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Population structure of algicidal marine bacteria targeting the red tide forming alga *Heterosigma akashiwo* (Raphidophyceae), determined by restriction fragment length polymorphism analysis of the bacterial 16S ribosomal RNA genes

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ABSTRACT: A total of 233 marine bacterial strains which killed a noxious marine microalga, *Heterosigma akashiwo*, were isolated from Hiroshima Bay, the Seto Inland Sea, Japan, during blooms of *H. akashiwo* in 1994 and 1995. Population structure and genetic diversity of the *H. akashiwo*-killing bacteria (HAKB) were analyzed by means of restriction fragment length polymorphism (RFLP) and partial sequences of 16S ribosomal RNA genes (16S rDNA) PCR (polymerase chain reaction) amplified from HAKB strains. The RFLPs were generated by separate digestion with 5 restriction enzymes, *Eco* RI, *Rsa* I, *Mbo* I, *Bst* UI and *Hha* I. Seventeen ribotypes were observed among 85 strains of HAKB isolated in 1994. Bacterial strains of 3 ribotypes, 2B, 2C and 2D, were dominant in the HAKB populations during the termination period of the *H. akashiwo* bloom concurrent with the increase in the number of HAKB. Partial sequences, almost 500 bp of nucleotides, and RFLP patterns of 16S rDNA from some HAKB strains revealed that the HAKB of 2B, 2C and 2D ribotypes are closely related to the γ-proteobacteria group. The HAKB strains belonging to 2C and 2D were repeatedly isolated from seawater collected at the end of a *H. akashiwo* bloom in 1995. These results suggest that 3 species of HAKB may play a role in the rapid termination of the *H. akashiwo* bloom in Hiroshima Bay.

KEY WORDS: Algicidal bacteria · Marine bacteria · 16S rRNA · Restriction fragment length polymorphism · *Heterosigma akashiwo* · Marine phytoplankton · Bloom

INTRODUCTION

In the last several decades, it has become well known that some bacteria in lakes, ponds and rivers kill and ingest blue-green algae, and these algicidal bacteria are considered to be one of the agents causing rapid disintegration of blue-green algal blooms (Stewart & Brown 1969, Shilo 1970, Stewart & Daft 1976, Yamamoto & Suzuki 1990). Gliding bacteria of the genera *Myxobacter*, *Lysobacter* and *Cytophaga* were the primary members of these algicidal bacterial populations (Yamamoto et al. 1993). Ecological studies relying on a soft agar overlay technique demonstrated that the dynamics of the algicidal gliding bacteria had a close relationship with the disappearance of blue-green algae at the end of blooms in freshwater environments (Daft et al. 1975, Yamamoto 1981, Mitsutani et al. 1987). In contrast, little information about the ecology of marine algicidal bacteria and their impact on marine microalgal communities has been available, since most marine microalgae (mainly phytoplankton) could not be cultivated on an agar plate and therefore were not amenable to the soft agar overlay technique (Imai et al. 1993).

We recently developed a most probable number (MPN) method for detecting and enumerating algicidal bacteria in seawater and found a close relationship

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between the dynamics of algicidal bacterial populations and of marine phytoplankton communities (Yoshinaga et al. 1995b, Imai et al. 1998, Kim et al. 1998, in this issue) An increase or decrease of the marine bacteria which inhibit the growth of *Gymnodinium mikimotoi* (Dinophyceae) correlated negatively with the development or decay, respectively, of a *G. mikimotoi* bloom in Tanabe Bay, Japan (Yoshinaga et al. 1995b) Moreover, we observed the rapid increase of algicidal bacteria targeting *Heterosigma akashiwo* (Raphidophyceae) at the end of *H. akashiwo* blooms which occurred in Hiroshima Bay, Japan, in 1994 and 1995 (Kim et al. 1998). These results strongly suggest that the marine algicidal bacteria may be one of the agents regulating the dynamics of the phytoplankton community in seawater in a way similar to the action of algicidal gliding bacteria in freshwater ecosystems (Yamamoto 1981, Mitsutani et al. 1987, Yoshinaga et al. 1995b, Kim et al. 1998).

Several researchers have isolated the bacteria algicidal to marine microalgae from nearshore seawater, sediment, an aquaculture pond for prawns and a mass culture of marine diatoms (Baker & Herson 1978, Sakata 1990, Imai et al. 1991, 1993, 1995, Fukami et al. 1992, Mitsutani et al. 1992, Yoshinaga et al. 1995b, 1997). The taxa of these marine algicidal bacteria are spread widely among genera; these genera are *Vibrio*, *Flavobacterium*, *Acinetobacter*, *Alteromonas* and *Pseudomonas* as well as gliding bacteria *Saprospira* and *Cytophaga*, in contrast to the algicidal bacterial populations in freshwater which are generally composed of the gliding bacteria. There seems to be a wide variety of algicidal bacteria existing in seawater as compared with freshwater.

Bacterial small subunit ribosomal RNA (16S rRNA) sequences have been analyzed frequently in the study of genetic diversity and structure of bacterial communities from various habitats (Woese 1987). It is commonly recognized that the 16S rRNA sequences provide more precise and informative characterization of bacterial taxa more rapidly than the physiological and chemical characteristics used in standard taxonomical protocol for bacteria. The recent dramatic increase in bacterial 16S rRNA databases and the development of molecular biological techniques, furthermore, has made it possible to analyze the bacterial community structure without isolating and culturing bacteria, by using PCR (polymerase chain reaction), dot hybridization and *in situ* hybridization (Giovannoni et al. 1990, 1995, Delong 1992, Mullins et al. 1995). Consequently, determination of 16S rRNA sequences of bacteria newly isolated from seawater is of great importance for marine microbial ecology.

Previously, we reported that the abundance of *Heterosigma akashiwo*-killing bacteria (HAKB) increased during decay periods of the *H. akashiwo* blooms in Hiroshima Bay in 1994 and 1995 (Kim et al. 1998). In this study, several strains of HAKB were isolated during the *H. akashiwo* blooms, and the 16S rRNA genes of the HAKB isolates were analyzed to determine the genetic diversity and structure of the HAKB populations.

MATERIALS AND METHODS

Microalgae. An axenic culture of *Heterosigma akashiwo* (893) was isolated from the Seto Inland Sea, Japan (Imai et al. 1993), and was cultivated in a SWM3 medium (Itoh & Imai 1987) at 20°C under an illumination of about 50 µmol photons m^{-2} s^{-1} with a 14 h light:10 h dark photo-cycle.

Isolation of HAKB. Strains of HAKB were isolated from surface and bottom seawater (1 m above the bottom) at 2 stations (Stns V and M) in Hiroshima Bay in 1994 and 1995 (Kim et al. 1998). Using the MPN procedure for counting HAKBs in seawater, strains of HAKB were isolated from the microplate well in which *Heterosigma akashiwo* cells were killed or lysed after inoculation of a seawater sample. In order to isolate the dominant bacterial strains of the HAKB population, isolates were obtained from those microplate wells highest in the dilution series in which *H. akashiwo* cells were killed or lysed. A portion (0.1 ml) of *H. akashiwo*-bacteria co-culture in the microplate well was spread onto a $ST10^{-1}$ agar plate (Yoshinaga et al. 1995b) and incubated at 20°C in the dark. To confirm their algicidal activities on *H. akashiwo*, the bacterial colonies on the agar plates were picked and inoculated again into the log phase cultures (3.5 ml) of *H. akashiwo* in test tubes (130 mm × 13 mm). No 2 separate strains were isolated from the same microplate well. In total, 96 and 127 strains of HAKB were isolated in 1994 and 1995, respectively, and were maintained in the $ST10^{-1}$ semi-solid culture medium (0.3% w/v of agar) at 20°C.

