The Journal of Microbiology, June 2008, p. 274-282 Vol. 46, No. 3
DOI 10.1007/s12275-007-0238-9

Monitoring of Algicidal Bacterium, *Alteromonas* sp. Strain A14 in its Application to Natural *Cochlodinium polykrikoides* Blooming Seawater Using Fluorescence *In Situ* Hybridization

Bo-Kyung Lee[1], Toshiya Katano[1], Shin-Ichi Kitamura[2,3], Myung-Joo Oh[2], and Myung-Soo Han[1]*

[1]*Department of Life Science, Hanyang University, Seoul 133-791, Republic of Korea*
[2]*Department of Aqualife Medicine, Chonnam National University, Chonnam 550-749, Republic of Korea*
[3]*Center for Marine Environmental Studies, Ehime University, Ehime 790-8577, Japan*

(Received December 4, 2007 / Accepted April 16, 2008)

The red tide of dinoflagellate, *Cochlodinium polykrikoides* has frequently occurred in coastal waters, causing severe damage to fisheries. In the present study, the algicidal bacterium *Alteromonas* sp. A14 isolated from the southern coast of Korea was applied to a red tide of *C. polykrikoides* in a laboratory experiment. In the experiment, the abundance of the strain A14 was monitored using fluorescence *in situ* hybridization. Inoculation of the A14 at a final cell density of 9.0×10^5 cells/ml caused a significant decrease in *C. polykrikoides* abundance from 1,830 to 700 cells/ml during 2 days, while abundances of harmless diatoms rapidly increased from 3 days. Abundances of both A14 and other bacteria increased to 1 day. After 1 day, with flagellate abundance increased, bacterial abundance decreased. Finally, algicidal bacterial abundance decreased to 3.5×10^4 cells/ml. In the biological control of harmful algal blooms, in addition to decrease in target algal abundance and not occurrence of other harmful blooms, decrease in abundance of utilized organism is also important. This study emphasizes the importance of monitoring the inoculated bacterium when applying bacterium to natural seawater.

***Keywords*:** algicidal bacteria, *Alteromonas*, *Cochlodinium polykrikoides*, harmful algal bloom (HAB), tyramide signal amplification-fluorescence *in situ* hybridization, biological control

Cochlodinium polykrikoides, which is one of the major harmful dinoflagellate species, cause fish mortalities by oxidative damage, such as the inactivation of gill transport-related enzyme activities, the fall in blood pO_2 and abnormal secretion of gill mucus (Kim *et al*., 1999, 2000). In the southern coast of Korea, blooms of *C. polykrikoides* have occurred almost every year from 1995 (Kim *et al*., 2007). Fisheries' losses by the bloom amounted to $60 million in 1995 (Kim *et al*., 2004). Their outbreaks appeared mainly in the temperate region of East Asia and the subtropical region of Central America, but it have increased worldwide (Kim *et al*., 2004; Lee and Lee, 2006). Therefore, the establishment of control of this harmful algal bloom (HAB) is urgently needed.

To mitigate harmful algal bloom several methods have been proposed such as killing algal cells by chemicals, absorption by clay particles, algal cell lysis by virus, bacteria or fungi (Anderson *et al*., 1997; Fukuyo *et al*., 2002). Among them, biological control using algicidal bacteria is one possible tool to remove harmful algal cells. In this technique, a specific species could be targeted. Up to the present, many bacteria have been isolated against harmful algal species, such as *Chattonella antique*, *Karenia mikimotoi*, *Heterocapsa circulatisquama*, *Gymnodinium catenatum*, and *Heterosigma akashiwo* (Imai *et al*., 1991, 1995; Kim *et al*., 1998; Lovejoy *et al*., 1998; Doucette *et al*., 1999; Nagasaki *et al*., 2000). In most studies, these algicidal bacteria were investigated with cultured algal strains to clarify the algicidal range, activity, and algicidal mode. However, field studies of application to natural blooming water are relatively limited.

Nagasaki *et al*. (2000) demonstrated that some bacteria prohibited the algicidal activity of *Cytophaga* sp. AA8-2, which has a killing effect on *Heterocapsa circularisquama.* Mayali and Doucette (2002) demonstrated that bacterial community in the cultures of *Karenia brevis* completely prohibited the algicidal activity of *Cytophaga* sp. strain 41-DBG2. Recently we demonstrated that grazing by flagellates on algicidal bacterium is another important factor in the expression of the activity in its application to natural water (Katano *et al*., 2006). These results imply that algicidal activity is possibly prohibited by natural microbial community. In addition, algal cell lysis by bacteria is generally noticeable when the bacterial abundance exceeds certain levels, such as $>10^6$ cells/ml (Mayali and Doucette, 2002). Therefore, monitoring of inoculated bacterial abundance is essential in its application to natural algal blooms.

Fluorescence *in situ* hybridization (FISH) has an advantage for the enumeration and observation of specific bacteria (Amann *et al*., 1990; Amann *et al*., 1995; Moter and Gobel, 2000). Pernthaler *et al*. (2002) introduced the tyramide signal amplification (TSA)-FISH that had enhanced fluorescence intensities and signal-to-background ratios compared to that

* To whom correspondence should be addressed.
(Tel) 82-2-2220-0956; (Fax) 82-2-2296-1741
(E-mail) hanms@hanyang.ac.kr

with monolabeled probes. Thus, TSA-FISH is a useful tool in the monitoring of specific algicidal bacterial abundance in natural seawaters.

In the present study, we developed a specific probe against an algicidal bacterial strain A14, which was isolated from the southern coast of Korea and possess algicidal activity against *C. polykrikoides*. We applied the strain A14 to the naturally occurring *C. plykrikoides* blooming seawater. In its application we monitored abundances of A14 using TSA-FISH. In addition to this, total bacteria, heterotrophic flagellates, phytoplankton were also monitored to examine the response of the microbial community. We found that the isolated bacterium caused a significant decrease in *C. polykrikoides* abundance. After 1 day, with decreasing of bacterial cell density, flagellate cell density increased. Finally, the abundance of algicidal bacterial decreased to 10^4 cell/ml. The present study emphasizes the importance of monitoring both the inoculated bacterium when applying bacterium to natural seawater.

Materials and Methods

Algal and bacterial cultures

Experimental algal cultures were incubated at the 20°C, 50 μmol photons/m^2/sec with 12:12 h light:dark cycle. Cultures, *Akashiwo sanguinea* (GnSg02, 03), *Gymnodinium catenatum* (GnCt-K01), *Prorocentrum minimum* (D-087), *Prorocentrum micans* (D-077), and *Heterosigma akashiwo* (NFHTS-AK-1), were obtained from Korea Ocean Research and Development Institute (KORDI) and were cultured in f/2 media without silicate (Guillard, 1975). *Cochlodinium polykrikoides* (CP-2001), *Heterocapsa triquetra* (HtTq-K01), *Nitzschia* sp., and *Skeletonema costatum* (SICs-K02) were kindly provided by South Sea Fisheries Research Institute. Former two algal species were cultured in f/2 media without silicate, and others were cultured in f/2 media (Guillard, 1975).

