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Fluctuations of the red tide flagellates *Chattonella* spp. (Raphidophyceae) and the algicidal bacterium *Cytophaga* sp. in the Seto Inland Sea, Japan

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Abstract A marine algicidal gliding bacterium *Cytophaga* sp. strain J18/M01 was isolated in 1990 from a station in northern Harima-Nada, the Seto Inland Sea, Japan, using the harmful red tide alga *Chattonella antiqua* (Hada) Ono as a susceptible organism. The bacterium can prey upon various species of microalgae. Temporal fluctuations of this bacterium and *Chattonella* spp. [*C. antiqua* and *C. marina* (Subrahmanyan) Hara et Chihara] were investigated weekly at the above station in the summer of 1997 and 1998, using immunofluorescence assay employing highly specific polyclonal antibodies for the bacterium. In the summer of 1997, the cell density of *Chattonella* spp. showed a maximum value (70 cells ml^{-1}) on 8 July, and decreased thereafter. The bacterium *Cytophaga* sp. J18/M01 was commonly detected around a few hundreds of cells per milliliter or less. The number of *Cytophaga* sp. J18/M01 increased after the peak of *Chattonella* spp., and the maximum cell number of the bacterium was 1350 ml^{-1}. This algicidal bacterium also followed the changes of total amounts of microalgal biomass (chlorophyll *a* + pheophytin) when *Chattonella* spp. were absent. In the summer of 1998, *Chattonella* spp. were relatively less abundant (maximum 21 cells ml^{-1}), and the algicidal bacterium *Cytophaga* sp. J18/M01 showed a close relationship with the change of total microalgal biomass. The present study suggests that the algicidal bacterium *Cytophaga* sp. J18/M01 preyed upon, not only harmful red tide microalgae, but also other common microalgae such as diatoms, and the bacterium presumably plays an important role in regulating microalgal biomass in natural marine environments.

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Introduction

The incidence of harmful algal blooms (HABs), or red tides, has recently increased in frequency as a long-term trend on a global scale (Hallegraeff 1993; Anderson 1997). HABs have caused mass mortalities of cultured fish and bivalves (Okaichi 1997), and shellfish poisonings in the coastal seas of the world. Therefore, there is an urgent need for the development of techniques for predicting and reducing the impacts of HABs.

A possibility is that algicidal bacteria and viruses could be useful tools in reducing the impacts of HABs. From natural marine environments of Japanese coastal waters, a number of algicidal bacteria have been isolated hitherto (Ishida and Sugahara 1994; Sakata 2000). These algicidal bacteria kill red tide–causing microalgae such as *Chattonella antiqua* (Hada) Ono (Raphidophyceae), *Heterosigma akashiwo* (Hada) Hada ex Hara et Chihara (Raphidophyceae), *Gymnodinium mikimotoi* Miyake et Kominami ex Oda (Dinophyceae), *Heterocapsa circularisquama* Horiguchi (Dinophyceae), *Coscinodiscus wailesii* Gran, and *Skeletonema costatum* (Greville) Cleve (Bacillariophyceae). Field studies on assessments of algicidal microorganisms and HAB species suggest that the killing of microalgae by algicidal bacteria is a significant factor influencing the population dynamics of harmful microalgae in nature and contributes to the termination of red tides in coastal seas (Yoshinaga et al. 1995; Fukami et al. 1996; Imai et al. 1998a, b, c; Kim et al. 1998). However, these studies were basically carried out by culturing the host microalgae with filtered

seawater samples which contain algicidal bacteria and viruses. Consequently, more precise and quantitative information obtained by the direct count technique is essential for more complete understanding of the relationships between the population dynamics of HAB species and algicidal bacteria in natural marine environments.

Specific recognition and enumeration of individual cells of bacterial serotypes in natural and mixed populations are feasible with immunofluorescence assay by using fluorochrome-labeled antibodies (Bohlool and Schmidt 1980; Hoff 1988). In the present study, we investigated temporal changes of the red tide flagellates *Chattonella* spp. (*C. antiqua* and *C. marina*) and the algicidal bacteria *Cytophaga* sp. J18/M01 (Imai et al. 1991, 1993) and *Alteromonas* sp. S (Imai et al. 1995) using immunofluorescence assay at a station in northern Harima-Nada, the Seto Inland Sea, Japan. As a result, a close relationship was detected between the fluctuations of *Cytophaga* sp. J18/M01 and the termination of small blooms of *Chattonella* spp. The ecological significance and implications of algicidal bacteria are discussed dealing with the termination of red tides in coastal seas.

