Distribution and Fluctuation of Bacteria Inhibiting the Growth of a Marine Red Tide Phytoplankton *Gymnodinium mikimotoi* in Tanabe Bay (Wakayama Pref., Japan)

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Distribution and seasonal fluctuation of the bacteria which inhibit the growth of a red tide marine dinoflagellate *Gymnodinium mikimotoi*, were surveyed in Tanabe Bay (Wakayama Pref., Japan), using the newly developed MPN method with an axenic culture of *G. mikimotoi*. *G. mikimotoi*'s growth inhibiting bacteria (Gm-GIB) were detected at 10³-10⁴ cells/ml before occurrences of huge red tides by *G. mikimotoi* at the beginning of August in 1990 and from the end of August to the beginning of September in 1991. The number of Gm-GIB fell by about two orders of magnitude at the blooming periods of *G. mikimotoi*, and then increased again after the blooms declined. These results suggest that the fluctuation of Gm-GIB counts in seawater is significantly related to the development and decline process of *G. mikimotoi* red tide. Forty strains of Gm-GIB isolated in this study all acted as killers against this dinoflagellate rather than as suppressers on the algal growth under laboratory conditions. The precise causes of the fluctuation of Gm-GIB in seawater environments remain unknown.

Key words: killer bacteria, growth inhibition, red tide, dinoflagellate, *Gymnodinium mikimotoi*

*Gymnodinium mikimotoi* (= *G. nagasakiense*) frequently causes huge red tides in the western region of Japan and severely damages nearshore fishery and aquaculture, since it was first reported in 1965 in Oomura Bay (Nagasaki Pref.). Many studies on the mechanism of *G. mikimotoi* blooming have been carried out on the physical aspects (water temperature, salinity and light intensity) and/or chemical (inorganic nutrients and other growth factors) environmental factors. However, there is little information about bacterial participation in the development and decline process of the red tide.

Close relationships between microalgae and bacteria have so far been observed under laboratory experiments and in natural environments. Fukami *et al.* carried out an AGP (algal growth potential) assay method with *in situ* bacterial populations during blooming periods of *G. mikimotoi* and *Skeletonema costatum* in Uranouchi Inlet (Kochi Pref.). They suggested that natural bacterial populations play an important role in the development and decline process of the red tides caused by *G. mikimotoi* and *S. costatum*.

Some bacteria which stimulate the growths of some marine algae through nutrients regeneration, vitamins production and other means, have been reported. A specific bacterium isolated from Maizuru Bay (Kyoto Pref.) was reported to produce a glycoprotein as a growth factor essential for a diatom *Asterionella glacialis*. On the contrary, the bacteria that suppress the algal growth or kill and/or lyse marine algae were also found and isolated from seawater and aquaculture. However, little information is available about the distribution of the killer bacteria against marine red tide algae.

Although ecological information of lysing bacteria on blue-green algae in freshwater has been obtained by a softagar-overlayer technique, it is too difficult to study on killer and/or lysing bacteria for marine red tide algae, because most marine phytoplankton cannot form any colonies on an agar plate. In this report, therefore, we developed a MPN (most probable number) method for detecting the bacteria which have inhibitory effects on the growth of *G. mikimotoi* (Gm-GIB; *G. mikimotoi*'s growth inhibiting bacteria), and investigated the distribution and seasonal fluctuation of Gm-GIB in Tanabe Bay (Wakayama Pref.) where the red tide caused by *G. mikimotoi* frequently occurs.

