Journal of Coastal Development Vol.3, No.1, October 1999: 501-506 ISSN : 1410-5217

RAPID GROUPING OF MARINE PSYCHROTROPHIC BACTERIA USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF PCR-AMPLIFIED 16S Rdna

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ABSTRACT

Grouping of 78 psychrotrophic bacteria isolated from surface and deep-sea waters of the north-western Pacific Ocean was carried out by RFLP analysis on the basis of PCR-amplified 16S rDNA. Six RFLP patterns from surface water and eight RFLP patterns from deep-sea water were obtained by Hha*I digestion. f pattern was only found in the surface water, while g, h, and i patterns exclusively existed in the deep-sea water. It was clearly shown that RFLP patterns of* Hha*I digestion were clear and distinguishable. This method is very simple and promises a rapid tool to group numerous isolates from natural environments.*

Key words: psychrotrophic bacteria, 16S rDNA, *Hha*I endonuclease, RFLP

I. INTRODUCTION

In the tropical and temperate zones, surface and deep-sea waters represent significantly different biosphere. Compared with the surface water which has warmer temperature, deep-sea water with the exception of waters in the vicinity of hydrothermal vents, belong to psychrosphere colder than 5°C. Somero (1998) mentioned that deep-sea is one of two regions of the world ocean which is characterized by extremely cold temperature. In addition, seawater at 0°C and seawater at 30°C vary in more than just temperature, and are quite different environment from the point an organism attempting to make a living there (Clarke, 1983).

Low temperature adapted microorganisms are classified into two categories: psychrophiles and psychrotrophs based on their cardinal temperatures. Psychrophiles are defined as those bacteria which have an optimum temperature for growth about 15°C, a maximum temperature for growth less than 20°C, and a minimum temperature for growth of 0°C, or lower. On the other hand, bacteria that could grow at low temperature such as 5°C, and lower, but with higher optimum growth temperatures, were defined as psychrotrophs (Morita, 1975).

In this study, a grouping of low temperature adapted bacteria in the surface and deep-sea waters in the open ocean was carried out. The work had been empashized to determine whether any differences occur in the group composition of bacterial community in the surface and deep-sea waters using RFLP patterns of PCR-amplified 16S rDNA.

II. MATERIAL AND METHODS

2.1. Bacterial Strains and DNA Extraction

A total of 78 strains isolated from surface water (0–200 m) and deep-sea water (1000– 8000 m) of the North-western Pacific Ocean were used. Isolation was carried out using plate medium of half-strength ZoBell 2216E at 4°C. Determination of psychrophily was based on growth at 4°C at and 20°C (Morita, 1975).

Strains were cultured at 20°C on 3-ml half-strength ZoBell 2216E broth medium. After harvest, cells were washed and suspended in sterile distilled water. 40µl suspension of DNAs were mixed with 10µl proteinase K (1 mg ml^{-1}) (Sigma Chemical Co, St. Louis, USA) and 50µl of 2 X K buffer (40 mM Tris buffer, 0.2% Nonidet P-40, 0.2 mM EDTA, 1% Tween 20, distilled water, pH 8.0). The mixture was heat treated at 60°C for 20 min, followed at 100°C for 10 min, then cooled rapidly on ice for 10 min and centrifuged at 8000 rpm for 5 min.

2.2. PCR Amplification

Primers (20F; position 8 to 27 and 1500 R; position 1510 to 1492 of *E coli* 16S rRNA numbering) described by Weisburg *et al* (1991) were used. PCR amplification was carried out in a DNA thermal cycler (Mini cycler TM, MJ Research Inc, Watertown, MA, USA) with the following temperature profile: an initial denaturation at 94°C for 2 min, 25 cycles of denaturation (2 min at 94°C), annealing (1.5 min at 45°C), and extension (2 min at 72°C), and final extension at 72°C for 3 min. Amplified DNA was examined by horizontal agarose gel in TAE electrophoresis buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA) with 1µl aliquots of PCR product.

2.3. RFLP Analysis

7 µl of PCR products were digested with restriction endonuclease *Hha*I (Toyobo Co, Ltd, Osaka, Japan) at 37°C for 180 min according to manufacturer's instructions. Restricted DNAs were analyzed by horizontal electrophoresis in 4% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME, USA). Electrophoresis was carried out at 50 V for 170 min with a Mupid mini-gel electrophoresis apparatus (Advance Co, Tokyo, Japan) in TAE electrophoresis buffer on ice. After electrophoresis, the gel was stained in ethidium bromide solution. Scanning image analysis of the gel and estimation of fragment size were carried out with a densitograph imaging analyzer (Atto Co, Tokyo, Japan) as described by Urakawa *et al* (1997).