Bacterial cultures were incubated on a shaker at 70 rpm at 20°C with 10% Zobell media and maintained on 10% Zobell gar plates (Zobell, 1946; Lovejoy *et al*., 1998). *Alteromonas* sp. strain JC2043, used for FISH experiments, was kindly provided by Prof. Chun Jong-Sik from Seoul National University. This strain JC2043 was cultured with Marine Broth medium (Difco 2216). Bacterial strains A14, A18, and G20 were isolated from the southern coast of Korea.

Bacterial isolation

For the isolation of bacteria, a water sample was collected at Masan Bay on 30 September, and a sediment sample was collected at Yeosu on 1 October 2005. At first, we targeted *Heterosigma akashiwo*. The 1 g of sediment sample was diluted using 10 ml of f/2 media (Park *et al*., 1999) and the water sample was filtered using GF/C filter (Whatman, USA). Subsequently 0.2 ml of each diluted sample was inoculated to 2 ml of logarithmic phase cultures of *H. akashiwo* in sterile 24 well tissue culture plates. Samples in the 24 well tissue culture plates were incubated at 20°C, 50 μmol photons m^2/sec with 12:12 h light:dark condition and the growth of *H. akashiwo* was monitored using an inverted microscope (Axtovert 100, Germany). A portion was taken from the wells in which *H. akashiwo* cells had lysis, and was re-inoculated into a growing *H. akashiwo* culture. From the above wells, several bacteria were cultured by spreading them on 10% Zobell agar plates (Zobell, 1946; Lovejoy *et al*., 1998) at 20°C for 2 weeks under dark conditions. Each colony was isolated and transferred to 100 ml of liquid 10% Zobell medium. Bacterial cultures were incubated until the stationary phase (approximately 3∼5 days) on a shaker at 70 rpm at 20°C. The 8 ml of each bacterial culture was inoculated to the 50 ml test tube containing 16 ml of *H. akashiwo* (NFHTS-AK-1) cultures. Cultures were incubated at the 20°C, 50 μmol photons/m^2/sec with 12:12 h light:dark for 7 or 15 days. The algal cell lysis was monitored with *in vivo* fluorescence using a 10-AU fluorometer (Turner Designs). Following the above methods, three bacteria strains A14 (from Yeosu coastal sediment), A18 and G20 (from Masan coastal waters) were isolated.

Algicidal range of strain A14

To examine the algicidal range of isolate, the bacterium was tested by a co-culture experiment with 9 species of marine phytoplankton (Table 2). Each phytoplankton species was cultured in f/2 medium without silicate for dinophytes and raphidophytes or with silicate for bacillariophytes (Guillard, 1975). Bacterium was added in duplicate to 30 ml of logarithmic phase algal culture at a concentration of approximately 10^7 cells/ml in the final incubation volume. They were incubated at the 20∼25°C, 50 μmol photons/m^2/sec with 12:12 h light:dark during 4∼5 days. The growth of phytoplankton in tubes was measured by *in vivo* chlorophyll fluorescence using a 10-AU fluorometer (Turner Designs). The algicidal activity of strain A14 was calculated by the following equation:algicidal activity (%)=(1-T*t*/C*t*)×100, C*t*, algal cell density in control treatment; T*t,* algal cell density in added treatment; t, the inoculation time.

Relationship between algicidal activity against *C. polykrikoides* cultures and bacterial cell density

To test the algicidal activity against *C. polykrikoides* at the different cell densities of the strain A14, the bacterial culture was inoculated in triplicate to 30 ml of *C. polykrikoides* culture in logarithmic phase at final bacterial densities of 10^5, 10^6, and 10^7 cells/ml. These test tubes were incubated at 20°C, 50 μmol photons/m^2/sec with 12:12 h light:dark for two days. The algal abundance in tubes was measured by *in vivo* chlorophyll fluorescence using a 10-AU fluorometer (Turner Designs). The activity was calculated as mentioned above.

Sequencing of 16S rDNA

For identification of the isolated strains A14, A18, and G20 using 16S rDNA sequences, bacterial chromosomal DNA was extracted using DNeasy Tissue Kit (QIAGEN). The 16S rDNA was amplified by PCR with 27F; 5'-AGAGTTTGATCATGGCTCAG-3' and 1492R; 5'-GGTTACCTTGTTACGACTT-3' in a 50 μl reaction containing, 1× PCR buffer, 5 mmol/L $MgCl_2$, 10 pmol/L dNTP, 10 pmol/L of each primer and 2.5 units of *Taq* DNA polymerase (TaKaRa). PCR product was sequenced with 27F, 341F; 5'-CCTACGGGAGGCAGCAG-3', 790F; 5'-ATTAGATACCCTGGTAG-3', 926F; 5'-GGTTAAAACT(CT)AAA(GT)GAATTGACGG-3'

primers. Sequences were aligned with a CLUSTAL X program (Thompson *et al.,* 1997). A phylogenetic tree was built by the neighbor-joining method (Saitou and Nei, 1987). The bootstrap analysis with re-sampling 1,000 times was also obtained. Tree drawing was performed with the TREE VIEW program (R.D.P. Page, Division of Environmental and Evolutionary Biology, Institute of Biomedical and Life Sciences, University of Glasgow, UK).

Probe design to enumerate strain A14 cells

A specific probe (ALTERO14 probe) targeting strain A14 was newly designed using ARB program (http://www.arb-home.de/). The probe sequence (5'-CAA GCA CAT CCT GCT ACC GTT-3') was determined with the probe design function of the program using a database of rRNA gene sequences with more than 24,000 sequences (Table 1). Before designing the probe, we added 16S rRNA gene sequences of strain A14 and its closely related strains to the rRNA database downloaded from the web site. The probe specificity was confirmed using a nucleotide-nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/BLAST). Melting temperature (Tm) value for the ALTERO 14 probe was 66.1°C calculated according to Hugenholtz *et al*. (2001) using the nearest neighbor method.

Fluorescence *in situ* hybridization and optimum FISH condition to detect strain A14

Horseradish peroxidase (HRP) conjugated probes were purchased from ThermoHybaid (Germany). Briefly, to avoid cell loss, filter samples were dipped in 0.2% low-gelling-point agarose (Sigma, USA) and dried, subsequently dehydrated in 96% ethanol for 1 min and dried according to Pernthaler *et al*. (2002). For cell wall permeabilization, filters were incubated in a lysozyme solution (10 mg/ml in 0.05 mol/L EDTA, 0.1 mol/L Tris-HCl; pH 7.5) at 37°C for at least 30 min. The filters were cut, and subsequently stored in 1.5 ml microtubes at -20°C.

