CHAPTER 4

DIFFERENTIATION OF BACTERIA AND HALOPHILIC ARCHAEA BY GRAM STAIN INDEX

Introduction

The phylogenetic tree based on ribosomal RNA sequencing indicates that evolution from the universal ancestor was originally in two directions, known as the Bacteria and Archaea / Eukarya line (Madigan *et al.*, 1999). Later, the Archaea and Eukarya lines diverged. Therefore, Archaea are phylogenetically more related to Eukarya relative to Bacteria. In present times, many Archaea inhabit extreme environments, such as high temperature, low pH, and high salinity (Madigan *et al.*, 1999).

In the particular case of saline environments, Bacteria in marine ecosystems are categorized as a group typical of slight halophiles. On the other hand, populations of the extremely halophilic Archaea have been isolated only from hypersaline lakes all over the world, such as the Dead Sea in Israel, Wadi Natrun in Egypt, and the Great Salt Lake in USA (DasSarma and Fleischmann, 1995; Imhoff *et al.*, 1979). Thus, the extreme halophiles are believed to thrive in extreme saline environments. However, Rodriguez-Valera *et al.* (1979) reported that extremely halophilic cocci were isolated from coastal waters in the Mediterranean Sea. They suggested possible survival of some extreme halophilic Archaea in the marine environment, even though they are unable to grow under laboratory conditions with

relatively low salinities as found in sea water. Therefore, for the biological analysis of a natural aquatic ecosystem, it is necessary to develop a characterization method to determine if a natural microbial population is composed of Bacteria or Archaea.

One of the promising methodologies for this purpose is to apply the Gram Stain Index (GSI) as applied recently by using a photometric image analysis method (Saida *et al.*, 1998; 2000) for the characterization of a natural bacterial population.

Our present study was firstly to characterize the GSI profile (Saida *et al.*, 1998) of a representative extreme halophilic archaeal organism. Further work was then performed to compare this profile to the GSI of natural microbial populations comprised of both Bacteria and Archaea. For this purpose, the coastal region near Shimoda, Japan was investigated as a case study of a typical marine environment.

Materials and Methods

Samples

Samples of water and salt were collected aseptically from three representative saline environments of Egypt, China and Japan. Salt samples were collected from a salt evaporation pan along the Red Sea at Oyon Moussa, Sinai (Egypt) on 11th August 1996. Surface water samples were collected aseptically from Aidingkol-Hu Lake, Urmuchi (China) on 19th September 1999. Coastal sea water samples and fresh water from the Inouzawa River flowing into Shimoda Bay (Japan) were collected at 6 stations on 8th and 9th December 1999. A sample of each water sample was fixed by adding 37% buffered formalin (sample:formalin = 20:1) immediately after the collection. Another portion was filtered immediately through Whatman GF/F glass-fiber filters, and kept at –20 °C until chemical analyses.

Culture and identification

One g of the salt sample was dissolved in 100ml sterile 4 M NaCl solution. Each 0.5 ml aliquot was transferred onto plates of TYS (tryptone-yeast extract salt) medium (containing NaCl 250 g, tryptone 5.0 g, yeast extract 4.0 g, $MgSO_4 \cdot 7H_2O$ 20.0 g, $CaCl_2 \cdot 6H_2O$ 0.2 g, and KCl 5.0 g per liter). The medium was then adjusted to pH 7.0. The samples were cultured at 30°C. Water samples from Aidingkol-Hu Lake were

cultured on agar plates of JCM166 Medium (containing Bacto-casamino acids (Difco) 15.0 g, Trisodium citrate 3.0 g, Glutamic acid 2.5 g, $MgSO_4 \cdot 7H_20$ 2.5 g, KCl 2.0 g, NaCl 250 g, and Agar 20.0 g per liter). The pH was adjusted to 8.5. Other portion of each water sample was cultured on a modified JCM 166 Medium with $MgSO_4$ increased up to 20 g per liter with pH adjusted at 7.0 for the purpose of isolating neutrohalophilic archaeal strains.

The isolates were identified according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). Morphological and physiological characteristics of isolates were determined and compared with those of standard archaeal strains for species identification. Biochemical activities were determined according to the recommended scheme of Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Identification was confirmed by the sequencing of 16S rRNA encoding genes for all archaeal isolates by the method described previously (Kamekura and Seno, 1993). PCR was performed to amplify 16S rRNA encoding genes with Gene Amp PCR system 9600 (Perkin Elmer Cetus). PCR products were cloned into pUC119 and sequenced by dideoxy methods in a sequencer ABI 373A. Alignment of the determined sequences with the standards of the gene bank was made for a full description of the archaeal isolates.