16S rDNA-RFLP analysis of HAKB. To investigate the genetic diversity among HAKB populations, restriction fragment length polymorphism (RFLP) of 16S rRNA genes PCR amplified from HAKB isolates were analyzed. Bacterial chromosomal DNAs were purified using the standard method (Sambrook et al. 1989) with a small modification. The bacterial cells cultivated in the $ST10^{-1}$ medium (100 ml) were collected by centrifugation (8000 × *g*, 10 min) and resuspended in 474 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). After incubation with 1% (w/v) sodium dodecyl sulfate (SDS) at 50°C for 30 min, 1 mg ml^{-1} of lysozyme (Sigma) was added and then the mixture was incubated at 37°C for 30 min. Bacterial cells were lysed by incubation with 0.1 mg ml^{-1} of proteinase K (Merck)

at 55°C for 30 min, and the bacterial proteins and cell debris were removed by phenol extraction (phenol: chloroform:isoamylalchol = 25:24:1). The contaminating polysaccharides and the residual proteins were aggregated by incubation with 1.2% hexadecyltrimethyl ammonium bromide (CTAB) and 0.7 M NaCl at 60°C for 60 min, and they were removed by extraction with an equal volume of chloroform:isoamylalcohol (24:1). The bacterial DNA was recovered by ethanol precipitation, washed twice with 70% ethanol, and resuspended into 500 µl of TE buffer. To remove contaminated RNA, 50 µg ml^{-1} of RNase A (Sigma) was added and then the mixture was incubated at 37°C for 30 min. The RNase A was inactivated and removed with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1), and the bacterial DNA was recovered by ethanol precipitation, washed twice with 70% ethanol and resuspended in distilled water (100 µl). The DNA was stored at –30°C until the PCR reaction was started.

Gene fragments of 16S rRNA were amplified from the bacterial chromosomal DNA by means of PCR with the primer set of 6F (5'GRAGAGTTTGATCMTGGC; corresponding to positions 6 to 23 of *Escherichia coli* 16S rRNA) and 1492R (5'GGTTACCTTGTTACGACTT; corresponding to positions 1474 to 1492 of *E. coli*) (Britschgi & Giovannoni 1991). All oligonucleotides used for PCR amplification of bacterial 16Sr DNA were synthesized on an automated DNA synthesizer (Pharmacia Gene Assembler Special, Pharmacia LKB Biotech., Uppsala, Sweden) and purified with an Oligonucleotides Purification Cartridge (Applied Biosystems Inc., Foster City, CA).

The PCR reaction was performed with a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) as follows: denaturation at 94°C for 1 min, annealing of primers at 53°C for 2.5 min, and extension at 72°C for 2.5 min, for a total of 30 cycles. The reaction mixture contained 100 pmol of primers, 200 µM of dNTPs (Takara, Otsu, Japan), 2 mM of $MgCl_2$, 2.5 units of Ampli Taq polymerase (Roche Molecular Systems, Inc.), 10 ng of bacterial DNA, and sterilized distilled water to a final volume of 100 µl.

Each PCR-amplified DNA fragment was purified by chloroform:isoamylalcohol (24:1) treatment followed by ethanol precipitation, and resuspended in 20 µl of distilled water. The purified PCR products (2 to 6 µl) were restricted with 2.5 units of 1 hexameric endonuclease, *Eco* RI (Takara), and 4 tetrameric endonucleases, *Rsa* I (Takara), *Mbo* I (Toyobo Co., Osaka, Japan), *Bst* UI and *Hha* I (New England Biolabs Inc., USA) individually, at 37°C for 4 h. The resulting restriction fragments were separated by gel electrophoresis in 1.5% agarose (Nippon Gene Co., Toyama, Japan) and the RFLP patterns were visualized under UV excitation after staining with 0.5 µg ml^{-1} of ethidium bromide.

Partial sequencing of 16S rDNA. Nucleotide sequences of the 5' end of the 16S rDNA of several HAKB isolates were determined. Almost 500 bp nucleotides from the 5' end of bacterial 16S rRNA genes were PCR amplified with the primer set of 6F and 536R (5'GWATTACCGCGGCKGCTG; corresponding to positions 519 to 536 of *Escherichia coli*). The PCR reaction was performed under the same conditions as specified above. The PCR products were excised from 1.5% agarose electrophoresis gels (Nippon Gene Co.) and eluted with SUPREC™-01 (Takara). The eluted PCR products were ligated in pCR™2.1 vector plasmids and transformed into *E. coli* JM109 by using a TA cloning Kit (Invitrogen, The Netherlands) according to the manufacturer's protocol. Inserted DNA fragments were sequenced by using a DyeDeoxy™ Cycle Sequencing Kit and DNA sequencing system (Model 373A; ABI) according to the manufacturer's recommended protocol. Two oligonucleotides, M13 reverse primer (5'CAGGAAACAGCTATGAC) and T7 promoter primer (5' TAATACTACTCACTATAGGG), were used as primers for sequencing.

The nucleotide sequences obtained were compared with the nucleotide sequence data in GenBank, EMBL (European Molecular Biology Laboratory) and DDBJ (DNA Data Base of Japan) using BLAST SEARCH, and were aligned with some sequences of well-known bacteria using the MALIGN program in computer software ODEN version 1.1.1 (National Institute of Genetics, Mishima, Japan) and DNASIS-Mac-0300 (Hitachi Software Engineering Co., Yokohama, Japan) to determine their phylogenetic relationships and taxonomic positions. Phylogenetic analyses were restricted to the comparison of highly and moderately conserved nucleotides positions, corresponding to residues 29 to 78, 101 to 454 and 480 to 516 (*Escherichia coli* numbering). The evolutionary distances between sequences were calculated according to the Jukes-Cantor method, using the DISTN program in ODEN, and a phylogenetic tree was constructed according to the neighbor-joining method, using CLUSTAL W (version 1.7). Bootstrap analyses of up to 1000 replicates were carried out with CLUSTAL W.

Bacterial 16Sr DNA sequences. The new nucleotide sequences reported in this paper are available under the following accession numbers in DDBJ, EMBL and GenBank: GY9 (1A), AB001332; MC8 (1A), AB001333; GY21 (2B), AB001335; MC27 (2B), AB001336; GY27 (2C), AB001334.

RESULTS

In total, 96 strains of HAKB were isolated from 34 seawater samples collected from Hiroshima Bay in 1994 (Tables 1 & 2). The RFLP patterns of 16S rRNA

Table 1. *Heterosigma akashiwo*-killing bacteria (HAKB) isolated from surface and bottom seawater at Stn V in Hiroshima Bay in 1994. Abundance of HAKB was determined by the most probable number (MPN) method (Kim et al. 1998). nd: not determined

