Ribotype Analysis of HAKB (*Heterosigma akashiwo*-Killing Bacteria) Strains Isolated during Termination Period of a *H. akashiwo* Bloom Occurred in Osaka Bay

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In recent years, many genera of marine bacteria which have algicidal effects on harmful algal bloom species of dinoflagellates, raphidophycean flagellates and diatoms have been isolated from near shore seawater of Japan and Australia.^{1–7)} In most of these cases, the algicidal bacteria were isolated at the end of algal blooms, and, therefore, the algicidal bacteria seem to be one of the agents which regulate and cause disintegration of algal blooms.⁸⁾

During *Heterosigma akashiwo* (Raphidophyceae) blooms occurred in Hiroshima Bay, Japan, in 1994 and 1995, we have enumerated and isolated *H. akashiwo*-killing bacteria (HAKB).⁹⁾ As a result in both years, abundance of HAKB increased at or just after the peak of *H. akashiwo* blooms and followed by rapid termination of the blooms. After restriction fragments length polymorphism (RFLP) analysis of 16S ribosomal RNA genes from the HAKB isolates, a total of 223 HAKB isolates were divided into 17 ribotypes.¹⁰⁾ Three ribotypes which were designated as ribotypes 2B, 2C and 2D were dominantly isolated during the termination periods of *H. akashiwo* blooms in both years, and they were considered to be novel species by the result of phylogenetic analysis with their 16S rRNA gene sequences.¹⁰⁾

In the present note, we describe the three ribotypes of HAKBs, 2B, 2C and 2D, that were also isolated abundantly during a *H. akashiwo* bloom occurred in Osaka Bay in 1997, as well as in Hiroshima Bay.

Seawater samples were collected in surface at 2 stations situated in Osaka Bay on May 26, June 3, June 9, June 16, and June 23 in 1997*⁴. Abundance of HAKB of each seawater sample was determined by using the MPN method with an axenic culture of *H. akashiwo* (893).⁹⁾ The seawater sample was filtered through a nucleopore filter (0.8 μ m of pore size) and diluted serially with autoclaved seawater. A portion of each diluted sample was inoculated into log phase cultures of *H. akashiwo* in 48-wells microtiter plates (Costar Co.). After incubation at 20°C under light, the survival of *H. akashiwo* cells in each well was assessed with an inverted microscope. MPN of HAKB was calculated from the number of wells in which more than 99% of *H. akashiwo* cells were destroyed, by using a microcomputer program.¹¹⁾ MPN of *Chattonella antiqua*-killing bacteria (CAKB) was also determined by the same

manner using an axenic culture of *C. antiqua* (NIES-1, Raphidophyceae).

Some strains of HAKB were isolated from the microtiter plate's wells in which the *H. akashiwo* cells were destroyed. Selecting the wells at as high a dilution factor as possible to isolate dominant species among HAKB populations, subsamples were spread onto a ST10⁻¹ agar plate.¹⁰⁾ Only one bacterial strain was isolated from one well. Eighteen HAKB strains were isolated and kept in the ST10⁻¹ semi-solid culture (0.3% w/v of agar) at 20°C.

To determine the ribotypes of HAKB isolates, restriction fragments length polymorphism (RFLP) of their 16S rRNA genes were analyzed as the same protocol which we reported previously.¹⁰⁾ Each PCR-amplified DNA fragment was purified and was digested with one hexameric endonuclease (*Eco* RI) and four tetrameric endonuclease (*Rsa* I, *Mbo* I, *Bst* UI and *Hha* I), individually, at 37°C for 4 h. The resulting restriction fragments were separated by 1.5% agarose gel electrophoresis. Some of the 16S rRNA gene fragments of HAKBs were cloned, and partial nucleotide sequences (almost 500 bp from the 5' end) of them were determined.¹⁰⁾

In 1997, abundance of *H. akashiwo* increased gradually in the end of May and bloomed on June 3 at the cell density of 3.4×10^4 cells/ml at Stn 13 and of 1.6×10^4 cells/ml at Stn 19, resulting in a red tide. Abundance of *H. akashiwo* decreased suddenly and the red tide disappeared on June 9, and no *H. akashiwo* cells were observed on June 16 at both sampling stations (Table 1). Abundance of HAKB determined by the MPN method increased at both stations after June 3 and accounted for 4.7×10^1 cells/ml at Stn. 13 and 1.0×10^2 at Stn. 19 on June 9 when the *H. akashiwo* cells diminished. On the other hand, abundance of CAKB did not increase during this period and ranged at a low MPN value under detectable. We observed the increases of HAKB abundance accompanied with the disintegrations of *H. akashiwo* blooms also in Hiroshima Bay in 1994 and 1995, and so that, it might be a common phenomenon, not be a limited case in Osaka Bay, that algicidal bacterial population dynamics influence the sudden termination of *H. akashiwo* blooms.⁹⁾

Eighteen strains of HAKB were classified by RFLP analysis of their 16S rRNA gene. As a result, they were divided

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Table 1. Abundance of *Heterosigma akashiwo*, *H. akashiwo*-killing bacteria (HAKB) and *Chattonella antiqua*-killing bacteria (CAKB) in surface seawater at Stn. 13 and Stn. 19 in Osaka Bay in 1997, and ribotypes of HAKB strains isolated from each sampling occasions

