細菌由来のChlamydomonas reinhardtiiおよびAlexandrium catenella接合阻害物質

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Bacterial Inhibitor for the Mating Reaction in *Chlamydomonas reinhardtii* and *Alexandrium catenella*

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Sexual agglutination and zygospore formation of *Chlamydomonas reinhardtii*, a heterothallic organism, were used for screening of mating inhibitors in culture supernatants and cell extracts of 150 bacterial strains. Both fractions of a gram-negative bacterium, strain NT4, inhibited the mating reaction. When algal vegetative cells were treated with the mating inhibitor, both sexual agglutination and zygospore formation were inhibited. However, when mature gametes were treated with the inhibitor, zygospore formation was inhibited but sexual agglutination was not. The mating inhibitor also blocked hypnozygote formation of a toxic dinoflagellate, *Alexandrium catenella*, at a concentration 128 times as high as that of *C. reinhardtii*.

Introduction

Sexual reproduction including zygote (cyst) formation have been shown for bloom forming microalgae, especially for dinoflagellates,[1] and their cysts are thought to provide the seed population for their blooms. Inhibition of sexual reproduction by bacteria, might therefore be a discreet method to repress the occurrence of hazardous algal blooms. As an assay organism for testing the inhibitory activity, the following three points are important: a high percentage of sexual reproduction, precise experimental conditions for induction of mating reaction, and synchronous mating reaction. However, most bloom algae have not been found to clearly exhibit the three conditions mentioned above.

Therefore, we used the mating reaction of *Chlamydomonas reinhardtii*, a unicellular green alga, as a first screening assay, because the sexual reproduction of this species is well established. *C. reinhardtii* has been studied biochemically and genetically[2] as a model of cell-cell recognition and sexual differentiation. There are two sexual mating types, plus (mt+) and minus (*mt*−). Under nitrogen starvation, vegetative cells of mt+ and mt− differentiate to their respective gametes.[3] When gametes of opposite mating type are mixed, gametes recognize the opposite mating type by specific agglutinins on the surface of their flagella.[4,5] Gametes produce agglutinins during sexual differentiation, while vegetative cells do not have any agglutinins. Agglutinins from both mating types were purified and found to be high molecular weight glycoproteins.[6,7] After removing a cell pair from a agglutinated clump, cell walls are shed and the naked cells rapidly fuse[8] and form a zygospore.[9]

A typical toxic dinoflagellate, *Alexandrium* (formerly *Protogonyaulax*) *catenella*, has been shown to be heterothallic and its mating reaction starts after mixing opposite mating types.[10] However, only poor mating efficiency was observed and the laboratory conditions and mechanism of its sexual reproduction has not been clarified yet.[11,12]

In this paper we screened bacterial inhibitors against the mating reaction of *C. reinhardtii* and examined the effects of mating inhibitors on the mating reactions in *C. reinhardtii* and *A. catenella*.

Materials and Methods

Organisms and Bioassays

Two sexual mating types (mt+ and mt−) of *C. reinhardtii* strain 137c[13] were used on the bioassay for screening of mating inhibitors. Standard cultures were maintained at 25°C under continuous light (5,000 lx) in TAP-medium.[14] When vegetative cells of mt+ and mt− reached to a concentration of $1\text{–}2\times10^6$ cells·ml^{-1}, the cells were harvested by centrifugation ($550\times g$, 5 min) and inoculated into a nitrogen deficient medium.[8] Gamete suspensions (100 μl) of mt+ and mt− were put into wells of microplates (96 wells, Nunc, Denmark), respectively, and bacterial samples (2–

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10 μl) were added to the wells. After 24 h incubation, the suspensions (50 μl) of mt+ and mt− treated with bacterial samples were mixed in another well. Whether sexual agglutination and zygospore formation occurred or not were observed under a inverted microscope. The inhibitory activity was determined by two-fold serial dilutions of samples in the bioassay. A mating inhibitory activity which inhibits the sexual agglutination of 10^6 cells·ml^{-1} in the standard mating assay of *C. reinhardtii* was defined as 1 unit.