Collection date	Surface			Bottom		
	H. akashiwo (cells ml^{-1})	HAKB (MPN ml^{-1})	Isolates	*H. akashiwo* (cells ml^{-1})	HAKB (MPN ml^{-1})	Isolates
May 24	2.8×10^{1}	2.6×10^{0}	GY1, GY2, GY3	2.0×10^{0}	nd	
June 1	1.4×10^{4}	3.5×10^{0}	GY4, GY6	1.7×10^{2}	nd	
June 3	1.8×10^{4}	5.4×10^{0}	GY7, GY8, GY9, GY10, GY11, GY12	2.9×10^{2}	nd	
June 6	<1	2.6×10^{2}	GY13, GY14, GY15, GY16, GY18, GY19, GY20, GY21, GY22	1.0×10^{4}	2.1×10^{1}	JM1
June 9	<1	5.1×10^{1}	GY23, GY27	1.3×10^{3}	2.7×10^{1}	JM2, JM4
June 13	3.5×10^{2}	1.1×10^{2}	GY28, GY29	2.0×10^{2}	9.3×10^{1}	JM5, JM6, JM7, JM8
June 16	3.0×10^{2}	1.0×10^{2}	GY31, GY32, GY34	8.3×10^{1}	7.1×10^{1}	JM9, JM10
June 20	2.2×10^{2}	3.8×10^{1}	GY35, GY36, GY37, GY38, GY39, GY40, GY41	<1	7.4×10^{1}	JM11, JM12
June 27	1.2×10^{2}	4.8×10^{0}	GY42, GY43, GY44	<1	4.2×10^{1}	JM13
July 4	3.7×10^{2}	6.6×10^{-1}		3.3×10^{1}	1.2×10^{1}	JM14, JM16, JM19

Table 2. *Heterosigma akashiwo*-killing bacteria (HAKB) isolated from surface and bottom seawater at Stn M in Hiroshima Bay in 1994. Abundance of HAKB was determined by the most probable number (MPN) method (Kim et al. 1998)

Collection date	Surface			Bottom		
	H. akashiwo (cells ml^{-1})	HAKB (MPN ml^{-1})	Isolates	*H. akashiwo* (cells ml^{-1})	HAKB (MPN ml^{-1})	Isolates
May 24	1.8×10^{1}	2.6×10^{0}	MC1, MC2	<1	1.5×10^{-1}	K1
June 1	3.2×10^{2}	3.6×10^{0}	MC3, MC4	3.2×10^{2}	1.0×10^{-1}	
June 3	4.4×10^{3}	2.2×10^{0}	MC7, MC8, MC9, MC10	5.8×10^{2}	2.3×10^{-1}	K3
June 6	2.1×10^{3}	1.9×10^{0}	MC13	3.5×10^{3}	1.9×10^{-1}	K4, K5
June 9	3.4×10^{3}	1.5×10^{1}	MC16, MC17	9.5×10^{2}	1.8×10^{-1}	K6, K7, K8
June 13	1.3×10^{3}	6.6×10^{1}	MC18	2.6×10^{3}	6.4×10^{0}	K9
June 16	4.7×10^{2}	5.0×10^{1}	MC21, MC23, MC24, MC25	3.3×10^{2}	1.4×10^{1}	K10
June 20	1.5×10^{2}	2.4×10^{2}	MC26, MC27, MC28, MC29, MC30, MC31	<1	7.5×10^{1}	K11, K12, K13, K14
June 27	3.3×10^{1}	1.2×10^{1}	MC32, MC33, MC34, MC37, MC38, MC39, MC40	<1	1.0×10^{2}	K15
July 4	8.7×10^{1}	1.7×10^{0}	MC41	<1	6.7×10^{1}	

gene (16S rDNA RFLP) amplified from 85 HAKB isolates were analyzed for studying genetical diversity of HAKB populations.

Table 3 lists the 16S rDNA RFLP patterns observed in the 85 HAKB strains after separate enzymatic treatment with the 5 endonucleases *Eco* RI, *Rsa* I, *Mbo* I, *Bst* UI and *Hha* I. The endonuclease *Eco* RI is a hexameric restriction enzyme, which has a 6-bp recognition site, and *Rsa* I, *Mbo* I, *Bst* UI and *Hha* I are tetrameric restriction enzymes, which have 4-bp recognition sites. The almost complete 16S rDNA (1500-bp nucleotide length) was digested into 1 or 2 fragments by *Eco* RI and into 1, 2, 3, 4, 5 or 6 fragments by *Rsa* I, *Mbo* I, *Bst* UI and *Hha* I. Three RFLP patterns were obtained among HAKB populations with *Eco* RI, and, in contrast, the 4 tetrameric restriction enzymes *Rsa* I, *Mbo* I, *Bst* UI and *Hha* I revealed 11, 15, 14 and 13 RFLP patterns, respectively. From the results of 16S rDNA RFLP by restriction with 5 endonucleases, we tentatively divided the HAKB strains into 17 ribotypes. Ribotypes 1A, 1B and 1C could be distinguished from each other by the treatment with *Mbo* I only and the RFLP of ribotype 2D with *Eco* RI, *Rsa* I and *Hha* I was identical to that of ribotype 2C. The RFLP patterns of 2B and 2D overlapped after restriction with *Eco* RI, *Mbo* I and *Hha* I.

Ribotype groups 2B and 2C together accounted for 55.3% of all the 85 HAKB strains isolated in 1994 (Table 4). Ribotype group 1A accounted for 11.8%, and was isolated only from the surface water at both Stns V and M. The remaining 28 strains were distributed among 14 ribotypes, and 9 ribotypes (1B, 1C, 1D, 1F, 1G, 1H, 1M, 2A and 2E) consisted of a single strain.

Table 3. Restriction fragment length polymorphisms of 16S rRNA gene (16S rDNA RFLP) of HAKB isolated in Hiroshima Bay in 1994, revealed by separate digestion with *Eco* RI, *Rsa* I, *Mbo* I, *Bst* UI and *Hha* I. The number of bacterial strains that were classified into each ribotype is given in parentheses

Restriction enzyme	Estimated size of 16S rDNA fragment (bp)	Ribotype in HAKB																
		1A (10)	1B (1)	1C (1)	1D (1)	1E (3)	1F (1)	1G (1)	1H (1)	1J (6)	1K (2)	1M (1)	2A (1)	2B (26)	2C (21)	2D (6)	2E (1)	3A (2)
Eco RI	1500	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
	850	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
	650	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–
	600	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Rsa I	900	–	–	–	+	+	–	–	–	–	–	–	+	+	+	+	–	–
	700	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
	650	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
	600	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–
	500	–	–	–	–	–	–	+	–	+	+	+	–	–	+	+	–	+
	450	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	+
	400	+	+	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–
	350	+	+	+	+	+	–	–	+	–	–	–	+	+	–	–	+	–
	250	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–
	150	+	+	+	–	–	–	–	–	+	+	+	–	–	–	–	+	–
	≤100	+	+	+	+	+	–	–	–	+	+	–	+	+	–	–	+	+
Mbo I	1500	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
	1200	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
	1100	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
	900	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
	850	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–
	800	–	–	–	–	–	–	–	–	+	–	+	–	+	–	+	–	+
	700	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
	600	–	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	450	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	400	–	+	–	–	–	+	+	–	+	+	+	–	+	–	+	–	–
	300	+	–	+	+	–	–	–	–	–	–	–	–	–	+	–	–	–
	250	–	+	–	–	+	+	–	–	+	–	–	+	+	–	+	–	–
	200	–	+	+	+	+	+	–	+	–	+	+	–	–	–	–	–	+
	150	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
	≤100	+	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	+
Bst UI	550	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
	525	+	+	+	–	–	–	–	–	–	–	–	–	+	+	–	–	–
	500	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
	475	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
	450	+	+	+	–	–	+	–	+	+	+	+	–	–	–	–	–	–
	400	–	–	–	+	+	–	–	–	+	–	+	+	–	–	–	+	+
	375	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
	350	–	–	–	–	–	–	+	+	–	+	–	+	+	+	+	–	+
	300	+	+	+	–	+	+	+	+	+	+	–	+	–	–	+	+	–
	250	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–
	225	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
	200	–	–	–	–	–	–	–	–	–	+	+	–	–	+	–	–	–
	150	–	–	–	–	–	+	–	+	–	–	+	–	–	–	–	–	–
	125	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
	≤100	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+
Hha I	1100	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
	1000	+	+	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–
	750	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
	550	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–
	500	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+
	400	+	+	+	–	+	–	–	+	–	+	–	+	+	+	+	+	+
	350	–	–	–	+	–	+	–	–	–	–	–	–	+	+	+	–	+
	300	–	–	–	–	–	–	+	–	–	–	–	+	+	+	+	+	–
	275	–	–	–	+	–	–	–	–	+	–	+	–	–	–	–	–	–
	250	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	+	–
	225	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–
	200	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
	150	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–
	≤100	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–

