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The Feasibility Study of Field Application on Lab Scale and Community Dynamics Associated with Algal Culture

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1. Introduction

Bacteria have the potential to act as biological control agents for HABs and there are tons of intriguing examples of killing bacteria having strong algicidal activities. Most algicidal bacteria are isolated from the ocean and have the advantage of being a native species already present in the HAB species' environment. Although the use of algicidal bacteria to control HABs may have great potential, there are significant environmental concerns that must be first addressed. First of all, the specificity and dynamics of bacteria and algal interactions have to be evaluated. Bacteria can have the potential to be highly specific and effective control agents. Since they are host-specific, suggesting that only harmful algal species are targeted, co-occurring organisms should not be affected. In reality, however, algicidal bacteria attack other organisms which are beneficial and important in the maintenance of the ecosystem. Therefore, the environmental impacts of any non-indigenous organism used in biological control also have to be assessed. This is mainly the reason there are still negative opinions to the concept of releasing one organism to treat another in the environment. The introduction of bacterial species in the environment possesses unknown risks and may cause irreversible damage. Second, we have to assess the viability and algicidal activity of algicidal bacteria when they are introduced to the environment. If they survive and display algicidal activity efficiently in the algal culture, the algicidal bacteria can play an important role in mitigating HABs blooms in the ocean ecosystem. In this study, the DGGE method has been used to monitor changes in the algae-associated microbial community over time. DGGE analysis was also used to identify the bacterial community. The main purpose of this study is to document the possibility of practical field application of algicidal bacteria. In order to do so, firstly, the viability of *M. luteus* SY-13 in the algal culture which is similar to the ocean environment was checked using PCR-DGGE. After introducing *M. luteus* SY-13 into the algal culture, the algicidal activity was evaluated using direct algal cell counting. Additionally, the

predominant bacterial community structure and diversity and the effect of *M. luteus* SY-13 to the bacterial community were assessed in the *C. polykrikoides* culture using PCR-DGGE fingerprinting and phylogenetic analysis.

2. Materials and Methods

2.1. Cultivation of *C. polykrikoides*

C. polykrikoides, the harmful dinoflagellate used in this study, was isolated from Kamak Bay in mid-September 2002. *C. polykrikoides* cultures were routinely maintained in f/2-Si medium made of GF/F-filtered seawater. The cultures were grown in disposable sterilized tissue culture flasks (Iwaki Inc., Chiba, Japan) under an illumination of 120$\mu Em^{-2}s^{-1}$and a 12h light : 12h dark cycle at 20°C.

2.2. Addition of *M. luteus* SY-13 to *C. polykrikoides* culture

M. luteus SY-13 had a negative effect on the growth of *C. polykrikoides* in the preliminary experiments. The bacteria incubated under optimal condition to the late exponential phase (approximately 24 hrs) were transferred to new sterile ZoBell 2216E medium and incubated at 25℃ until mid to late exponential phase before being harvested (approximately 12 hrs). The culture was then centrifuged for 20 min at 5,000 g and rinsed with sterile f/2-Si medium three times, followed by re-centrifugation to harvest it. Bacteria were added in triplicate to 50 ml of *C. polykrikoides* culture at a concentration of approximately $10^3$and 10^6cells ml^{-1} in the final incubation volume. An equal volume of sterile f/2-Si medium was added to the controls. Culture were incubated at optimal culture conditions of C. polykrikoides (under an illumination of 120 $\mu Em^{-2}s^{-1}$and a 12h light : 12h dark cycle at 20°C) for 12 or 16 days. Samples for algal cell count were preserved on Days 0, 2, 4, 6, 8, 10, 12, 14, and 16. Also, samples for bacterial community analysis were collected on Days 0, 2, 4, 6, 8, 10, 12, 14, and 16.

2.3. DNA extraction

Collected samples were filtered through the 0.2 ㎛ pore-size filters before bacterial DNA extraction. Bacterial community DNA was extracted from the filtered samples using Fast DNA SPIN Kit, as recommended by the manufacturer (Bio 101, Vista, CA). DNA extracts were examined by agarose (0.8%w/v) gelelectrophoresis and the resulting DNA extracts were used as the template for PCR.

2.4. PCR amplification of 16S rDNA fragments for DGGE

Amplification of bacterial 16S rRNA genes from the extracted DNA was performed using the following primer combinations in polymerase chain reaction (PCR) with 27F (5′

-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′- GATTACCTTGTTACGACTT-3′). For bacterial community analysis using denaturing gradient gel electrophoresis (DGGE), the DNA extracts were amplified with 341F-GC (5′-CGCCCGCCGCGCGC GGCGGGCGGGGCGGGGGCACGGGGGCCTACGGGAGGCAGCAG-3′) and 518R (5′-ATTACCGCGGCTGCT-3′) primer pair as described.

2.5. PCR-DGGE analysis of bacterial diversity

Samples were collected on Days 0, 2, 4, 6, 8, 10, 12, 14, and 16 to evaluate the diversity of the bacterial community in control and bacterial addition cultures of *C. polykrikoides* growth together with *M. luteus* SY-13 and *Janibacter brevis* SY-47 using PCR-DGGE.

3. Results and Discussion

In this study, we investigated how the initial algal cell and bacterial cell concentrations affect algicidal activity. The effect of initial algal cell concentrations revealed that the algicidal effect was greater when the concentration was higher than the low concentration treatments. Our results also indicated that the higher initial algicidal bacteria killed *C. polykrikoides* significantly faster than the lower initial inoculation of algicidal bacteria in the high concentration of algal cells. However, in low initial algal concentrations, there were no significant differences between the low initial algicidal bacteria concentration treatments. From the result, we assumed that algicidal bacterium *M. luteus* SY-13 must reach a certain concentration level to activate algicidal activity and the number of initial algal cell strongly influences the algicidal activity. Bacterial community dynamics associated with *C. polykrikoides* cultures were also analyzed. Since the most algicidal activity was present in high algae and high bacteria treatment, we analyzed the bacterial community after inoculation of algicidal bacteria *M. luteus* SY-13. PCR-DGGE profiles showed dramatic changes in the microbial community structure. The bands from the DGGE profile were cut and analyzed. With time, the number and composition of the dominants bands changed. However, some bands remained as dominant from Day 0 until the end of the experiment. The phylogenetic identification was conducted from the DGGE profile. 22 bands were excised and sequenced. The dominant bands present in all the experiments most closely matched that of *Devosia sp.*, *Alteromonas tagae*, uncultured bacterium clone GN01-8.128, and uncultured alpha *Proteobacterium*. In this study, we did not elucidate the mechanism of the bacterial community change after exposure to algicidal bacteria so further study will be needed.

Although this study was conducted on a lab scale, the results suggest the potential

of *M. luteus* SY-13 as a substitute of clay or chemical algicide to control HABs.

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