing 50 µg of ampicillin per ml, IPTG, and X-GAL) were isolated, recombinant plasmid DNAs extracted and digested with *Kpn*I and *Bam*HI, and the fragments produced were run on an agarose gel to select recombinants harboring plasmids with the correct insert. For determining the nucleotide sequence, single-stranded DNAs of recombinant plasmids were prepared as described earlier[9] and sequencing reactions were performed by the dideoxy methods with dye-labeled M13 universal primer (−21; Applied Biosystems Inc.) and run in a DNA sequencer ABI 373A.

From one of the transformants, 1–34, a 670 bp-insert was cut out and isolated by electro-elution. The fragment was labeled with digoxygenin-dUTP using a non-radioactive DNA labeling and detection kit (Boehringer Mannheim), and used as a probe in Southern blot and colony hybridization experiments.

16S ribosomal DNA amplification and sequencing

Four primers, each 20 nucleotides long were designed from very conserved regions of 16S rRNAs from halophilic archaea: sense primer-1, 5′ATTCCGGTTGATCCTGCCGG3′ (1–20, numbering according to *H. halobium* 16S rRNA[15]); sense primer-5, 5′CCGACAGTGAGGGACGAAAG3′ (689–708); antisense primer-4, 3′TGGCCTAATCTATGGGCCCA5′ (721–740); antisense primer-6, 3′GACGCCGACCTAGTGGAGGA5′ (1454–1473). With combinations of primers 1 + 4 and 5 + 6, partially overlapping rDNAs, approximately 740 and 785 bp respectively, were synthesized by PCR, cloned into pUC119, and sequenced as described above.

Alignment of the determined sequences and construction of a phylogenetic tree by UPGMA (unweighted pair group method with arithmetic mean) was performed using GeneWork, IntelliGenetics, Inc. USA.

Results

Purification of serine protease from Hf. mediterranei R4

Hf. mediterranei R4 grew extremely well in the chemically defined medium, producing high amounts of extracellular protease. Kauri et al.[11] reported a chemically defined synthetic medium which supported good growth of *Hf. volcanii* and some other strains of extreme halophiles. They mentioned that *Hf. mediterranei* did not grow in their medium which contained NH_4Cl and glycerol as nitrogen and carbon sources, respectively. In our hands a synthetic medium with NH_4Cl and glucose supported good growth of *Hf. mediterranei*, and the strain required no vitamin. Later we noticed that replacement of NH_4Cl and glucose by sodium glutamate and sucrose gave better growth and protease production.

Though the final preparation of the protease seemed almost homogeneous as judged from the symmetrical elution pattern on gel permeation chromatography, nondenaturing polyacrylamide gel electrophoresis gave more than one protein band stained with Coomassie brilliant blue, and no activity was detected after flooding the gel with a substrate solution[6], indicating that the enzyme had denatured (autodigested) during the run at 5 °C.

Characterization of the R4 protease

The instability of the R4 protease was shown by the following experiments. Incubation at 37 °C in the presence of 2.5 M NaCl resulted in linear inactivation of the R4 protease with complete loss of activity in 4 h, while no decrease in the halolysin activity was observed during the incubation. The R4 protease retained about 80% activity, however, after incubation at 5 °C for 20 h in the presence of 3 M NaCl.

Table. Substrate specifications of halolysin and R4 protease

Substrate	Halolysin	R4 Protease
Pyr-phe-leu-pNA	+	−
Z-ala-ala-leu-pNA	++	−
Z-gly-gly-leu-pNA	++	++
α-NBz-arg-pNA	−	−
Suc-ala-pNA	−	−
Suc-ala-ala-pNA	+	−
Suc-ala-ala-ala-pNA	++	−
Suc-leu-leu-val-tyr-MCA	++	++
Suc-ala-ala-pro-phe-MCA	++	++
Suc-arg-pro-phe-his-leu-leu-val-tyr-MCA	+	−

Abbreviations: Z, carbobenzoyl; Bz, benzoyl; pNA, para-nitroanilide; MCA, methylcoumarylamide.

On the other hand the R4 protease was quite halophilic. With a synthetic substrate succinyl-ala-ala-pro-phe-MCA, increasing activity was observed as the NaCl concentration in the reaction mixture was increased up to 4.3 M. Approximately 12 fold higher activity was observed at 25% NaCl than at 5% NaCl.

Both halolysin and R4 protease were completely inhibited with 1 mM phenylmethane sulfonylfluoride and 5 μg/ml chymostatin, and slightly inhibited with antipain, whereas no inhibition was observed with pepstatin or leupeptin.

Substrate specifications of the two enzymes are compared in the table.

Partial sequencing of the R4 protease gene

During the course of our efforts to determine the N-terminal amino acid sequence of the R4 protease, Stepanov et al.[21] reported purification and characterization of a protease from *Hf. mediterranei* strain 1538. They purified the enzyme to homogeneity by affinity chromatography on bacitracin-Sepharose, and determined the N-terminal amino acid sequence. Though the strain they used might not be the same as the strain ATCC 33500, we adopted the underlined part of their sequence (NH_2-Asp-Thr-Ala-Asn-Asp-Pro-Lys-Tyr-Gly-Ser-Gln-Tyr-Ala-Pro-Gln-Lys-Val-Asn) to synthesize a sense primer for a probe to clone the gene of R4 serine protease. The underlined sequence is the same as that of halolysin except that lysine is replaced by glutamine in halolysin. An antisense primer was synthesized from the very well conserved sequence around the serine residue which comprises the active center of subtilisin-type serine proteases.