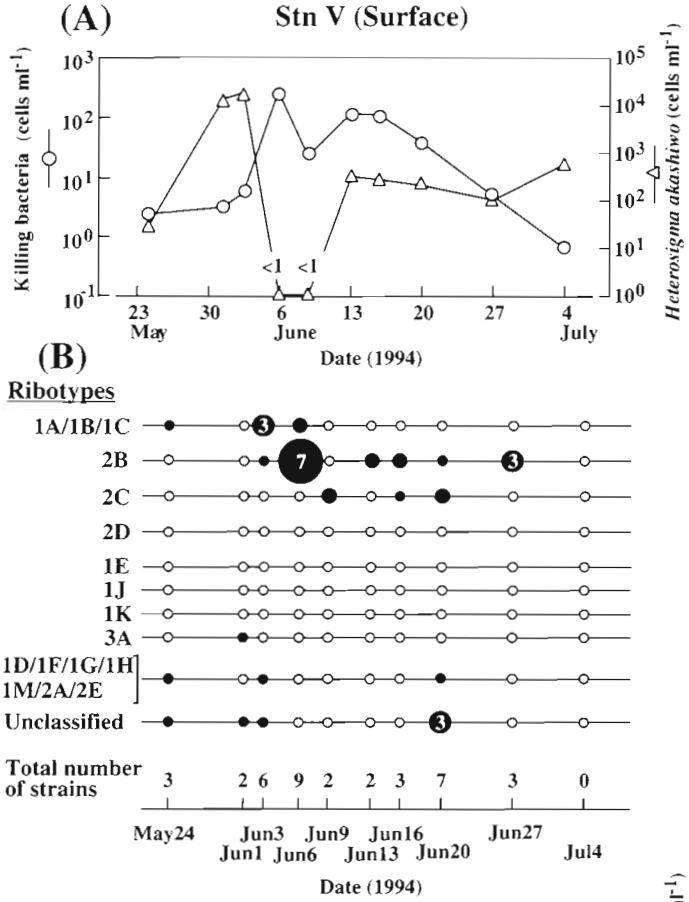

Fig. 1. (A) Abundance of *Heterosigma akashiwo* and HAKB in the surface seawater at Stn V in Hiroshima Bay in 1994 and (B) bacterial ribotype distribution among HAKB populations isolated on each sampling occasion. The abundance of HAKB was determined by the MPN method (Kim et al. 1998). The ribotypes were determined using restriction fragment length polymorphism of the 16S rRNA gene PCR amplified from HAKB isolates. Number of strains belonging to each ribotype is represented by the size of the circles as follows: (o) 0 strains, (•) 1 strain, (●) 2 strains, (❸–❼), 3–7 strains. 'Unclassified' bacteria for which ribotypes could not be determined

Algal cell densities greater than 10^4 cells ml^{-1} occurred in the surface water at Stn V during blooms of *Heterosigma akashiwo* from June 1 through June 5, 1994 (Kim et al. 1998) (Fig. 1A). The bloom terminated suddenly and *H. akashiwo* cells disappeared at the surface on June 6. At that time the MPN of HAKB rapidly increased 50-fold from 5.4 cells ml^{-1} on June 3 to 2.6×10^2 cells ml^{-1} on June 6 in 1994. The abundance of HAKB in the bottom water was more than 7.1×10^1 cells ml^{-1} during the gradual decrease of *H. akashiwo* after June 6, 1994 (Fig. 2A). At Stn M, an increase of HAKB abundance was observed during the disintegration period in 1994, similar to as at Stn V (Figs. 3A & 4A).

Fig. 1B shows the changing ribotype distribution (genetic diversity) of HAKB populations during the development and decay process of the *Heterosigma akashiwo* bloom that occurred in the surface water at Stn V in 1994. Ribotype 2B was largely dominant in the HAKB population on June 6, when the *H. akashiwo* bloom was terminated coincident with the rapid increase in HAKB abundance. Afterwards, HAKB populations in the surface consisted mainly of ribotype 2B and 2C until June 27, 1994. Ribotypes 2B, 2C and 2D were dominant throughout the collapse of the *H. akashiwo* bloom in the surface at Stn M (Fig. 3B). Ribotypes 1A, 1B and 1C, however, dominated the HAKB populations at the surface at both Stns V and M before *H. akashiwo* blooms were

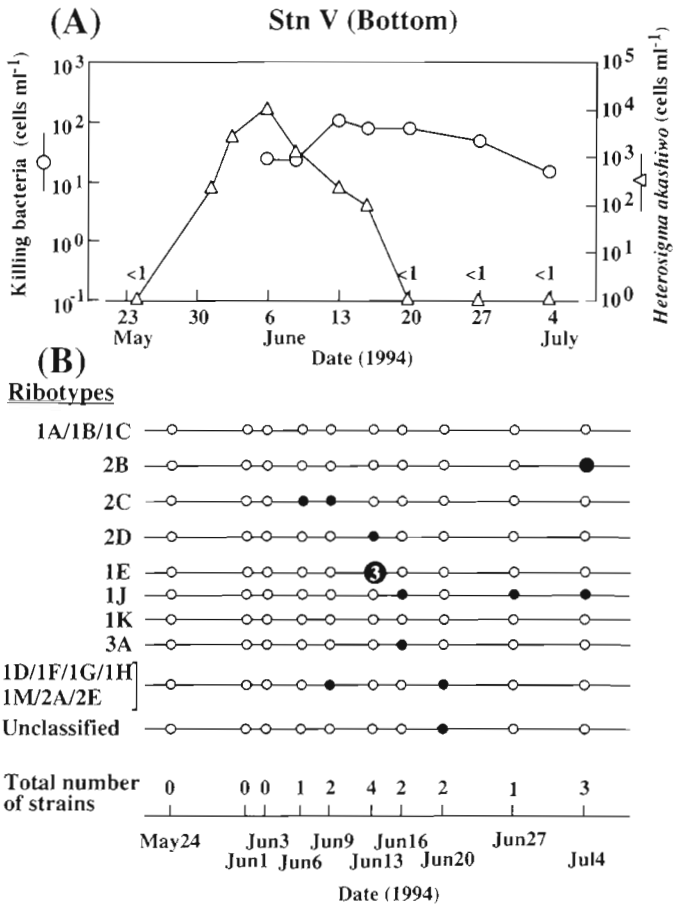

Fig. 2. (A) Abundance of *Heterosigma akashiwo* and HAKB in the bottom seawater at Stn V in Hiroshima Bay in 1994 and (B) bacterial ribotype distribution among HAKB populations isolated on each sampling occasion. Refer to the legend of Fig. 1 for explanation of symbols in (B)

Table 4. Number of HAKB strains classified into ribotypes. The HAKB strains were isolated in surface and bottom water at Stns V and M in Hiroshima Bay in 1994. Ribotypes were estimated using restriction fragment length polymorphisms of the 16S rRNA gene from HAKB (see Table 3)

