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Producing mechanism of an algicidal compound against red tide phytoplankton in a marine bacterium *γ-proteobacterium*

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Abstract Strain MS-02-063, *γ-proteobacterium*, isolated from a coast area of Nagasaki, Japan, produced a red pigment which belongs to prodigiosin members. This pigment, PG-L-1, showed potent algicidal activity against various red tide phytoplanktons in a concentration-dependent manner. An understanding of a mechanism of PG-L-1 production by this marine bacterium may yield important new insights and strategies for preventing blooms of harmful flagellate algae in natural marine environments. Therefore, we analyzed the mechanisms of PG-L-1 production. In our previous study, the pigment production by this marine bacterium was completely inhibited at 1.56 μg/ml of erythromycin or 3.13 μg/ml of chloramphenicol, while minimal inhibitory concentrations for cell growth of erythromycin and chloramphenicol against this bacterium were >100 and 25 μg/ml, respectively. It is interesting to note that the ability of the pigment production in erythromycin-treated bacterium recovered by an addition of homoserine lactone. In fact, the pigment production was inhibited by β-cyclodextrin that inhibits autoinducer activities by a complex with *N*-acyl homoserine lactones. *N*-acyl homoserine lactones with autoinducer activities are ubiquitous bacterial signaling molecules that regulate gene expression in a cell density dependent process known as quorum sensing. Therefore, it was suggested that PG-L-1 produced by strain MS-02-063 is controlled by the homoserine lactone quorum sensing. It is speculated that this quorum sensing is involved in the production of algicidal agents of other marine bacteria. This bacterium and other algicidal bacteria might be concerned in regulating the blooms of harmful flagellate algae through the quorum sensing system.

Keywords Algicidal activity · Harmful algal bloom · Prodigiosin · *γ-proteobacterium* · Homoserine lactone

Abbreviations

N-AHL	*N*-acyl homoserine lactones
DAPI	4,6-Diamidino 2-phenylindole dihydrochloride
ESM	Erd–Schreiber modified
HABs	Harmful algal blooms
HPLC	High performance liquid chromatography
MIC	Minimal inhibitory concentration
YPG medium	Yeast extract-peptone-glucose medium

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Introduction

The mass mortality of marine living organisms due to harmful algal blooms (HABs) generally results in further pollutions of local and regional economies as well as loss of marine food resources. In the recent years, they appear to be more frequent and extensive throughout the world's coastal area (Anderson 1997; Hallengraeff 1993). Many studies on HABs problems have been conducted to eradicate massive blooms of harmful algal. For instance, monitoring of harmful algal cells and toxins (Sellner et al. 2003), satellite remote sensing (Chang et al. 2005), and application of chemical compounds such as copper sulfate (Steidinger 1993) and clays (Na et al. 1996). Thus, there are a number of strategies that can reduce or mitigate the impacts from HABs. One such strategy includes studies on bacteria with algicidal or inhibitory activities against red tide phytoplankton.

Several lines of evidence suggest that certain marine bacteria may play an important role in regulating microalgal biomass in natural marine environments (Furuki and Kobayashi 1991; Imai et al. 2001; Iwata et al. 2004). It has been reported that some marine bacteria selectively promote formation of algal bloom environments (Furuki and Kobayashi 1991), while other marine bacteria are able to inhibit the growth of red tide phytoplankton and are involved in the termination of algal bloom (Imai et al. 2001; Iwata et al. 2004). The latter findings have raised the possibility of bacterial control of HABs, and the algicidal bacteria could be useful tools for reducing the impacts of HABs. Algicidal bacteria inhibit algal growth effectively through direct attack that is required for cell-to-cell contact (Imai et al. 1993) or indirect attack by the production of extracellular compounds (Fukami et al. 1992; Imai et al. 1993; Wang et al. 2005). Although the extracellular algicidal compounds for red tide phytoplankton by marine bacteria are considered to be useful, the bacterial mechanisms activated to produce the extracellular compounds are largely unknown.

