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Mode of action of an antialgal agent produced by a marine gammaproteobacterium against *Chattonella marina*

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ABSTRACT: A marine gammaproteobacterium, strain MS-02-063, was able to kill *Chattonella marina,* a noxious red tide phytoplankton. However, the algicidal activity of bacterial cells washed with the planktonic medium was significantly reduced. These results suggest that strain MS-02-063 produces an extracellular substance, the pigment, PG-L-1, that showed a potent algicidal effect on *C. marina*. The LD_{50} value of PG-L-1 was calculated to be approximately 8.5 µg ml^{-1}. At the approximate LD_{50} concentration of 10 µg ml^{-1}, a morphological change, which seemed to be due to the inhibition of cell division, was observed in *C. marina*. Almost all cells of *C. marina* were destroyed readily at 100 µg ml^{-1} of PG-L-1, and the cytostatic activity of PG-L-1 against this phytoplankton was observed at a concentration of 1 µg ml^{-1} during the 5 d of incubation. A sublethal concentration of PG-L-1 of 10 µg ml^{-1} significantly inhibited the reactive oxygen species (ROS) production by *C. marina.* ROS production has been previously reported to be essential for normal growth of *C. marina* (Oda et al. 1995; Biosci Biotechnol Biochem 59:2044–2048). Therefore, the inhibitory effect of PG-L-1 on ROS production may lead to growth inhibition of *C. marina,* at least in part. The pigment, PG-L-1, may be a useful compound not only as an applicable agent for the mitigation of harmful algal blooms, but also as an experimental tool to analyse the ROS production system in a red tide phytoplankton such as *C. marina*.

KEY WORDS: Prodigiosin · Algicidal activity · Gammaproteobacterium · Red tide phytoplankton · *Chattonella marina* · Reactive oxygen species

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INTRODUCTION

Harmful algal blooms (HABs) due to flagellated algae such as *Chattonella marina* (Raphidophyceae) are associated with the mass mortality of natural marine living organisms and have frequently caused serious damage to the aquaculture industry (Landsberg 2002). Several lines of evidence suggest that marine bacteria play an important role in regulating microalgal biomass in natural marine environments (Furuki & Kobayashi 1991, Imai et al. 2001, Iwata et al. 2004). Some marine bacteria selectively promote bloom formations by certain algal species (Furuki & Kobayashi 1991), whereas other bacteria have algicidal effects, and they even cause the termination and decomposition of algal blooms (Imai et al. 2001, Iwata et al. 2004). The latter findings have raised the possibility of bacterial control of HABs, and the algicidal bacteria have been considered as useful tools to reduce the impacts of HABs. The underlying algicidal mechanisms seem to be dependent on the species of bacteria, and 2 modes of algicidal activity depending on the bacterial species have been proposed. Some bacteria have been reported to affect red tide phytoplankton through direct cell-to-cell contact (Imai et al. 1993). On the other hand, certain bacteria are known to release extracellular compounds that affect the growth of red tide phytoplankton (Lovejoy et al. 1998, Seong-Yun et al. 2003).

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During our screening of useful bacteria that produce anti-microbial agents from the coastal area of Nagasaki, Japan, we discovered a red-pigmented bacterium, designated as strain MS-02-063. Strain MS-02-063 was phylogenetically closely related to gamma-proteobacterium MBIC 3957; however, there were differences in the physiological and biochemical properties (Nakashima et al. 2005b). The red pigment produced by strain MS-02-063 had potent antifungal, antibacterial, and *in vitro* antitumor activities (Nakashima et al. 2005a, 2005b, 2005c). Our chemical structural and biochemical analysis revealed that this red pigment belongs to the prodigiosin family, and we named it PG-L-1. Other marine bacteria such as *Pseudoalteromonas bacteriolytica* and *Vibrio ruber* also produce prodigiosin-like pigments (Sawabe et al. 1998, Shieh et al. 2003). We found that PG-L-1, a prodigiosin analogue pigment, shows potent algicidal activity against several red tide phytoplankton (Nakashima et al. 2006) and, therefore, conducted a detailed analysis of the algicidal mechanism of PG-L-1 in *Chattonella marina*. To search for new aspects of biochemical activity of PG-L-1 against *C. marina*, we also examined the effect of PG-L-1 on reactive oxygen species (ROS) production by *C marina*, which is essential for the growth of this red tide phytoplankton.