For hybridization, 200 μl of hybridization buffer [0.9 mol/L NaCl, 20 mmol/L Tris-HCl; pH 7.5, 10% dextran sulfate, 0.02% sodium dodecyl sulfate (SDS), 50% of formamide, 2% blocking reagent (Roche)] and 2 μl of HRP probe working solution (50 ng DNA/μl, ThermoHybaid, Germany) were pipetted onto the filter sections in microtubes. The filters in microtubes were incubated at 35°C for 2 h. The filters were removed from the hybridyzation mixture and then incubated in pre-warmed washing buffer [19 mmol/L NaCl, 5 mmol/L EDTA; pH 8.0, 20 mmol/L Tris-HCl; pH 7.5, 0.01% (w/v) SDS] according to Pernthaler *et al*. (2001) at 35°C for 10 min.

To equilibrate the probe-delivered HRP, sections were incubated in phosphate-buffered saline (PBS, pH 7.4) supplemented with 0.05% Triton X-100 for 15 min at room temperature. Composition of the PBS was followed by Biegala *et al*. (2003). The sections were dabbed onto a blotting paper and immediately transferred to a substrate mix containing one part of tyramide-fluorescein (TSA, NEN Life Science, USA), 50 parts of amplification diluents and 50 parts of 10% dextran sulfate. Filter sections were incubated at room temperature in the dark for 30 min and placed onto a blotting paper to remove excess tyramide-fluorescein, and washed for 15 min in PBS supplemented with 0.05% Triton X-100. Filter sections were subsequently washed with DW and 96% ethanol for 1 min. After filter sections were air dried, filters were incubated in DAPI solution at a final concentration of 1 μg/ml in PBS (pH 7.4) for 5 min, and washed with DW, and mounted on a slide glass with immersion oil.

To test the specificity of the ALTERO14 probe, we used strains A14, A18, G20, and JC2043. There is a two-base mismatch between 16S rRNA of strain JC2043 and the ALTERO14 probe. We tested the ALTERO14 probe against these four strains under various formamide and probe concentrations. Two probes, EUB338; 5'- GCTGCCTCCCGTA GGAGT-3' (Amann *et al*., 1995) and NONEUB338; 5'-ACT CCTACGGGAGGCAGC-3' (Amann *et al*., 1995) were also used as positive and negative controls, respectively. To determine the optimum conditions for the hybridization, series of five formamide concentrations 40, 45, 50, 55, 60%. Composition of the hybridization buffer followed Pernthaler *et al*. (2001). Hybridization buffer containing 60% of the formamide was prepared by reducing the volume of 40% dextran sulfate solution.

All strains tested showed high fluorescence when hybridized with EUB338, and showed no fluorescence with NONEUB (data not shown). A14, hybridized with ALTERO14, showed fluorescence under all formamide concentrations tested (40~60%). Strain JC2043, hybridized with ALTERO14 probe, showed fluorescence under the 40 and 45% of formamide concentrations, but no fluorescence under 50~60%. Strain A18 and G20 have no fluorescence with ALTERO14 probe under all formamide concentrations. Therefore, we fixed 50% of formamide concentration to detect strain A14 using ALTERO14 probe.

Microcosm experiments

Sampling and experimental setting

A seawater sample was collected from the surface in the southern coast in Korea on 12 August 2006 when *C. polykrikoides* bloom occurred (http://www.nfrdi.re.kr./www06/home/

Table 1. Theoretical specificity of the probe ALTERO14. Target site of the probe is 64~84 in 16S rRNA position

Strain	Sequences (5'→3')	Accession number
Probe target	AACGGTAGCAGGATGTGCTTG	
Alteromonas sp. A14	----------	AB 295494
Alteromonas sp. JC2043	-------A-----A-------	AY 207503
Alteromonas sp. A18	-------A--TTTCTA-----	AB 295495
Glaciecola sp. G20	-------A--TTTCTA-----	AB 295496

ocean/redtide.php). Water temperature and salinity were, respectively, 24.9°C and 32.4 and cell density of *C. polykrikoides* was ca. 2×10^3 cells/ml. The sample was treated within 0.5~1.0 h after the collection. The 600 ml of seawater containing natural *C. polykrikoides* was prepared in 6 flasks (1 L) and isolate A14 was added in triplicate at a final concentration of ca. 10^6 cells/ml. Each flask was added nutrients at final concentrations of 60 μmol N/l, 4 μmol P/L for maintaining bloom condition. All flasks were incubated at 25°C on a 12:12 h light:dark cycle with a approximately 40~80 μmol photons/m^2/sec on the shaker table at 35 rpm for 6 days.

Chlorophyll *a* measurement

Samples for measurements of chlorophyll *a* (chl. *a*) concentrations were collected and filtered through a glass fiber filter (Whatman GF/F), and the filters were kept at -20°C in the dark. These pigments were extracted in 90% acetone and determined by the method of Jeffrey and Humpherey (1975) with a spectrophotometer (Hewlett Packard, Germany).

Phytoplankton cell counts

Phytoplankton samples were collected everyday and preserved with Lugol's solution at a final concentration of 1% and stored in the dark at 4°C until analysis. Cells of three dominant algae, *C. polykikoides*, *Chaetoceros* spp., and *S. costatum*, were counted with a light microscope (Axiolab, Zeiss, Germany) under 400× magnifications with a Neubauer hemocytometer.

Bacterial cell counts

Samples for bacterial counting were collected everyday and preserved with glutaraldehyde at a final concentration of 1% and stored at 4°C in the dark. A portion of the sample was filtered on a 0.2 μm polycarbonate filter (GTTP, Millipore, USA) pre-stained with Sudan Black. Bacterial cells were stained with DAPI method according to Porter and Feig (1980). Cells were enumerated with an epifluorescence microscope (Axioplan, Zeiss, Germany) under UV excitation at 1,000× magnification. At least 400 cells were counted for each sample.

Strain A14 cell counts

Samples for enumeration of the strain A14 with TSA-FISH were collected everyday and preserved with formaldehyde at a final concentration of 2% for 1~24 h at 4°C. For precise bacteria cell counting, samples were gently sonicated for 5~10 min with a washing type sonicator (55 W 35 kHz, UT-53N, Sharp, Japan) with ice. Portions of water samples were filtered onto white polycarbonate filters (GTTP, 0.2 μm in pore diameter, Millipore, USA), washed with 5 ml of DW, and stored in a -20°C freezer. Probe-positive cells after FISH were counted with an epifluorescence microscope (Axioplan, Zeiss, Germany and Nikon eclipse E800) under blue excitation, equipped with a 100 W mercury lamp. At least 400 DAPI positive cells were counted for each sample. For the counting of DAPI positive cells, UV excitation was used.