Materials and methods

Water samples were collected at stn. NH3 (34°42.8′N; 134°41′E) located in northern Harima-Nada, eastern Seto Inland Sea of Japan (Imai et al. 1998a, b), from three depths (0.5, 10, and 1 m above the bottom = ~20 m). Samplings were made once a week as a general rule from 8 July to 18 August in 1997, and from 29 June to 17 August in 1998. From each depth, approximately 100 ml seawater sample was taken into a 100 ml volume plastic bottle, fixed with glutaraldehyde at a final concentration of 0.5%, and stored at 5 °C in a refrigerator. Numbers of microalgae, including *Chattonella* spp. (*C. antiqua* and *C. marina*), in 1 ml were counted using unfixed live samples on the same sampling day without concentration treatments. If *Chattonella* spp. were < 10 cells ml^{-1}, countings were carried out for an additional 2 ml. As the total biomass of microalgae, chlorophyll *a* and pheophytin were determined. According to Cole (1982), the peak in microbial activity or biomass tends to occur during or near the death-phase of an algal bloom in a given water body. Hence we thought that pheophytin should be included in algal biomass data. Appropriate amounts of each seawater sample were filtered through a glass fiber filter (Whatman GF/C), and the filters were kept at ca. −20 °C in the dark. These pigments were extracted in 90% acetone and determined by the method of Yentsch and Menzel (1963) with a spectrofluorometer (Shimazu RF-5000).

The total number of bacteria in each water sample was enumerated by epifluorescence microscopy. The water samples were stained for at least 5 min with 4′6-diamidino-2-phenylindole (DAPI) at a final concentration of 0.5 μg ml^{-1} and filtered on Sudan Black B–stained Nuclepore polycarbonate filters (0.2 μm pore size) (Zimmermann et al. 1978), according to the procedure of Porter and Feig (1980) with some modification by Imai (1987). Observations and countings were made at ×1250 magnification using an ultraviolet excitation system. More than 300 bacterial cells were enumerated in at least ten randomly selected fields.

Detection and enumeration of the algicidal bacteria *Cytophaga* sp. J18/M01 (originally isolated from the same stn. NH3 in the summer of 1990, Imai et al. 1991) and *Alteromonas* sp. S (originally isolated from northern Hiroshima Bay in 1991, Imai et al. 1995) were carried out by an immunofluorescence assay method for double staining of bacteria with DAPI and fluorescein isothiocyanate (FITC)-labeled antibodies based on the method by Hoff (1988). The basic protocol for the immunofluorescence assay is shown in Fig. 1. Fixed seawater samples (5–15 ml) were filtered on black Nuclepore carbonate filters (described above), stained with DAPI, and washed once with 0.2 μm filtered distilled water (DW). Then, incubation was made with primary polyclonal antibody against *Cytophaga* sp. J18/M01 (at a dilution of 1:4000 with PBS) or *Alteromonas* sp. S (at a dilution of 1:800) for 15 min at room temperature. Antibodies against *Cytophaga* sp. J18/M01 and *Alteromonas* sp. S were produced by the immunization of mice. After washing twice with phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 in 1 l DW), incubation was made with secondary antibody (FITC-conjugated rabbit anti-mouse antibody, at a dilution of 1:3200 with PBS) for 15 min at room temperature. The filters were again washed twice with PBS. Subsequently, the algicidal bacteria *Cytophaga* sp. J18/M01 and *Alteromonas* sp. S were enumerated by epifluorescence microscopy at ×1250 magnification. At least 30 optical fields were observed for the enumeration.

Cross reactivity of the polyclonal antibody against *Cytophaga* sp. J18/M01 was tested using phylogenetically close bacteria (analyzed by 16S rDNA sequences, Kondo et al. 1999), phylogenetically close algicidal bacteria, and algicidal bacteria isolated from Japanese coastal waters (Table 1).