MATERIALS AND METHODS

Bacteria strains and growth conditions. A marine bacterium, strain MS-02-063, was isolated from the coastal area of Omura Bay, Nagasaki, Japan (Nakashima et al. 2005b). The bacterial clone was cultured at 28°C for 40 h, with shaking, in a medium composed of 12.5 g yeast extract (Becton Dickinson), 12.5 g peptone (Becton Dickinson), and 30 g glucose l^{-1} of 50% artificial seawater. Since strain MS-02-063 is a slime bacterium, the method of viable bacterial cell counts on nutrient agar was unsuitable; therefore, the bacterial DNA was stained with the dye, 4,6-diamidino 2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich), and total cell number was determined as described by Porter & Feig (1980).

Red tide phytoplankton. *Chattonella marina* was generously provided by Kagoshima Prefectural Fisheries Experimental Station, Japan. An axenic culture of this algal strain was maintained at 26°C in Erd-Schreiber modified (ESM) medium (pH 8.2) under illumination from a fluorescent lamp (30 µmol photons m^{-2} s^{-1}) with a cycle of 12 h light and 12 h dark.

Preparation of PG-L-1. Pigment PG-L-1 produced by strain MS-02-063 was purified as described in Nakashima et al. (2005b). The maximal PG-L-1 production was achieved by incubation for 40 h at 28°C (Nakashima et al. 2006). The PG-L-1 was dissolved in methanol at a concentration of 10 mg ml^{-1} and stored in the dark at –20°C until use.

Mixed algal-bacterial cultures. Effects of strain MS-02-063 on *Chattonella marina* were examined by mixed algal–bacterial cultures. *C. marina* in logarithmic growth phase and strain MS-02-063 that was at the maximal PG-L-1 production (cultivation for 40 h) were diluted with ESM medium to densities of 2×10^4 and 2×10^7 cells ml^{-1}, respectively. Subsequently, strain MS-02-063 was added into ESM medium in serial 10-fold dilutions at bacterial cell densities ranging from 0 to 2×10^7 cells ml^{-1}. Aliquots (500 µl) of the strain MS-02-063 and *C. marina* cell suspension were dispensed in the wells of 24-well plates and then incubated at 26°C under light illumination at 30 µmol photons m^{-2} s^{-1} with a cycle of 12 h light and 12 h dark. After incubation for 24 h, the number of viable algal cells that were not lysed was determined with a haemacytometer at a magnification of ×100.

Algicidal effect of strain MS-02-063 on *Chattonella marina*. To test the algicidal effect of strain MS-02-063 on *C. marina* through direct cell-to-cell contact, the bacterial cells were harvested by centrifugation (10 000 × *g* for 10 min), and the pellet was vigorously washed 3 times with ESM medium. The washed bacterial cells were resuspended in ESM medium to a density of 2×10^5 cells ml^{-1}. The algicidal activity of washed bacterial cells was measured using a haemacytometer at a magnification of ×100.

Algicidal effect of PG-L-1 on *Chattonella marina*. To elucidate the algicidal activity of PG-L-1, 2 experiments that differed in exposure times of *C. marina* to PG-L-1 were performed. The stock solution of PG-L-1 was diluted 50-fold in ESM medium, and serial 2-fold dilutions in ESM medium were prepared. *C. marina* in the logarithmic growth phase was diluted in ESM medium to a final density of 1×10^4 cells ml^{-1} for a short exposure time or to 1×10^3 cells ml^{-1} for a long exposure time. Aliquots (500 µl) of PG-L-1 in ESM medium and the *C. marina* cell suspension were dispensed into the wells of 24-well plates. The final concentrations of PG-L-1 ranged from 100 to 0.1 µg ml^{-1}. After 1, 3, and 6 h or 1, 2, 3, 4, and 5 d of incubation, the algicidal activity of PG-L-1 was measured using a haemacytometer at a magnification of ×100.