Flagellate cell counts

The samples for the counting of flagellates were collected on 0, 2, and 5 days and preserved with a glutaraldehyde at a final concentration of 1%. The samples were filtered onto 1.2 μm polycarbonate filters (GTTP, Millipore, USA) pre-stained with Sudan Black. Flagellate cells were stained by the primulin method (Caron, 1983). Cells were enumerated with an epifluorescence microscope (Axioplan, Zeiss, Germany) under UV excitation at 1,000× magnification. Generally, at least 100 cells were counted for each sample.

Statistical analysis

Two-way ANOVA test was carried out using Microsoft Excel v. X to test for differences between treatments (control and A14 added treatment) and among incubation time (days) on data about phytoplankton cell densities and chlorophyll *a* concentration in the microcosm experiment. Subsequently, Tukey's multiple comparison tests was conducted by hand with a discrimination level of $P<0.05$.

Results

Algicidal strain A14

A strain A14 showed algicidal activity on dinophycean strains, such as *Akashiwo sanguinea*, *Cochlodinium polykrikoides*, *Gymnodinium catenatum*, and *Heterocapsa triquetra* (Table 2). However, the growth of *Prorocentrum minimum* was stimulated by the addition of a strain A14. *Prorocentrum micans* was not killed by the addition. The killing activity of the stain A14 was not so strong on two strains of *Heterosigma akashiwo* and the algicidal activity on each strain was slightly different. Two diatom strains tested in the present study (*Nitzschia* sp. and *Skeletonema costatum*) were not killed by the addition of strain A14.

Table 2. The algicidal activity of *Alteromonas* sp. strain A14 against various marine algal strains. The activity was evaluated on 4 or 5 days after the inoculation

Strains	Algicidal activity (%)[a]
Dinophyceae	
Akashiwo sanguinea GnSg02	95.8±0.6
Akashiwo sanguinea GnSg03	97.3±0.1
Cochlodinium polykrikoides CP-2001	90.2±9.0
Gymnodinium catenatum GnCt-K01	74.3±6.8
Heterocapsa triquetra HtTq-K01	99.3±0.0
Prorocentrum minimum D-087	-18.8±4.7
Prorocentrum micans D-077	4.8±2.4
Raphidophyceae	
Heterosigma akashiwo NFHTS-AK-1	19.4±20.8
Heterosigma akashiwo CCMP1912	2.6±10.1
Bacillariophyceae	
Nitzschia sp.	3.0±3.6
Skeletonema costatum SICs-K02	0.5±0.5

[a] Algicidal activity (%)=(1-T*t*/C*t*)×100, C*t*, algal cell density in control treatment; T*t*, algal cell density in added treatment; t, the inoculation time.

The algicidal activity was detected at the final bacterial cell densities of 10^6 and 10^7 cells/ml (Fig. 1). When the final cell density was 10^7 cells/ml, *in vivo* fluorescence of *C. polykrikoides* was decreased by 91.46% within 2 days. The activity became low with decreasing inoculated bacteria cell densities. However, we found significant decrease in the fluorescence at the 10^6 cells/ml. At the 10^5 cells/ml, any algicidal activity was detected.

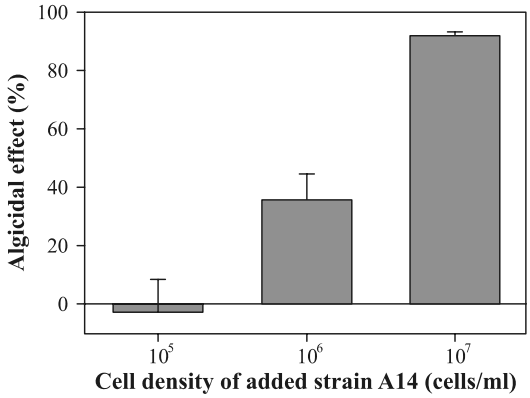

Fig. 1. Algicidal activity against *Cochlodinium polykrikoides* in cultures inoculated with different concentrations of algicidal strain A14.

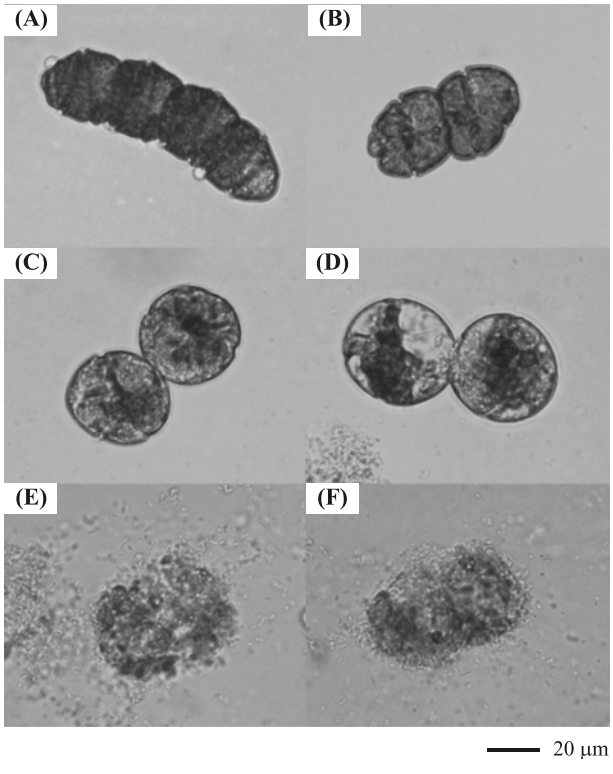

Fig. 2. Micrographs of *Cochlodinium polykrikoides* cells before the inoculation of strain A14 (A, B), still alive cells on 1 day after the inoculation of strain A14 (C, D), and killed cells on 1 day after the inoculation of strain A14 (E, F). The bar indicates 20 μm.

We confirmed the algal cell lysis by the addition of the strain A14 under the microscope (Fig. 2). After 1 day of the inoculation, we found both dead and still alive cells. However, morphology of the still alive cells apparently differed from cells before the addition. After 2 days, most cells were degraded.

The strain A14 (accession no. AB295494) was 98% similar to *Alteromonas marina* (AY881234) by 16S rDNA sequence, and was identified as the genus *Alteromonas*. This bacterium formed cream-colored colonies and circular form, convex elevation and entire margin. In other strains, A18 (accession no. AB 295495) and G20 (accession no. AB295496) were identified as the genera *Alteromonas* and *Glaciecola*, respectively.