Results

The bacterial cells recognized by the polyclonal antibody of the algicidal bacterium *Cytophaga* sp. J18/M01 could be detected by the immunofluorescence assay method

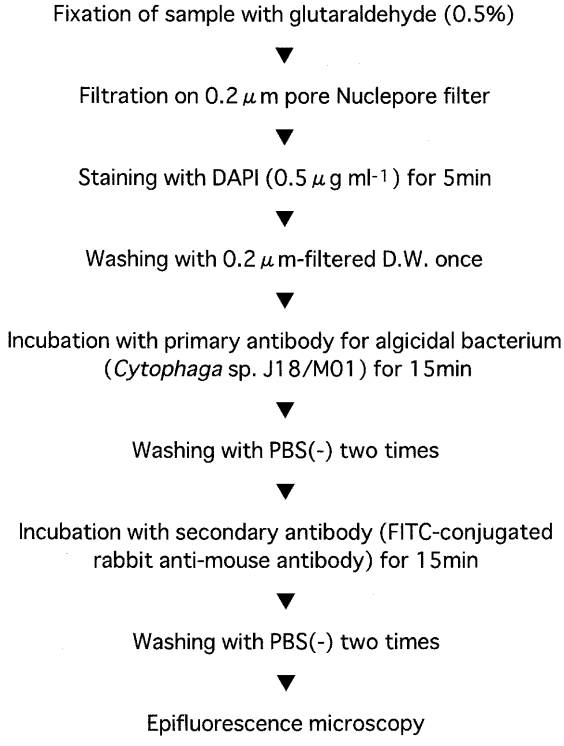

Fig. 1 *Chattonella* spp. and *Cytophaga* sp. Procedure of the immunofluorescence assay for double staining of algicidal bacteria with DAPI and FITC-conjugated antibodies

Table 1 *Chattonella* spp. and *Cytophaga* sp. Cross reactivity of the polyclonal antibody for the algicidal bacterium *Cytophaga* sp. J18/M01 to phylogenetically close bacteria analyzed by 16S rDNA sequences and to some algicidal bacteria isolated from the coastal Sea of Japan

Species and strain	Reactivity
Phylogenetically close bacteria	
Cytophaga johnsonae IFO 14942T	−
Cytophaga lytica IFO 14961T	−
Cytophaga marinoflava IFO 14170T	−
Cytophaga succinicans IFO 14905T	−
Cytophaga uliginosa IFO 14962T	−
Cytophaga latercula ATCC 23177T	−
Flavobacterium aquatile IAM 12316T	−
Flavobacterium branchiophilum IFO 15030 T	−
Flavobacterium odoratum IFO 14945T	−
Flavobacterium salegens ATCC 51522T	−
Flavobacterium gondowanense ATCC 51278T	−
Flexibacter maritimus ATCC 43398T	−
Phylogenetically close algicidal bacteria	
Flavobacterium sp. 5N-3[a] (from Uranouchi Inlet, Kochi)	−
Cytophaga sp. AA8-2[b] (from Ago Bay, Mie)	+
Algicidal bacteria	
Alteromonas sp. S (from Hiroshima Bay, Seto Inland Sea)	−
Alteromonas sp. K (from Hiroshima Bay)	−
Alteromonas sp. D (from Hiroshima Bay)	−
Pseudoalteromonas sp. R (from Hiroshima Bay)	−
Pseudomonas sp. A18 (from Ariake Sea, Fukuoka)	−
Pseudomonas sp. A22 (from Ariake Sea)	−
Cytophaga sp. A5Y (from Ariake Sea)	−
Cytophaga sp. A11 (from Ariake Sea)	−
Cytophaga sp. A12 (from Ariake Sea)	−
Cytophaga sp. A13 (from Ariake Sea)	−
Cytophaga sp. A14 (from Ariake Sea)	−
Cytophaga sp. A32 (from Ariake Sea)	−
Cytophaga sp. A35 (from Ariake Sea)	−
Cytophaga sp. A37 (from Ariake Sea)	−
Cytophaga sp. A43 (from Ariake Sea)	−
Cytophaga sp. A47 (from Ariake Sea)	−
Cytophaga sp. A50 (from Ariake Sea)	−
Flavobacterium sp. A23 (from Ariake Sea)	−
Flavobacterium sp. A48 (from Ariake Sea)	−

[a] Phylogenetic homology of 89.5% to *Cytophaga* sp. J18/M01
[b] Phylogenetic homology of 98.5% to *Cytophaga* sp. J18/M01

employed in this study. Figure 2 shows examples of epifluorescence microphotographs of positively reacting bacterial cells in a seawater sample collected from 10 m depth at stn. NH3 on 14 July 1997. Under observations with blue-light excitation epifluorescence microscopy, these positively reacting bacterial cells were easily detectable due to the clear cell surface outline with the green-fluorescence of FITC (Fig. 2), despite some detrital materials being stained by this method. Positively reacting bacterial cells recognized by the polyclonal antibody of *Alteromonas* sp. S could also be detected in seawater samples collected on 8 July 1997 (photographs not taken).

