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Isolation of halophilic and halotolerant bacteria from a Japanese salt field and comparison of the partial 16S rRNA gene sequence of an extremely halophilic isolate with those of other extreme halophiles

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Halophilic and halotolerant bacteria were isolated from soil samples of a Japanese salt field, an environment where salt concentrations vary annually. From 1 g of each of the five samples collected, over 1×10^3 bacterial colonies (colony forming units (cfu)g^{-1}) grew on agar medium containing 2M Na^+. In contrast, 0–4 bacterial colonies (cfu g^{-1}) were observed on agar medium containing 4M Na^+. Two of the five samples contained numerous bacteria (10^2–10^3 cfu g^{-1}) capable of growth on a 2M Na^+ alkaline (pH = 9.5) medium, while few bacterial colonies were observed from the other three samples. Only one of the five samples was shown to contain bacteria capable of growth on a 4M Na^+ alkaline medium. Most of the bacteria isolated on 4M Na^+ agar were eubacteria, but one extreme halophile (TR-1, already described as *Haloarcula japonica* JCM7785) was also isolated. The 16S rRNA sequence of TR-1 was determined and shows high homology (94.4–98.5%) to *Ha. marismortui* and *Ha. sinaiiensis*. These results suggested that: 1) environments with seasonally varying salinity can harbour halotolerants as well as halophiles and, 2) closely related halophiles can be isolated from geographically distant habitats.

Keywords: extreme halophile; halotolerant; *Haloarcula japonica*; 16S rRNA sequence

Introduction

Hypersaline environments, e.g. salt mines, salt lakes, soda lakes, salt fields, are environments where extremely halophilic archae have been found to flourish (Grant, 1992; Norton *et al.*, 1993). Highly saline environments also contain taxonomically heterogeneous halotolerant and moderately halophilic eubacterial populations that often are more numerous than halophilic archae. Environments in which the salinity varies seasonally may be particularly suitable habitats for such eubacteria. To examine this possibility, a study of the distribution of halophilic and halotolerant bacteria in a salt field was undertaken. The production of salt from this salt field results in seasonal variation in salinity at this site. Salt production occurs only in the summer, by the spreading of sea water over wet sand. The salinity is increased by evaporation which results in a

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thick salt solution, although the salt concentration of the salt field surface is not assumed to be extremely high, as subsequent filtering and boiling is performed to obtain salt. During winter, the salt field typically is covered with snow, greatly lowering salinity prior to the summer salt production. In this study, the distribution of halotolerants and extreme halophiles in samples collected in and around the salt field in Japan is reported. In addition, the 16S rRNA gene of an extreme halophile isolated from the salt field is reported and compared with those of other known extreme halophiles.

Materials and methods

Bacterial isolation and culture

Soil samples were collected from a salt field located on the coast of Japan Sea in Ishikawa Prefecture, Japan. The samples were collected directly into plastic sample bags using a scoop and were then brought back to the laboratory. One gram of each sample was then suspended in a series of isolation media (2M Na^+ neutral, 2M Na^+ alkaline, 4M Na^+ neutral, and 4M Na^+ alkaline). The composition, per litre, of the 2M Na^+ and 4M Na^+ neutral media was as follows: casamino acid, 7.5 g; yeast extract (Difco), 10 g; $MgSO_4.7H_2O$, 20 g; trisodium citrate, 3 g; KCl, 2 g; $MnCl_2.4H_2O$, 0.36 mg; $FeSO_4.7H_2O$, 1.6 mg; agar, 15 g; and either 120 g or 240 g of NaCl. For alkaline pH media, $MgSO_4.7H_2O$ was reduced to 1.0 g and 18.5 g Na_2CO_3 was supplemented into the corresponding neutral media. Cultivation of the isolated extreme halophile was done by a method previously described (Takashina *et al.*, 1990). Bacterial growth was monitored by optical density at 660 nm.

Electron microscopy

Staining was carried out by a conventional glutaraldehyde fixation and phosphotungstic acid shadowing method, as described previously (Nishiyama *et al.*, 1992). Cells were grown in 4M Na^+ neutral liquid medium and were harvested during log growth phase by centrifugation. Electron micrography was done using a JEM 2000FX electron microscope (JEOL Co., Tokyo, Japan).