Analysis

The salinity of water samples was measured by EC meter (CM 14P, TOA Electronics Ltd., Tokyo). The DOC measurement was made with the high temperature combustion technique (Greenberg *et al.*, 1992) on a Shimadzu TOC-5000, equipped with an ASI-5000A.

Each fixed sample was filtered onto a polycarbonate Nuclepore filter (pore size, 0.2 μm; diameter, 25mm). Bacterial cells on half of each filter were counted by epifluorescent microscopy after staining with the acridine orange solution (acridine orange at 1:10,000 in 6.6 mM phosphate buffer of pH 6.6).

Bacterial cells on the other half of each filter were stained by the basic Gram method (Rodina, 1972). They were examined photometrically by using an optical microscope with a Fluor 100/1/30 oil Ph4DL 160/0.17 objective lens (Optiphot-2; Nikon, Tokyo). The image of bacterial cells was recorded (total magnification, ×2,500) by a 3-CCD charge-couple device video camera (XC-009; Sony, Tokyo). For each image, three primary colours (red, green, and blue) were transferred separately to an image analyzer (Luzex III U; Nireco, Tokyo) and light intensity was digitized on each pixel at 8-bit resolution (256 levels). The intensities of only red and blue were used to determine the characteristics of bacterial GSI (Saida *et al.*, 1998; 2000). The GSI histograms of 300 bacterial cells in each sample were compared based on their

modes and ranges.

Theoretical peak positions, intensities, and widths of those histograms were determined by Gaussian fitting to a normal curve and compared with each other. Statistical analyses were conducted by t test.

Results and Discussion

Pysico-chemical and biological conditions of each sampling site.

The water temperature (mean ± SD, n=3) during the investigations was 25.3 ± 0.7 °C at Aidingkol-Hu Lake, 12.0 ± 0.9 °C at the Inouzawa River, 14.7 ± 1.2 °C at the riverine portion of estuary at Shimoda Bay, 14.5 ± 1.6 °C at the middle estuary at Shimoda Bay, 16.0 ± 0.2 °C where the river enters Shimoda Bay, 17.8 ± 0.1 °C at the center of Shimoda Bay, and 18.0 ± 0.1 °C immediately outside of Shimoda Bay in the Kuroshio counter current, respectively (Table 4-1). The salinity of water in Aidingkol-Hu Lake was 316‰, which is categorized as hypersaline. Samples in Shimoda Bay and estuary were colleted along a salt gradient, but there was no gradient of pH and Eh (Table 4-1). Based on the DOC concentration of waters in this region, the water masses were categorized as eutrophic in the Inouzawa River, the riverine and middle estuary of Shimoda Bay, the entrance and center of Shimoda Bay, and mesotrophic immediately outside of Shimoda Bay. They were classified by the definitions of eutrophic and mesotrophic levels (i.e., Wetzel *et al.*, 1975; Seki and Nakano, 1981; Søndergaard and Schierup, 1982). According to the categorization above, The GSI profiles of natural microbial communities in those water masses were classified into 4 groups: river water, estuarine water, bay water, and coastal water.

Identification

The isolate from evaporated pan in the coast of the Red Sea at Oyon Moussa in Egypt was identified as *Halobacterium salinarum*, an extremely halophilic Archaea. Eleven reddish isolates among 12 strains from the salty Aidingkol-Hu Lake in China were identified *Haloterrigena sp.*, *Haloarcula sp.*, and *Natrialba sp.*, respectively, whereas a colorless isolate was identified as *Natrialba sp.*

GSI

The isolate of *H. salinarum* from the Red Sea, was used as a standard of Gram stain for halophilic Archaea, and was compared with a typical Gram negative Bacteria (*E. coli*) and a typical Gram positive Bacteria (*B. subtilis*).