Changes in phytoplankton abundance and composition

Cell density of *C. polykrikoides* in the control treatment was relatively stable up to 4 days, but decreased between 4 to 5 days (Fig. 3). In the A14 added treatment, cell density of *C. polykrikoides* decreased from 1,830 cells/ml to 700 cells/ml within 4 days. In the ANOVA test, interaction between treatment and time was not detected. Multiple comparison tests revealed that the inoculation of A14 induced significant difference (decrease) in the cell density ($P<0.05$, data not shown). The cell densities of *S. costatum* and *Chaetoceros* spp. in the A14 added treatment increased from 80 cells/ml to 14,000 cells/ml and from 50 cells/ml to 1,700 cells/ml, respectively. In the ANOVA test, interaction between treatment and time was detected in both *S. costatum* and *Chaetoceros* spp. Multiple comparison tests showed significant differences between A14 added and control treatments on 4 and 5 days in both *S. costatum* and *Chaetoceros* spp. (data not shown).

Chlorophyll *a* concentration in the control treatment increased until 1 day and then reduced gradually. In A14 added treatment, the concentration also increased until 1 day and reduced until 3 days and then steadily increased until the end of the incubation. ANOVA and subsequent multiple comparison tests revealed significant difference between 5 days and other days, but did not show any difference between A14 added and control treatments.

On the 0 day, *Nitzschia* spp., *Prorocentrum mimimum*, *P. triestnum*, *Dictyocha* spp., and *Protoperidinium* sp. were found in addition to *C. polykrikoides*, *Skeletonema* spp., and *Chaetoceros* sp. However, these algal cell densities were quite low (<140 cells/ml) during the incubation experiment in both control and added treatments.

Changes in cell densities of total bacteria, strain A14, and flagellates

Total bacterial abundance in the control treatment ranged between 2.0×10^6 cells/ml and 6.0×10^6 cells/ml (Fig. 4). The cell density was the highest on 1 day, and decreased to the same level as the 0 day. In A14 added treatment, total bacterial cell density ranged between 4.6×10^6 cells/ml and 1.4×10^7 cells/ml. All bacterial cells showed negative signal against ALTERO14 probe in the control treatment, indicating that the detected cells using the probe in A14 added treatment were cells of strain A14 (Fig. 5). Cell density of A14 increased from ca. 9.0×10^5 cells/ml on 0 day to 1.5×10^6

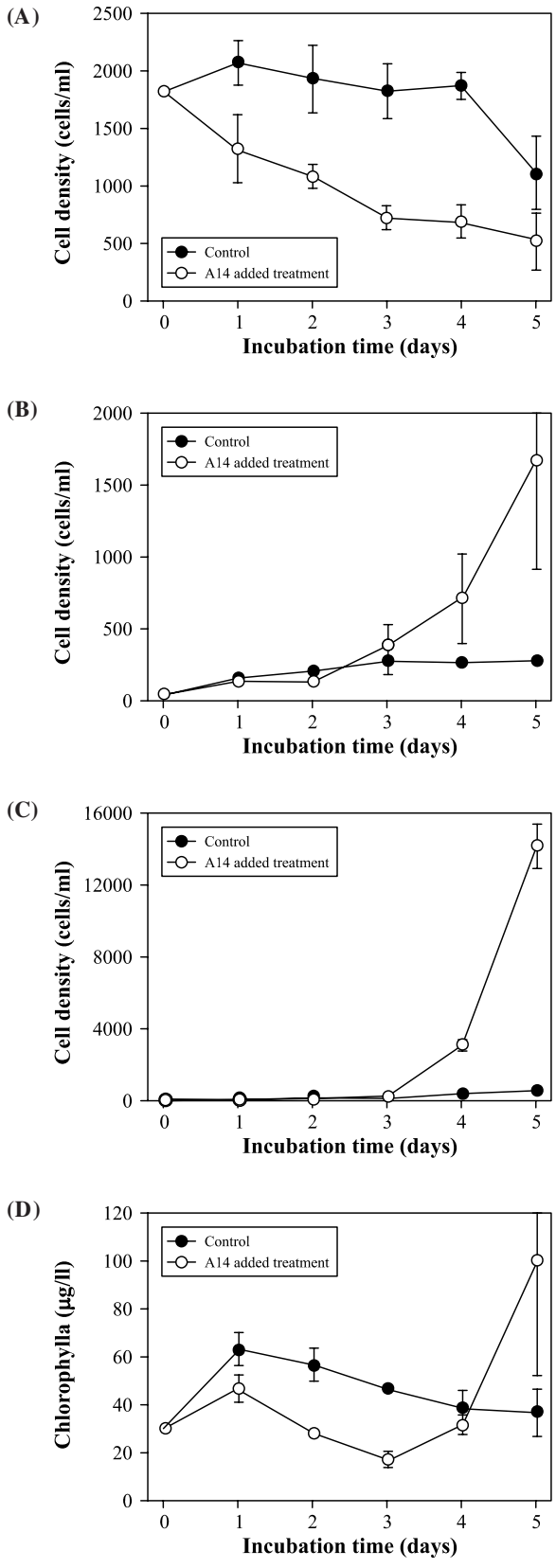

Fig. 3. Changes in cell densities of *Cochlodinium polykrikoides* (A), *Chaetoceros* spp. (B), *Skeletonema costatum* (C), and concentration of chlorophyll *a* (D).

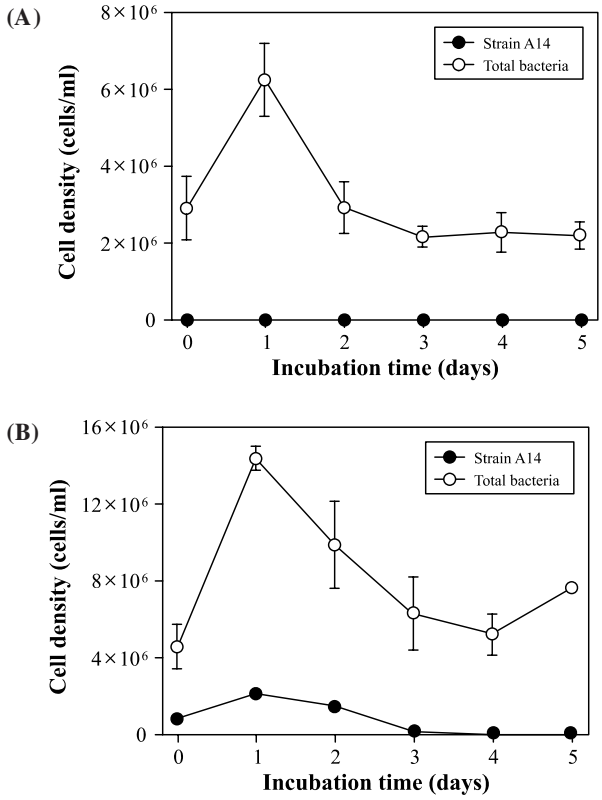

Fig. 4. Changes in total bacterial cell densities and strain A14 cell densities in the control treatment (A) and in the added A14 treatment (B).