Morphological change in *Chattonella marina*. PG-L-1, at a final concentration of 10 µg ml^{-1}, was added to the cell suspensions of *C. marina* (1×10^4 cells ml^{-1}) in the wells of a 12-well plate. The plates were incubated at 26°C for 24 h under the growth conditions described previously. After incubation, the *C. marina* cells treated with PG-L-1 were observed at a magnification of 400× using a Axiovent 200 Microscope (Carl Zeiss).

Chemiluminescence assay for detection of ROS. In the chemiluminescence analysis for the detection of ROS produced by *Chattonella marina*, we employed L-012 (Wako Chemical), which is a highly sensitive chemiluminescence probe for analyzing ROS (Imada et al. 1999). The L-012 was dissolved in distilled water and stored at –80°C. An aliquot of various concentrations of PG-L-1 or superoxide dismutase (SOD) at a final concentration of 10 U ml^{-1} in ESM medium were added to *C. marina* cell suspensions (final cell density from 1.5×10^3 to 5×10^4 cells $well^{-1}$) in wells of a 96-well white microplate (Dynex MicroliteTM 1; Dynex Technologies). The final concentrations of PG-L-1 ranged from 10 to 0.1 µg ml^{-1}. After incubation of the mixture for 30 s at 26°C, the reaction was started by the addition of 10 µl of L-012 solution (final concentration of 10 µM). During the incubation, chemiluminescence intensity of each well was recorded continuously for 2 min by using a multilabel recorder Mithras LB940 (Berthold Technologies).

Statistical analysis. Individual treatment groups were compared with appropriate controls using Dunnett's multiple comparison test. Data points were represented as triplicate means ± SD.

RESULTS

Algicidal activity of strain MS-02-063 against *Chattonella marina*

As shown in Fig. 1A, the suspension of strain MS-02-063 inhibited the growth of *Chattonella marina* in a density-dependent manner. In the presence of 1×10^5 cells ml^{-1} of strain MS-02-063, almost all *C. marina* cells died within 24 h. In contrast to the potent algicidal activity of the culture suspension, the algicidal activity against *C. marina* was significantly reduced in the bacterial cells that were vigorously washed with ESM medium (Fig. 1B).

Algicidal activity of PG-L-1 against *Chattonella marina*

As shown in Fig. 2, PG-L-1 had potent algicidal effects on *Chattonella marina* in a concentration-dependent manner. The LD_{50} value of PG-L-1 against *C. marina* was calculated to be approximately 8.5 µg ml^{-1} from the dose-response curve.

Morphological change in *Chattonella marina*

Chattonella marina has no rigid cellular structure and the algal cells often undergo morphological