Table 1 shows the results of cross reactivity tests for the polyclonal antibody of the algicidal bacterium *Cytophaga* sp. J18/M01 to phylogenetically close bacteria (analyzed by 16S rDNA sequences) and to some strains of algicidal bacteria isolated from the coastal seas of western Japan. Twelve phylogenetically close strains of bacteria revealed no positive reactivity to the polyclonal antibody of *Cytophaga* sp. J18/M01. Other algicidal bacteria (Imai et al. 1995; Mitsutani 1997), belonging to the genera *Cytophaga*, *Flavobacterium*, *Alteromonas*, *Pseudoalteromonas*, and *Pseudomonas*,

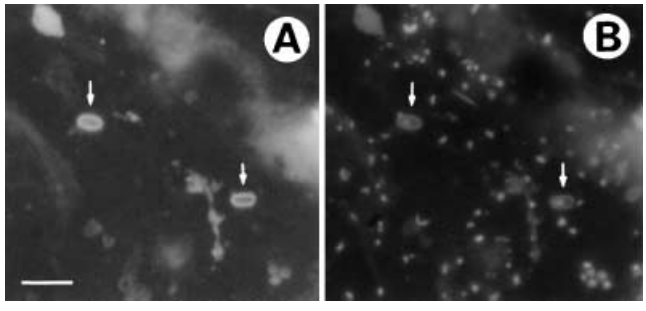

Fig. 2A, B *Chattonella* spp. and *Cytophaga* sp. Epifluorescence microphotographs of bacterial cells in a seawater sample observed by immunofluorescence assay method for double staining with DAPI and FITC-conjugated antibodies. The seawater sample was collected at 10 m depth at stn. NH3 on 14 July 1997. **A** Observation under blue-light excitation. Two bacterial cells recognized by the polyclonal antibody of the algicidal gliding bacterium *Cytophaga* sp. J18/M01 (indicated by *white arrows*) are visible with clear outline. *Scale bar* = 5 μm. **B** The same field as in A observed under ultraviolet excitation. Many bacterial cells stained with DAPI are visible as blue spots. Two positively reacting bacterial cells are indicated by *white arrows*