The nucleotide sequence of the PCR product, 672 bp in total, is shown in figure 1. Tentative translation of the nucleotide to the amino acid sequence (data not shown) suggested that the enzyme was more closely related to halolysin than to thermitase. At present cloning of the entire gene for the enzyme is in progress, using the 672 bp-DNA fragment as a probe.

```
      10        20        30        40        50
1234567890 1234567890 1234567890 1234567890 1234567890
CAGTACGCTC CACAGAAGGT CAACGCCGAC TCCGCGTGGG ACACCACGCT   50
CGGAAGTTCC TCCGTGAAGA TTGCCGTCGT CGACCAGGGT GTCAAGTACG  100
ACCACCCNGA NCTTTCGAGC CAGTTCGGCT CGAACAAAGG TCGAGACTTC  150
GTTGATATCG ACGGAGAACC TGTATCCGGA CCTGATTATT CGAACGANTA  200
CCACGGAACC AACGTCGCAG GTATCGCCGC CGGGACCACC GATAACAACG  250
AAGGTATTGG CGGAATCAGT AACTCCACGC TCCTCTCTGG ACGCGCCCTT  300
TCCGAATCCG GAAGCGGTTC GACGAGTGAC ATTGCGAACG CTATCGAGTG  350
GGCCGCAGAC CAGGGTGCAG ACGTTATCAA CCTCTCCCTG GGTGGTGGCG  400
GCTACTCCTC GACGATTAAA AACGCCGTAT CCTACGCGAC GCAGCAAGGT  450
TCGCTCGTCG TCGCCGCTGT TGGTAATGAC GGCCGGCAAA GCGTCTTCCT  500
ACCCCGCGGC ATACAGCGAG TGTGTCCCGT CTCGGCGCTC GACCCCGACG  550
AGACGTTCGC ATCGTACTCG AACTACGGGT CGGAAATCGA CCTCGCAGCA  600
CCGGGGACGA ACGTCCTCTC GTGCTGGACG ACCAGTACCG AGTACAACGA  650
AATCTCCGGC ACGTCGATGG CG                                672
```

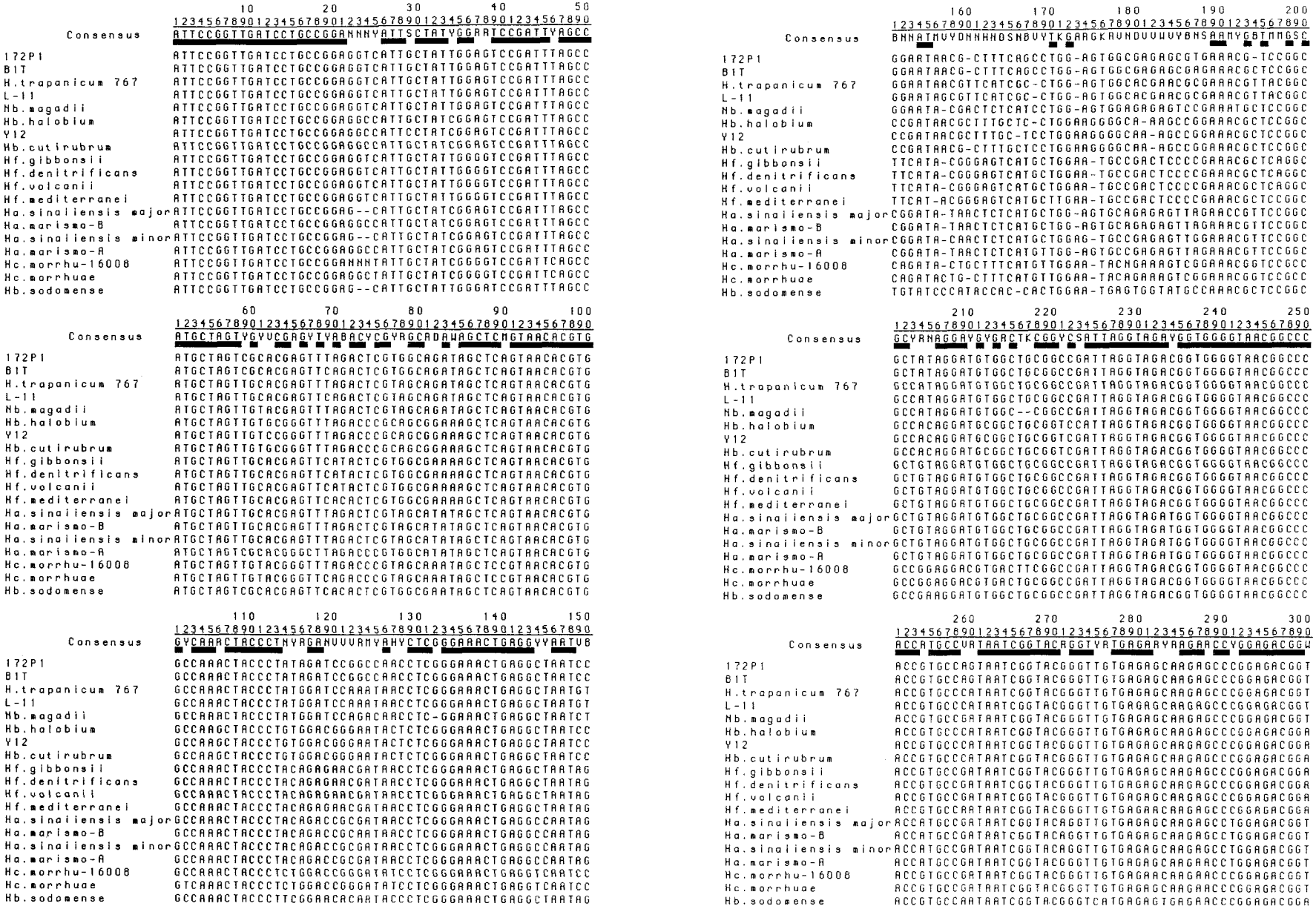
Figure 1. Nucleotide sequence of part of the gene encoding for *Hf. mediterranei* R4 protease amplified by PCR.