III. RESULTS AND DISCUSSION

All of the 4°C –culturable bacteria grew well at 4°C and 20°C indicating that they were psychrotrophic bacteria (Morita, 1975). Among these bacteria, habitat-associated difference was revealed in the bacterial abundance with maximum growth temperature and shown in Figure 1. Surface bacterial groups showed high growth rate at 20-35°C, while deep-sea groups grew well in narrower temperature range(20-30°C). The member of group with maximum growth rate at 35°C was only found in the surface water. This also reflects one of the habitat-associated differences that affects the growth of psychrotrophs. The dominant groups in the surface(47.7%) and deep-sea(47%) waters had maximum growth rate at 25°C. This result clearly shows how psychrotrophs based on their optimum temperature, can be distingushed from psychrophiles, which have optimum temperature for growth about 15°C.

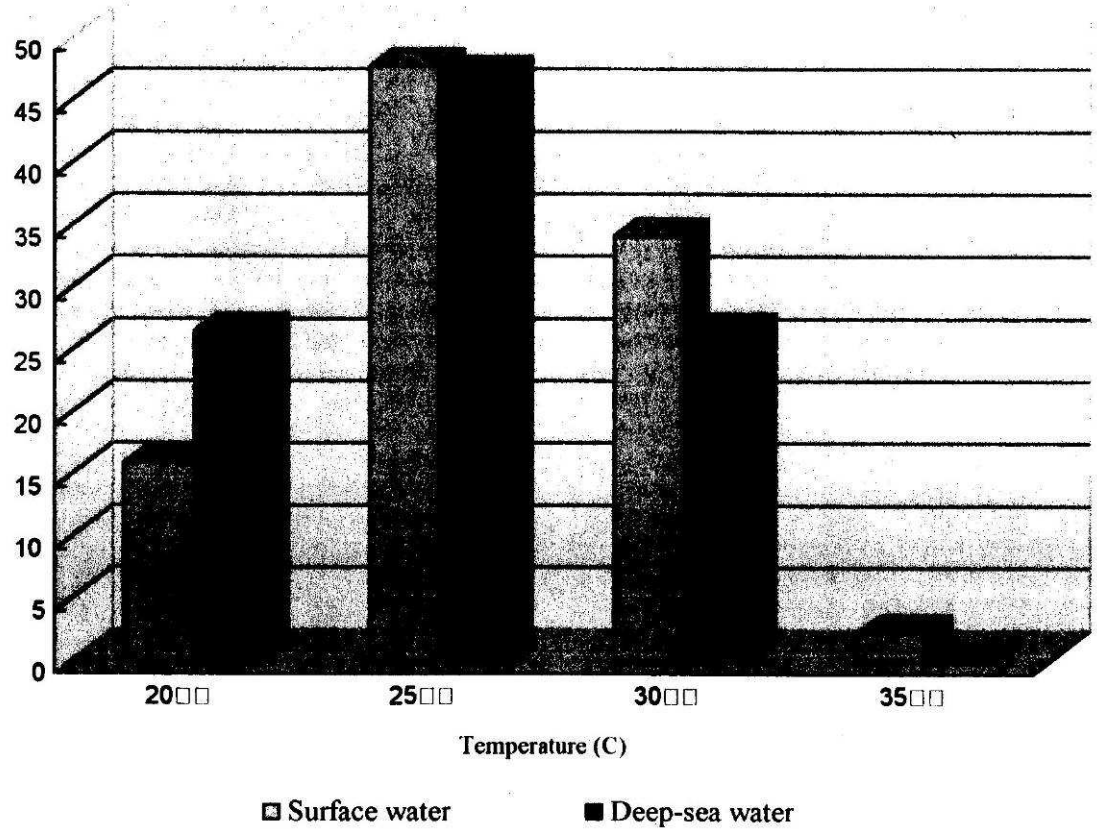

Figure 1. Relative abundance of psychrotrophic bacteria based on their maximum growth rate at different temperatures.

DNAs of 78 strains examined were amplified with the ribosomal primers, and produced a single band of about 1500 bp (data not shown). This size corresponded to the expected size of the 16S rDNA among bacteria.

From 3 to 5 informative bands were detected with the digestion of *Hha*I endonuclease (Fig.2). Restriction fragments shorter than 100 bp were not well resolved by electrophoresis in 4% NuSieve agarose, thus the size of PCR products estimated by summing the size of restricted fragments range from 1230 to 1545 bp (Table. 1).