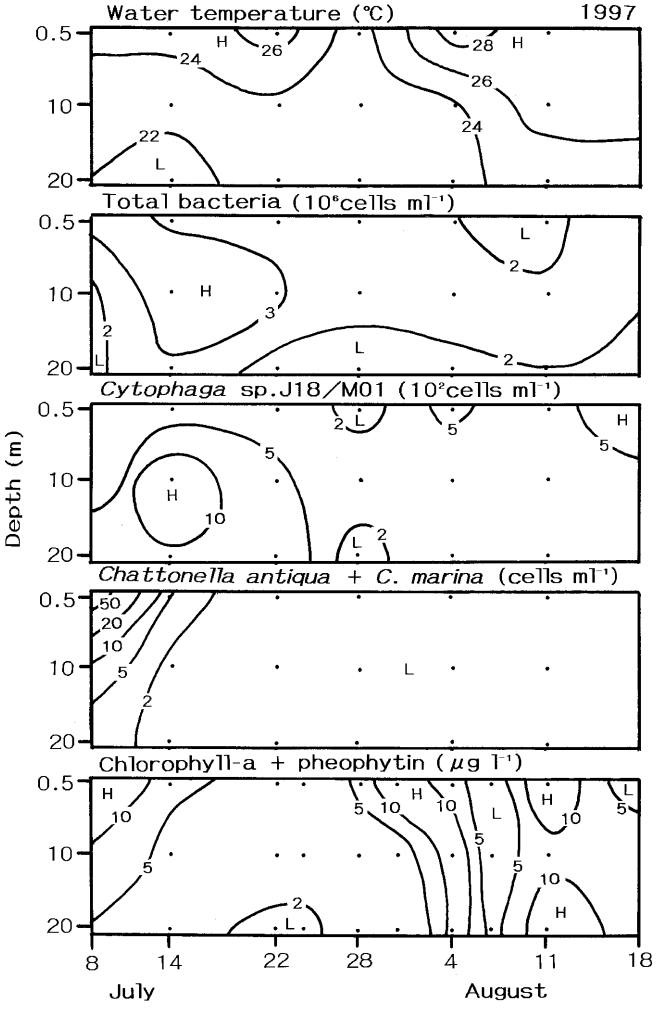

Fig. 3 *Chattonella* spp. and *Cytophaga* sp. Changes in vertical profiles of water temperature, total bacteria, algicidal bacterium *Cytophaga* sp. J18/M01, *Chattonella* spp. (*C. antiqua* + *C. marina*), and microalgal biomass (chlorophyll *a* + pheophytin) at stn. NH3 in northern Harima-Nada during the summer of 1997

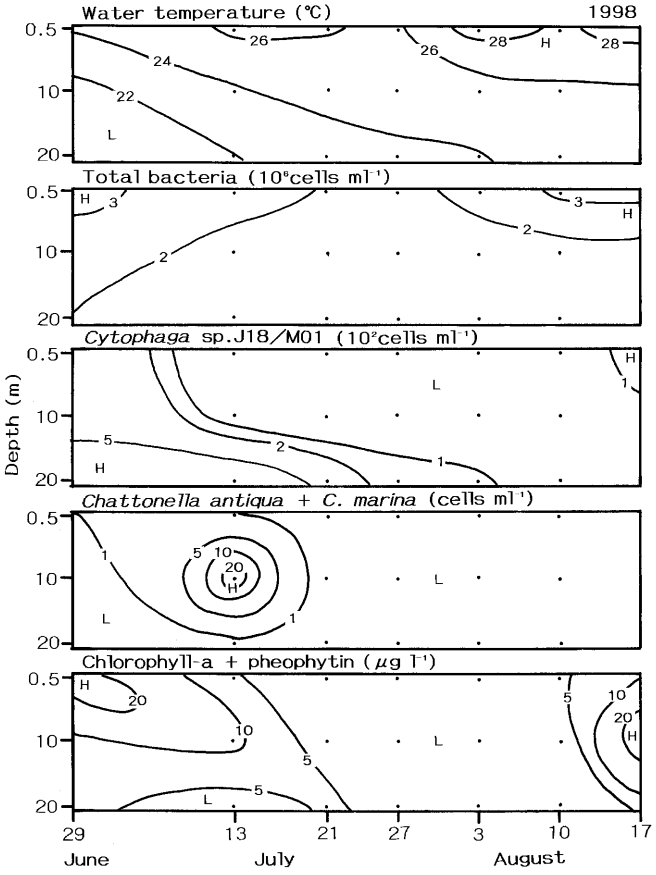

Fig. 4 *Chattonella* spp. and *Cytophaga* sp. Changes in vertical profiles of water temperature, total bacteria, algicidal bacterium *Cytophaga* sp. J18/M01, *Chattonella* spp. (*C. antiqua* + *C. marina*), and microalgal biomass (chlorophyll *a* + pheophytin) at stn. NH3 in northern Harima-Nada during the summer of 1998

also showed no positive reactivity to this antibody. Only a single strain *Cytophaga* sp. AA8-2, which was isolated from Ago Bay, Mie Prefecture, in the summer of 1995 (Imai et al. 1999), showed positive reactivity. This strain is close (phylogenetic homology of 98.5%, Kondo et al. 1999) to *Cytophaga* sp. J18/M01 at the level of being the same species.

Fluctuations of vertical profiles in water temperature, total bacteria, *Cytophaga* sp. J18/M01, *Chattonella* spp. (*C. antiqua* + *C. marina*), and total microalgal biomass (chlorophyll *a* + pheophytin) at stn. NH3 in northern Harima-Nada during the summer of 1997 are presented in Fig. 3, and for 1998 in Fig. 4.