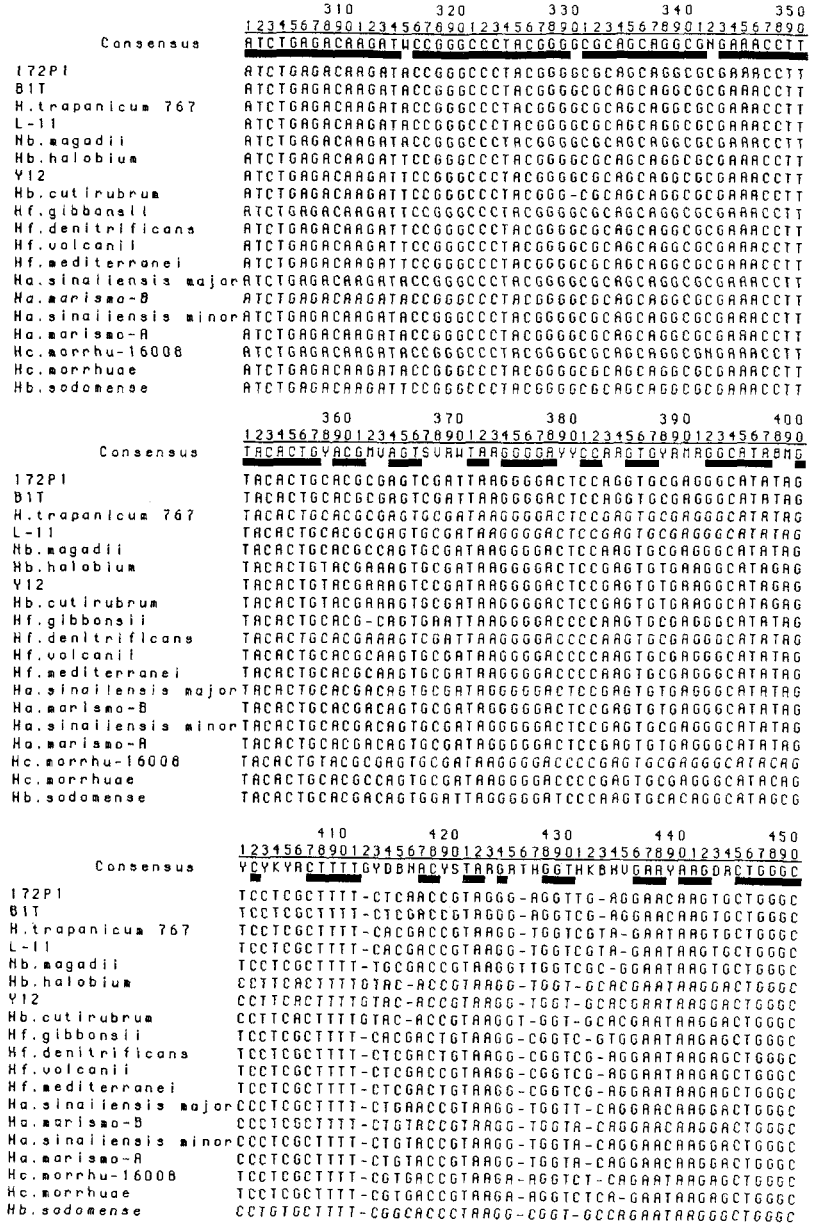
Nucleotide sequences of 16S rRNA encoding-genes from halophilic archaea

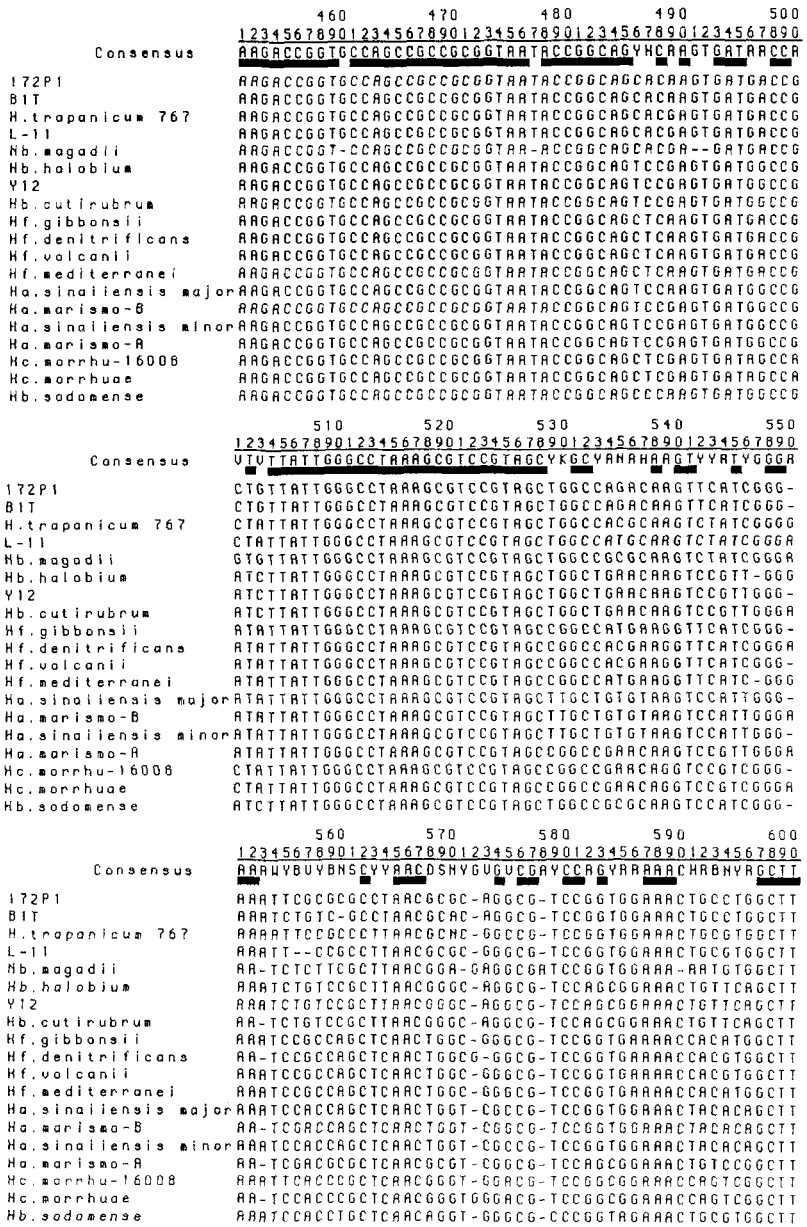
The ribosomal RNA-encoding genes were amplified by PCR using two pairs of sense and antisense primers. The nucleotide sequences of the PCR products, 5′-part fragment of 740 bp, and 3′-part fragment of 785 bp, were determined from both directions by reading at least five preparations each of single-stranded DNA of the recombinant plasmids. No notable heterogeneity was detected in the sequences determined from most halophiles; there were 3 base substitutions at most. An exception was *Haloarcula sinaiiensis* ATCC 33800, in which at least two different sequences were detected, major and minor, which had base substitutions or addition-deletions at 41 out of 1468 bases (2.8%). Twenty five out of 41 were concentrated in the 176 bases from 708 to 883.