Table 3. Changes in flagellates cell densities

(Unit, $\times 10^2$ cells/ml)

Time (days)	Control	A14 added treatment
0	6.89±5.67	6.89±5.67
2	28.69±4.63	234.41±5.41
5	33.28±1.24	88.53±60.15

cells/ml on 2 days but from day 2 the cell density decreased until 3.5×10^4 cells/ml on day 5 (Fig. 4). Cell density of flagellates in control treatment increased from 690 cells/ml on 0 day to 3,300 cells/ml on day 5 (Table 3). In A14 added treatment, cell density of flagellates increased rapidly to 23,400 cells/ml on day 2 but decreased to 8,850 cells/ml until day 5.

Discussion

Up to the present, few studies have examined the response of microbial community against the algicidal bacterial inoculation to natural blooming water. We investigated the efficiency of algal cell lysis, succession of phytoplankton community, and dynamics of the inoculated bacterium after the inoculation. In the experiment, abundance of the applied bacterium was monitored using specific probe in TSA-FISH. Information on abundance of algicidal bacterium as well as

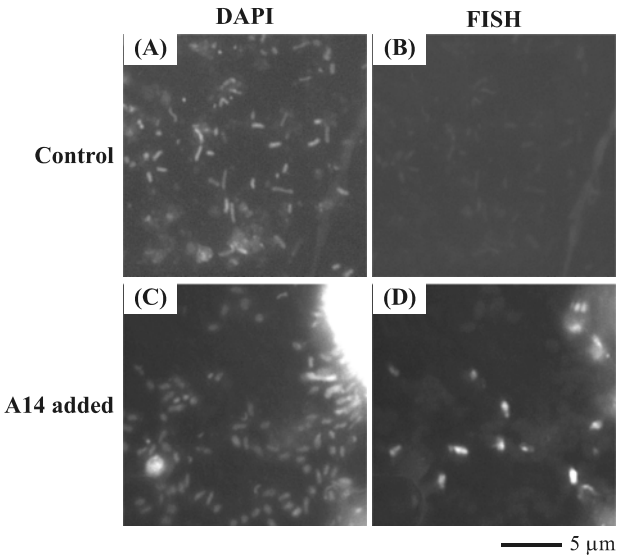

Fig. 5. Micrographs of the control treatment (A and B) and the added A14 treatment (C and D) after FISH using ALTERO14 probe. Left and right panels, respectively, show DAPI fluorescence under UV excitation and probe fluorescence under blue excitation.

algal cell lysis is essential to evaluate the effect of the bacterium in its application.

In the microcosm experiment, inoculation of A14 caused significant decrease in abundance of *C. polykrikoides* as compared to control (Fig. 3). However, the algicidal activity of A14 (Fig. 1) seemed lower than those of other algicidal bacteria belonged to *Alteromonas* or *Pseudoalteromonas,* although target algal species were different (Imai *et al*., 1995; Lovejoy *et al*., 1998; Lee *et al*., 2000). For example, the inoculation of algicidal bacterium, *Alteromonas* sp. strain S, at a final cell density of 10^3 cells/ml caused significant algal cell lysis of *Chattonella antiqua*, *C. marina*, *Gymnodinium mimotoi* (*Karenia mikimotoi*) within six days (Imai *et al*., 1995). Recently, Imai and Kimura tested algicidal bacteria against *C. polykrikoides* (Imai and Kimura, 2008). Some algicidal bacteria inoculated at the final cell density of 10^3 cells/ml showed killing activity against certain strains of *C. polykrikoides* although algal killing appeared 10 days after the inoculation. In contrast, algicidal activity of A14 was not obvious when cells were inoculated at 10^5 cells/ml. Thus, other algicidal bacteria, which show stronger algicidal activity against *C. polykrikoides* than the strain A14, should be isolated in future to develop the biological control of this harmful algal bloom.

There are two types of algicidal action (Mayali and Azam, 2004). One is direct attack. In this type of action, algicidal bacteria require direct contact to target algal cells (Imai *et al*., 1993; Yoshinaga *et al*., 1998; Kang *et al*., 2005; Jung *et al*., in press). Most algicidal bacteria belonged to *Cytophaga* and *Pseudomonas* possess this type of mode. The other is indirect attack. Many algicidal bacteria belonged to *Alteromonas*/*Pseudoalteromonas* produce dissolved compound responsible to the killing activity to the environment (Imai *et al*., 1995; Yoshinaga *et al*., 1998; Kim *et al*., 1999). Our isolates A14 belonged to genus *Alteromonas* also showed indirect attack (data not shown). Therefore, we applied cultured cells directly to the natural seawater without washing.

Total bacterial abundance increased in both control and treatment although the increased cell numbers were different between the two (Fig. 4). The growth rates of bacteria without isolate A14 were 0.76±0.15/day and 1.78±0.03/day in control and A14 added treatment, respectively. Increase in control suggests that the experimental operation on natural seawater caused positive effect on natural bacterial assemblage, although the critical reason is unknown. Higher increase in the abundance found in the A14 added treatment is probably due to added culture containing both organic and inorganic nutrients, and algal cell lysis. Increase in bacterial abundance after the application of algicidal bacteria were obtained by Kamiyama *et al*. (2000) and van Boekel *et al*. (1992). Kamiyama *et al*. (2000) demonstrated bacterial abundance increased depending on elevated dissolved organic matter (DOM) produced by lysis of *H. akashiwo* and *Prorocentrum* spp.. Van Boekel *et al*. (1992) also reported that an increased release of DOM through lysis during the decline of the *Phaeocystis* bloom would facilitate a strong increase of biomass in the microbial food web. It is likely that DOM from the degrade *C. polykrikoides* cells induced the high abundance of other bacteria as demonstrated in these studies.

To clarify the fate of the inoculated bacterium is quite important to evaluate the application to control algal blooms. FISH targeting the A14 provided us important insight. The strain A14 also grew well (growth rate; 0.98/day) from 0 to 1 day as well as other bacteria, although the growth rate of A14 was lower than that of other bacteria in A14 added treatment. Since A14 was cultured in 10% Zobell media, DOM from the lysed algal cells may be efficiently utilized by other bacteria that were adapted diluted nutrients rather than inoculated bacterial strain. In summary, we detected active growth of the algicidal strain A14 in seawater collected from the natural environment. If these inoculated bacterial cells could not grow by their algicidal activity, continuous application is required. Therefore, the growth capability in natural environments is important for the algicidal bacteria as the biological control agent.