	<i>H. akashiwo</i> (cells / ml)	HAKB (MPN / ml)	CAKB (MPN / ml)	HAKB strains (ribotypes)
Stn. 13				
May 26	7.6×10^2	—	—	
June 3	3.4×10^4	8.4×10^0	$< 10^{-2}$	DH1 (2B), DH2 (2X)
June 9	1.2×10^2	4.7×10^1	$< 10^{-2}$	DH4 (2B), DH5 (2C), DH6(2C), DH7 (2C)
June 16	< 1	4.9×10^0	$< 10^{-2}$	DH9 (2X), DH11 (2B)
June 23	1.5×10^1	$< 10^{-2}$	$< 10^{-2}$	DH12 (2B)
Stn. 19				
May 26	1.3×10^3	—	—	
June 3	1.6×10^4	1.2×10^1	$< 10^{-2}$	OK1 (2C), OK2 (2C), OK3 (2D), OK4 (3Y)
June 9	2.3×10^2	1.0×10^2	$< 10^{-2}$	OK5 (2D), OK6 (2C), OK7 (2C)
June 16	< 1	4.9×10^1	$< 10^{-2}$	OK8 (2C)
June 23	< 1	$< 10^{-2}$	1.3×10^0	OK9 (2X)

Number of HAKB and CAKB were counted by the most probable number (MPN) method.⁹⁾ The ribotypes were determined from the results of restriction fragment length polymorphism (RFLP) analysis of 16S ribosomal RNA genes after digesting with *Eco* RI, *Rsa* I, *Mbo* I, *Bst* UI and *Hha* I. —: not examined.

Table 2. Restriction fragment length polymorphisms of 16S rRNA gene (16S rDNA-REL) of *H. akashiwo*-killing bacteria (HAKB) isolated in Osaka Bay in 1997, after digesting individually with *Eco* RI, *Rsa* I, *Mbo* I, *Bst* UI and *Hha* I

Restriction enzyme	Estimated size of 16S rDNA fragment (bp)	Ribotypes in HAKB				
		2B (4)*	2C (8)*	2D (2)*	2X (3)*	3Y (1)*
<i>Eco</i> RI	850	+	+	+	+	+
	650	+	+	+	+	—
	600	—	—	—	—	+
<i>Rsa</i> I	900	+	+	+	+	—
	500	—	+	+	—	+
	450	—	—	—	—	+
	350	+	—	—	+	—
	150	—	—	—	+	—
	≤ 100	+	—	—	—	+
<i>Mbo</i> I	1200	—	+	—	—	—
	1100	—	—	—	—	+
	800	+	—	+	—	—
	500	—	—	—	+	—
	450	—	—	—	+	—
	400	+	—	+	—	—
	300	—	+	—	—	—
	250	+	—	+	+	+
<i>Bst</i> UI	150	—	—	—	+	+
	525	+	+	—	—	+
	500	—	—	+	—	—
	400	—	—	—	+	—
	375	+	—	—	—	+
	350	+	+	+	—	+
	300	—	—	+	+	—
	200	—	+	—	—	—
<i>Hha</i> I	≤ 100	+	+	+	+	+
	600	—	—	—	+	+
	400	+	+	+	+	—
	350	+	+	+	—	+
	300	+	+	+	—	—
	250	—	—	—	+	—
	225	+	+	+	—	—
	200	—	—	—	+	+
	150	—	—	—	—	+
	≤ 100	+	+	+	—	—

*The number of bacterial strains examined.

into 5 ribotypes, 2B, 2C, 2D, 2X and 3Y (Table 2). The ribotypes 2B, 2C and 2D, which were dominant ribotypes in the HAKB population during the termination periods of *H. akashiwo* blooms occurred in Hiroshima Bay in 1994 and 1995,¹⁰⁾ amounted to 77.8% (14 of 18 strains) of HAKB population in Osaka Bay (Table 1). Especially on June 9, when abundance of HAKB increased, the ribotype 2C was dominantly recovered (Table 1). Partial nucleotide

sequences of 16S rRNA gene of HAKB strains DH4, DH5 and OK5 were almost identical (more than 99.6% homology) to those of the HAKB isolates from Hiroshima Bay which ribotypes were 2B, 2C and 2D, respectively (Data not shown). On the other hand, ribotypes 2X and 3Y were not appeared and detected in Hiroshima Bay and they may be unique bacteria in Osaka Bay, and the 14 ribotypes except 2B, 2C and 2D detected in Hiroshima Bay could not be recovered in Osaka Bay.¹⁰⁾ This result suggests that, although indigenous HAKB species exist in Osaka Bay and Hiroshima Bay, respectively, ribotypes 2B, 2C and 2D generally exist and might play a role on a termination of *H. akashiwo* red tides in both areas.

Although Osaka bay is separated from Hiroshima Bay by about 300 km, the bays are situated at the ends of Seto Inland Sea and therefore algicidal bacteria of ribotypes 2B, 2C and 2D can transfer and exchange between the areas. It would be interesting to investigate whether the same species of algicidal bacteria are recovered or not at the termination periods of *H. akashiwo* blooms occurred out of Seto Inland Sea in the western area of Japan, for example Kagoshima Bay and Uranouchi Bay etc.

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