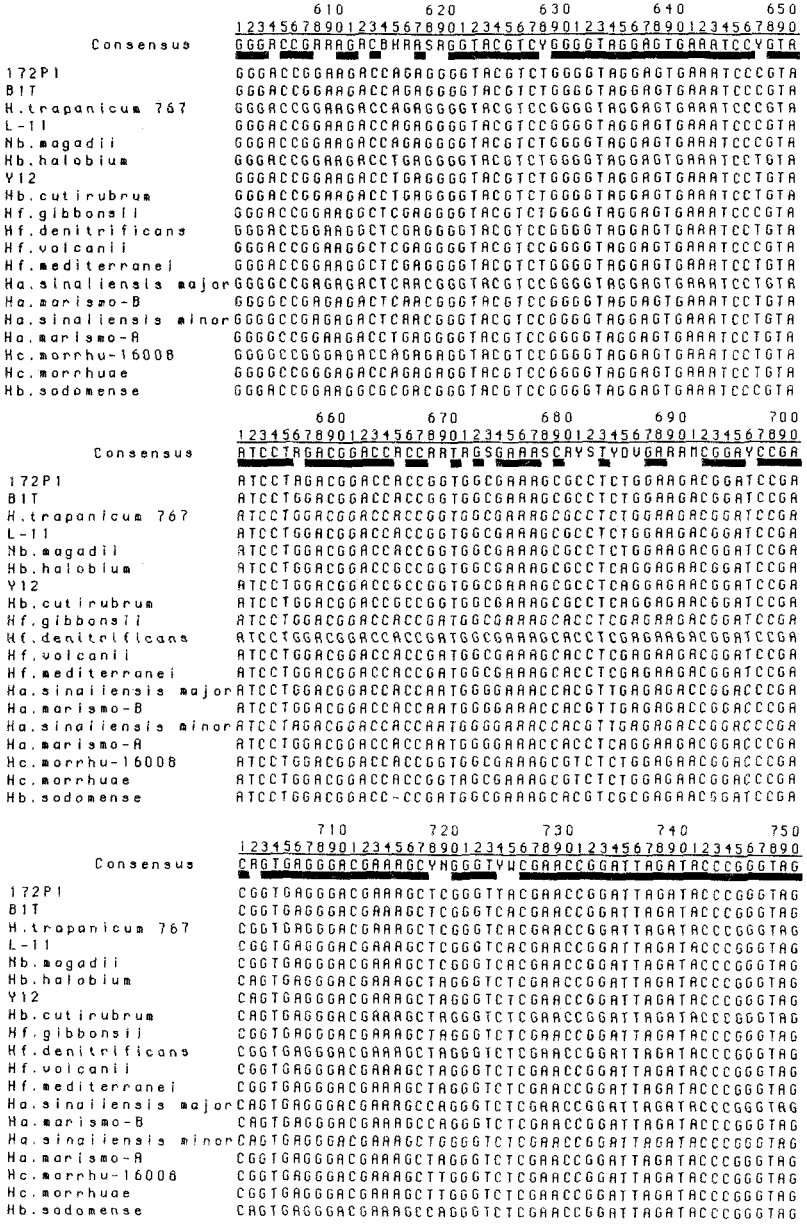
Nineteen complete nucleotide sequences, including the published sequences, are aligned in figure 2. A phylogenetic tree (fig. 3) constructed from the aligned sequences showed that species of the same genus clustered very closely. The strain 172P1 associated with B1T, suggesting it may form a new genus together with the strain B1T. A detailed taxonomic study is now in progress, including physiological and DNA-DNA hybridization comparison, and determination of the structure of the novel glycolipid. *Hb. sodomense* showed the least relationship to other halophiles. Taxonomy of the whole halobacteria will be discussed elsewhere.

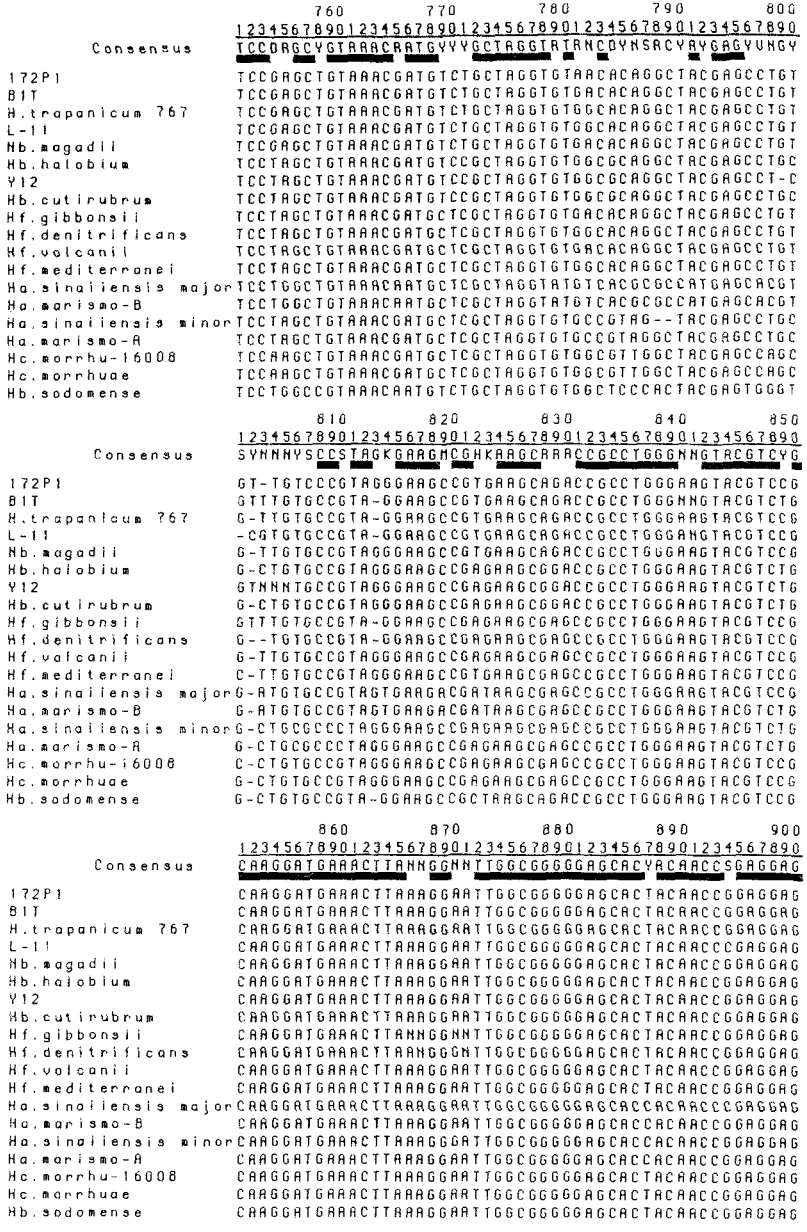
Figure 2. Nucleotide sequences of 16S rRNA encoding genes from halophilic archaea. Sequences from the following halophiles were determined by us. 172P1, B1T, *Hb. trapanicum* NCMB 767, L-11, *Halobacterium* sp.Y12, *Hf. gibbonsii* ATCC 33959, *Hf. denitrificans* ATCC 35960, *Haloarcula sinaiiensis* ATCC 33800, and *Hb. sodomense* ATCC 33755. The following sequences were taken from references and the EMBL Gene Bank; Nb. magadii (*Natronobacterium magadii* NCMB 2190, ref. 14), Hb. halobium (*Halobacterium halobium* R1, ref. 15), Hb. cutirubrum[4], Hf. volcanii (*Haloferax volcanii*, ref. 3), Ha. marismo-A and -B (*Haloarcula marismortui* Ginzburg, ref. 17), and Hc. morrhuae (*Halococcus morrhuae* ATCC 17082, ref. 13). Sequences for *Hf. mediterranei* and *Hc. morrhuae* NRC 16008 have been reported elsewhere by the authors[8].