The GSI spectrum of *H. salinarum* was distinctly different (Fig. 4-1) from those of bacteria (Fig. 4-3; from Saida *et al.*, 1998). In contrast to a standard Gram negative bacteria, *E. coli*, showing its peak at –0.049 with the range of 0.041, the GSI of *H. salinarum* (sample no: 307 cells) showed narrower range (0.29) with the peak at –0.064 (theoretical peak by Gaussian fitting with the 95% confidence limits). The GSI profile had the following two distinct features compared to the profiles of *E. coli* and *B. subtilis*: Firstly, a single sharp peak appeared on the more negative side of the GSI spectrum, that is, *H. salinarum* shifted 0.012 to the negative side. Secondly,

fluctuation of peak position of GSI profiles in the logarithmic phase of growth has been shown to be smaller (Saida *et al.*, 1998). Each fluctuation range was 0.01 for *H. salinarum*, 0.14 for *E. coli*, and 0.11 for *B. subtilis*. Those features are apparently different from the GSI of typical Bacteria such as *E. coli* and *B. subtilis*. Therefore, the GSI of halophilic Archaea can be differentiated clearly from those of Bacteria.

The GSI spectra of natural microbial populations in the extremely saline, Aidingkol-Hu Lake, also showed the typical profile, characterized with a sharp single peak (Fig. 4-2). The spectrum ranged from –0.09 to –0.045 having a sharp maximal peak at –0.070 (theoretical peak by Gaussian fitting with 95% confidence limits). The GSI profile was similar to those of a typical halophilic Archaea (*H. salinarum* and *Haloterrigena* sp) in shape, both width and peak position. Even though more than 4 genera of halophilic Archaea should have been present in the sample water, a sharp profile was observed. This may have been because most archaeal species show a narrow range profiles due to lack of peptidoglycan (Beveridge and Schultze-Lam, 1996), and partly because the profiles were formed by only a few species of dominant Archaea. Therefore, if archaeal species dominate a certain microbial community in an extreme environment, the GSI profile may show a sharp profile which tends to the Gram negative side.

All the GSI profiles of the natural bacterial communities in Shimoda Bay and adjacent regions were characterized by a wider range tending towards the Gram positive side, when compared with the profile from the extremely saline environment of Aidingkol-Hu Lake. The peaks in the Gram negative range were shifted more to the Gram negative side, compared to the peaks for *E. coli* (Fig. 4-3). Each profile included ranges of –0.120 to 0.030 in the river water, -0.105 to 0.095 in the estuary, -0.110 to 0.020 in the bay, and –0.115 to 0.010 in the coastal water. The width and theoretical peaks of the bacterial communities in these sampling sites were 0.030 and –0.065, 0.031 and –0.067, 0.039 and –0.062, and 0.038 and –0.072, respectively. Therefore, when the GSI spectra were compared with the standards for *E. coli* and *B. subtilis*, it was apparent that all the water environments in the coastal region showed a tendency to natural bacterial communities dominated generally by Gram negative bacterial populations. The bacterial community in the estuarine water, however, showed a GSI profile extended to the more Gram positive side, possibly because of the influence of a Gram positive bacterial population in the estuarine sediment carried up by the tide. The broadest GSI spectrum for the coastal bacterial community was detected in bay water, possibly because the diversity of bacterial species in this water mass was the greatest, with a mixture of both freshwater and marine bacteria. The GSI spectrum

of the natural bacterial community in the coastal water showed a profile shifting more towards the Gram negative side than that in any other water mass. This is theoretically reasonable because bacteria having greater Gram negative characteristics should be dominant in more oligotrophic water in the marine ecosystem (Seki, 1992). Statistical differences of the frequency distribution of GSI values of bacterial communities in those different water environments were significant as $p<0.001$ between the coastal water and the river water, $p<0.004$ between the coastal water and the estuarine water, $p<0.001$ between the coastal water and the bay water, respectively.

In conclusion, it was shown by our present study that the photometric image analysis of GSI spectra of natural microbial cells existing in different saline environments can be useful for characterizing each bacterial community structure in the environment.

Table 4-1. Some important physico-chemical parameters at each sampling site.

	Water temperature (°C)	Salinity (‰)	pH	Eh (mV)	DOC (mgC l^{-1})
river water	12.0 ± 0.9	0.1 ± 0.0	7.3 ± 0.6	570 ± 32	5.5 ± 0.1
estuarine	14.5 ± 1.6	20.4 ± 7.6	8.1 ± 0.1	520 ± 83	10.1 ± 2.4
bay water	16.0 ± 0.2	31.4 ± 0.2	8.2 ± 0.1	505 ± 12	8.1 ± 1.4
coastal water	18.0 ± 0.1	32.9 ± 0.1	8.3 ± 0.2	497 ± 9	3.0 ± 0.6