Nucleotide sequencing of 16S rRNA gene

In general, molecular manipulation techniques were employed as described in Sambrook *et al.*, 1989. DNA was isolated from the extreme halophile as described previously (Takashina *et al.*, 1990). The gene coding for the 16S rRNA was cloned into a *Bam*HI digested pBR322 from a DNA library partially cleaved by *Sau*3A. To clone the gene, colony hybridization was performed with two primers deduced from the sequences of the 5′- and 3′-termini of the *Halococcus morrhuae* 16S rRNA sequence, corresponding to the 254–333 (80mer) and 1259–1355 (97mer) in the sequence determined by Leffers and Garret (1984). Subclonings for nucleotide sequencing were performed with pUC118 and pUC119 vectors and transformed into *E. coli* MV1184. The nucleotide sequence was determined by the dideoxy termination method (Sambrook *et al.*, 1989), using a DNA sequencer (Applied Biosystems Model 373A) with 18 synthetic primers deduced from consensus regions of the archaeal 16S rRNA genes. The primer sequences are; 120F, 5′-GCTCAGTAACACGTGG-3′; 170F, 5′-CTCGGGAAACTGAGG-3′ and its complementary sequence (170R); 350F, 5′-CTACGGGGCGCAGCAG-3′ and its com-

plementary sequence (350R); 520F, 5′-GCCAGCCGCCGCGGTA-3′ and its complementary sequence (520R); 700F, 5′-GGGGTAGGAGTGAAAT-3′ and its complementary sequence (700R); 800F, 5′-ATTAGATACCCGGGTAG-3′ and its complementary sequence (800R); 1100F, 5′-CAACGAGCGAGACCCG-3′ and its complementary sequence (1100R); 1240F, 5′-ACGCGGGCTACAATGG-3′ and its complementary sequence (1240R; 1400F, 5′-GCACACACCGCCCGT-3′; 1400R, 5′-GCAGGGACGTATTCACCGCG-3′; and 1510R, 5′-GGCTCCCTTGTTACGT-3′. All the primers were synthesized by an Applied Biosystems Model 380B DNA synthesizer.