Materials and Methods

Axenic culture of *G. mikimotoi* G303, which was kindly offered by Dr. M. Yamaguchi, Nansui National Fisheries Research Institute, was used for an assay of the bacterial inhibitory effects on the algal growth. *G. mikimotoi* was cultivated preliminarily in 2 l of a SWM3 medium at 20°C with an L: D cycle of 14:10 under 8000 lx. Immediately before experiments were conducted, the culture of *G. mikimotoi* at the logarithmic growth state was dispensed.
Sampling for counting and isolation of Gm-GIB was carried out in Tanabe Bay, Wakayama Pref., Japan, from June to September in 1990, 1991 and 1992. The sampling stations in Tanabe Bay are illustrated Fig. 1. Stns. 4, 6, 7, and 12 were situated in the inner area of the bay where the red tide of *G. mikimotoi* often occurs, and Stn. 17 was in the entrance of the bay. Seawater was collected from 0 m, 5 m and 10 m depths and at 1 m above the bottom (B-1 m) at Stns. 6, 7, 12 and 17 with a Ban-Dorn sampler. At Stn. 4 (ca 12 m water depth), water samples at 0 m, 3 m, 8 m and B-1 m depths were collected in the same manner.

Numbers of Gm-GIB were estimated by the MPN method with an axenic culture of *G. mikimotoi* (Fig. 2). After filtration with a sterilized membrane filter (1.2 μm, Millipore Co.) for exclusion of larger predators against phytoplankton, seawater samples were diluted serially with sterilized seawater. The diluted samples (0.1 ml) in each dilution series were inoculated in 5 replicates into the MPN tubes prepared previously as mentioned above. In parallel, 0.1 ml aliquots of autoclaved seawater were inoculated in 10 replicates into the MPN tubes as a control culture. Incubation was carried out at 20°C with an L:D cycle of 14:10 under 8000 lux, and the algal growth in each MPN tube was monitored by *in vivo* autofluorescence excited by blue-light, using a Turner Fluorometer Model 112 (Turner Inc.). After 2 weeks' incubation, the MPN tubes in which the algal growth was less than 50% of that in the control culture were regarded as "Gm-GIB-positive". MPN values of Gm-GIB in the seawater samples were determined from a series of numbers for "Gm-GIB-positive" tubes.

Total bacterial number and viable bacterial number in seawater samples were determined by direct microscopic observation with DAPI stain and by colony formation on a ST10-1 agar medium (trypticase peptone 0.5 g; yeast extract 0.05 g; agar 1.2%; in 1 l of aged sea water), respectively.

Several Gm-GIB were isolated from the "Gm-GIB-positive" test tubes in the MPN experiment by a ST10-1 agar medium. The Gm-GIB isolates were cultivated in a ST10-1 liquid or a semi-liquid (0.3% agar) agar medium. To study their inhibitory effects on *G. mikimotoi*'s growth, they were inoculated at an initial density of about 10^4 cells/ml into the algal culture and algal growths were monitored as mentioned above.
Results

Fluctuations of cell densities of *G. mikimotoi* at the sampling stations in Tanabe Bay during the sampling periods are shown in Fig. 3. Red tides by *G. mikimotoi* occurred from the beginning to the middle of August in 1990 and from the end of August to the beginning of September in 1991, but did not occur in 1992. In 1990, *G. mikimotoi* cells which were observed at a small number (<1 cell/ml) around the bay during July started increasing rapidly in the southern area from July 24, and then formed a huge red tide both in the southern and eastern areas till August 14. A maximum cell density of $2.0 \times 10^4$ cells/ml was observed on the surface (0 m) at Stn. 12 on August 14, and the bloom of *G. mikimotoi* disintegrated till August 24. In 1991, cell density increased gradually in the southern area from August 12, and attained a maximum ($2.0 \times 10^4$ cells/ml) in the surface water at Stn. 7 on September 2. In Stn. 4 in the eastern area, the algal cell density attained $7.0 \times 10^3$ cells/ml on September 10 and then disintegrated.

Total bacterial numbers, viable bacterial numbers and the numbers of Gm-GIB determined by the MPN method in the seawater samples from Stns. 4, 6, 12 and 17 during the sampling periods in 1990 are shown in Table 1. As a whole, the total bacterial counts in untreated seawaters in Stn. 4 did not change significantly at $2-4 \times 10^6$ cells/ml except for the increase at the period of the red tide. By the filtration treatment with a 1.2 řm filter, which was carried out in order to remove predators from seawater samples, 23.6%-74.5% (mean 53.1%, n=23) of the total bacteria remained. When a 0.8 řm membrane filter was used, more than 90% of total bacteria was removed from filtered seawater samples (data not shown).