It has been reported that some γ-*proteobacteria* showed a broad spectrum of algicidal activity against bloom-forming red tide phytoplankton (Clinton et al. 2005; Lee et al. 2000; Lovejoy et al. 1998; Wang et al. 2005). We also isolated a member of the algicidal γ-*proteobacterium*, strain MS-02-063, that produced a prodigiosin member from the soil of coastal area of Omura Bay, Nagasaki, Japan (Nakashima et al. 2005a). In this study, we found that the prodigiosin pigment, PG-L-1, had algicidal activity against various red tide phytoplanktons. In addition to this marine bacterium, it has been reported that other marine bacteria such as *Serratia* sp., *Pseudoalteromonas bacteriolytica*, and *Vibrio ruber* also produce prodigiosin-like pigments (Lewis and Corpe 1964; Sawabe et al. 1998; Shieh et al. 2003). The production of prodigiosin in *Serratia* sp. is controlled by *N*-acyl homoserine lactone (AHL) quorum sensing. The complete prodigiosin biosynthetic gene cluster is located in *Serratia* chromosome (Thomson et al. 2000). In our previous study, the production of PG-L-1 by strain MS-02-063 was strongly inhibited by erythromycin and chloramphenicol at lower concentrations than the minimal inhibitory concentration (MIC) (Nakashima et al. 2005a). Both antibiotics are known to inhibit protein synthesis by binding to the 50S ribosomal subunit (Cundliffe and McQuillen 1976; Fernandez-Munoz et al. 1971). The objective of this study is to determine whether a *N*-AHL quorum sensing system is involved in the mechanism of pigment production in strain MS-02-063.

Materials and methods

Bacteria strain and growth conditions

A marine bacterium, strain MS-02-063, was isolated from Omura-Bay of Nagasaki, Japan and phylogenetically closely related to γ-*proteobacterium* MBIC 3957, however, there were difference in the physiological and biochemical properties between both bacteria (Nakashima et al. 2005a). Strain MS-02-063 was grown at 28 °C with shaking in culture medium which was composed of 12.5 g yeast extract, 12.5 g peptone, and 30 g glucose per 1 l of 50% artificial seawater (YPG medium). Because this strain was a slime-producing bacterium, the number of viable bacterial cells could not be determined from colony counts on nutrient agar. Therefore, the bacterial cell number was quantified from dry weights of 10 ml aliquots of strain MS-02-063 culture broth. Total cell count was made from preparations that were stained with dye 4,6-diamidino 2-phenylindole dihydrochloride (DAPI) as previously described (Kogure et al. 1979).

Red tide phytoplankton

Heterosigma akashiwo (NIES-6) was obtained from the National Institute for Environmental Studies, Japan. *Heterocapsa circularisquama*, *Gymnodinium impudicum*, and *Alexandrium tamarense* were provided by National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Japan. *Cochlodinium polykrikoides* was generously provided by Nagasaki Prefectural Fisheries Experimental Station, Japan. An axenic culture of each clonal strain of these algae except *A. tamarense* 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 as previously described (Oda et al. 1992). *A. tamarense* was cultured in modified SWM-3 medium (pH 8.0) (Itoh and Imai 1987) at 19 °C 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

The rate of PG-L-1 secretion from strain MS-02-063 was measured at 536 nm, which is a maximal absorption of this pigment (Nakashima et al. 2005a,b), with a U-2001 spectrophotometer (Hitachi Let., Tokyo, Japan). PG-L-1 production was confirmed by reversed-phase high performance liquid chromatography (HPLC) as modified methods described previously (Nakashima et al. 2005b). An ODS C-18 column (YMC-Pack Pro C18, 250×10 mm, 5 μm) was used with the following mobile phase; solution A was 0.1% trifluoroacetic acid, solution B was 100% methanol. After a 5-min flow with 100% solution A, a 20-min linear gradient to 100% solution B was run at a flow rate of 1 ml/min. Analysis of PG-L-1 was performed with 3-dimensional (3D) chromatogram. PG-L-1 was dissolved in methanol at a concentration of 10 mg/ml and stored in the dark at −20 °C until use.

Inhibitory effect of PG-L-1 on red tide phytoplankton

To determine the algicidal activities of PG-L-1 against red tide phytoplankton, *H. akashiwo*, *H. circularisquama*, *C. polykrikoides*, *G. impudicum*, and *A. tamarense*, the stock solution of PG-L-1 (10 mg/ml) was diluted 50-fold in ESM medium and serial twofold dilutions in ESM medium were prepared. Red tide phytoplankton in the logarithmic growth phase was diluted with ESM medium to a final density of 2×10^4 cells/ml. ESM medium (500 μl) with various concentrations of PG-L-1 were added to each tested algal cell suspension (500 μl) in the well of 24-well plate. The final concentrations of PG-L-1 used were 0.1 to 100 μg/ml. After incubation at 26 °C (at 19 °C for *A. tamarense*) for 24 h, the number of viable algal cells, which was not lysed nor destructed, was determined using a hemacytometer at a magnification of ×100.