	Ribotype																	Total
	1A	1B	1C	1D	1E	1F	1G	1H	1J	1K	1M	2A	2B	2C	2D	2E	3A	
Stn V																		
Surface	5	1	0	0	0	1	0	0	0	0	1	1	16	5	0	0	1	31
(%)	(16.1)	(3.2)	(0.0)	(0.0)	(0.0)	(3.2)	(0.0)	(0.0)	(0.0)	(0.0)	(3.2)	(3.2)	(51.6)	(16.1)	(0.0)	(0.0)	(3.2)	(100.0)
Bottom	0	0	0	0	3	0	0	1	3	0	0	0	2	2	1	1	1	14
(%)	(0.0)	(0.0)	(0.0)	(0.0)	(21.4)	(0.0)	(0.0)	(7.1)	(21.4)	(0.0)	(0.0)	(0.0)	(14.3)	(14.3)	(7.1)	(7.1)	(7.1)	(100.0)
Stn M																		
Surface	5	0	1	1	0	0	1	0	0	1	0	0	6	8	5	0	0	28
(%)	(17.9)	(0.0)	(3.6)	(3.6)	(0.0)	(0.0)	(3.6)	(0.0)	(0.0)	(3.6)	(0.0)	(0.0)	(21.4)	(28.6)	(17.9)	(0.0)	(0.0)	(100.0)
Bottom	0	0	0	0	1	0	0	0	2	1	0	0	2	6	0	0	0	12
(%)	(0.0)	(0.0)	(0.0)	(0.0)	(8.3)	(0.0)	(0.0)	(0.0)	(16.7)	(8.3)	(0.0)	(0.0)	(16.7)	(50.0)	(0.0)	(0.0)	(0.0)	(100.0)
Total	10	1	1	1	4	1	1	1	5	2	1	1	26	21	6	1	2	85
(%)	(11.8)	(1.2)	(1.2)	(1.2)	(4.7)	(1.2)	(1.2)	(1.2)	(5.9)	(2.4)	(1.2)	(1.2)	(30.6)	(24.7)	(7.1)	(1.2)	(2.4)	(100.0)

terminated, that is, 4 of 11 strains of HAKB isolated at Stn V up to June 3, 1994, and 6 of 11 strains isolated at Stn M upto June 6, 1994, were classified as ribotypes 1A, 1B and 1C (Figs. 1B & 3B). In the bottom water at both Stns V and M, HAKBs belonging to a comparatively wide variety of ribotypes were recovered throughout the sampling period.

From May 30 through July 10 in 1995, 102 and 25 strains of HAKB were isolated from the surface at Stn V and Stn M, respectively. The ribotypes of 47 strains from Stn V and 11 strains from Stn M were determined in the same manner in 1994. In 1995, the HAKB members of 2B, 2C and 2D accounted for 52 strains and corresponded to 89.7% of all 16S rDNA RFLPs analyzed (data not shown). The HAKB strains of ribotype 2C and 2D were especially dominant in the HAKB populations isolated at Stn V on June 30 (19 strains of 2C and 8 strains of 2D were observed in 29 strains of HAKB), when the MPN of HAKB dramatically increased and was followed by disintegration of the *Heterosigma akashiwo* bloom (Kim et al. 1998).

To strengthen the 16S rDNA RFLP classification, we determined the partial nucleotide sequences of 16S rRNA genes from several HAKB strains isolated on different sampling occasions in 1994 (Fig. 5). Strains GY21 and MC17 belonged to the ribotype 2B, and strains GY9 and MC8 belonged to the ribotype 1A. The nucleotide sequence of the 16S rDNA of GY21, which was isolated from the surface water collected at Stn V on June 6 in 1994, was almost identical (99.6% similarity) to that of MC17, which was isolated from the surface water at Stn M on June 9, 1994 (Fig. 5). The nucleotide sequences of strains GY9 and MC8, which belong to ribotype 1A,

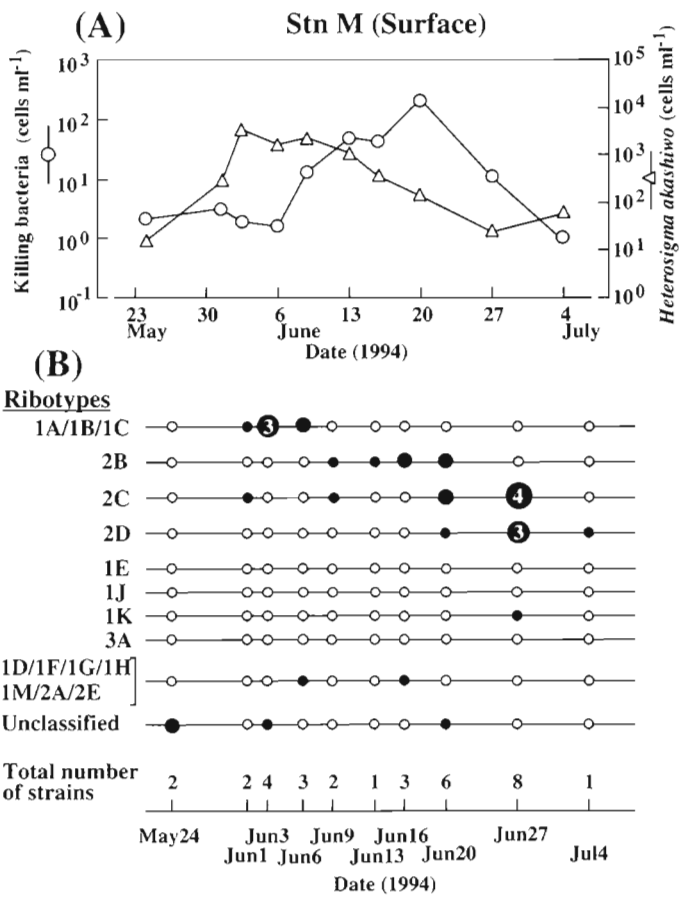

Fig. 3. (A) Abundance of *Heterosigma akashiwo* and HAKB in the surface seawater at Stn M in Hiroshima Bay in 1994 and (B) bacterial ribotype distribution among HAKB populations isolated on each sampling occasion. Refer to the legend of Fig. 1 for explanation of symbols in (B)

were also nearly identical (99.4% similarity). Partial (almost 500 nucleotides) sequences of 3 strains of ribotype 2C were also identical, showing more than 99% similarity (data not shown). Comparing the partial 16S rDNA nucleotide sequences between different ribotypes, the similarity value between 2B (GY21) and 1A (GY9) was significantly low (77.4%), although the similarity between 2B and 2C (GY27) was 94.7%.

A phylogenetic tree demonstrating the relationship among 3 ribotypes (1A, 2B and 2C) of HAKB strains (GY9, GY21 and GY27) and 15 species of well-known bacteria was constructed from the partial sequences of 16S rDNA (Fig. 6). The tree's topology was determined by the neighbor-joining method with *Prochloron* sp. as an outgroup. Five species of bacteria belonging to the *Cytophaga/Flexibacter* group and 10 species belonging to the γ-proteobacteria group were phylogenetically compared with 3 HAKBs on the tree. The HAKB strain GY9 (1A) formed a monophyletic lineage with some gliding bacteria belonging to the genera *Cytophaga* and *Flexibacter*. Two strains (GY21 and GY27) of ribotypes 2B and 2C formed a single cluster neighbor to *Alteromonas macleodii* and *Aeromonas* sp., which belong to the γ-proteobacteria group.