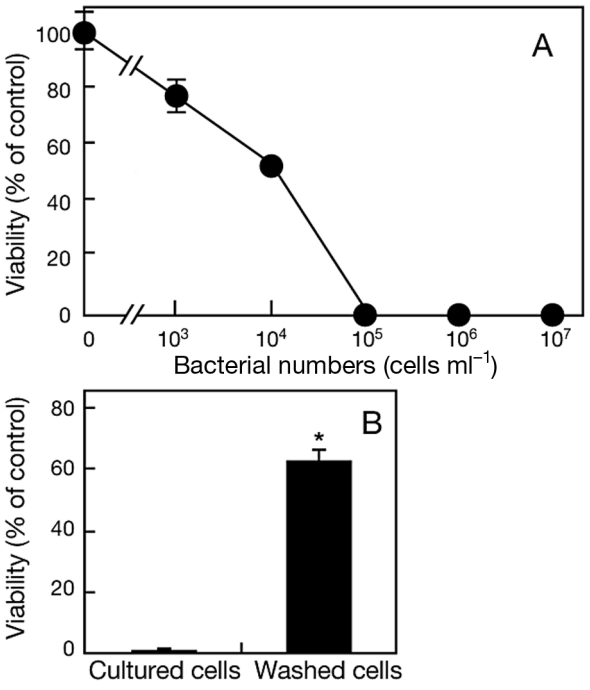

Fig. 1. *Chattonella marina*. Algicidal effect of strain MS-02-063. (A) *C. marina* cell suspension (1×10^4 cells ml^{-1}) in ESM (Erd-Schreiber modified) medium was mixed with various concentrations of strain MS-02-063 cells (0 to 10^7 cells ml^{-1}). Then the mixtures were cultured under normal culture conditions as described in 'Materials and methods'. After incubation for 24 h, the number of viable cells of *C. marina* were counted. (B) The bacterial cell suspension washed with ESM medium was added to *C. marina* cell suspension (1×10^5 cells ml^{-1}). Non-washed bacteria cell suspension (cultured cells) was used as a positive control. After incubation for 24 h the number of viable cells of *C. marina* were counted. Experiments were performed in triplicate. Error bars represent SD. Significant differences between the groups of washed and non-washed bacteria cell suspensions are indicated by *: $p < 0.01$

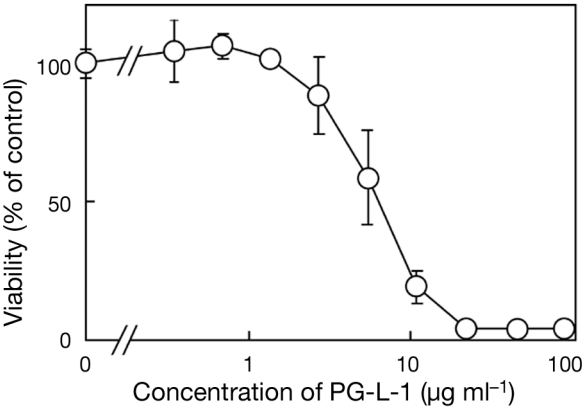

Fig. 2. *Chattonella marina*. Algicidal effect of PG-L-1. The indicated final concentrations of PG-L-1 were added to *C. marina* cell suspension (1×10^4 cells ml^{-1}). After incubation for 24 h, the number of viable cells of *C. marina* were counted. Experiments were performed in triplicate. Error bars represent SD

changes under unfavorable culture conditions or when this alga is exposed to exogenous toxic stress. In agreement with these findings, PG-L-1 (10 µg ml^{-1}) induced dramatic morphological changes in *C. marina* cells (Fig. 3B) that were easily distinguishable from normal cells (Fig. 3A). The morphologically changed cells were also observed at even lower concentrations (0.1 and 1 µg ml^{-1}; data not shown). In addition, the same kind of morphological changes were observed with the addition of the bacterial cell suspension but not with the addition of the washed bacterial cells (data not shown). These dimeric cells (Fig. 3B) may be attributed to the depression of cell division by PG-L-1. For comparison, normal cell division is shown in Fig. 3C. Although a few surviving *C. marina* cells that exhibited morphological changes were still swimming, their moving speed was abnormally decreased.