Mixed algal-bacterial cultures

Effects of strain MS-02-063 on *H. akashiwo* were examined by using mixed algal–bacterial cultures. *H. akashiwo* in the logarithmic growth phase was diluted with ESM medium to a density of 2×10^4 cells/ml. In our previous study, the ability of strain MS-02-063 to produce pigment was completely inhibited by 10 μg/ml of erythromycin and chloramphenicol. Both of these concentrations were lower than the MIC values for this bacterium. The concentrations of penicillin G, vancomycin, and tetracycline necessary to inhibit pigment production were not less than the MIC values for these antibiotics (Nakashima et al. 2005a). Therefore, strain MS-02-063 was cultivated on YPG broth medium with or without 10 mg of erythromycin per liter. Strain MS-02-063 that was cultured with or without erythromycin diluted with ESM medium to 2×10^7 cells/ml. Subsequently, the diluted strains were incorporated into ESM medium in serial tenfold dilutions at final densities ranging from 1×10^7 to 10^3 cells/ml. Equal volume (0.5 ml) of the bacterial and algal cell suspensions were mixed and further incubated at 26 °C under 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 was determined as described above.

Autoinducer activities of quorum sensing in strain MS-02-063

To investigate whether a functional quorum sensing system is involved in the mechanism of pigment production by this bacterium, two experiments were performed. In the first experiment, strain MS-02-063 was inoculated with density of 1×10^5 cells/ml into YPG agar medium containing 10 μg of erythromycin per ml in petri dishes. Five microliters of various concentrations of L-homoserine lactone (0.5, 1, and 2 μmol) was spotted on the agar plate. After cultivation at 28 °C for 48 h, the change of color surrounding the spots was observed.

In the second experiment, the effect of β-cyclodextrin, which is known to inhibit autoinducer activities by a complex with *N*-AHL (Ikeda et al. 2002), on the pigment production was examined. The YPG agar medium without erythromycin inoculated with density of 1×10^5 cells/ml of the strain MS-02-063 was prepared, and then paper disks (∅ 8 mm, thick type, Advantec Toyo Kaisha, Tokyo, Japan) containing varying concentrations of β-cyclodextrin (2.5, 5, and 10 μM) were put on the YPG agar. The diameter of the nonpigment circle surrounding a paper disk was measured after incubation at 28 °C for 48 h.

Statistical analysis

Data points represent triplicate means±standard deviation. Statistical analysis was performed using the Student's *t* test for independent samples.

Result

Conditions of growth and production of red pigment in strain MS-02-063

Results of growth and pigment production in strain MS-02-063 in YPG broth are shown in Fig. 1. The optimal conditions of temperature and pH were attained at 28 °C