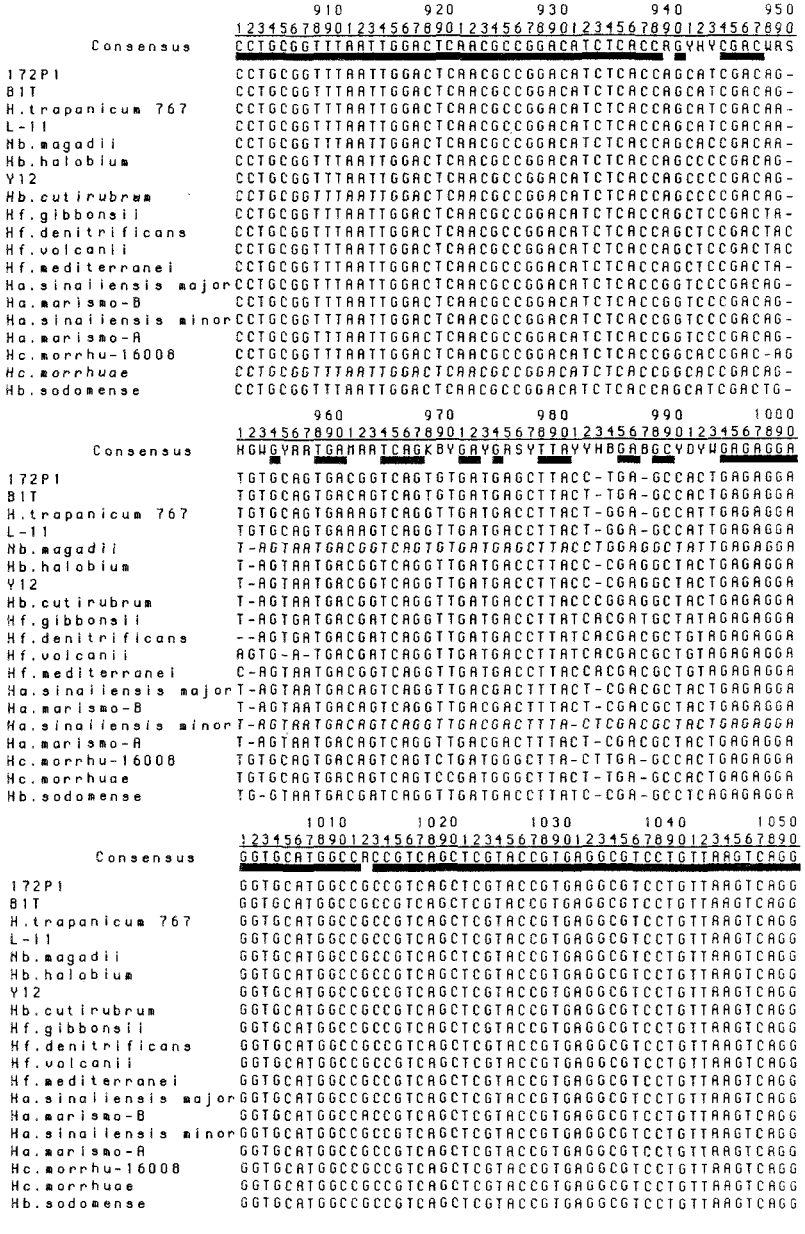

Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense

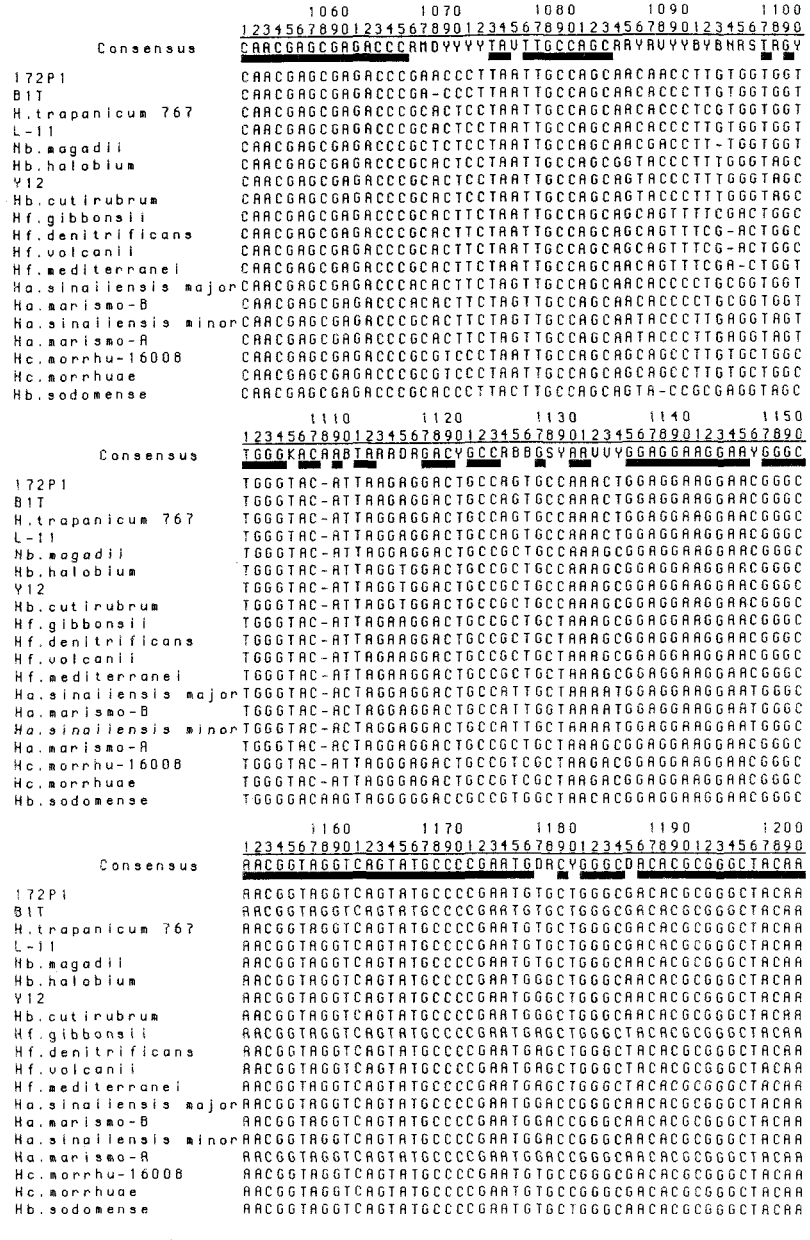

Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense

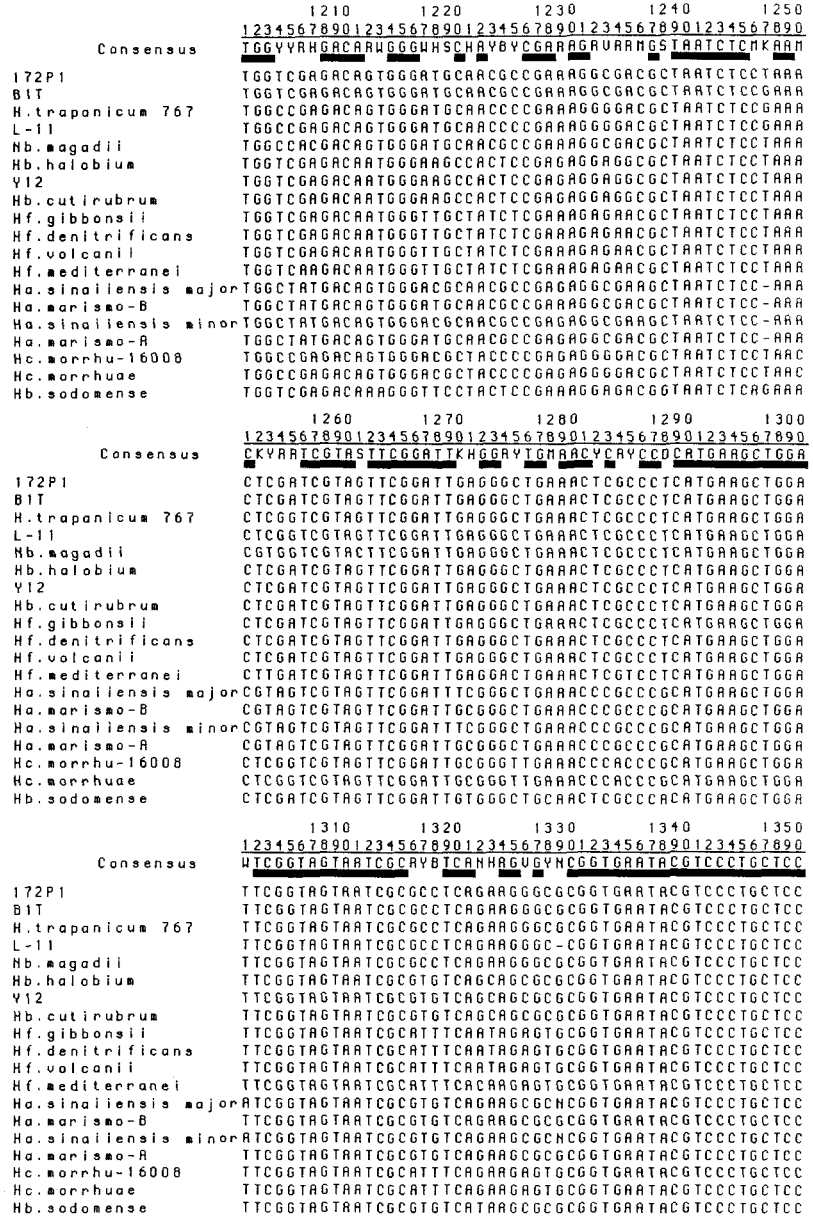

Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense


610 620 630 640 650
Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense
660 670 680 690 700
710 720 730 740 750


760 770 780 790 800
Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense
810 820 830 840 850
860 870 880 890 900


910 920 930 940 950
Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense
960 970 980 990 1000
Consensus
1010 1020 1030 1040 1050
Consensus


1060 1070 1080 1090 1100
Consensus
1110 1120 1130 1140 1150
Consensus
1160 1170 1180 1190 1200
Consensus


1210 1220 1230 1240 1250
Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense
1260 1270 1280 1290 1300
Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense
1310 1320 1330 1340 1350
Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense


1360 1370 1380 1390 1400
Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense
1410 1420 1430 1440 1450
Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense
1460 1470 1480
Consensus
172P1
B1T
H.trapanicum 767
L-11
Nb.magadii
Hb.halobium
Y12
Hb.cutirubrum
Hf.gibbonsii
Hf.denitrificans
Hf.volcanii
Hf.mediterranei
Ha.sinaiiensis major
Ha.marismo-B
Ha.sinaiiensis minor
Ha.marismo-A
Hc.morrhu-16008
Hc.morrhuae
Hb.sodomense