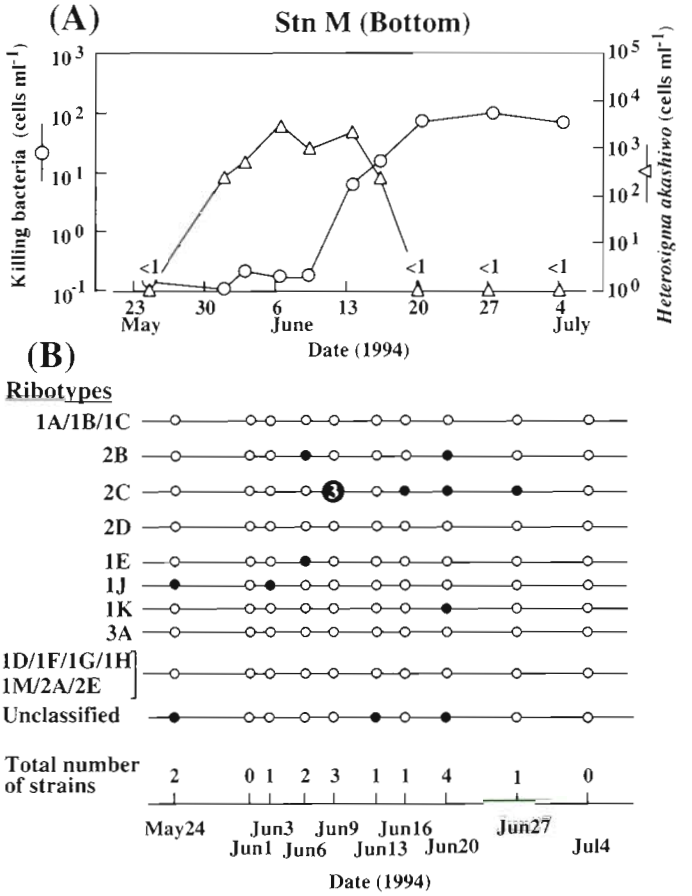

Fig. 4. (A) Abundance of *Heterosigma akashiwo* and HAKB in the bottom seawater at Stn M in Hiroshima Bay in 1994 and (B) bacterial ribotype distribution among HAKB populations isolated on each sampling occasion. Refer to the legend of Fig. 1 for explanation of symbols in (B)

DISCUSSION

In this study HAKB were defined as those which killed and/or lysed growing cells of *Heterosigma akashiwo* and multiplied by using the organic nutrients derived from the dead microalgal cells. In the procedure for isolation of HAKBs, their killing activities were confirmed by inoculating into the log phase culture of *H. akashiwo* at the initial bacterial density of ca 10^3 cells ml^{-1}. Most HAKB isolates completely destroyed the microalgal culture within 48 h but a few HAKB isolates took a week to destroy the microalgal culture. The cell densities of all HAKBs after the destruction of the microalgal culture were more than 10^7 cells ml^{-1}, although SWM3, the culture medium for *H. akashiwo*, contained no organic nutrients. Our results indicate that all HAKB strains in this study killed *H. akashiwo* and utilized the dead algal cells for their multiplication.

RFLP analysis of PCR-amplified DNA fragments of the 16S rRNA gene has been useful for studying genetic diversity and structure of microbial communities in natural environments (Moyer et al. 1994, Wilson et al. 1995, Suzuki et al. 1997). The RFLP analysis is normally easier and more economical than sequencing 16S rDNA; hence it is frequently used to describe the diversity of microbial populations and to identify the most abundant and hence important species. We used 16S rDNA RFLP analysis to investigate the diversity and dynamics of HAKB populations during *Heterosigma akashiwo* blooms and to determine which HAKB species were most abundant in the disintegration of *H. akashiwo* blooms.

RFLP analysis of 16S rRNA gene fragments, using the 5 endonucleases *Eco* RI, *Rsa* I, *Mbo* I, *Bst* UI and *Hha* I separately, revealed 17 ribotypes in HAKB populations in 1994 (Table 3). This result indicates that many species of bacteria inhabiting seawater have killing activities on *Heterosigma akashiwo*. Ribotypes 1A, 1B and 1C are probably a closely related bacterial group because they could be separated from each other by the treatment with *Mbo* I only. Members of ribotype 2D are probably close relatives of ribotypes 2B and 2C since the RFLP of 2D with 3 endonucleases overlapped those of 2B and 2C (Table 3).