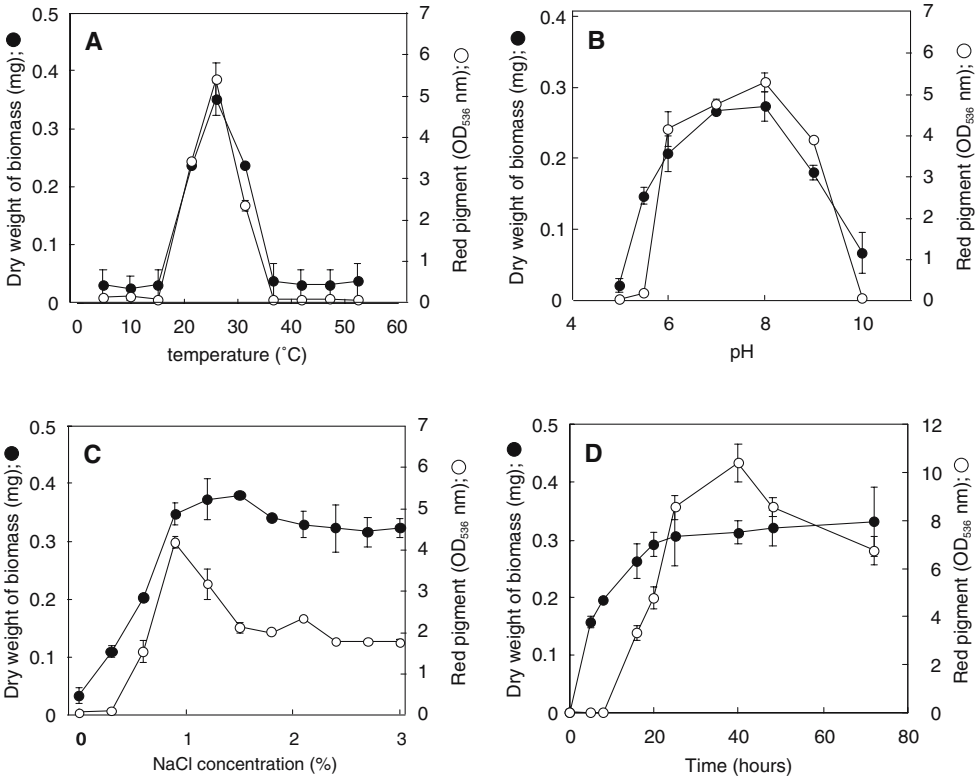

Fig. 1 Growth conditions of strain MS-02-063. **a** Optimal temperature of growth and pigment production on YPG broth in 50% artificial seawater adjusted to pH 7 for 24 h. **b** Optimal pH of growth and pigment production on YPG broth in 50% artificial seawater with various pH adjusted by NaOH or HCl at 28 °C for 24 h. **c** Optimal salt concentration of growth and pigment production on YPG broth adjusted to pH 8 with addition of NaCl at 28 °C for 24 h. **d** Time courses of growth and pigment production on YPG broth in 30% artificial seawater (about 1% as NaCl) adjusted to pH 8 at 28 °C. Experiments were performed in triplicate. *Error bars* represent standard deviation

(Fig. 1a) and pH 8 (Fig. 1b). This strain optimally produced PG-L-1 in medium containing 1% NaCl (Fig. 1c), but the pigment production decreased by half at a concentration of 1.5% NaCl and the bacterial growth was suppressed at >5% NaCl (data not shown). This strain produced the red pigment after cultivation at 28 °C for 10 h (pH 8). At this time, the cell count was 10^5 cells/ml and the dry weight of the biomass was 0.2 mg per 10 ml of culture broth. The bacterial growth and pigment production were achieved by incubation for 20 and 40 h, respectively (other conditions; 28 °C, pH 8, 30% seawater). The red pigment was confirmed to be PG-L-1 by 3D chromatogram in HPLC analysis (Nakashima et al. 2005b).

Algicidal activity of PG-L-1 against red tide phytoplankton

H. akashiwo, *H. circularisquama*, *G. impudicum*, *C. polykrikoides*, and *A. tamarense* exhibited similar dose responses to PG-L-1 in a concentration-dependent manner (data not shown). The LD_{50} and LD_{90} values of PG-L-1 against these red tide phytoplanktons were estimated to be 5.0–12.5 and 25.8–50.2 μg/ml, respectively (Table 1).

Effect of erythromycin on algicidal activity of strain MS-02-063

The algicidal effect of the strain MS-02-063 culture on *H. akashiwo* is shown in Fig. 2. This bacterium culture showed potent algicidal activity against *H. akashiwo*, and the minimum cell density of strain MS-02-063 required to kill 90% of *H. akashiwo* cells was 1×10^5 cells/ml within 24 h (Fig. 2). However, when the bacterium was treated with 10 μg/ml of erythromycin, the red pigment production was inhibited and the algicidal activity toward *H. akashiwo* was completely lost. Although both bacteria (erythromycin-treated and erythromycin-nontreated) were living cells in mixed culture for 24 h, the bacterial cell number did not increase after MS-02-063 was added to *H. akashiwo* (data not shown).

Table 1 Algicidal activity of PG-L-1 toward several red tide phytoplanktons

Phytoplankton	Algicidal activity (μg/ml)	
	LD_{50}	LD_{90}
Heterosigma akashiwo	5.0±0.0	38.6±5.3
Heterocapsa circularisquama	8.2±0.3	50.2±2.6
Cochlodinium polykrikoides	6.8±0.1	30.1±3.6
Gymnodinium impudicum	5.1±0.1	25.8±7.3
Alexandrium tamarense	12.5±0.4	48.3±6.8

Red tide phytoplankton was mixed with various concentrations of PG-L-1 and incubated at 26 °C (at 19 °C for *A. tamarense*) for 24 h. The algicidal activity of PG-L-1 was measured by visual counts of viable algal cells. The 50 and 90% lethal dose were calculated with the dose response curves of PG-L-1 toward each phytoplankton. Experiments were performed in triplicate. The values represent the mean±SD

