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An efficient method to obtain axenic cultures of *Alexandrium tamarense*—a PSP-producing dinoflagellate

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Abstract

The fact that species of harmful algae maintained in the laboratory harbor a complex bacterial flora increases the difficulties involved in the study of the relationship between bacteria and algae. An efficient method to remove bacteria from a laboratory culture of the marine dinoflagellate *Alexandrium tamarense* is presented in this paper. The alga was subjected to repeated washing, lysozyme/SDS and antibiotic treatment with a mixture of gentamycin, streptomycin, cephalothin and rifampicin. Axenic status was confirmed after subculturing three times in sterile f/2 medium without antibiotics. Bacteria could not be detected in various media, both solid and liquid, nor by epifluorescence microscopy and PCR amplification of 16S rDNA of both eubacteria and archaea. Bacterial presence was monitored throughout a full growth cycle and, following subculture, no bacteria were detected using the above methods. This method is more efficient and less time-consuming than other methods and the resultant axenic *A. tamarense* cultures would provide a simpler system for further study of bacteria–alga interactions.

Keywords: *Alexandrium tamarense*; Axenic culture; Epifluorescence microscopy; Polymerase chain reaction (PCR)

1. Introduction

The apparent global increase in the occurrence of harmful algal blooms (HABs) has been accompanied by both an enlargement of the areas affected by red tide and more harm to the environment and humans. There is an urgent need, therefore, to develop practical measures for predicting and reducing the impacts of HABs.

Recently there has been discussion concerning the important role of bacteria–alga interactions in algal bloom dynamics. Such interactions range from beneficial trophic relationships to the more negative impacts of bacteria on algal growth (Doucette, 1995a,b; Doucette et al., 1998). In recent years, efforts have been made to investigate the relationships between marine bacteria and harmful bloom-causing microalgae in order to understand both the outbreak and termination mechanisms of algal blooms. With these efforts, several bacteria capable of inhibiting and killing various HAB species have been isolated. Such bacteria that inhibit algal growth have their effect through direct (physical contact with algal cells) or indirect attack (excretion of bioactive compounds into the water) (Kodani et al., 2002; Su et al., 2005; Wang et al., 2005; Zheng et al., 2005). However, the mechanisms by which the bacteria influence HAB dynamics and algal growth remain largely unstudied, partly due to the complexity of the bacteria–alga system.

Reports have shown that species of harmful algae harbor an attached bacterial flora, and also various free-living bacteria coexist in algal cultures maintained in the laboratory (Kopp et al., 1997; Hold et al., 2001b; Green et al., 2004). These bacteria are an inherent part of the physical environment of algae and thus can be considered as symbionts. Cultures of *Alexandrium tamarense*, like other dinoflagellates, often contain a considerable amount of bacteria from the original samples. These bacteria can produce substances which are either stimulatory or inhibitory to algae and thus change some characteristics of the algae, such as toxin production (Gallacher and Smith, 1999; Uribe and Espejo, 2003). Hold et al. (2001a) found that the

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elimination of bacteria did not affect the growth and toxin profile of *Alexandrium lusitanicum* and *A. tamarense*, whereas it affected the amount of toxins. Removal of bacteria did not have any effect on the growth of *Alexandrium catenella* either, but the total toxicity was diminished to about one-fifth of that in nonaxenic cultures (Uribe and Espejo, 2003).

Bacteria associated with several *Karenia brevis* strains were found to be responsible for their resistance to *Cytophaga* strain 41-DBG2, a bacterium algicidal to *K. brevis*. Resistance and susceptibility to the algicidal activity of 41-DBG2 could be transferred between *K. brevis* strains with the exchange of their respective unattached bacterial communities (Mayali and Doucette, 2002). Three bacterial strains isolated from *A. catenella* culture, which were closely associated with *A. catenella* and which survived extensive antibiotic treatment, were found to be potentially lytic to algae in high nutrient media (Amaro et al., 2005).

These reports show that the bacterial community in algal cultures interact with the algae, and thus, removal of bacteria from stock algal cultures would provide a simpler system for the study of bacteria–alga interactions. Here, we present an efficient method to remove bacteria from cultures of *A. tamarense*, a PSP-producing HAB specie with global distribution.

2. Material and methods

2.1. Algal cultures

A. tamarense cultures (provided by the Institute of Hydrobiology, Jinan University, Guangzhou 510632, China), were maintained in f/2 medium (without silicate) prepared with natural seawater (Guillard, 1975) at 20±1 °C under a 12:12 h light–dark cycle with a light intensity of 50 μmol photons m^{-2} s^{-1}.

2.2. Antibiotic sensitivity of bacteria isolated from algal cultures

In order to obtain bacterial isolates from algal cultures, 1 mL samples were taken during the early and late exponential phase and during the stationary phase. Samples were serially diluted (10-fold dilution), and aliquots (0.1 mL) of each dilution were spread onto Zobell 2216E agar plates and incubated for 7 days at 25 °C. Single colonies were streaked onto individual 2216E agar plates for purification.

Pure isolates were inoculated into 2216E broth and incubated for 24 h in a shaking incubator (25 °C, 180 rpm). Aliquots (0.1 mL) of each bacterial culture were spread evenly onto 2216E agar plates and air-dried to form a bacterial lawn. Sterile antibiotic sensitivity discs, containing gentamycin 50 μg, neomycin 30 μg, streptomycin 25 μg, kanamycin 25 μg, cephalothin 10 μg and rifampicin 5 μg, were laid onto the agar surface. Plates were incubated at 25 °C until the inhibition zones became clear and stable. Antibiotic sensitivity was identified by formation of an inhibition zone of 5 mm in diameter or larger.

2.3. Removal of bacteria from algal cultures

Mid-exponential phase algal cultures, 100 mL, in triplicate, were filtered through a 10 μm pore size membrane and subjected to the following treatments: The algal cells were suspended in 50 mL sterile f/2 medium before sequential centrifuging (1000×*g*, 10 min) and washing three times. The washed cells were suspended in 50 mL sterile f/2 medium containing 0.005% Tween-80 and 0.1 M EDTA (at 20 °C for 1 h) before lysozyme (0.5 mg mL^{-1}, 20 °C for 10 min) and SDS (0.25%, 20 °C for 10 min) were added sequentially. The algal cells were centrifuged and washed twice to remove lysozyme and SDS and then resuspended in 50 mL sterile f/2 medium. The antibiotic cocktail containing gentamycin, streptomycin, cephalothin (each 100 μg mL^{-1}) and rifampicin (10 μg mL^{-1}) were added to the treated algal cultures followed by incubation at 20 °C with a 12:12 light–dark cycle for 7 days. Assessment for bacterial presence was carried out after subculturing three times in order to remove the antibiotics.

2.4. Assessment of bacterial presence

Three methods were used to detect the presence of bacteria in the *A. tamarense* cultures. The treated cultures were inoculated

Table 1
Antibiotic sensitivity of bacterial isolates

Strains of bacterial isolates	Diameter of inhibition zone (mm)					
	Cephalothin	Gentamycin	Kanamycin	Neomycin	Rifampicin	Streptomycin
AT1	21	33	22	25	37	27
AT2	22	32	19	22	37	26
AT3	30	26	22	17	15	22
AT4	27	29	18	16	26	–
AT5	45	24	11	7	17	–
AT6	30	16	17	17	19	12
AT7	30	7	17	14	–	19
AT8	37	29	22	26	26	24
AT9	23	20	26	26	25	24

– means antibiotic resistant.