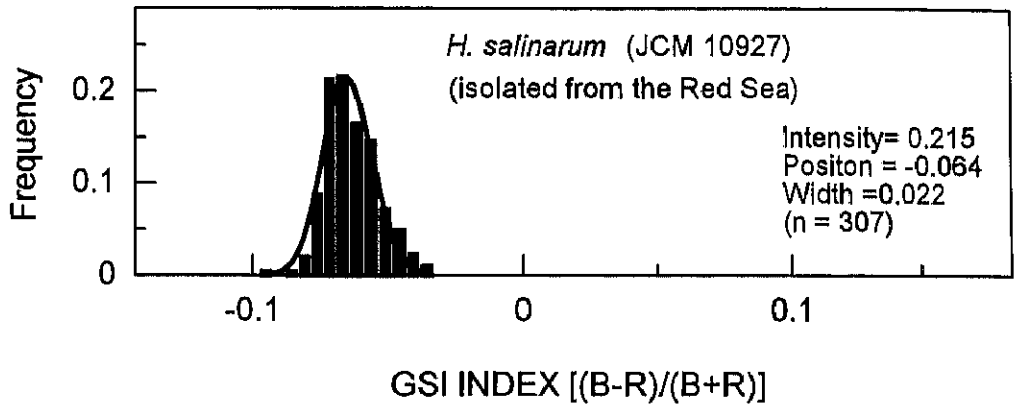

Fig. 4-1. GSI spectrum of an archaeal strain, *Halobacterium salinarum* (JCM 10927).

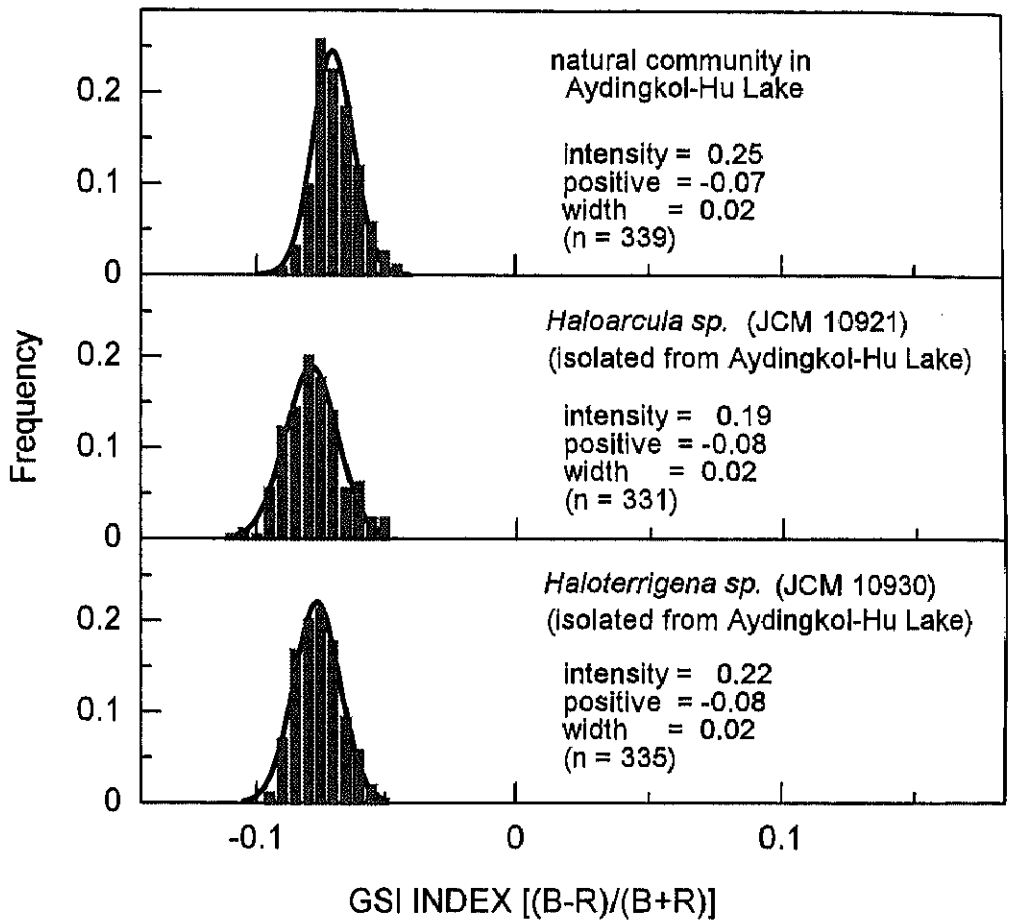

Fig. 4-2. GSI on natural microbial community in an extreme saline lake (Aidingkol-Hu Lake, China) and 2 isolates from the lake, *Haloarcula sp.* (JCM 10921) and *Haloterrigena sp.* (JCM 10930).