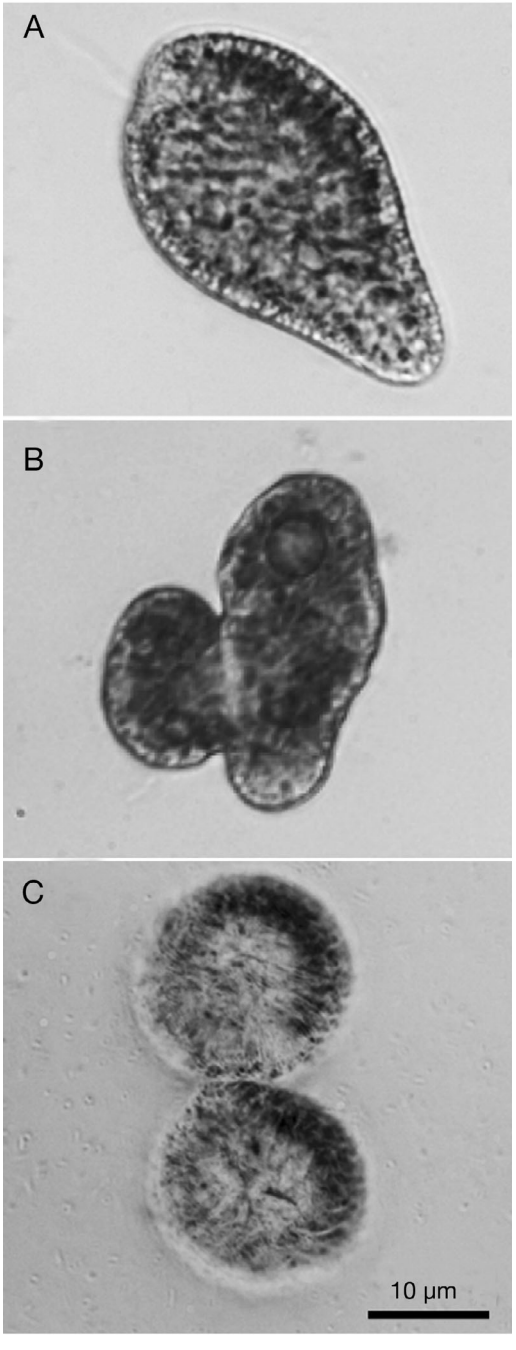

Fig. 3. *Chattonella marina*. Morphological change induced in *C. marina* after treatment with PG-L-1 (10 µg ml^{-1}) for 24 h. (A) Normal *C. marina* cell; (B) PG-L-1-treated *C. marina* cells; (C) normal cells during cell division

Kinetic analysis of algicidal activity of PG-L-1 against *Chattonella marina*

As shown in Fig. 4A, PG-L-1 had algicidal activity against *Chattonella marina* in a time-dependent manner. Almost all cells of *C. marina* were destroyed within 1 h in the presence of 100 µg ml^{-1} of PG-L-1. A gradual decrease in the viability of *C. marina* was observed at 10 µg ml^{-1} of PG-L-1, but no significant algicidal activity of PG-L-1 was shown at less than 1 µg ml^{-1} after 6 h of incubation. During the longer incubation time (1 to 5 d), cytostatic activity of PG-L-1 against *C. marina* was observed even at 0.1 µg ml^{-1}, and the increase in viable cell counts was completely suppressed in the presence of 1 µg ml^{-1} of PG-L-1 (Fig. 4B).

Effect of PG-L-1 on ROS production by *Chattonella marina*

Chattonella marina produces ROS under normal growth conditions without the addition of exogenous stimuli, suggesting that ROS production by *C. marina* is essential for its growth (Oda et al. 1995). Furthermore, we found in past studies that PG-L-1 had an inhibitory effect on ROS production by phorbol myristate acetate-stimulated inflammatory cell lines (Nakashima et al. 2005b,c). Therefore, we examined the inhibitory effect of PG-L-1 on ROS production by *C. marina*. As shown in Fig. 5A, the relationship between the cell number of *C. marina* and the chemiluminescence intensity that represented the ROS level showed good linearity (R^2 = 0.9519). The ROS production by *C. marina* was significantly reduced by the addition of SOD (10 U) ($p < 0.01$, Fig. 5B). In the SOD-inhibitable reduction system, PG-L-1 inhibited ROS production by *C. marina* in a concentration-dependent manner (Fig. 5B). In the presence of 10 µg ml^{-1} of PG-L-1, the chemiluminescence level by ROS was decreased to less than 50% of the control level.