Gonium pectorale, a heterothallic Volvocales, was also used for testing the mating inhibitory activity. Strain N4–4 (mating type +) and N4–3 (mating type −) were isolated from Nepal by S. Saito and grown in M6 medium.[15] Culture conditions and mating assay procedures were the same as that of *C. reinhardtii* except that gametogenesis was induced in MT medium.[15]

Alexandrium catenella TN 22 (mating type +) and TN 7 (mating type −) isolated from Tanabe Bay, Japan (1987), were maintained at 20°C on 16 h light and 8 h dark cycles, at 5000 lx in ESJ medium (ES+Jarmarin S).[16] Each 100 μl (2–5×10^4 cells·ml^{-1}) of late logarithmic phase cells of the both strains were mixed in a well of a microplate with a mating inhibitor (MI), which was partially purified from bacterial strain NT 4. After 2–3 weeks incubation, the number of hypnozygotes in the well was counted with a inverted microscope. The experiments were achieved with triplicate. Effect of MI on cell growth of *A. catenella* in test tubes was duplicately estimated by chlorophyl measurement (Turner, Fluorometer model 110). Inhibition ratio of hypnozygote formation and growth by MI were calculated using the following formula:

$$\text{Inhibition ratio of hypnozygote formation} = \left(1-\frac{\text{Hypnozygote number in a treated well}}{\text{Hypnozygote number in a untreated well}}\right)\times 100$$

$$\text{Inhibition ratio of growth} = \left(1-\frac{\text{Chlorophyl quantity of a treated culture}}{\text{Chlorophyl quantity of a untreated culture}}\right)\times 100$$

Bacterial Strains and Culture

One hundred fifty bacteria isolated from the surface sediment soil of high latitude lakes (about 5000 m), Lake Tsumoso and Pond Ongre, in Nepal were used for screening. Bacteria were grown at 30°C while being shaken (220 rpm) in test tubes containing 10 ml liquid medium. The medium consisted of peptone (Daigo Co., Osaka, Japan) 5 g and yeast extract (Nakarai tesque. Inc., Kyoto, Japan) 0.5 g in 1 l of tap water. The cells in the early stationary phase were harvested by centrifugation (4500×g, 10 min) in order to obtain the culture supernatant. The cells were disrupted in deionized distilled water by ultrasonication (UR200-P, Tomy Seiko Co., Japan) at 0°C. The suspension was centrifuged at 4500× g for 10 min at 4°C. Mating inhibitory activity of culture supernatants and cell extracts of bacteria were assayed under the standard bioassay conditions as mentioned above.

Ammonical nitrogen in bacterial cultures was determined by titlation with 0.005 N H_2SO_4 after steam-distillation.[17]

Partial Purification of MI

Culture supernatant of strain NT 4 was used for the purification of MI. Strain NT 4 was grown at 30°C for 48 h while being shaken (220 rpm) in a 2 l flask containing 0.5 l of the liquid medium. The supernatant was applied to Amberlite IRC-50 (Rhom and Haas, U.S.A.) H^+ form. After washing the resin with distilled water, MI was eluted with 1 N NH_4OH. Active fractions were evapolated at 30°C under reduced pressure. Portions of the active fractions from Amberlite IRC-50 were ultrafiltrated employing membranes (Amicon YM2 and YM5, 1000 and 5000 dalton cut-off, respectively) under pressure of 4 kg·cm^{-2}. Fractions retained on the membranes were suspended in deionized distilled water.

Effects of MI on the Mating Reaction and Growth of C. reinhardtii

When the cell density of *C. reinhardtii* cultures reached $1–2\times10^6$ cells·ml^{-1}, vegetative cells were treated with MI at the concentrations of 0.5, 1, 2, and 4 units·ml^{-1}. After 5 min, treated cells were centrifuged at 550×g for 5 min. The cells were suspended in nitrogen-free medium and 10 h later they were mixed with untreated opposite mating type gametes. Agglutination and zygospore formation were observed under a inverted microscope at 10–20 min and at 24 h later from mixing, respectively. MI treatment was also performed 1 and 10 h after introduction to nitrogen-free medium.

