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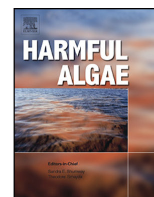

The impact of associated bacteria on morphology and physiology of the dinoflagellate *Alexandrium tamarense*

Cécile Jauzein[a,*], Andrew N. Evans[b], Deana L. Erdner[a]

[a] *University of Texas Marine Science Institute, Port Aransas, TX, USA*
[b] *Department of Coastal Sciences, University of Southern Mississippi, Ocean Springs, MS, USA*

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ABSTRACT

Despite their potential impact on phytoplankton dynamics and biogeochemical cycles, biological associations between algae and bacteria are still poorly understood. The aim of the present work was to characterize the influence of bacteria on the growth and function of the dinoflagellate *Alexandrium tamarense*. Axenic microalgal cultures were inoculated with a microbial community and the resulting cultures were monitored over a 15-month period, in order to allow for the establishment of specific algal–bacterial associations. Algal cells maintained in these new mixed cultures first experienced a period of growth inhibition. After several months, algal growth and cell volume increased, and indicators of photosynthetic function also improved. Our results suggest that community assembly processes facilitated the development of mutualistic relationships between *A. tamarense* cells and bacteria. These interactions had beneficial effects on the alga that may be only partly explained by mixotrophy of *A. tamarense* cells. The potential role of organic exudates in the establishment of these algal–bacterial associations is discussed. The present results do not support a role for algal–bacterial interactions in dinoflagellate toxin synthesis. However, variations observed in the toxin profile of *A. tamarense* cells during culture experiments give new clues for the understanding of biosynthetic pathways of saxitoxin, a potent phycotoxin.

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1. Introduction

Phytoplankton forms the base of the food web and plays a key role in carbon cycling in marine environments. Interest in the carbon cycle has increased recently owing to problems such as climate change and coastal eutrophication. Fertilization of large-scale areas of the ocean has even been proposed to mitigate climate change by stimulating the growth of phytoplankton in order to sequester carbon dioxide (Denman, 2008; Glibert et al., 2008). Impressive advances have been made in assessing environmental forcing of phytoplankton dynamics during the last decades, mainly focusing on physico-chemical controlling factors (Tian, 2006; Irwin et al., 2012). However, some mechanisms, such as biological associations between microorganisms, are still poorly understood, and their role in phytoplankton dynamics and biogeochemical cycles of marine ecosystems could be crucial.

The activities and fates of algae and aquatic bacteria are closely linked (Amin et al., 2012), yet the ecological significance of most naturally occurring algal–bacterial associations is still unclear. The most well-known connection is via their roles in energy fluxes and nutrient cycling, as bacteria utilize algal-derived organic matter and, in turn, regenerate inorganic nutrients (Legendre and Rassoulzadegan, 1995). There is some evidence that differences in the quality of organic matter produced by different types of phytoplankton cause shifts in the species composition of bacterial communities utilizing these algal exudates (van Hannen et al., 1999). Whilst such trophic relationships can structure microbial communities, specific interactions between algae and bacteria can alter this dynamic in subtle ways, potentially influencing bloom dynamics and the cycling of matter in the ocean (Mayali et al., 2011).

Over the last several decades, coastal areas throughout the world have experienced an escalating threat associated with occurrence of "harmful algal blooms" (HABs). This trend comes from a global expansion of harmful algal species, aggravated by an increase in the use of the coastal zone for fisheries and recreational activities (Anderson, 2009). Among potential factors controlling outbreak and persistence of HABs, algal–bacterial interactions

* Corresponding author. Present address: Sorbonne Universités, UPMC Univ Paris 06, INSU-CNRS, Laboratoire d'Océanographie de Villefranche, Villefranche sur mer, France. Tel.: +33 4 93 76 38 43.
E-mail address: cjauzein@gmail.com (C. Jauzein).