Analysis of RFLP patterns of PCR-amplified 16S rDNA digested by *Hha*I among marine psychrotrophic bacteria produced six patterns in the surface and eight patterns in the deep-sea waters. A schematic summary of fragment patterns is shown in Fig. 2. These patterns indicated that bacterial diversity in the deep-sea water was higher than surface water. According to Maruyama *et al* (1997), deep-sea bacterial groups consist of members possessing a higher adaptability to low-temperature environments than the surface bacterial group. a, b, c, d, e patterns were found both

in the surface and deep-sea waters, and this suggests the wide distribution of psychrotrophic bacteria in the marine environments. According to Gounot (1991), psychrotrophs which are regarded as more adaptable than psychrophiles to a wider temperature range play an important role in the biodegradation of organic matters in marine environments .

Among the surface bacterial groups, only f pattern did not exist in the deep-sea water and this pattern could be less adapted to low temperature environment. In the contrary, among the deep-sea group, g, h, and i patterns were only found in the deep-sea water. These patterns may represent a group psychrotrophic bacteria that have adapted to deep-sea environment in such a way that metabolic processes, reproduction and survival strategies are optimal. e pattern dominated the abundance of psychrotrophic bacteria in the surface(27.3%) and deep-sea(20.6%) waters, and could be the most common psychrotrophic species distributed in the marine environment (Fig 3).