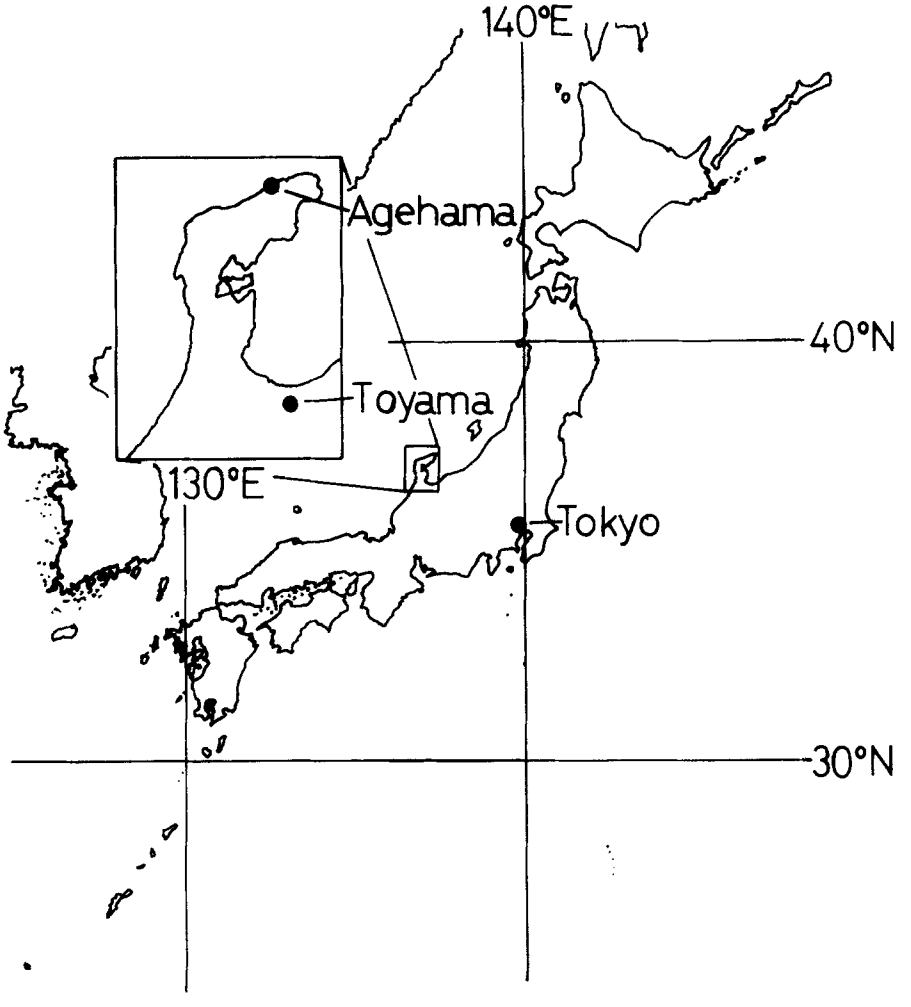


Figure 1. Location of Agehama salt field where sample collections were made. Noto Peninsula, which locates almost in the centre of Japan, is enlarged. The locations of Tokyo, the city of Toyama and Agehama are indicated.

Results

Isolation of halotolerant and halophilic bacteria

Isolation of halotolerants and extreme halophiles at neutral and alkaline pHs was carried out in media containing either 2M Na^+ or 4M Na^+. Samples were taken from various sites in the Agehama salt field located on the north shore of the Noto Peninsula, Ishikawa Prefecture, Japan (Fig. 1).

Colony counts were determined after 10 days incubation at 37°C (Table 1). Sample 1, collected from the centre of the salt field, resulted in 1×10^3 cfu g^{-1} on 2M Na^+ neutral medium, but only one colony on either 2M Na^+ alkaline or 4M Na^+ neutral medium. Sample 2, collected from the salt field periphery, resulted in 1×10^3, 21, and 4 cfu g^{-1} on 2M Na^+ neutral, 2M Na^+ alkaline, and 4M Na^+ neutral media, respectively. Two samples were collected from just outside of the salt field, a surface sample (sample 3) and a submerged sample (sample 4). The surface sample (3) resulted in the largest number of colonies observed: 5×10^3, 4×10^3, 1, and 3 cfu g^{-1} on 2M Na^+ neutral, 2M Na^+ alkaline, 4M Na^+ neutral and 4M Na^+ alkaline media, respectively. The submerged sample (4) gave somewhat fewer colonies: 4×10^3 and 12 cfu g^{-1} on 2M Na^+ neutral and 2M Na^+ alkaline media, respectively. Sample 5, collected from the soil near an evaporating pot, resulted in 4×10^3, 5×10^2, and 1 cfu g^{-1} on 2M Na^+ neutral, 2M Na^+ alkaline and 4M Na^+ neutral media, respectively.

Growth characteristics of halophiles grown on 4M Na^+ media

Seven colonies were obtained on the 4M Na^+ neutral media. However, after examination of morphological and growth characteristics, all four of the colonies from sample 2 appeared to be of the same bacterial species. The four bacterial species isolated on 4M Na^+ neutral medium were designated as strains: HT1103 (sample 1), HT1215 (sample 2), HT1308 (sample 3) and TR-1 (sample 5). Three colonies grew on 4M Na^+ alkaline medium, but all appeared to be of the same bacterial species, designated as strain HT1310 (sample 4).