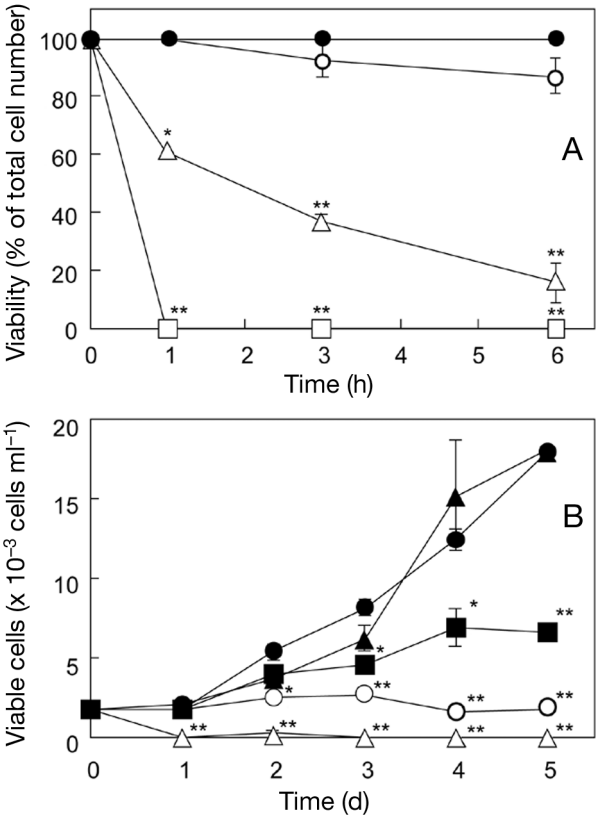

Fig. 4. *Chattonella marina*. Time-dependent algicidal activity of PG-L-1. (A) In short-term exposure experiments (0 to 6 h), final concentrations of 0 µg ml^{-1} (●), 1 µg ml^{-1} (○), 10 µg ml^{-1} (△), and 100 µg ml^{-1} (□) of PG-L-1 were added to *C. marina* cell suspensions (1×10^4 cells ml^{-1}). After incubation for the indicated periods of time, the number of viable cells of *C. marina* were counted. (B) In the long-term exposure experiments (0 to 5 d), final concentrations of 0 µg ml^{-1} (●), 0.01 µg ml^{-1} (▲), 0.1 µg ml^{-1} (■), 1 µg ml^{-1} (○), 10 µg ml^{-1} (△) of PG-L-1 were added to *C. marina* cell suspensions (1×10^3 cells ml^{-1}). After incubation for the indicated periods of time, the number of viable cells of *C. marina* were counted. Experiments were performed in triplicate. Error bars represent SD. Significant differences between the groups with and without PG-L-1 are indicated by *: $p < 0.05$; **: $p < 0.01$

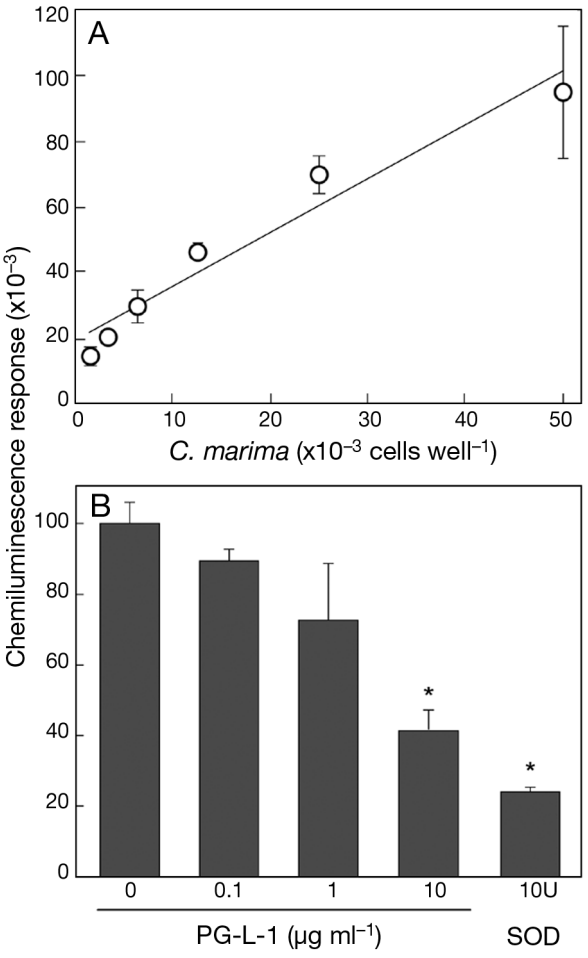

Fig. 5. *Chattonella marina*. Inhibitory effect of PG-L-1 on ROS production. (A) Relationship between the number of *C. marina* cells and chemiluminescence intensity. Various concentrations of *C. marina* were inoculated into each well of a 96-well white microplate. After the addition of chemiluminescence probe L-012 (10 µM) to each well, chemiluminescence intensity was measured immediately during the initial 2 min as described in 'Materials and methods'. (B) Various concentrations of PG-L-1 (0 to 10 µg ml^{-1}) or SOD (10 U ml^{-1}) were added to *C. marina* cell suspensions (5×10^4 cells well^{-1}) in each well of a 96-well white microplate. Value of the control was taken as 100%, and the data represented percent of the ratio of the values in the presence of PG-L-1 or SOD. Experiments were performed in triplicate. Significant differences between the groups with and without PG-L-1 or SOD are indicated by *: $p < 0.01$