The number of Gm-GIB at 8 m depth at Stn. 4 on June 26 was $1.1 \times 10^4$ cells/ml and the number did not change greatly till July 24. Gm-GIB then decreased to $1.4 \times 10^4$ cells/ml on August 7 when the huge red tide by *G. mikimotoi* occurred. Also in the other sampling sites, even in Stn. 17 where the red tide did not appear, the numbers of Gm-GIB declined to about 10 cells/ml on August 7, then increased on September 4.

Fluctuations of Gm-GIB counts at Stn. 4 and Stn. 7 during the sampling periods (July–September) in 1990, 1991 and 1992 are summarized in Fig. 4. Both in 1990 and 1991, the number of Gm-GIB which changed in the range of $10^2$–$10^3$ cells/ml before the occurrences of red tide of *G. mikimotoi*, fell by one or two orders of magnitude as the red tide expanded. After disintegration of the bloom, the number of Gm-GIB recovered to the same level as that before the red tide. The number of Gm-GIB in 1992 when red tide by *G. mikimotoi* did not appear fluctuated at a rel-

![Fig. 3. Fluctuation of numbers of Gymnodinium mikimotoi at several depths of sampling stations in Tanabe Bay during sampling periods (June-September) in 1990 (A), 1991 (B) and 1992 (C).](image-url)
Table 1. Numbers of total bacteria, viable bacteria and the bacteria which have an inhibitory effect on the growth of *G. mikimotoi* (Gm-GIB) in Tanabe Bay from June to September in 1990

<table>
<thead>
<tr>
<th>Station</th>
<th>Date</th>
<th>Total Bacteria</th>
<th>Viable Bacteria</th>
<th>Gm-GIB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>untreated (A)</td>
<td>&lt;1.2μm (B)</td>
<td>(B)/(A)</td>
</tr>
<tr>
<td>Stn. 4</td>
<td>0 m</td>
<td>3.9 x 10^8</td>
<td>2.8 x 10^8</td>
<td>72.4</td>
</tr>
<tr>
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<td>0 m</td>
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<td>1.5 x 10^8</td>
<td>69.7</td>
</tr>
<tr>
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<td>4.3 x 10^8</td>
<td>3.2 x 10^8</td>
<td>74.5</td>
</tr>
<tr>
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<td>3.5 x 10^8</td>
<td>62.3</td>
</tr>
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</tr>
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<td>Stn. 6</td>
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<td>4.1 x 10^6</td>
<td>63.6</td>
</tr>
<tr>
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<td>3.6 x 10^6</td>
<td>1.2 x 10^6</td>
<td>34.2</td>
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</tbody>
</table>

Total bacteria and viable bacteria were counted by direct microscopic observation with DAPI stain and by colony formation on a ST10^-1 agar medium, respectively. The numbers of Gm-GIB were estimated by MPN method with an axenic culture of *G. mikimotoi*. ND: not determined.
Fig. 5. Effects of bacterial assemblages in seawater samples from Tanabe Bay in 1992 on the growth of Gymnodinium mikimotoi in six tubes (A-F) of the MPN method for detecting Gm-GIB.

○ : control (without bacteria), □ : with bacteria.

In 1992, the growth of G. mikimotoi in a tube for the MPN experiment was monitored in detail. The algal growth with bacterial assemblage shown in Fig. 5A was better than the control (without bacterial assemblage). Also in Fig. 5B, 5C and 5D, the bacterial assemblages promoted the algal growth till 10–12 days after bacterial inoculation, but after 2 weeks algal chlorophyll (autofluorescences) diminished. In the tubes shown in Fig. 5E and 5F, on the other hand, G. mikimotoi's autofluorescences diminished quickly.

Forty test tubes in the highest dilution series were selected among the "Gm-GIB positive" tubes in the MPN experiment, and 40 strains of Gm-GIB were isolated from each of them. Strains D6 and T26 as representative strains of Gm-GIB were inoculated into the mid log-phase culture of G. mikimotoi at the initial cell density of about 10^6 cells/ml in each. The algal autofluorescences diminished in 2–4 days' incubation (Fig. 6). Microscopic observation indicated that the decrease of autofluorescence meant the death and breakdown of algal cells. The other 38 isolates of Gm-GIB killed G. mikimotoi within a week after bacterial inoculum.