The growth characteristics of all five strains at various salt concentrations are shown in Fig. 2. Growth was measured by optical density at 660 nm after 48 h of incubation at 37°C, except for TR-1 which was measured after five days of incubation. The growth characteristics of strains HT1103, HT1215, HT1308 and HT1310 show that they are halotolerants, rather than halophiles. This contrasts greatly with the preferential growth of

Table 1. Distribution of halotolerant bacteria in the samples taken from Agehama salt field. Numbers of colonies from 1 g of samples on 4 different media are shown

Isolation media	2M Na^+		4M Na^+	
	neutral	alkaline	neutral	alkaline
Sample-1	1×10^3	1	1	0
Sample-2	1×10^3	21	4	0
Sample-3	5×10^3	4×10^3	1	3
Sample-4	4×10^3	12	0	0
Sample-5	4×10^3	5×10^3	1	0

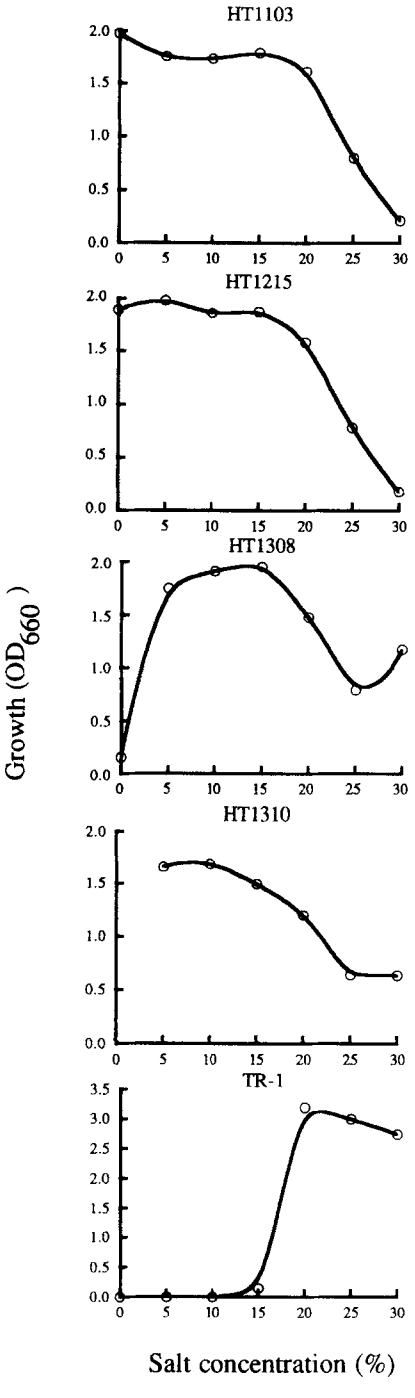


Figure 2. Growth of representative halotolerant strains in various salt concentrations. The growth after 2 days (for TR-1, 5 days) of incubation are shown by the optical density values measured at 660 nm. The neutral (pH 7.0) growth medium was used for HT1103, HT1215 and HT1308 and for HT1310, the alkaline media (pH9.0) were used.