DISCUSSION

Over the past few decades, HABs have tended to increase worldwide, which may be correlated partly with the deterioration of marine environments and global warming. The red tide events often lead to acute shellfish poisonings or mass mortalities of aquatic organisms (Friedman & Levin 2005). In Japan, red tide blooms, especially those due to *Chattonella* spp., have repeatedly caused fish mortalities (Yamamoto 2003). Therefore, control of red tide organisms or mitigation of the HABs impact is now one of the most important concerns forthe management of marine environments. Based on recently accumulating knowledge of the microbial community in natural marine environments (Imai et al. 2001, Iwata et al. 2004), applications of naturally occurring algicidal bacteria and viruses have been proposed as an effective and practicable strategy for the control of HABs. Several researchers have focused on isolation and characterization of bacteria with algicidal or growth inhibitory

activities against red tide phytoplankton. Thus, several bacteria capable of inhibiting the growth of certain phytoplanktons have been isolated from natural marine environments (Furuki & Kobayashi 1991, Fukami et al. 1992, Imai et al. 1993, Lovejoy et al. 1998, Seong-Yun et al. 2003, Iwata et al. 2004).

A number of bacteria with algicidal activities belong to the class of gammaproteobacteria and include *Pseudoalteromonas* sp., *Alteromonas* sp., *Shewanella* sp. and *Pseudomonas aeruginosa* (Lovejoy et al. 1998, Lee et al. 2000, Hare et al. 2005, Wang et al. 2005). Since the algicidal effects were detected in the cell-free culture supernatants of some of these bacteria, researchers have suggested that some marine bacteria, e.g. *Pseudoalteromonas* sp. strain Y, *Pseudomonas* sp. strain T827/2B, and *Flavobacterium* sp. strain 5N-3, indirectly attack red tide phytoplankton (Baker & Herson 1978, Fukami et al. 1992, Lovejoy et al. 1998). *Pseudoalteromonas* sp. strain Y (Lovejoy et al. 1998) and *Pseudomonas* sp. strain T827/2B (Baker & Herson 1978) killed the microalgae through heat-labile bacterial metabolic compounds having relatively high molecular weight. *Flavobacterium* sp. strain 5N-3 produced an algicidal compound with a low molecular weight (<500 Da) against the dinoflagellate *Gymnodinium nagasakiense* (Fukami et al. 1992). Nevertheless, few of these compounds have been identified, and the algicidal mechanisms are still unclear.

We found that strain MS-02-063 isolated from the coastal area of Omura Bay, Nagasaki, Japan had potent algicidal activity against *Chattonella marina*. However, the algicidal activity of bacterial cells washed with the planktonic medium was significantly reduced. Therefore, we concluded that extracellular substances produced by this bacterium are responsible for the algicidal activity, rather than strain MS-02-063 having an algicidal effect on *C. marina* through direct cell-to-cell contact. A biological characteristic of strain MS-02-063 is that this bacterium produces a large amount of a prodigiosin analogue, PG-L-1, as a secondary metabolic substance. The amount of the pigment produced by strain MS-02-063 in YPG broth was approximately 1.1 mg ml^{-1} after incubation for 48 h at 28°C (Nakashima et al. 2005b). Nakashima et al. (2006) showed that a cell density of $>10^5$ cells ml^{-1} was required to produce the pigment, suggesting that PG-L-1 produced by strain MS-02-063 is controlled by homoserine lactone quorum sensing. In that study, when this bacterium was treated with erythromycin, the red pigment production was inhibited and the algicidal activity against *Heterosigma akashiwo* was completely lost. Therefore, the results of this study together with those previous findings suggest that PG-L-1 is the main algicidal compound.