Portions of the vegetative culture treated with MI for 5 min at the concentrations of 2 or 4 units·ml^{-1} were re-suspended in TAP medium to de-

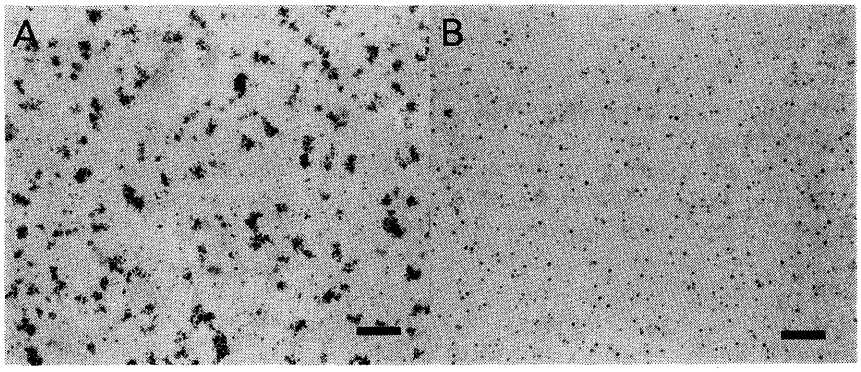

Fig. 1. Inhibition of sexual agglutination by the culture supernatant of strain NT4. A: Untreated mt+ and mt− gametes mixture. **B**: mt+ and mt− gametes mixture treated with the culture supernatant of strain NT4 at a concentration of 20 $\mu l \cdot ml^{-1}$, respectively, at early stage of differentiation. Bars represent 50 μm.

termine the effect of MI on the growth of cells. Gametogenesis periods, which began at nitrogen starvation, of 1 and 10 h were also employed. Growth of cells was measured by absorbance at 660 nm.

Results

Screening for Bacterial Strains Inhibiting the Mating Reaction of C. reinhardtii

Among tested bacterial strains, culture supernatants of three strains, strain NT4 isolated from Lake Tsumoso, and strains NO4 and NO9 isolated from Pond Ongre, inhibited the mating reaction of *C. reinhardtii*. The cell extract of strain NT4, a gram negative bacterium, also strongly inhibited sexual agglutination and zygospore formation. When either mt+ or mt− induced cell suspensions of 1 m*l* were pre-treated with 20 μl of the culture supernatant of strain NT4, they did not display sexual agglutination (Fig. 1).

The growth and change of the mating inhibitory activity of NT4 with culture period are shown in Fig. 2. The bacterium aerobically grew very well in the liquid medium containing peptone and yeast extract. MI activity increased during logarithmic growth phase. The maximum mating inhibitory activity was 0.4 unit $\cdot \mu l^{-1}$ of the culture supernatant.

As for strains NO4 and NO9, at least 100 μl of their culture supernatants was required to inhibit the mating reaction of 1 m*l* *C. reinhardtii* cells, indicating that activities of the two strains were weaker than that of strain NT4. Even at the inhibitory concentration, cells treated with the culture supernatants of strains NO4 and NO9 gradually formed zygote. Ammonium concentrations of strains NO4 and NO9 cultures were 18 and 11 mM, respectively. Gametogenesis of *C. reinhardtii* were prevented by NH_4Cl, arginine, glutamine, histidine, at a concentration of 5, 25, 5 and 5 mM. respectively.

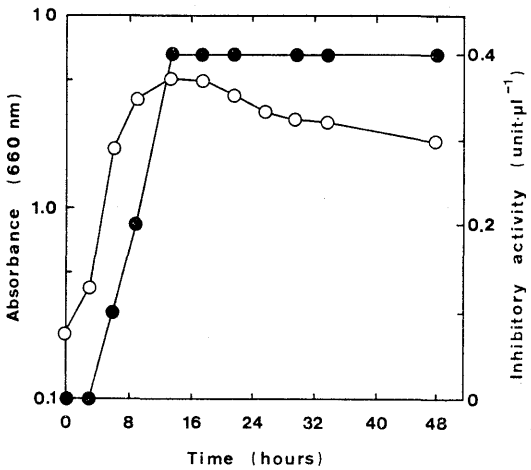

Fig. 2. Mating inhibitory activity in the culture supernatant of strain NT4 and the cell growth. Inhibitory activity (●) was determined by the mating assay. Growth of strain NT4 (○) was measured by absorbance at 660 nm.