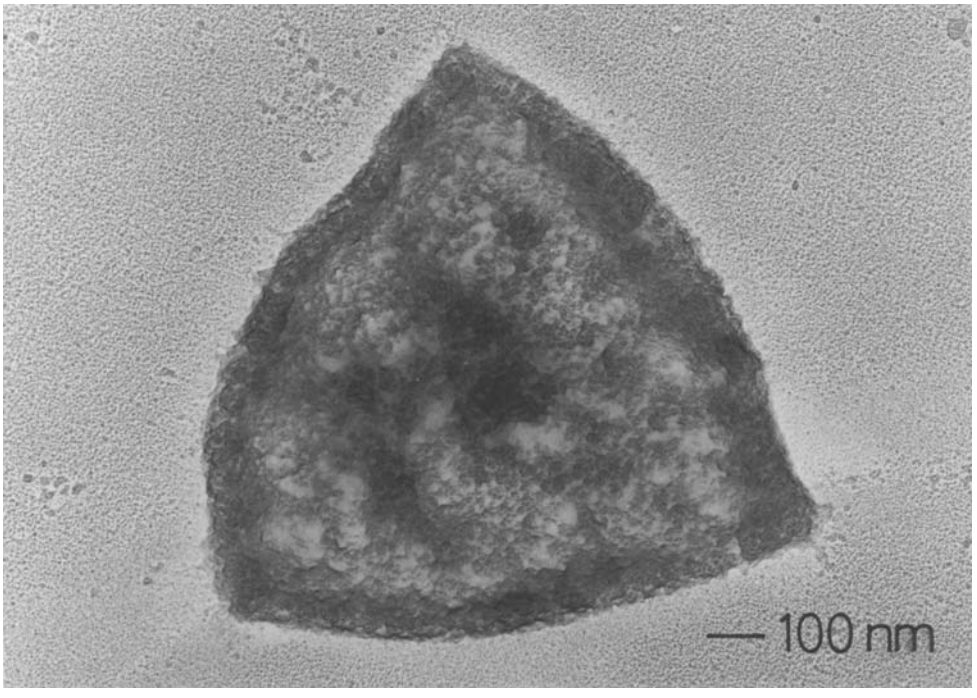


Figure 3. Electron micrograph of a cultured cell of *Ha. japonica*. Bar indicates 100 nm.

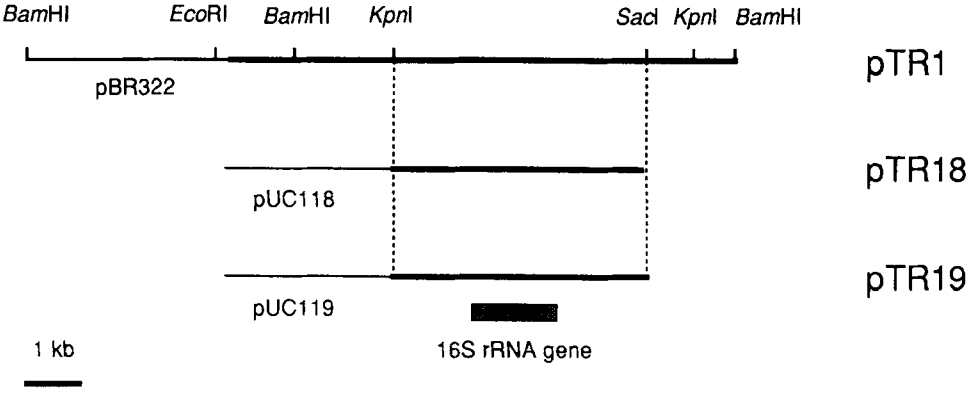


Figure 4. Physical maps of recombinant plasmids harbouring the 16S rRNA gene of *Ha. japonica*. The inserted fragments of recombinant plasmids, pTR1, pTR18 and pTR19 are shown by thick lines with the locations of restriction enzyme sites indicated above. The location of the 16S rRNA gene revealed by the nucleotide sequencing is shown by a dotted box. A bar at the left bottom indicates 1 kb.

Table 2. Homology values of 16S rRNA genes among extreme halophiles

	Ha. japonica	*Ha. sinaiiensis* (major)	*Ha. marismortui* (B)	*Ha. sinaiiensis* (minor)
Ha. sinaiiensis (major)	70 (94.8)			
Ha. marismortui (B)	68 (94.9)	20 (98.5)		
Ha. sinaiiensis (minor)	56 (95.8)	31 (97.7)	40 (97.0)	
Ha. marismortui (A)	38 (97.2)	75 (94.4)	63 (95.3)	52 (96.1)
Hb. cutirubrum[a]	141 (89.5)			
Hf. volcanii[b]	167 (87.5)			
Hc. morrhuae[c]	157 (88.3)			

Numbers indicate the different nucleotides within the 1341 bp we have determined in the sequence. Numbers in parenthesis show the homology % between the two strains. The sequences of; [a] 16s rRNA gene from *Halobacterium cutirubrum*; [b] *Haloferax volcanii*; and [c] *Halococcus morrhuae* are taken from Kamekura and Seno (1993).

strain TR-1 at high salt concentrations in agreement with our previous reports, where we described the characteristic triangular shape of strain TR-1 (Hamamoto *et al.*, 1988; Takashina *et al.*, 1990; Nishiyama *et al.*, 1992; Horikoshi *et al.*, 1993). An electron micrograph of TR-1 is shown in Fig. 3. The TR-1 type strain is deposited as *Ha. japonica* JCM7785 in the Japan Collection of Microorganisms at Riken Institute (Saitama 351-01, Japan).