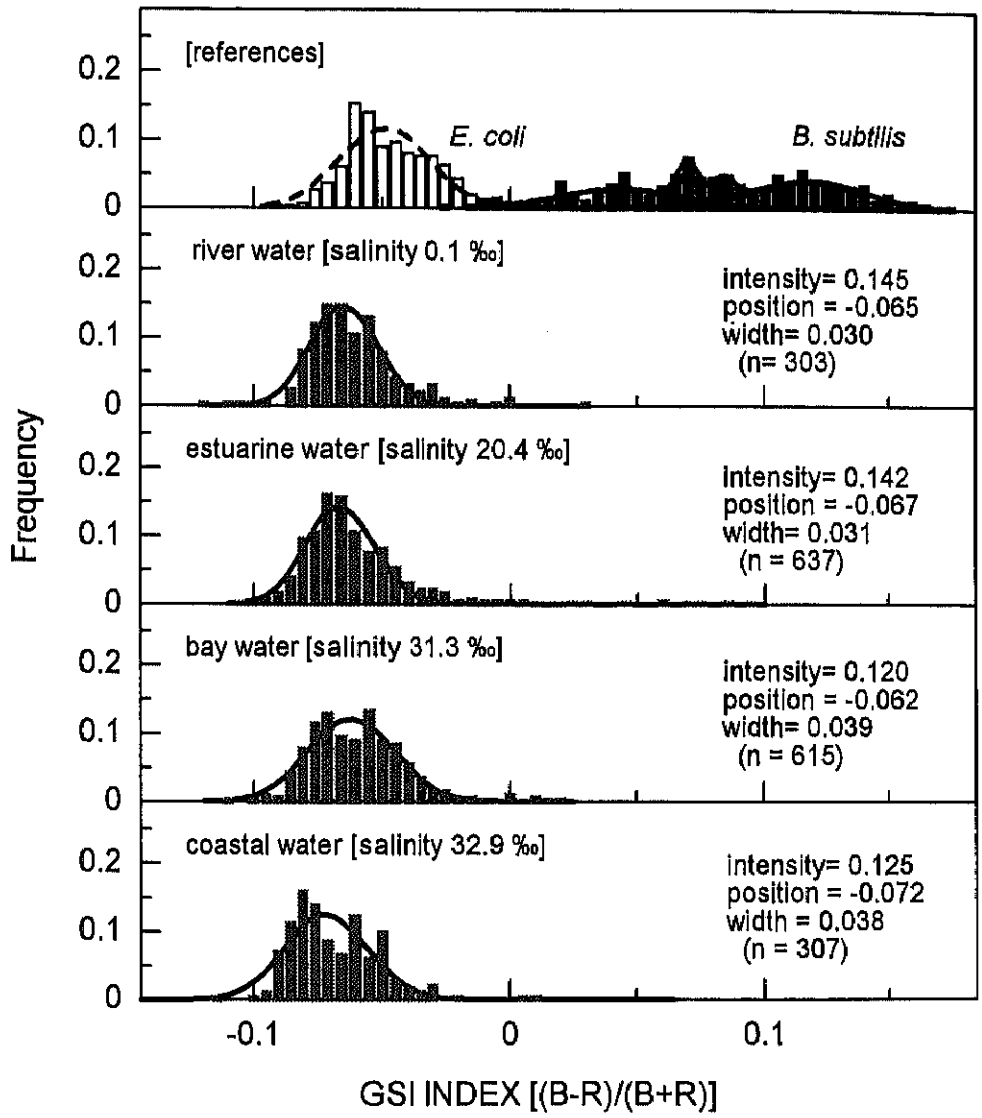

Fig. 4-3. GSI of natural microbial communities in the saline gradient of Shimoda Bay and adjacent region, Japan with references (*E. coli* and *B. subtilis*). Intensities, positions and widths of references are as follows: *E. coli*, intensity = 0.116, position = -0.049, width = 0.041 *B. subtilis*, 1:intensity = 0.029, 1:pososition = 0.043, 1:width = 0.056, 2:intensity = 0.053, 2:posisition = 0.070, 2:width=0.009, 3:intensity = 0.030, 3:pos = 0.083, 3:width=0.010, 4:intensity = 0.040, 4:position = 0.118, 4:width = 0.053.