In the summer of 1997 (Fig. 3), water temperature was in the optimal range for growth of *Chattonella* spp., around 25 °C at the surface (0.5 m) and 10 m depth. Total counts of bacteria were 1.3×10^6–3.8×10^6 cells ml^{-1}. *Chattonella* spp. formed a small bloom (maximum density of 70 cells ml^{-1}) at the surface on 8 July, which corresponded to a maximal value (14 μg l^{-1}) of chlorophyll *a* + pheophytin on the same day. Microalgal biomass (chlorophyll *a* + pheophytin) fluctuated and revealed two peaks higher than 10 μg l^{-1}. Accompanying the decline of *Chattonella* spp., the algicidal bacterium *Cytophaga* sp. J18/M01 increased to a maximum of 1350 cells ml^{-1} together with that of total bacteria, 3.8×10^6 cells ml^{-1}. During August, cell density of *Chattonella* spp. was lower than 1 cell ml^{-1}. Microalgae other than *Chattonella* spp. showed two peaks in August, and *Cytophaga* sp. J18/M01 followed these microalgal peaks after several days and also showed two peaks of 530 cells ml^{-1} on 4 August and 990 cells ml^{-1} on 18 August. Cells of *Alteromonas* sp. S were detected only on 8 July at densities of 304 cells ml^{-1} at 0.5 m, 76 cells ml^{-1} at 10 m, and 508 cells ml^{-1} at 1 m above the bottom of stn. NH3, and were undetectable (<40 cells ml^{-1}) thereafter.

In the summer of 1998 (Fig. 4), water temperature was also optimal for the growth of *Chattonella* spp. Total bacteria ranged between 1.4×10^6 and 3.5×10^6 cells ml^{-1}, showing higher density in surface layers. *Cytophaga* sp. J18/M01 fluctuated from 40 to 890 cells ml^{-1}. *Chattonella* spp. were most abundant (21 cells ml^{-1}) at 10 m depth on 13 July, and disap-

peared thereafter. Total microalgal biomass revealed a maximal value of 24 μg l^{-1} at 0.5 m depth on 29 June, decreased thereafter to less than 5 μg l^{-1}, and again increased to 37 μg l^{-1} at 10 m depth on 17 August. Cell densities of *Cytophaga* sp. J18/M01 were 890 cells ml^{-1} at 1 m above the bottom on 29 June, and 760 cells ml^{-1} at the same depth on 13 July 1998. These relatively high values of the bacterium corresponded and/or followed the high microalgal biomass at surface and middle layers, resembling the tendency observed in the summer of 1997. Accompanying the increase of microalgal biomass on 17 August, *Cytophaga* sp. J18/M01 increased again. Cell densities of *Alteromonas* sp. S were lower than the detection level (40 cells ml^{-1}) during the entire period of research in 1998.

Discussion

The polyclonal antibody against *Cytophaga* sp. J18/M01 exhibited cross reactivity to the algicidal bacterium *Cytophaga* sp. AA8-2. The phylogenetic homology between these two strains of algicidal bacteria was 98.5% (Table 1). Levels of DNA relatedness were determined by the hybridization method of Ezaki et al. (1989). *Cytophaga* sp. J18/M01 shared high levels of DNA relatedness with *Cytophaga* sp. AA8-2, exceeding 70.1%. This fact indicates that these two strains of algicidal bacteria are conspecific, despite the differences in the host algae from which they were isolated, and the locality and year of isolation. Consequently, it can be concluded that the bacterial cells recognized by the polyclonal antibody of *Cytophaga* sp. J18/M01 may be regarded as this species and that this antibody has a high specificity. Although the low possibility of cross reactivity of this antibody to cells of different bacterial species cannot be discarded entirely, this antibody is proved suitable for practical use in field investigations. Concerning the antibody of *Alteromonas* sp. S, no cross reactivity was observed to the species or strains of algicidal bacteria shown in Table 1.

In the summer of both 1997 and 1998, bacterial cells reacting to the polyclonal antibody of *Cytophaga* sp. J18/M01 were detected with a maximum number of 1350 cells ml^{-1}. The algicidal bacterium *Cytophaga* sp. J18/M01 was originally isolated from stn. NH3 in a sample from 1990 (Imai et al. 1991). These facts strongly suggest that the bacterium *Cytophaga* sp. J18/M01 is a common member of the bacterial community in Harima-Nada, the Seto Inland Sea. Another algicidal bacterium, *Cytophaga* sp. AA8-2, which was isolated from Ago Bay, a small inlet opened to Pacific Ocean, in the summer of 1995, was identified to be the same species as *Cytophaga* sp. J18/M01. Hence, the algicidal gliding bacterium presumably occupies an important niche as "microalga killers" in coastal ecosystems.