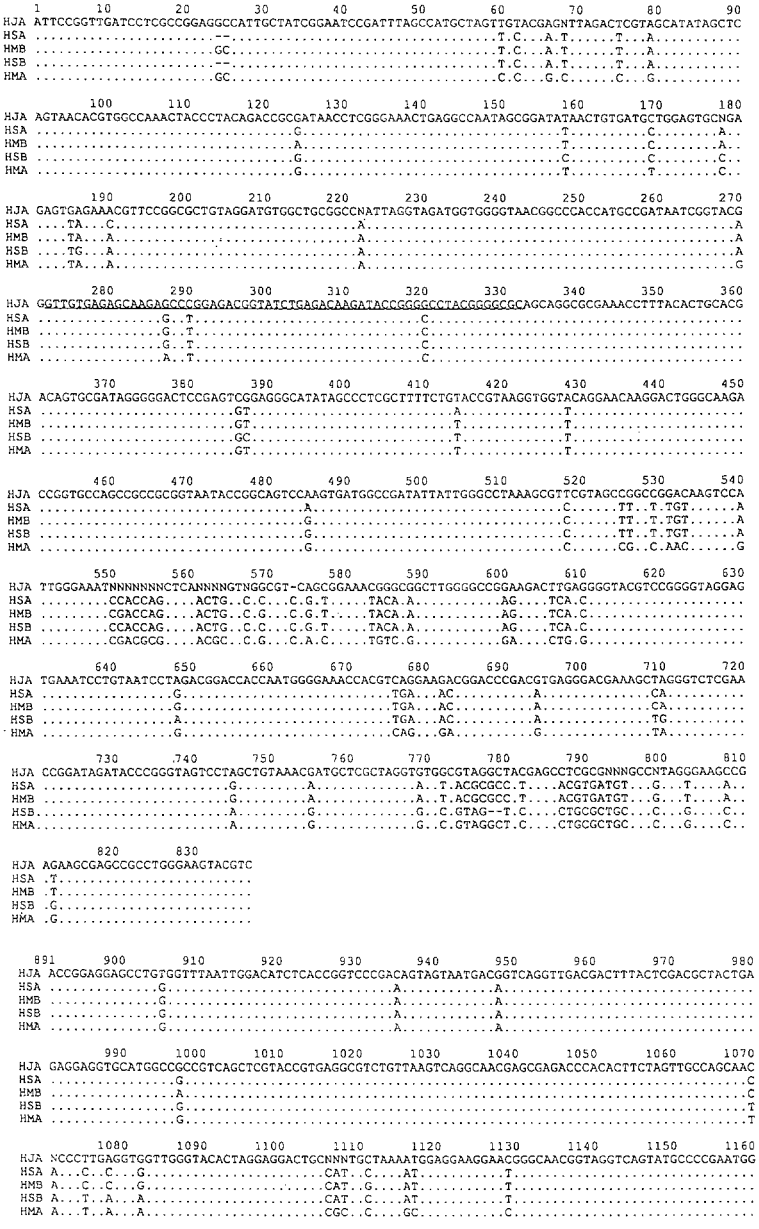
Partial 16S rRNA sequence of strain TR-1

We previously reported that the results of biochemical characterization and DNA/DNA homology suggested that strain TR-1 is a new species (Takashina *et al.*, 1990). To confirm this result, the 16S rRNA gene of TR-1 was cloned and sequenced. A gene library of the *Ha. japonica* chromosome DNA was partially digested with *Sau*3A. A DNA fragment cloned from the gene library cloned into *E. coli* MV1184 was shown to contain a sequence corresponding to the 5′- and 3′-termini of the *Halococcus morrhuae* 16S rRNA gene by colony hybridization. We subsequently constructed a recombinant plasmid, pTR1, which contains an 8 kb DNA fragment from *Ha. japonica* chromosome (Fig. 4). Subcloning experiments indicated that the DNA region of pTR1 which hybridized with the two probes is located within the *Kpn*I-*Sac*I fragment of the 8 kb insert. For sequencing purposes, recombinant plasmids pTR18 and pTR19 were constructed using pUC118 and pUC119 as vector plasmids. Nucleotide sequencing of the partial 16S rRNA gene resulted in 1341 bases (Fig. 5). Figure 5 also shows the alignment of the partial 16S rRNA sequence with other published sequence of *Haloarcula* species (Kamekura and Seno, 1993). Sequence similarity of the 16S rRNA genes of *Ha. japonica* with some halophilic archae is given in Table 2. As expected, sequence similarity with *Halococcus*, *Halobacterium* and *Haloferax* species is relatively low (88.3–89.5%). In contrast, the sequence similarity with *Haloarcula* species is considerably high (>94.8%), and is within the range of sequence similarity for the 16S rRNA gene sequences of *Haloarcula* species.