Partial Purification of MI

MI was not heat denatured (100°C, 10 min) and freezing also did not affect its activity. Mating inhibitory activity was absorbed with Amberlite IRC-50 in water and eluted with 1 N NH_4OH. The recovery of this procedure was about 90%.

In the ultrafiltration with the membrane for M.W. 5000 dalton, mating inhibitory activity was recognized in the filtrate. The activity in the fraction less than 1000 dalton was as strong as that

in the higher fraction.

Effects of MI on C. reinhardtii and G. pectorale

Sexual agglutination and zygospore formation were determined when either mt+ or mt− was treated with MI before or after induction of gametes, as shown in Table 1. When vegetative cells and immature gametes (gametogenesis period of 1 h) were treated with MI at a concentration of 2–4 units·ml^{-1}, sexual agglutination and zygospore formation did not occur. However, when mature gametes (gametogenesis period of 10 h) were treated with MI at a concentration of 2–4 units·ml^{-1}, sexual agglutination occurred normally, but zygospore formation was inhibited. Difference in inhibitory effects between mt+ and mt− was not recognized, except for the case of 1 unit·ml^{-1} MI treatment at a gametogenesis period of 1 h.

Vegetative cells of mt+ treated with MI for 5 min at a concentration of 2 or 4 units·ml^{-1} grew approximately the same as untreated cells (Fig. 3A). When gametes were treated with MI at the concentrations of 2 and 4 units·ml^{-1} at gametogenesis periods of 1 and 10 h and resuspended in TAP medium, the cells showed one and three days longer lag period, respectively, than that of untreated gametes. (Fig. 3B, C). Mt− cells showed a little longer lag period than that of mt+ on the same treatment (data are not shown).

Without removal of MI, vegetative cells of mt+ and mt− maintained their motility but lost their growth ability at a concentration of 1 unit·ml^{-1}.

In a case of *G. pectorale*, a mating inhibitory concentration of MI was a quarter of that of *C. reinhardtii*.

Effect of MI on A. catenella

Ninety eight percent and 79% inhibition of hypnozygote formation of *A. catenella* were observed in the presence 128 and 64 units·ml^{-1} of MI, respectively (Fig. 4). Cell growth of algal strain TN22 and TN7 with 128 units·ml^{-1} of MI only reduced by 35% and 7%, respectively.

Discussion

It was found that three bacterial strains had mating inhibitory activities in their culture supernatants by the mating inhibitory bioassay with *C. reinhardtii*. In particular the culture supernatant and the cell extract of strain NT4 strongly inhibited the mating reaction. The inhibitor of strain NT4 also inhibited hypnozygote formation of *A. catenella*, but the inhibitory concentration was 128 times as high as that of *C. reinhardtii*. Vegetative cell growth of *A. catenella* was little affected by MI. From these results, the mating assay of *C. reinhardtii* can be used for the screening of mating inhibitors against *A. catenella* and to quantify an activity of mating inhibitors in the process of their purification. Comparing the sensitivity of the mating reactions to MI, the freshwater microalgae, *C. reinhardtii* and *G. pectorale*, are seen to be more sensitive than the marine dinoflagellate. It is not clear that this result is derived from the difference in their salt requirement or in taxonomical status.

Table 1. Effects of MI on sexual agglutination and zygospore formation of *C. reinhardtii*

Mating type	MI concentration (units·ml^{-1})	Sexual agglutination			Zygospore formation		
		Time[a]			Time		
		0 h	1 h	10 h	0 h	1 h	10 h
(+)	0	−[b]	−	−	−	−	−
	0.5	−	−	−	−	−	−
	1	−	+	−	−	+	−
	2	+	+	−	+	+	+
	4	+	+	−	+	+	+
(−)	0	−	−	−	−	−	−
	0.5	−	−	−	−	−	−
	1	−	−	−	−	−	−
	2	+	+	−	+	+	+
	4	+	+	−	+	+	+

Cells were treated with MI for 5 min at three different stages in gamete induction process. The total induction time was 10 h.

[a]: Time from induction to nitrogen-free medium to treatment with MI. Cells at 0, 1 and 10 h denote vegetative cell, immature and mature gamete stages, respectively.

[b]: +, Sexual agglutination or zygospore formation was not observed; −, Sexual agglutination or zygospore formation was observed.