In northern Hiroshima Bay, temporal fluctuations of algicidal microorganisms against the red tide–causing flagellate *Heterosigma akashiwo* were investigated using the microplate MPN method in 1992 and 1993 by Imai et al. (1998b) and in 1994 and 1995 by Kim et al. (1998). The method is based on co-culturing of host alga (*H. akashiwo*) and filtered (< 0.8 μm) seawater samples (Imai et al. 1998a). Red tides of *H. akashiwo* occurred in June of every year. The dynamics of killer microorganisms against *H. akashiwo* revealed a close relationship with that of *H. akashiwo* populations. Killers against *H. akashiwo* followed the increase of *H. akashiwo* populations, reached a maximum level after the beginning of decline of *H. akashiwo*, maintained a high level for a while after the termination of red tide, and then decreased. Similar dynamics were observed for *Cytophaga* sp. J18/M01 and *Chattonella antiqua* in Harima-Nada in the summer of 1997 (Fig. 3). The algicidal gliding bacterium *Cytophaga* sp. J18/M01, therefore, presumably plays an important role in the rapid crash of *Chattonella* red tides in Harima-Nada, although cyst formation after the depletion of nutrients also contributes to the termination of the blooms of *Chattonella* (Imai et al. 1998d).

Cell encounter of bacteria and phytoplankton is the first step in the algicidal process. Shimeta (1993) predicted the sum and ratio of normalized encounter rates by direct interception and Brownian diffusion for a 100 μm, nonswimming, spherical heliozoan. The total rates of encounter between the heliozoan and 1 μm diameter particles were ca. 1×10^{-6} and 1×10^{-5} ml h^{-1} at low (10^{-6} cm^2 s^{-3}) and high (10^{-2} cm^2 s^{-3}) levels of turbulence, respectively. These values of encounter rates are roughly applicable to the raphidophycean flagellate *Chattonella antiqua* (length, 100 μm or more) and the algicidal bacterium *Cytophaga* sp. J18/M01. Given a cell density of 10^3 cells ml^{-1} for *Cytophaga* sp. J18/M01 at the encounter rate of 1×10^{-5} ml h^{-1}, *C. antiqua* encounters the bacterium at the rate of 10^{-2} cells h^{-1}. In a case of cell density of 10^2 cells ml^{-1} for *C. antiqua*, the total population of *C. antiqua* in 1-ml seawater encounters the algicidal bacterium at a rate of 1 cell h^{-1}. Consequently, in situ cell densities of *C. antiqua* and *Cytophaga* sp. J18/M01 are considered to be enough for the occurrences of algicidal processes in nature.

The cell densities of *Cytophaga* sp. J18/M01 increased several days after the peaks of total microalgal biomass (chlorophyll *a* + pheophytin) in the summer of both 1997 and 1998 (Figs. 3, 4). These phenomena can be explained by the ability of the bacterium as a predator possessing a wide prey range (Imai et al. 1993). However, other types of algicidal bacteria exist, such as *Alteromonas*, *Pseudoalteromonas*, *Pseudomonas*, etc., in coastal seas (Imai et al. 1995; Mitsutani 1997; Lovejoy et al. 1998; Yoshinaga et al. 1998; Sakata 2000). Accordingly, not only *Cytophaga* sp. J18/M01 but also these other types of algicidal bacteria probably play a crucial role in the crash of blooms in the coastal sea, together with algicidal viruses (Nagasaki et al. 1994; Elbrächter and Schnepf 1998; Imai et al. 1998b).

Marine algicidal bacteria tend to have a wide prey range (Imai et al. 1993; Ishida and Sugahara 1994; Mitsutani 1997; Sakata 2000). Algicidal bacteria such as *Cytophaga* sp. J18/M01 would prey upon various species of microalgae, and may control microalgal biomass within some homeostatic level of moderate range in natural marine environments. Additionally, a dominant species of blooming microalgae will be killed with much higher frequencies as compared to other less abundant species during bloom periods by algicidal bacteria with wide prey range. As a result, this type of algicidal activity implies species-specific predation of "bloom-forming algae". Algicidal activities might provide an answer to the paradox of the plankton: "how it is possible for a number of species to coexist in a relatively isotopic or unstructured environment all competing for the same sorts of nutrients" (Hutchinson 1961).

Before algicidal bacteria can be applied as a tool for exterminating noxious red tides, several problems remain to be solved. Firstly, risk assessments are necessary as to whether these bacteria have harmful effects on other microalgae, zooplankton, fish, or other important organisms. Secondly, the fate of algicidal bacteria in water columns and sediments should be understood after the termination of red tides.

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