The pigment PG-L-1 had potent algicidal activity against *Chattonella marina* in a concentration-dependent manner, and almost all cells of *C. marina* were readily destroyed at 100 µg ml^{-1}. At concentrations around the LD_{50} values, PG-L-1 induced a characteristic morphological change in *C. marina* that seemed to inhibit cell division in *C. marina*. At low concentration ranges (0.1 to 1.0 µg ml^{-1}), PG-L-1 showed static growth-inhibitory activity against *C. marina*. The increase in the number of viable cells of *C. marina* was completely prevented in the presence of 1 µg ml^{-1} of PG-L-1 during the 5 d of incubation. These results suggested that the underlying toxic actions differ with concentrations of PG-L-1. Since PG-L-1 is hydrophobic, the prompt action of PG-L-1 at a high concentration may be attributed to the hydrophobic interaction between PG-L-1 and the cell membrane of *C. marina*. At low concentrations, PG-L-1 may suppress certain specific intracellular metabolic pathways in *C. marina*, resulting in the inhibition of cell division.

In our previous study, PG-L-1 showed potent cytotoxic activities against various cultured mammalian cell lines. We suggested that the cytotoxic mechanism of PG-L-1 is the induction of apoptotic cell death through activation of p38 mitogen-activated protein kinase in human myeloid leukemia (U937) cells (Nakashima et al. 2005c). Since PG-L-1 showed cytostatic activity against *Chattonella marina*, the cytotoxic mechanism of action of PG-L-1 against this raphidophyte may be different from that of mammalian cells.

Oda et al. 1998 showed that *Chattonella. marina* produced ROS such as superoxide anions, hydrogen peroxide, and hydroxyl radicals under normal growth conditions without specific triggers or stimuli. Although the biological significance of ROS production in *C. marina* is still unclear, it has been suggested that ROS produced by *C. marina* play essential roles as autocrine growth factors or as mitogenic stimuli in its own survival (Oda et al. 1995). During the course of searching for new aspects of biological activity of PG-L-1, we found that PG-L-1 potently inhibited ROS production by phorbol myristate acetate-stimulated mouse macrophage and human myeloid leukemia cell lines (Nakashima et al. 2005b,c). Similarly, sublethal concentration of PG-L-1 inhibited ROS production by *C. marina*. Although the underlying mechanisms of the inhibitory activity of PG-L-1 against *C. marina* are still unclear, these results together with previous findings suggest that cytostatic activity of PG-L-1 against *C. marina* may be caused, at least in part, by the inhibitory effect of PG-L-1 on ROS production by this raphidophyte. It was proposed that the ROS-producing system of *C. marina* is similar to NADPH oxidase, which is present in the plasma membrane of mammalian phagocytic cells (Kim et al. 2000). In prelimi-

nary studies, PG-L-1 led to the suppression of activation of NADPH oxidase in a macrophage cell line (data not shown). In addition, since no scavenging effect of PG-L-1 on ROS produced by hypoxanthine/xanthine oxidase system was observed (Nakashima et al. 2005c), PG-L-1 may directly act on NADPH oxidase-like enzyme in *C. marina*. Detailed analyses of the effects of PG-L-1 on both NADPH oxidase in *C. marina* and macrophage cell lines are now in progress.

Dinoflagellates such as *Cochlodinium polykrikoides* (Landsberg 2002) and *Karenia* (formerly *Gymnodinium*) *mikimotoi* (Yamasaki et al. 2004) are also known to produce ROS. Thus, it seems that ROS production by marine phytoplankton is not a rare phenomenon. In fact, PG-L-1 showed potent algicidal activity against these dinoflagellates (Nakashima et al. 2006). In addition, it has been reported that phlorotannins, which are antioxidants, isolated from the brown alga *Eisenia bicyclis* showed algicidal effect on dinoflagellates such as *C. polykrikoides* and *G. mikimotoi* (Nakamura et al. 1996, Nagayama et al. 2003). These findings suggest a possibility that scavenging or elimination of ROS lead to the inhibition of the growth of certain algal species, especially ROS-producing phytoplankton.

In conclusion, PG-L-1 may not only be a promising candidate for control of HABs, but also a useful experimental tool to analyse the biological mechanisms of ROS production. In addition, strain MS-02-063, as well as other algicidal bacteria, may play an important role in regulating microalgal biomass in natural marine environments.

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