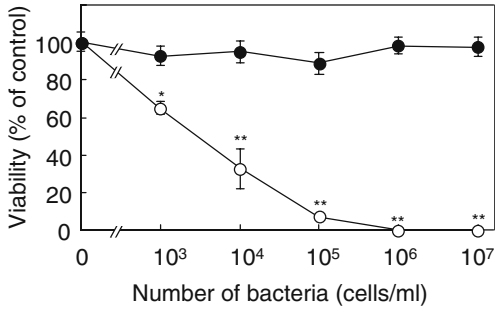

Fig. 2 Effect of erythromycin on algicidal activity of strain MS-02-063. Strain MS-02-063 culture was grown on YPG broth in 50% artificial seawater adjusted to pH 8 at 28 °C for 40 h in the presence (•) and absence (○) of 10 μg/ml of erythromycin. Strain MS-02-063 that was cultured with or without erythromycin was diluted with ESM medium for the growth of phytoplankton. Equal volume of *Heterosigma akashiwo* and the bacterial cell suspensions were mixed and further incubated at 26 °C for 24 h. Experiments were performed in triplicate. *Error bars* represent standard deviation. Significant differences between the groups with and without erythromycin are indicated by $*P<0.05$; $**P<0.01$

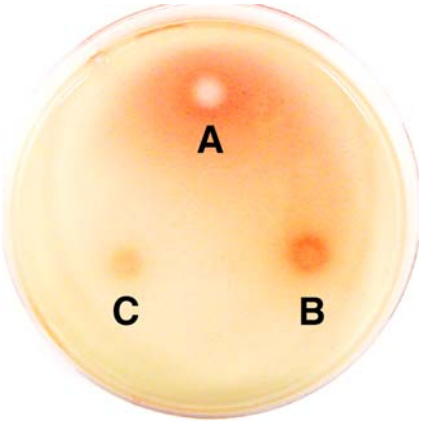

Fig. 3 Induction of PG-L-1 production in strain MS-02-063 by L-homoserine lactone. PG-L-1 production was inhibited by 10 μg/ml of erythromycin that was incorporated into the growth medium. The bacterium was inoculated with density of 1×10^5 cells/ml into the medium. Various concentrations of L-homoserine lactone (*A* 2.0 mM, *B* 1.0 mM, and *C* 0.5 mM) were spotted on the medium. After incubation at 28 °C for 48 h, the pigment production of this bacterium was visually observed. *White point* of *A* is an area that inhibited the growth of this bacterium by L-homoserine lactone

Involvement of *N*-acyl homoserine lactone quorum sensing in the production of PG-L-1

As erythromycin (3.13–100 μg/ml) completely inhibited the pigment production by strain MS-02-063 (Nakashima et al. 2005a), subeffective concentration of this antibiotic (10 μg/ml) was used for detection of *N*-AHL quorum sensing system as a new method. Although a high concentration of L-homoserine lactone (2 mM) partially inhibited the growth of strain MS-02-063, the pigment production was significantly induced by L-homoserine lactone in a concentration-dependent manner even in the presence of erythromycin (Fig. 3). The minimum induction concentration of L-homoserine lactone was 0.5 mM.

Recently, it was reported that cyclodextrin form a complex with *N*-acyl-homoserine lactone in the bacterial culture medium and show the effect on autoinducers' activities of quorum sensing (Ikeda et al. 2002). The clear pigment product inhibition by β-cyclodextrin was observed in the bacterium grown on YPG ′ manner (Table 2).

Discussion

Recent reports demonstrated that many marine bacteria have a significant algicidal effect on many species of red tide phytoplankton (Fukami et al. 1992; Imai et al. 2001, 1993; Iwata et al. 2004), and interactions between marine bacteria and phytoplankton have been found to play a major role in HABs dynamics (Furuki and Kobayashi 1991; Fukami et al. 1997). Some marine bacteria with algicidal activities were reported to belong to γ-*proteobacteria* (Holmstrom and Kjelleberg 1999; Mayali and Azam 2004). We isolated a marine bacterium belonging to the γ-*proteobacterium* group, which produced a prodigiosin-like pigment, from Omura-Bay, Nagasaki, Japan, where there have been HABs. The prodigiosin-like pigment, PG-L-1, showed potent algicidal effect on several red tide phytoplanktons (Table 1).

An understanding of the mechanism for production of algicidal compounds produced by marine bacteria may yield important new insights and strategies in natural marine environments. Therefore, we analyzed the mechanisms of PG-L-1 production by strain MS-02-063.