Moyer et al. (1996) assessed the availabilities of 10 tetrameric restriction enzymes for 16S rDNA RFLP

```
          1                                                          60
GY21(2B)  AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAAC
MC17(2B)  .......... .......... .......... .......... .......... ..........
GY9(1A)   .......... .C........ GA........ A......... T......... ........GG
MC8(1A)   .......... .C........ GA........ A......... T......... ........GG
          61                                                         120
GY21(2B)  GGTAACAGGA ATTAGCTTGC TAATTTGCTG ACGAGTGGCG GACGGGTGAG TAATGCTTG-
MC17(2B)  .......... .......... .......... .......... .......... .........-
GY9(1A)   .......TTG G.-------- --.CCA.A.. ....CC.... C.......C. ...C..G.AT
MC8(1A)   .......TTG G.-------- --.CCA.A.. ....CC.... C.......C. ...C..G.AT
          121                                                        180
GY21(2B)  GGAACTTGCC TTTGCGAGGG GGACAACAGT TGGAAACGAC TGCTAATACC GCATAACGTC
MC17(2B)  .......... .......... .......... .......... .......... ..........
GY9(1A)   .A...C.A.. .AATAC.... ...T.G.CCA GA....TTTG GAT....... C...GGTACT
MC8(1A)   .A...C.A.. .AATAC.... ...T.G.CCA GA....TTTG GAT....... C...GGTACT
          181                                                        240
GY21(2B)  TTCGGACCAA ACGG------ GGCTTAGGCT CTGGCGCAAA GAGAGGCCCA AGTGAG--AT
MC17(2B)  .......... ....------ .......... .....C.... .......... ......--..
GY9(1A)   G.T.A.T.GC CT.ATTCAAT A.T.A.A.A. T.AT.GGN.T T...T.GT.. T.C.TTCT..
MC8(1A)   G.T.A.T.GC CT.ATTCAAT A.T.A.A.A. T.AT.GGT.T T...T.GT.. T.C.TTCT..
          241                                                        300
GY21(2B)  TAGCTAGTTG GCGAGGTAAA GGCTCACCAA GGCGACGATC TCTAGCTGTT CTGAGAGGAA
MC17(2B)  .......... .T........ .......... .......... .......... ..........
GY9(1A)   ...T...... .TA......C ....T..... .A.CG....A GA...GG.CC ........GG
MC8(1A)   ...T...... .TA......C ....T..... .A.CG....A GA...GG.CC ........GG
          301                                                        360
GY21(2B)  GATCAGCCAC ACTGGGACTG AGACACGGCC CAGACTCCTA CGGGAGGCAG CAGTGGGGAA
MC17(2B)  .......... .......... .......... .......... .......... ..........
GY9(1A)   ....CC.... .....T.... ........A. .......... .......... .....A....
MC8(1A)   ....CC.... .....T.... ........A. .......... .......... .....A....
          361                                                        420
GY21(2B)  TATTGCACAA TGGGGGAAAC CCTGATGCAG CCATGCCGCG TGTGTGAAGA AGGCCTT-CG
MC17(2B)  .......... .......... .......... .......... .......... .......-..
GY9(1A)   ...T.G.... ...A..C... T.....C... ....-..... ..AAG..... CT...C.AT.
MC8(1A)   ...C.G.... ...A..C... T.....C... ....-..... ..AAG..... CT...C.AT.
          421                                                        480
GY21(2B)  GGTTGTAAAG CACCTTCAGT TGTGAGGAAG GGTTGTTGGT TAATACCCAA CAGCATTGAC
MC17(2B)  .......... .......... .......... .......... .......... ..........
GY9(1A)   .........C TT.T..T.-- .A-..... AAAC..-.A. ..-CGTGTN. TCAT-.....
MC8(1A)   .........C TT.T..T.-- .A-..... AAAC..-.A. ..-CGTGTN. TCAT-.....
          481                                                        533
GY21(2B)  GTTAGCAACA GAAGAAGCAC CGGCTAACTC CGTGCCAGCA GCCGCGGTAA TAC
MC17(2B)  .......... .......... .......... .......... .......... ...
GY9(1A)   .G..CTCTAC ...T...G.T .......... .......... .......... ...
MC8(1A)   .G..CTCTAC ...T...G.T .......... .......... .......... ...
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Fig. 5. Alignment of partial nucleotide sequences of 16S ribosomal RNA genes (16S rDNA) of several strains of HAKB (GY21, MC17, GY9 and MC8) isolated in 1994. The ribotype of each bacterial strain analyzed by restriction fragment length polymorphism of the 16S rRNA gene is given in parentheses. A dot indicates the identical nucleotide as that of GY21, and a dash indicates an introduced gap

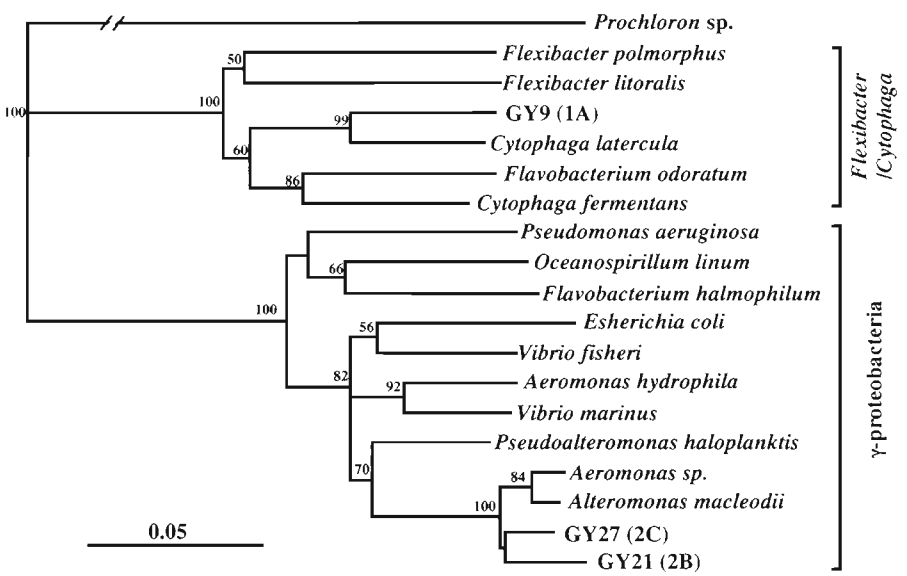

Fig. 6. Phylogenetic tree showing relationships of HAKB strains belonging to ribotypes 1A, 2B and 2C to representative bacteria. The tree was inferred from partial sequences of 16S rRNA genes by the neighbor-joining method. The number at each node represents the bootstrap value (percent) for that node, based on 1000 bootstrap replicates. An outgroup is represented by *Prochloron* sp. (accession number X63141 in GenBank). Bootstrap values below 50% are not shown

analysis using a computer simulation. They analyzed 16S rDNA RFLPs from 100 closely and distantly related bacteria in Genbank and recommended *Hha* I, *Bst* UI and *Rsa* I as the most useful enzymes for a tentative classification of bacteria. They also suggested that treatment with more than 3 different tetrameric restriction enzymes could theoretically distinguish between closely related bacteria (Moyer et al. 1996). Moreover, we determined the partial sequences of 16S rRNA genes from some representatives of HAKB to verify the ribotyping by RFLP. The 533-bp nucleotides sequences of the 16S rRNA genes were almost identical between strains of the same ribotype isolated at different sampling times (Fig. 5). These results strongly support the fact that the ribotyping by the 5 endonucleases used in this study was sufficiently powerful to resolve the population structure and dynamics of the HAKB.

At Stn V in Hiroshima Bay in 1994 and 1995, *Heterosigma akashiwo* blooms occurred at the beginning of June and at the end of June, respectively (Kim et al. 1998). In the both cases, the bloom at the surface dramatically disintegrated within a few days after the climax. Use of the MPN method with an axenic *H. akashiwo* culture demonstrated the rapid increase in the abundance of HAKB during the dramatic termination of the blooms. Hundreds of HAKB strains were isolated at the same time from the MPN cultures containing HAKB. In 1994, strains of ribotypes 2B, 2C and 2D were dominant in HAKB populations during the disintegration period of the *H. akashiwo* bloom. Also, in 1995, 93% of HAKB strains (27 of 29) isolated at the end of the *H. akashiwo* bloom at Stn V (June 30) belonged to ribotypes 2C and 2D. These results strongly suggested that the HAKB of ribotypes 2B, 2C and 2D, which are closely related phylogenetically, played a significant role in the dramatic termination of *H. akashiwo* blooms in Hiroshima Bay.

Ribotype 1A was the third most abundant group among the HAKB populations, but the members of this group were isolated mainly before the blooming of *Heterosigma akashiwo*, when the MPN of HAKB was not especially high (Figs. 1 & 3). This result might not mean that the HAKBs of 1A disappeared during the disintegration periods of *H. akashiwo* blooms, but they might become a minority among HAKB populations during these periods. In any case, 1A may not be the primary agent causing the termination of *H. akashiwo* blooms.

Several HAKB strains which belong to ribotypes 1E, 1H, 1J, 2D and 2E were isolated only from the bottom water (seawater samples 1 m above the bottom); however, in contrast, the HAKB strains of ribotype 1A were not isolated from the bottom water at both Stns V and M. In Hiroshima Bay, the peak number of *Heterosigma akashiwo* in the bottom water occurred just after the climax of the algal blooms in the surface water, and most *H. akashiwo* cells in the bottom water were swimming less actively than in the surface water. We suppose these less active algal cells would soon be killed because microscopic observations have shown that *H. akashiwo* cells stop swimming and then are killed by HAKB. Therefore the physiological state of target algal cells possibly effects the HAKB population structure, although no evidence for this is available.

A phylogenetic tree (Fig. 6) inferred from the partial 16S rRNA gene sequences of some HAKB strains revealed a relatively close affiliation of ribotype 1A with the *Cytophaga/Flexibacter* group. In contrast, 2B and 2C clearly belong to the γ-proteobacterial group, which includes many marine bacteria. However, the values of similarity between these ribotypes and their closest relatives in the GenBank, EMBL and DDBJ databases were not very high (less than 92%), and, consequently, the HAKB strains of 1A, 2B and 2C seem to be newly isolated marine bacteria.