The bacterial cell density decreased after 1 day (Fig. 4), while decrease in *C. polykrikoides* continued (Fig. 3). On 2 days, the cell density of A14 (1.5×10^6 cells/ml) was still high as compared to those on the 0 day (9.0×10^5 cells/ml). Thus, it is possible that *C. polykrikoides* reduced because of the A14, although the bacterial abundance decreased. On 3 days, when the abundance of A14 reduced to $<10^6$ cells/ml, *C. polykrikoides* abundance became stable. The decrease in *C. polykrikoides* from 0 to 3 days may be due to the algicidal activity of A14.

The decrease in A14 abundance coincided with rapid increase in flagellates abundance (Table 3). Therefore, decrease in bacterial abundance may be due to grazing by flagellates. The growth rate of flagellates between 0 and 2 days in the A14 added treatment (1.85±0.15/day) was 1.8 times higher that of control treatment (1.00±0.05/day) due to higher bacterial abundance in A14 added treatment. Consequently, these flagellate cells were probably grazed by ciliate, although we did not count after 2 days. Indeed, we found high abundance

of ciliates on 5 days (data not shown). Finally, cell density of A14 was reduced to 3.5×10^4 cells/ml, which was ca. 5% to total bacterial abundance. These results indicate that the applied algicidal bacterium is quickly removed by the natural microbial community. This reducing of the applied algicidal bacterium abundance is important in the aspect of environmental conservation. For example, copper and clay have a remained effect after reducing a HAB bloom (Anderson *et al*., 1997).

Generally, the heterotrophic flagellates and microzooplankton play a major role in re-mineralization in the sea (Azam *et al*., 1983). In our study, the increased flagellates due to increased bacterial abundance in A14 added treatment might produce much more dissolved inorganic matter. And it might be efficiently utilized by diatoms. Indeed, an apparent increase in cell densities of *Skeletonema costatum* and *Chaetoceros* spp. in A14 added treatment was found from 3 days after the rapid increase of flagellate cell density (Fig. 3 and Table 3).

The algicidal range of bacterium has a strong impact on the succession of phytoplankton community after the inoculation. When the microcosm experiment started, several species of dinoflagellates and diatoms were found at a low cell density as mentioned above. Among them, *Skeletonema costatum* and *Chaetoceros* spp. that were out of the algicidal range of A14 grew rapidly in A14 added treatment from 3 days after the inoculation. Banse (1982) concluded that size-based diatom growth rates would exceed dinoflagellate rate by up to 3 fold. Smayda (1997) also stated that diatom growth rates are generally much higher than those for dinoflagellates based on equivalent body mass. Therefore, in addition to algicidal range, potential growth rate of each algal species is also important for algal succession.

The rapid increase in abundance of harmless diatoms such as *S. costatum* and *Chaetoceros* spp. is probably important to prevent other harmful algal bloom after the application of algicidal bacteria. In the aligicidal bacterium application, inorganic nutrients were probably re-mineralized from lysed algal cells and were quickly utilized by diatoms, although we do not have information about nutrient concentration. If so, in addition to algal cell lysis, these increases may also be responsible for the termination of *C. polykrikoides* bloom and be important to prevent other harmful algal bloom after the application of algicidal bacteria. Fukuyo *et al*. (2002) proposed possible control of *Chattonella* sp. red tides using diatoms, since the growth of diatoms induces nutrient exhaustion in the surface layer, which prevents the growth of *Chattonella* spp.. Therefore, it is probably important in the control HAB that the algicidal bacterium does not have algicidal activity against these harmless diatoms.

In conclusion, the present study demonstrated that the fate of algicidal strain A14 in its application to naturally occurring *C. polykrikoides* bloom. The abundance of the strain increased with decreasing of its target alga. These increased bacterial cells decreased probably due to grazing by flagellates, and finally the abundance decreased to the negligible level (0.5% of total bacterial abundance). Although we could not examine the effect of cultured media on the bacterial growth in the experiment, however, we would like to emphasize the importance of monitoring algicidal bacterium in the application to natural environments. In this point of view, FISH using the specific probe is quite powerful. Therefore, FISH should be more employed in the application of algicidal bacteria to accumulate the knowledge for development in biological control of harmful algal blooms using bacteria.

Acknowledgements

We are grateful to Dr. Do Yong Kim and members of The Ministry of Marine Affairs and Fisheries of Yeosu for their help in sampling seawater. Thanks are also to Prof. A. Turner for his correction of the English and constructive comments on the manuscript.

References

Amann, R.I., B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux, and D.A. Stahl. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* 56, 1919-1925.

Amann, R.I., W. Ludwig, and K.H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143-169.

Anderson, D.M. 1997. Turning back the harmful red tide. *Nature* 388, 513-514.

Azam, F., T. Fenchel, J.G. Field, J.S. Gray, L.A. Meyer-Reil, and F. Thingstad. 1983. The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.* 10, 257-263.

Banse, K. 1982. Cell volumes, maximal growth rates of unicellular algae and ciliates, and the role of ciliates in the marine pelagial. *Limnol. Oceanogr.* 27, 1059-1071.

Biegala, I.C., F. Not, D. Vaulot, and N. Simon. 2003. Quantative assessment of picoeukaryotes in the natural environment by using taxon-specific oligonucleotide probes in association with tyramide signal amplification-fluorescence *in situ* hybridization and flow cytometry. *Appl. Environ. Microbiol.* 69, 5519-5529.

Caron, D.A. 1983. Technique for enumeration of heterotrophic and phototrophic nanoplankton using epifluorescence microscopy and comparison with other procedures. *Appl. Environ. Microbiol.* 46, 491-498.

Doucette, G.J., E.R. McGovern, and J.A. Babinchak. 1999. Algicidal bacteria active against *Gymnodinium breve* (Dinophyceae). I. Bacterial isolation and characterization of killing activity. *J. Phycol.* 35, 1447-1454.

Fukuyo, Y., I. Imai, M. Kodama, and K. Tamai. 2002. Red tides and other harmful algal blooms in Japan. Harmful algal blooms in the PICES region of the North Pacific. (http://www.pices.int/publications/scientific_reports/report23/HAB_Japan.pdf)

Guillard, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates, p. 29-60. *In* W.L. Smith and M.H. Chanley (eds.), Cultures of marine invertebrate animals. Plenum Press, New York, NY, USA.

Hugenholtz, P., G.W. Tyson, and L.L. Blackall. 2001. Design and evaluation of 16S rRNA targeted oligonucleotide probes for fluorescence *in situ* hybridization. *Methods Mol. Biol*. 76, 29-41.