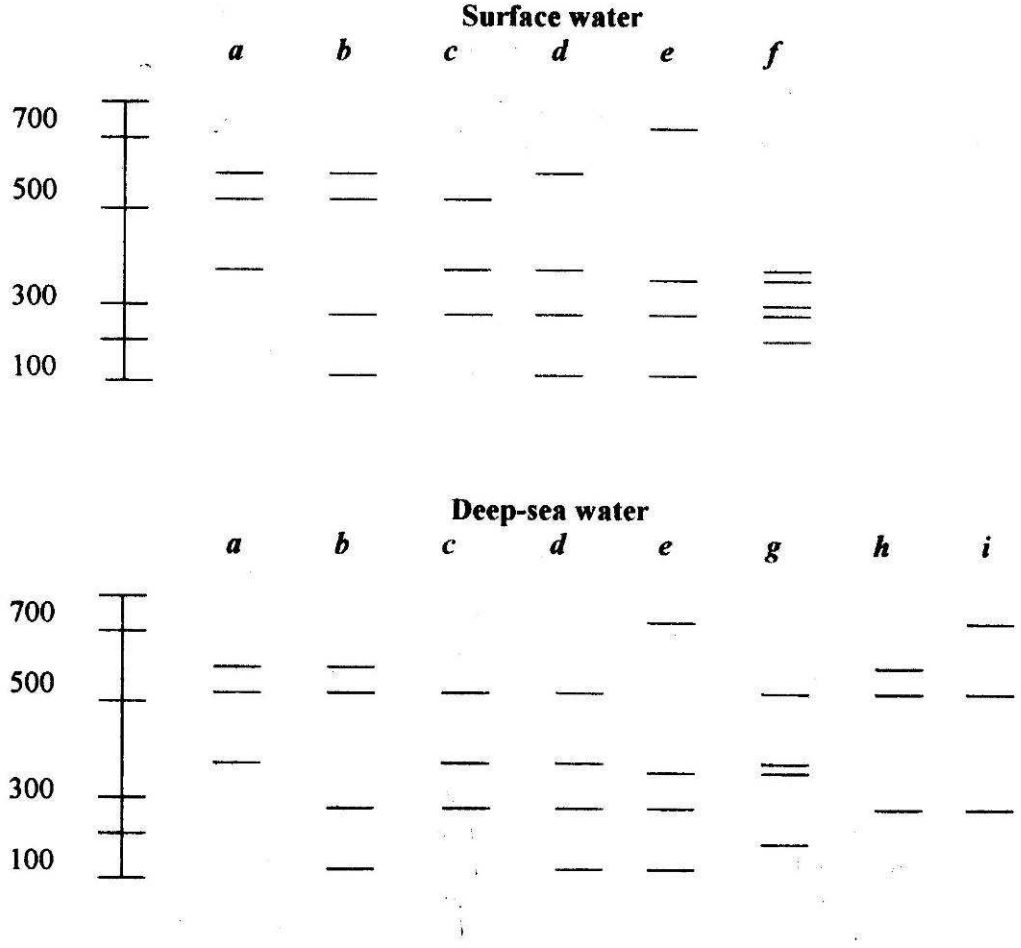

Figure 2. Restriction pattern of PCR-amplified 16S rDNA digested with *Hha*I

Table 1. Restriction fragment sizes of the strains digested with *Hha*I

Habitat	Pattern	Fragment size (bp)	Sum of fragment (bp)
Surface water	a	590, 535, 400	1525
Surface water	b	570, 535, 270, 140	1515
Surface water	c	565, 400, 265	1230
Surface water	d	580, 415, 270, 140	1405
Surface water	e	730, 375, 280, 135	1520
Surface water	f	405, 350, 280, 250, 205	1490
Deep-sea water	a	565, 540, 410	1515
Deep-sea water	b	570, 530, 275, 135	1510
Deep-sea water	c	560, 410, 270	1240
Deep-sea water	d	540, 405, 265, 135	1345
Deep-sea water	e	730, 365, 270, 135	1500
Deep-sea water	g	530, 410, 360, 215	1515
Deep-sea water	h	580, 545, 280	1405
Deep-sea water	l	730, 550, 265	1545

[a] fragment size in base pairs are rounded to nearest 5

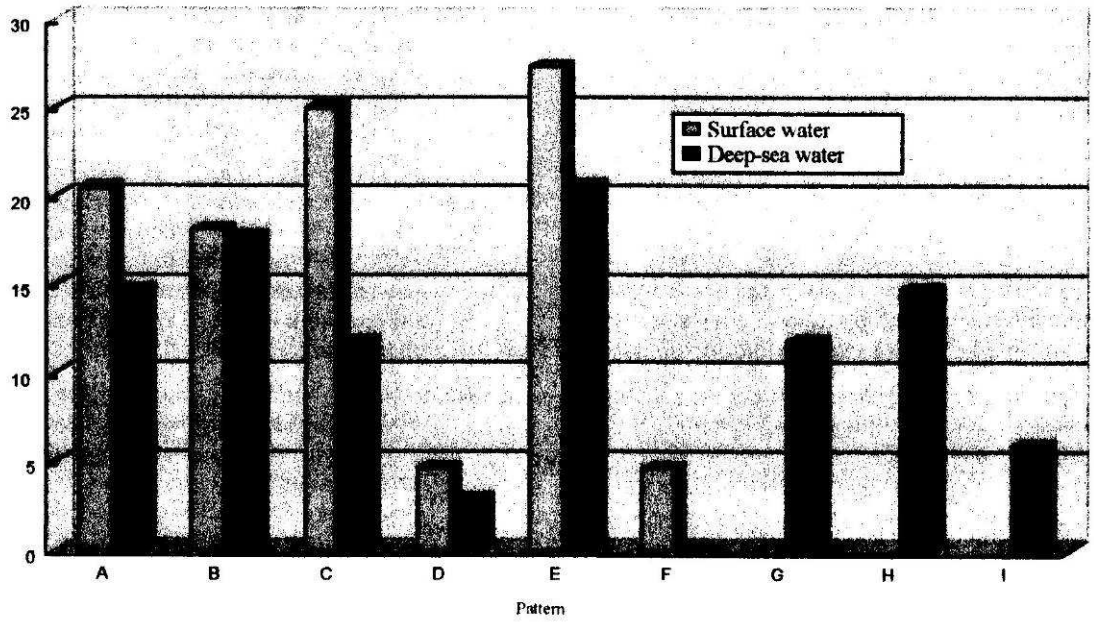

Fig. 3. Group composition of psychrotrophic bacteria generated from total number of each pattern

Several studies have reported the generic composition of psychrotrophic bacteria from cold environments. Maruyama *et al* (1997) described the generic composition of psychrotrophic bacteria from the Japan Trench. In addition, Urakawa *et al* (1999) compared the diversity of psychrotrophic and psychrophilic *Vibrio* and *Photobacterium* in the coastal and deep-sea waters. However, less information is available about the comparative diversity of bacterial species that inhabit in the surface and the deep-sea of the open ocean based on phylogenetic analysis.

Respresentative strains within each group based on RFLP analysis have now been characterized by 16S rDNA sequence analysis, and the results of this analysis will be used to determine the phylogenetic position of these bacteria.

Analysis of restriction patterns of 16S rDNA by *Hha*I digestion among marine psychrotrophic bacteria showed that the method is simple and distinguishable. Urakawa *et al* (1999) reported that among four base-specific restriction endonucleases (*Hha*I, *Dde*I, *Rsa*I, and *Sau*3AI) used in their study, *Hha*I digestion gave the clearest RFLP patterns, which did not produce any double bands.

In conclusion, the PCR-RFLP method used in this study promises a rapid tool to group isolates from environments since DNAs could be amplified directly from cells, and particularly useful in routine identification requiring rapid examination of numerous isolates.

ACKNOWLEDGEMENTS

This work was supported by a grant from Ministry of Education, Sports, Science and Culture of the Japanese government. We thank the Captain and crews of R/V Hakuho-maru for their kind helps during the cruise.

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