Several strains of bacteria algicidal to marine diatoms, raphidophycean flagellates and dinoflagellates have been isolated from the nearshore seawater of Japan (Sakata 1990, Imai et al. 1991, 1993, 1995, Fukami et al. 1992, Mitsutani et al. 1992, Yoshinaga et al. 1995a, b, 1997). These algicidal marine bacteria can generally be divided into 2 groups by their algicidal mechanisms; the first group directly attacks and lyses target microalgae after cell-to-cell attachment, and the second produces and excretes algicidal substances to kill microalgae, killing them without direct attachment to the microalgal cells (Ishida et al. 1997). It is well known that gliding algicidal bacteria, including *Cytophaga*, generally attach directly to microalgal cells and lyse them. In contrast, most algicidal bacteria belonging to the genera *Vibrio*, *Flavobacterium*, *Alteromonas* and *Pseudomonas*, which belong to the γ-proteobacterial group, are thought to kill microalgae by means of algicidal substances. Microscopic observations and experiments using a diffusion chamber cultivation system (Yoshinaga et al. 1995a) suggest that the HAKB isolates of 2B and 2C do not need to directly attach to a microalgal cell surface to kill *Heterosigma akashiwo*, although strains of 1A do (I. Yoshinaga et al. unpubl.). The phylogenetic analysis is consistent with these observations of the mechanisms by which HAKB kill.

The newly improved MPN method (Kim et al. 1998), using an axenic culture of *Heterosigma akashiwo* as an MPN medium, and the 16S rDNA RFLP analysis suggested that the HAKB of ribotypes 2B, 2C and 2D regulate the rapid termination of *H. akashiwo* blooms. It would be interesting to utilize DNA probes, which recognize members of the 2B, 2C and 2D ribotypes, to obtain information about the dynamics of HAKB popu-

lations during blooms in natural environments (Amann et al. 1995, Lanoil & Giovannoni 1997).

Furthermore, the HAKB strains may be useful agents for the prevention and extermination of *Heterosigma akashiwo* blooms, which frequently damage aquaculture and nearshore fisheries. More detailed studies on the mechanisms by which HAKB kill and the ecology of HAKB populations are needed.

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LITERATURE CITED

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Baker KH, Herson DS (1978) Interactions between the diatom *Thallasiosira pseudonanna* and an associated pseudomonad in a mariculture system. Appl Environ Microbiol 35:791–796

Britschgi T, Giovannoni SJ (1991) Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. Appl Environ Microbiol 57: 1707–1713

Daft MJ, McCord SB, Stewart WDP (1975) Ecological studies on algal-lysing bacteria in fresh waters. Freshwat Biol 5: 577–596

Delong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci USA 89:5685–5689

Fukami K, Yuzawa A, Nishijima T, Hata Y (1992) Isolation and properties of a bacterium inhibiting the growth of *Gymnodinium nagasakiense*. Nippon Suisan Gakkaishi 58: 1073–1077

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345:63–65

Giovannoni SJ, Mullins TD, Field KG (1995) Microbial diversity in oceanic systems: rRNA approaches to the study of unculturable microbes. In: Joint I (ed) Molecular ecology of aquatic microbes. Springer, Berlin, p 217–248

Imai I, Ishida Y, Hata Y (1993) Killing of marine phytoplankton by a gliding bacterium *Cytophaga* sp., isolated from the coastal sea of Japan. Mar Biol 116:527–532

Imai I, Ishida Y, Sakaguchi K, Hata Y (1995) Algicidal marine bacteria isolated from northern Hiroshima Bay, Japan. Fish Sci 61:624–632

Imai I, Ishida Y, Sawayama S, Hata Y (1991) Isolation of a marine gliding bacterium that kills *Chattonella antiqua* (Raphidophyceae). Nippon Suisan Gakkaishi 57:1409

Imai I, Kim MC, Nagasaki K, Itakura S, Ishida Y (1998) Detection and enumeration of killer microorganisms against harmful phytoplankton in the coastal sea. Plankton Biol Ecol 45:15–25

Ishida Y, Yoshinaga I, Kim MC, Uchida A (1997) Possibility of bacterial control of harmful algal blooms. In: Martius MT, Sato MIZ, Tiedje JM, Hagler LCN, Dobereiner J, Sanchez PS (eds) Progress in marine ecology (Proc 7th Int Symp Microb Ecol). Brazilian Society for Microbiology, Santos, p 495–500

Itoh K, Imai I (1987) Raphido so (Raphidophyceae). In: Japan Fisheries Resource Conservation Association (ed) A guide for studies of red tide organisms. Shuwa, Tokyo, p 122–130 (in Japanese)

Kim MC, Yoshinaga I, Imai I, Nagasaki K, Itakura S, Ishida Y (1998) A close relationship between algicidal bacteria and termination of *Heterosigma akashiwo* (Raphidophyceae) blooms in Hiroshima Bay, Japan. Mar Ecol Prog Ser 170:25–32

Lanoil B, Giovannoni SJ (1997) Identification of bacterial cells by chromosomal painting. Appl Environ Microbiol 63: 1118–1123

Mitsutani A, Takesue K, Kirita M, Ishida Y (1992) Lysis of *Skeletonema costatum* by *Cytophaga* sp. isolated from the coastal water of the Ariake Sea. Nippon Suisan Gakkaishi 58:2158–2167

Mitsutani A, Uchida A, Ishida Y (1987) Occurrence of blue-green algae and algal lytic bacteria in Lake Biwa. Bull Jpn Soc Microb Ecol 2:21–28

Moyer CL, Dobbs FC, Karl DM (1994) Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. Appl Environ Microbiol 60:871–879

Moyer CL, James M, Tiedje M, Dobbs FC, Karl DM (1996) A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficiency of selected tetrameric restriction enzymes for studies of microbial diversity in nature. Appl Environ Microbiol 62:2501–2507

Mullins TD, Britschgi TB, Krest RL, Giovannoni SJ (1995) Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. Limnol Oceanogr 40:148–158

Sakata T (1990) Occurrence of marine *Saprospira* sp. possessing algicidal activity for diatoms. Nippon Suisan Gakkaishi 56:1165

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shilo M (1970) Lysis of blue-green algae by Myxobacter. J Bacteriol 104:453–461

Stewart JR, Brown RM (1969) *Cytophaga* that kills or lyses algae. Science 164:1523–1524

Stewart WDP, Daft MJ (1976) Algal lysing agents of freshwater habitats. In: Skinner FA, Carr JG (eds) Microbiology in agriculture, fisheries and food. Academic Press, London, p 63–90

Suzuki MT, Rappe MS, Haimberger ZW, Winfield H, Adair N, Strobel J, Giovannoni SJ (1997) Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl Environ Microbiol 63: 983–989

Wilson MJ, Wade WG, Weightman AJ (1995) Restriction fragment length polymorphism analysis of PCR-amplified 16S ribosomal DNA of human *Capnocytphaga*. J Appl Bacteriol 78:394–401

Woese CR (1987) Bacterial evolution. Microbiol Rev 51. 221–271

Yamamoto Y (1981) Observation on the occurrences of microbial agents which cause lysis of blue-green algae in Lake Kasumigaura. Jpn J Limnol 42:20–27

Yamamoto Y, Nishizawa S, Kuroda N, Sakamoto M (1993) Occurrence of heterotrophic bacteria causing lysis of cyanobacteria in a eutrophic lake. Jpn J Phycol 41. 215–220

Yamamoto Y, Suzuki K (1990) Distribution and algal-lysing activity of fruiting *Myxobacteria* in Lake Suwa. J Phycol 26:45–51

Yoshinaga I, Kawai T, Ishida Y (1995a) Lysis of *Gymnodinium nagasakiense* by marine bacteria. In: Lassus P, Arzul G, Erard E, Gentien P, Marcaillou C (eds) Harmful marine algal blooms. Lavoisier Publishing, Paris, p 687–692

Yoshinaga I, Kawai T, Ishida Y (1997) Analysis of algicidal ranges of the bacteria killing the marine dinoflagellate, *Gymnodinium mikimotoi*, isolated from Tanabe Bay, Wakayama Pref Japan. Fish Sci 63:94–98

Yoshinaga I, Kawai T, Takeuchi T, Ishida Y (1995b) Distribution and fluctuation of bacteria inhibiting the growth of a marine red tide phytoplankton *Gymnodinium mikimotoi* in Tanabe Bay (Wakayama Pref Japan). Fish Sci 61:780–786

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