Seawater is weakly alkaline and the temperatures of seawater in Omura-Bay range from 26 to 28 °C during summer (Iizuka 1972). The seasonal conditions when HABs, such as red tides, have been occurring in frequency were correlated with the optimal conditions of growth or pigment production in strain MS-02-063. Therefore, this isolate may be also involved in the regulation of algal blooms as reported for other algicidal bacteria (Furuki and Kobayashi 1991; Fukami et al. 1997).

The amount of the pigment produced by strain MS-02-063 in YPG broth was approximately 1.1 mg/ml after 48 h at 28 °C (Nakashima et al. 2005a). The pigment was found to be a secondary metabolic substance (Fig. 1). As shown in Fig. 2, a cell density of $>10^5$ cells/ml was required to produce PG-L-1. This was also the minimum cell density that was required to kill 90% of the cells of *H. akashiwo*. The killing effect of PG-L-1 on *H. akashiwo* increased depending on the number of bacterial cells per milliliter, suggesting that the killing effect increases depending on the concentration of PG-L-1 produced by the strain MS-02-

Table 2 Inhibitory effect of β-cyclodextrin on PG-L-1 production in strain MS-02-063

Concentrations of β-cyclodextrin (μM per paper disk)	Diameter of prevention zone of producing pigment (mm)
2.5	8.5±0.1
5	10.2±0.4
10	12.4±0.3

The bacterium was inoculated with density of 1×10^5 cells/ml into the growth medium. Paper disks containing various concentrations of β-cyclodextrin were put on the medium. After incubation at 28 °C for 48 h, the zones of pigment product inhibition were measured. Experiments were performed in triplicate. The values represent the mean±SD. Paper disk diameter: ∅ 8 mm

063. Moreover, the production of PG-L-1 was inhibited by erythromycin, resulting in the loss of the algicidal activity for strain MS-02-063 (Fig. 2).

The bacterial mechanisms that activate to produce algicidal compounds are largely unknown. Chemicals that are produced by Gram-negative bacteria such as acetylated homoserine lactones can be used to regulate production of secondary metabolic substances and communicate among bacteria (Bassler et al. 1993; Riedel et al. 2001). It is interesting to note that the bacterium treated with L-homoserine lactone recovered from the loss in the ability of the production of PG-L-1 by erythromycin, an inhibitor of protein synthesis (Fig. 3). Moreover, the pigment production was inhibited by β-cyclodextrin, an inhibitor of *N*-AHL (Table 2).

It has been reported that algicidal or inhibitory activity was not activated via AHL, but bacterial quorum sensing for algicidal bacteria was shown by means of the auto-inducer (AI)-2 mechanism (Skerratt et al. 2002). However, the results in this study strongly suggest that PG-L-1 production by strain MS-02-063 is controlled by *N*-AHL quorum sensing, in which the signal is a bacterial intercellular communication device for controlling gene expression in response to population density (Fuqua et al. 1994; Salmond et al. 1995; Swift et al. 1996). Quorum sensing systems generally consist of three genes; the I-gene codes for an AI synthetic enzyme, the R-gene codes for a transcriptional activator and the target genes. *N*-AHL is synthesized by the AI synthetic enzyme, and then the complex between *N*-AHL and the transcriptional activator is formed. Subsequently, the complex connects with the transcriptional control region of the target genes (Fuqua et al. 1994; Salmond et al. 1995; Swift et al. 1996). Thus, it seems likely that protein synthesis inhibition by erythromycin that binding to the 50S ribosomal subunit leads to the arrest of quorum sensing-related gene expression which results in suppression of pigment production. Prodigiosin pigments are produced by numerous bacteria, including marine bacteria, as secondary metabolic substance. Moreover, *Pseudoalteromonas* sp. strain A28 which belongs to the *γ-proteobacterium* as does strain MS-02-063, has been reported to lyse marine algae (Lee et al. 2000). It has been proposed that the components responsible for expression of this lytic activity are regulated by *N*-AHL quorum sensing (Kato et al. 1999).

Hahella chejuensis, which is closely related to strain MS-02-063, produced extracellular polysaccharides (Lee et al. 2001). Strain MS-02-063 also produced abundant extracellular polysaccharides and was a slime bacterium (data not shown). These strains may be able to form communities of microorganisms attached to a solid surface such as biofilms. It is speculated that biofilm formation is a favorable condition of pigment production for this bacterium in natural environments. Although the relationship between this bacterium and other marine bacteria is not clear, the *N*-AHL quorum sensing system may be used for cell-to-cell communication among bacteria, resulting in the regulation of blooms of harmful flagellate algae in natural marine environments.

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