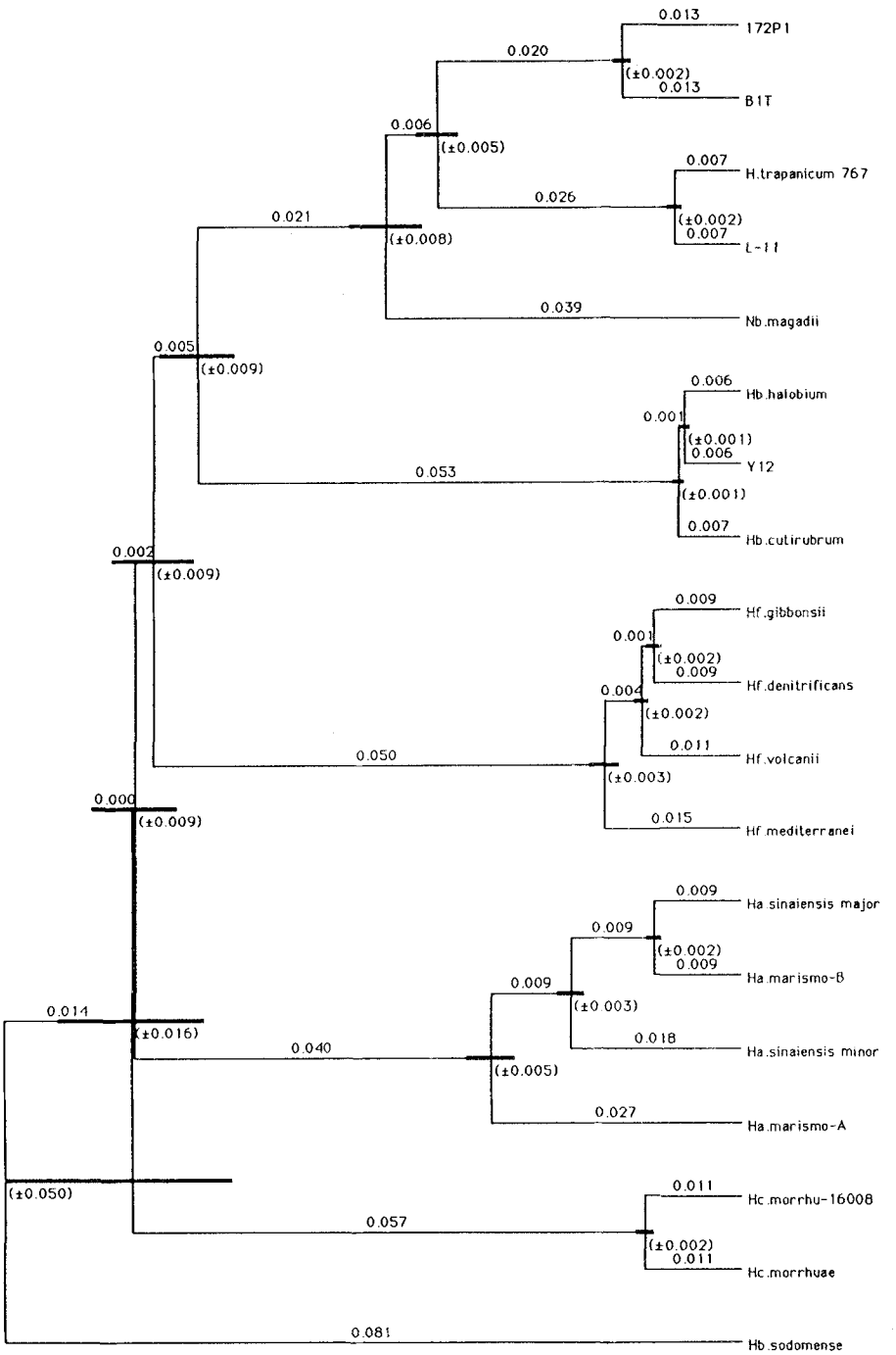


Figure 3. Clustering of halobacterial strains based on sequence alignment of 16S rRNA-encoding genes by the UPGMA method.

thermitase as a model molecule would be possible. Cloning of the whole gene of the protease from *Hf. mediterranei* R4 is now in progress. Once the complete amino acid sequence of the R4 protease is known, we aim to engineer mutations in both enzymes to identify the regions that are responsible for halophilicity.

The authors initiated sequencing of the 16S rRNA-encoding genes from halophilic archaea with the aim of identifying the strain 172P1. This strain, isolated from sea sands, is an extreme halophile, not pigmented, and has a novel bis-sulfated diglycosyldiether (manuscript in preparation). According to the classification based on the glycolipid composition[10], the strain may be distinct enough to be designated as a new genus. For the purpose of confirming the taxonomic position of strain 172P1 on the basis of its 16S rRNA sequence, the sequences of all the established halophilic archaeal strains are required.

It is generally observed that multiple rRNA-encoding operons in the genome of most organisms exhibit perfect or near perfect sequence identity. This has been the basis for determining the sequences of 16S-like rRNA by reverse transcription[12] or direct sequencing of rDNA amplified by PCR[22] for phylogenetic study. The only notable exceptions to the sequence identity reported so far were in the eucaryotic parasites *Plasmodium berghei* and *P. falciparum*[2,16]. The genomes of these organisms contain two types of small rRNA genes that differ by about 5.2% and 17%, respectively, by nucleotide substitution or deletion-insertion.

When the authors started work on sequencing of rDNA, five complete sequences from the following halophilic archaea had been reported; *Hf. volcanii*, *Halococcus morrhuae* ATCC 17082, *Hb. halobium* R1, *Hb. cutirubrum*, *Natronobacterium magadii*. All these sequences were determined from the genes cloned into plasmid vectors. Although the *Hf. volcanii* genome is known to contain two rRNA operons, the sequence of the two 16S rRNA genes are identical or nearly identical[17]. Moreover *Hb. cutirubrum* and *Hc. morrhuae* ATCC 17082 have been shown to contain only a single rRNA operon. Sanz et al., however, showed that many strains of halophilic archaea contained three or four rRNA operons[19]; for example, *Ha. californiae* ATCC 33799 and *Hf. gibbonsii* ATCC 33959 contained at least 4 genes. This suggests that simple sequencing by the reverse transcriptase method might be misleading if applied to systematic studies. Quite recently Mylvaganam and Dennis[17] showed that *Haloarcula marismortui* contained two non-adjacent ribosomal RNA operons, designated *rrn*A and *rrn*B. The 16S genes within these operons differed by nucleotide substitutions at 74 positions out of 1472 nucleotides (5.0%). By comparing their sequences with that which had been determined by Oren et al.[18] using reverse transcriptase, they showed that at 50 positions of *rrn*A and *rrn*B DNA sequence heterogeneity, where Oren et al. were able to specify a base, 24 corresponded to the *rrn*A sequence and 26 corresponded to the *rrn*B sequence. Thus they pointed out a problem that could be encountered when the reverse transcriptase sequencing method is used in phylogenetic and systematic studies, particularly when the target organism has more than two rRNA genes.

Since the alignment of the reported sequences showed that there were several well conserved regions among the halophilic archaea including the 5'- and 3'-termini, we decided to amplify the genes by PCR using two pairs of sense and antisense primers rather than to clone 2 or more entire coding genes from each halophile. After cloning the PCR product into plasmid pUC119, we would be able to sequence many clones to check for detectable heterogeneities, if any, among the ribosomal genes.