Discussion

Fukami et al. suggested through AGP experiments with bacterial fractions (<0.8 μm fraction) in seawater of
Uranouchi Inlet (Kochi Pref.) that natural communities of bacteria might influence the succession of algal population which dominate in situ. The bacterial population which stimulated the growth of G. mikimotoi was often inhibitory or non-effective against the growth of S. costatum, and stimulative or inhibitory effects of bacterial population on the growth of each algal species was positively or negatively associated with the fluctuation of algal population.

Our results obtained from the field survey in 1990 and 1991 in Tanabe Bay show that Gm-GIB generally exist in seawater at 10–10^3 cells/ml, and strongly suggest that the fluctuation of the Gm-GIB is related to the development and decay process of the red tide caused by G. mikimotoi. Especially, the tentative stagnation in the development of G. mikimotoi bloom observed at the beginning of August in 1991 could be explained by the appearance of Gm-GIB on August 13 at relatively higher density than that on the corresponding period in 1990, because G. mikimotoi numbers at Stn. 4 and Stn. 7 surged again at the end of August in accordance with decline of Gm-GIB numbers (Fig. 4). Forty isolates of Gm-GIB in this study all killed and lysed G. mikimotoi cells rather than only suppressing the algal growth (Fig. 6). Algicidal bacteria against H. akashiwo, C. antiqua and S. costatum and other some microalgae were reported to exist generally at 10–10^3 cells/ml in natural seawater, and some of them were isolated.22-24) Cytophaga sp.,22) which was a strong killer against C. antiqua, was reported to be able to grow in an amended seawater medium as marine oligotrophs do.25) Because most of the Gm-GIB isolates could also grow in an amended seawater medium, they may be normally alive in marine environments independent of the killing of red tide algae. Although it is probable that algicidal bacteria negatively influence the occurrences of a variety of algal blooms, it is unclear what regulates the fluctuation of algicidal bacterial populations in nature. Hence, the population dynamics of algicidal bacteria in natural seawater must be studied in more detail to facilitate the prediction of red tide and its effects on the algal blooming.

Growth stimulation of G. mikimotoi by bacteria was frequently observed through the MPN experiment for counting Gm-GIB (Fig. 5A and B). Probably, the succession of bacterial population occurred in each MPN tube during incubation. Influences on algal growth by bacterial population in a MPN tube must be changeable during incubation, because the effects of bacterial population on the growth of algae must reflect the balance of stimulatory and inhibitory effects of individual bacterium. Actually, the inhibitory processes on algal growth among MPN tubes were variable (Fig. 5). In the MPN tubes shown in Fig. 5 (C and D), for example, bacteria which stimulated the growth of G. mikimotoi were probably predominant, followed by Gm-GIB, and the bacterial population in these tubes represents the inhibitory activities of the bacteria on algal growth after two weeks’ incubation. In other cases, two-weeks’ incubation for the MPN experiment might be not enough for detection of Gm-GIB. If an algal-bacterial mixed culture in the MPN tube (Fig. 5B) which was assessed “Gm-GIB negative” was incubated more than two weeks, Gm-GIB would become predominant, thus inhibiting algal growth. The MPN of Gm-GIB in this study should perhaps be underestimated.

The MPN method in this study was unique and applicable for detecting and counting the bacteria which influence algal growth, and the method showed the importance of algicidal bacteria such as Gm-GIB during the development and decline process of red tide in natural environments. However, this method needs a long incubation period and a complicated treatment in addition to several problems mentioned above. For monitoring Gm-GIB directly, new methods with DNA probes or antibodies which can recognize them must be developed. If the fluctuation of these bacteria and the intensity of their inhibitory activities can both be estimated, then, the importance of Gm-GIB in the development and decay process of red tide could be fairly assessed.

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References

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