Discussion

The distribution of halotolerants and moderate halophiles in a salt field was examined. All samples resulted in a far larger number of colonies on 2M Na^+ neutral medium than on 4M Na^+ neutral medium. This was not unexpected given that high salt concentration should be stressful even for halotolerants, and that the salt field is operated

Figure 5. Nucleotide sequences of 16S rRNA genes from *Haloarcula* species. The nucleotide sequence of the gene for 16S rRNA from *Ha. japonica* is shown and numbered on the right of HJA. The 16S rRNA gene sequences are shown on the rows of; HSA, *Ha. sinaiiensis* ATCC33959 (major); HMB, *Ha. marismortui* Ginzberg (B); HSB, *Ha. sinaiiensis* ATCC33959 (minor); HMA (A), *Ha. marismortui* Ginzberg. The sequences are shown with dots for the identical, bars for absence and letters for the different bases. Letters "N" denote ambiguous bases. The corresponding regions (base No. 271 to 334 and No. 1253 to 1349) to the probe sequences derived from *Halococcus morrhuae* were shown by underlines. The nucleotide sequence data for *Ha. japonica* was deposited in DDBJ/EMBL/GenBank with the accession number D28872.



only seasonally resulting in seasonal variation in salinity. Moreover, the seasonal variation in salinity may have also contributed to the low occurrence of high halotolerants. The growth characteristics of strains isolated from the 4M Na^+ medium indicated that these isolates grew better in 5% salt medium (ca 0.8 M). We also isolated alkali-tolerant halotolerant bacteria from the salt field. It is known that non-halotolerant alkaliphiles were isolated not only from alkaline samples but also from neutral environments (Hamamoto and Horikoshi, 1992). Therefore it appears that alkali-tolerant bacteria can be isolated from both saline and non-saline neutral environments.

Despite the large numbers of halotolerant colonies observed, we also isolated an extreme halophile, TR-1. [The strain has been previously identified, (Takashina *et al.*, 1990) and descriptions of the strain's cell shape and cell surface have also been previously reported (Hamamoto *et al.*, 1988; Horikoshi *et al.*, 1993).] We sequenced the TR-1 16S rRNA gene, using a cloned gene to avoid difficulties arising from multiple 16S rRNA genes which some halophilic archae are known to contain (Kamekura and Seno, 1993). The TR-1 16S rRNA sequence has high sequence similarity (>94.9%) with 16S rRNA sequences of *Haloarcula* species, which is consistent with our previous findings that TR-1 is a species belonging to the *Haloarcula* group (Takashina, *et al.*, 1990). Moreover, the high sequence similarity of the 16S rRNA gene among the *Haloarcula* is consistent with the taxonomic grouping of these strains in general.

Ionic sensitive extreme halophiles are not commonly thought to be good candidates for high frequency migration between geographically distant habitats (e.g., the Japan and Red seas). The high sequence similarity (94.8–97.2%) between *Ha. japonica* and other *Haloarcula* isolated from geographically distant locations is clearly not high enough to suggest that the Japanese isolate is a recent immigrant. The range of sequence similarity of *Ha. japonica* to *Haloarcula* of geographically distant locations is roughly equal to that of *Haloarcula* isolated from the Red and Dead seas (94.4–98.5%), locations which are relatively close (Grant and Larsen, 1989). Examination of the factors which may have affected genetic diversity among the *Haloarcula* will be particularly interesting.

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