As expected, the phylogenetic tree constructed from the 16S rRNA sequences showed that species in the same genus formed separate clusters. A closer look at the aligned sequences revealed that in many positions base substitutions are observed which are specific for a particular genus. For example species of the genus *Halobacterium* have 25 specific bases scattered throughout the molecule, while *Haloarcula* has 22, *Haloferax* 24, and *Halococcus* 29. On the other hand, the strains 172P1 and B1T are similar phenotypically in that both are unpigmented and have the same novel glycolipid (bis-sulfated diglycosyl diether); the two strains share 6 of these specific nucleotides.

Haloarcula sinaiiensis was the only exception in that it showed at least two rRNA genes with considerable heterogeneity. Determination of 16S rRNA sequences will be required from other species of the genus *Haloarcula*, e.g. *Ha. vallismortis*, *Ha. hispanica*, *Ha. japonica* etc. to be sure that high heterogeneity is a particular feature of the genus.

1 Benachenhou, N., and Baldacci, G., The gene for a halophilic glutamate dehydrogenase: sequence, transcription analysis and phylogenetic implications. Mol. gen. Gen. *230* (1991) 345–352.

2 Gunderson, J. H., Sogin, M. L., Wollett, G., Hollingdale, M., De La Cruz, V. F., Waters, A. P., and McCulchen, T. F., Structurally distinct, stage specific ribosomes occur in *Plasmodium*. Science *238* (1987) 933–937.

3 Gupta, R., Lanter, J. M., and Woese, C. R., Sequence of the 16S risosomal RNA from *Halobacterium volcanii*, an archaebacterium. Science *221* (1983) 656–659.

4 Hui, I., and Dennis, P. P., Characterization of the ribosomal RNA gene clusters in *Halobacterium cutirubrum*. J. biol. Chem. *260* (1985) 899–906.

5 Ivanova, I. I., Zvyagintseva, I. S., Tarasov, A. L., and Lysogorskaya, E. N., Extracellular enzymes of extremely halophilic archaebacteria. Mikrobiologiya (Eng. ed.) *58* (1989) 194–198.

6 Kamekura, M., and Seno, Y., A halophilic extracellular protease from a halophilic archaebacterium strain 172P1. Biochem. Cell. Biol. *68* (1990) 352–359.

7 Kamekura, M., and Seno, Y., Lysis of halobacteria with bile acids and proteolytic enzymes of halophilic archaeobacteria, in: General and Applied Aspects Halophilic Microorganisms, pp. 359–365. Ed. Rodriguez-Valera, Plenum Press, New York 1991.

8 Kamekura, M., and Seno, Y., Nucleotide sequences of 16S rRNA encoding genes from halophilic archaea *Halococcus morrhuae* NRC16008 and *Haloferax mediterranei* ATCC33500. Nucl. Acid Res. *20* (1992) 3517.

9 Kamekura, M., Seno, Y., Holmes, M. L., and Dyall-Smith, M. L., Molecular cloning and sequencing of the gene for a halophilic alkaline serine protease (halolysin) from an unidentified halophilic archaea strain (172P1) and expression of the gene in *Haloferax volcanii*. J. Bact. *174* (1992) 736–742.

10 Kates, M., Archaebacterial lipids: structure, biosynthesis and function, in: The archaebacteria: Biochemistry and Biotechnology, pp. 51–72. Eds M. J. Danson, D. W. Hough, and G. G. Lunt, Biochemical Society Symposium 58, Portland Press, London 1992.

11 Kauri, T., Wallace, R., and Kushner, D., Nutrition of the halophilic archaebacterium, *Haloferax volcanii*. Syst. appl. Microbiol. *13* (1990) 14–18.

12 Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., and Pace, N. P., Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. natl Acad. Sci. USA. *82* (1985) 6955–6959.

13 Leffers, H., and Garett, R. A., The nucleotide sequence of the 16S ribosomal RNA gene of the archaebacterium *Halococcus morrhuae*. EMBO J. *3* (1984) 1613–1619.

14 Lodwick, D., Ross, H. N. M., Walker, J. A., Almond, J. W., and Grant, W. D., Nucleotide sequence of the 16S ribosomal RNA gene from the haloalkaliphilic archaeon (archaebacterium) *Natronobacterium magadii*, and the phylogeny of halobacteria. Syst. appl. Microbiol. *14* (1991) 352–357.

15 Mankin, A. S., Kagramanova, V. K., Teterina, N. L., Rubtsov, P. M., Belova, E. N., Kopylov, A. M., Baratova, L. A., and Gogdanov, A. A., The nucleotide sequence of the gene coding for the 16S rRNA from the archaebacterium *Halobacterium halobium*. Gene *37* (1985) 181–189.

16 McCutchan, T. F., De La Cruz, V. F., Lal, A. A., Gunderson, J. H., Elwood, H. J., and Sogin, M. L., Primary sequence of two small subunit ribosomal RNA genes from *Plasmodium falciparum*. Mol. Biochem. Parasitol. *28* (1988) 63–68.

17 Mylvaganam, S., and Dennis, P. P., Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. Genetics *130* (1992) 399–410.

18 Oren, A., Lau, P. P., and Fox, G. E., The taxonomic status of "*Halobacterium marismortui*" from the Dead Sea: a comparison with *Halobacterium vallismortis*. Syst. appl. Microbiol. *10* (1988) 251–258.

19 Sanz, J. L., Marin, I., Ramirez, L., Abad, J. P., Smith, C. L., and Amils, R., Variable rRNA gene copies in extreme halobacteria. Nucl. Acid Res. *16* (1988) 7827–7832.

20 Siezen, R. J., de Vos, W. M., Leunissen, J. A. M., and Dijkstra, B., Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. Protein Engng. *4* (1991) 719–737.

21 Stepanov, V. M., Rudebskaya, G. N., Revina, L. P., Gryaznova, Y. B., Lysogorskaya, E. N., Filippova, I. Y., and Ivanova, I. I., A serine proteinase of an archaebacterium, *Halobacterium mediterranei*. A homologue of eubacterial subtilisins. Biochem. J. *285* (1992) 281–286.

22 Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J., 16S ribosomal DNA amplification for phylogenetic study. J. Bact. *173* (1991) 697–703.

23 Zaccai, G., and Eisenberg, H., Halophilic proteins and the influence of solvent on protein stabilization. Trends Biochem. Sci. *15* (1990) 333–337.

24 Zusman, T., Rosenshine, I., Bochm, G., Jaenicke, R., Leskiw, B., and Mevarech, M., Dihydrofolate reductase of the extremely halophilic archaebacterium *Halobacterium volcanii*. The enzyme and its coding gene. J. biol. Chem. *264* (1989) 18878–18883.