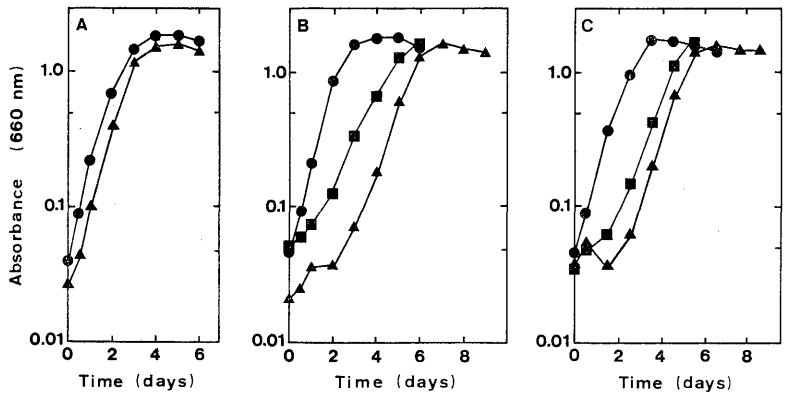

Fig. 3. Growth of *C. reinhardtii* (mt+) treated with MI at three different stages. Cell concentrations after transfer into TAP medium were measured by the absorbance at 660 nm. Growth curves of cells treated with MI at a concentration of 4 units·ml^{-1} for 5 min (▲), and 2 units·ml^{-1} for 5 min (■). Growth curves of untreated cells (●). **A**: Vegetative cells. **B**: Immature gametes (treated with MI at gametogenesis period 1 h). **C**: Mature gametes (treated with MI at gametogenesis period 10 h).

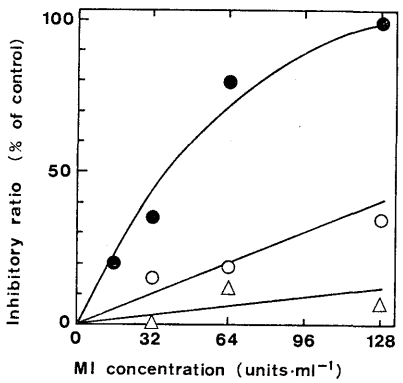

Fig. 4. Effects of MI on hypnozygote formation and growth of *A. catenella*. (●), inhibition ratio (% control) of hypnozygote formation by MI at various concentrations. Inhibition ratio (% control) of vegetative cell growth, TN22 (○) and TN 7 (△).

Vegetative cells and immature gametes of *C. reinhardtii* treated with MI did not show the mating reactions, and mature gametes treated with MI after gametogenesis was able to do sexual agglutination but not zygospore formation. These suggest that agglutinability of gametes was prevented indirectly by MI; probably through inhibition of agglutinin synthesis and that zygote formation itself was also affected by MI. However, thinking about the growth inhibitory experiments, cells at gametogenesis period seemed to be more sensitive to MI than vegetative cells, since the longer lag periods indicated an extent of the cell damage due to the treatment of MI. Dedifferentiation from gamete to vegetative cell might be more or less blocked.

The effect of MI on *C. reinhardtii* was similar to those of streptomycin and cycloheximide, which blocked gametogenesis through protein synthesis inhibition.[18,19] MI prevented sexual agglutination and fusion of *C. reinhardtii*, as well as tunicamycin (TM)[20] and dithiothreitol (DTT).[21] However, MI inhibited agglutinabilities of both mating type, while TM or DTT showed the inhibitory effect only on mt+ gamete. MT is an inhibitor of glycosylation of proteins, and DTT reduces disulfide bonds of agglutinin.[22]

Since both the culture supernatant and the cell extract of strain NT4 have mating inhibitory activity, strain NT4 probably produced MI and secreted it out of the cell. Total activity of the culture supernatant of strain NT4 in the early stationary phase was 1.5 folds as high as that of the cell extract. The results of ultrafiltration suggests a molecular weight of MI was about 1000 dalton.

Culture supernatants of strains NO4 and NO9 showed a low inhibitory effect. High concentrations of ammonical nitrogen as well as other organic nitrogen sources in the fractions might deterred *C. reinhardtii* from differentiation.[3]

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