Imai, I., Y. Ishida, and Y. Hata. 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., Y. Ishida, K. Sakaguchi, and Y. Hata. 1995. Algicidal marine bacteria isolated from northern Hiroshima bay, Japan. *Fish. Sci.* 61, 628-636.

Imai, I., Y. Ishida, S. Sawayama, and Y. Hata. 1991. Isolation of a marine gliding bacterium that kills *Chattonella antiqua* (Raphi-

dophyceae). *Nippon Suisan Gakkaishi* 57, 1409.

Imai, I. and S. Kimura 2008. Resistance of the fish-killing dinoflagellate *Cochlodinium polykrikoides* against algicidal bacteria isolated from the coastal sea of Japan. *Harmful Algae* 7, 360-367.

Jeffrey, S.W. and G.F. Humphrey. 1975. New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1, c_2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanzen* 167, 191-194.

Jung, S.W., B.H. Kim, T. Katano, D.S. Kong, and M.S. Han. *Pseudomonas fluorescens* HYK0210-SK09 offers species-specific biological control of winter algal blooms caused by freshwater diatom *Stephanodiscus hantzschii*. *J. Appl. Microbiol*. Article in Press.

Kang, Y.H., J.D. Kim, B.H. Kim, D.S. Kong, and M.S. Han. 2005. Isolation and characterization of a bio-agent antagonistic to diatom, *Stephanodiscus hantzschii*. *J. Appl. Microbiol*. 98, 1030-1038.

Kamiyama, T., S. Itakura, and K. Nagasaki. 2000. Changes in microbial loop components: effects of a harmful algal bloom formation and its decay. *Aquat. Microb. Ecol.* 21, 21-30.

Katano, T., B.K. Lee, B.H. Kim, H.O. Lee, and M.S. Han. 2006. Population dynamics of *Pseudomonas* sp. HYK0210-SK09 in microbial communities using the genus specific probe in tyramide signal amplification-fluorescence *in situ* hybridization, p. 44-51. Proceedings of 2nd Japan-Korea Joint Symposium for Limnology.

Kim, C.J., H.G. Kim, C.H. Kim, and H.M. Oh. 2007. Life cycle of the ichthyotoxic dinoflagellate *Cochlodinium polykrikoides* in Korean coastal waters. *Harmful Algae* 6, 104-111.

Kim, C.S., S.G. Lee, and H.G. Kim. 2000. Biochemical responses of fish exposed to a harmful dinoflagellate *Cochlodinium polykrikoides*. *J. Exp. Mar. Biol. Ecol.* 254, 131-141.

Kim, C.S., S.G. Lee, C.K. Lee, A.G. Kim, and J. Jung. 1999. Reactive oxygen species ascausative agents in the ichthyotoxicity of the red tide dinoflagellate *Cochlodinium polykrikoides*. *J. Plankton. Res.* 21, 2105-2115.

Kim, D.I., Y. Matsuyama, S. Nagasoe, M. Yamaguchi, Y.H. Yoon, Y. Oshima, N. Imada, and T. Honjo. 2004. Effects of temperature, salinity and irradiance on the growth of the harmful red tide dinoflagellate *Cochlodinium polykrikoides* Margalef (dinophyceae). *J. Plankton. Res.* 26, 61-66.

Kim, M.C., I. Yoshinaga, I. Imai, K. Nagasaki, S. Itakura, and Y. Ishida. 1998. A close relationship between algicidal bacteria and termination of *Heterosigma akashiwo* (raphidophyceae) blooms in Hiroshoma bay, Japan. *Mar. Ecol. Prog. Ser.* 170, 25-32.

Lee, S.O., J. Kato, N. Takiguchi, A. Kuroda, T. Ikeda, A. Mitsutani, and H. Ohtake. 2000. Involvement of an extracellular protease in algicidal activity of the marine bacterium *Pseudoalteromonas* sp. strain A28. *Appl. Environ. Microbiol*. 66, 4334-4339.

Lee, Y.S. and S.Y. Lee. 2006. Factors affecting outbreaks of *Cochlodinium polykrikoides* blooms in coastal areas of Korea. *Mar. Pollut. Bull.* 52, 626-634.

Lovejoy, C., J.P. Bowman, and G.M. Hallegraeff. 1998. Algicidal effects of a novel marine *Pseudoalteromonas* isolate (class Proteobacteria, gamma subdivision) on harmful algal bloom species of the genera *Chattonella*, *Gymnodinium*, and *Heterosigma*. *Appl. Environ. Microbiol.* 64, 2806-2813.

Mayali, X. and F. Azam. 2004. Algicidal bacteria in sea and their impact on algal blooms. *J. Eukaryot. Microbiol.* 51, 139-144.

Mayali, X. and G.J. Doucette. 2002. Microbial community interactions and population dynamics of an algicidal bacterium active against *Karenia brevis* (Dinophyceae). *Harmful Algae* 1, 277-293.

Moter, A. and U.B. Gobel. 2000. Fluorescence *in situ* hybridization (FISH) for direct visualization of microorganisms. *J. Microbiol. Methods* 41, 85-112.

Nagasaki, K., M. Yamaguchi, and I. Imai. 2000. Algicidal activity of a killer bacterium against the harmful red tide dinoflagellate *Heterocapsa circularisquama* isolated from ago bay, Japan. *Nippon Suisan Gakkaishi* 66, 666-673.

Park, K.Y., M.R. Kim, and S.K. Kim. 1999. Isolation and characterization of algicidal bacteria KY1. *J. Korean Fish. Soc.* 32, 452-457.

Pernthaler, J., F.O. Glockner, W. Schonhuber, and R. Amann. 2001. Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes. *Methods Microbiol.* 30, 207-226.

Pernthaler, A., J. Pernthaler, and R. Amann. 2002. Fluorescence *in situ* hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl. Environ. Microbiol.* 68, 3094-3101.

Porter, K.G. and Y.S. Feig. 1980. The use of DAPI for identification and counting aquatic microflora. *Limnol. Oceanogr.* 25, 943-948.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406-425.

Smayda, T.J. 1997. Harmful algal blooms; Their ecophysiology and general relevance to phytoplankton blooms in the sea. *Limnol. Oceanogr.* 42, 1137-1153.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876-4882.

Van Boekel, W.H.M., F.C. Hansen, R. Riegman, and R.P.M. Bak. 1992. Lysis-induced decline of *Phaeocystis* spring bloom and coupling with the microbial foodweb. *Mar. Ecol. Prog. Ser.* 81, 269-276.

Yoshinaga, I., M.C. Kim, N. Katanozaka, I. Imai, A. Uchida, and Y. Ishida. 1998. 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. *Mar. Ecol. Prog. Ser.* 170, 33-44.

ZoBell, C.E. 1946. Marine microbiology. Chronica Botanica, Waltham, Massachusetts, USA.