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have received increasing attention in the recent past (Doucette et al., 1998; Mayali and Azam, 2004; Hasegawa et al., 2007). Algicidal marine bacteria have even been considered as potential biological control agents for regulation of HABs in natural seawaters (Doucette et al., 1999; Kim et al., 2008). A better understanding of algal–bacterial interactions could help to improve management strategies for HAB events, including mitigation and forecasting.

Algal–bacterial interactions are complex, partly because they can be species-specific and vary with changes in environmental conditions (Mayali and Azam, 2004; Grossart and Simon, 2007; Danger et al., 2007). For the toxic dinoflagellate *Alexandrium* spp., which produces paralytic shellfish toxins (PSTs), various effects of bacteria have been reported, including promotion or inhibition of growth (Ferrier et al., 2002; Wang et al., 2012), stimulation or inhibition of encystment (Adachi et al., 1999; Adachi et al., 2002), and modulation of toxicity (Hold et al., 2001; Uribe and Espejo, 2003). The mode of interaction varies from indirect effects, through release of organic exudates (Wang et al., 2012), to symbiosis, such as intracellular bacteria within *Alexandrium* cells (Palacios and Marín, 2008). During bloom development, algal–bacterial interactions may lead to selection of specific bacteria phylotypes in the phycosphere of *Alexandrium* cells (Hasegawa et al., 2007), showing their importance in microbial community dynamics in the field. The aim of the present work was to characterize the influence of bacteria on *Alexandrium tamarense* using culture experiments in controlled conditions. In addition to analyzing the gross effects of bacteria on growth and toxin production of *A. tamarense*, specific biological functions, including photosynthesis, inorganic carbon (C) uptake, and phagotrophy of algal cells, were examined.

Most of the laboratory studies conducted on phytoplankton–bacteria interactions are dependent on the production and maintenance of axenic cultures. Comparison of axenic and xenic clones are generally done on short time scales, e.g. after a few days or weeks of culture maintenance in newly axenic medium (e.g. Uribe and Espejo, 2003) or after inoculation with bacteria (e.g. Grossart and Simon, 2007). To our best knowledge, our study is the first to analyze these processes over the longer time scales that may be required for the establishment of specific, functional algal–bacterial associations. Experiments were run using clonal cultures of *Alexandrium tamarense* that have been maintained under either axenic or bacterized conditions over several years. Effects of bacteria inoculation in axenic cultures were considered over a 15 month period. The results of this study provide new insights into the influence of bacteria on biological functions of phytoplankton cells, with implications for understanding phytoplankton dynamics and organic matter cycling. In addition, our results suggest that beneficial algal–bacterial associations can contribute to the success of toxic dinoflagellates in various environments.

2. Materials and methods

2.1. Cultures and maintenance

Two clones of the same strain of *Alexandrium tamarense*, CCMP 1493 and CCMP 1598, were obtained from the National Center for Marine Algae and Microbiota (NCMA). CCMP 1493 was isolated from the China Sea in 1991 and has been maintained under bacterized conditions since then; an axenic clone of this strain, CCMP 1598, was created a few years later and has since been grown under axenic conditions. In our laboratory, stock cultures of the two clones were grown in f/2 medium (minus Si) (Guillard and Ryther, 1962), at 16 °C, under ~100 μmol photons m^{-2} s^{-1}, with a 12:12 h light:dark cycle. All culture transfers were conducted under sterile conditions, from an initial cell concentration of ~200 cells mL^{-1}. Sterility of axenic cultures was periodically determined by direct bacteria observations with DAPI (4′,6-diamidino-2-phenylindole) staining and epifluorescence microscopy (see details below). Cultures were grown without bubbling but with a gentle stirring every other day.

2.2. Experimental design

Triplicate 1L batch cultures were used to characterize growth and toxin production of the two parent and donor clones, CCMP 1598 and 1493. Analyses were conducted during exponential growth, when cell density exceeded 2500 cells mL^{-1}. Characterization of net growth was based on measurements of growth rate and cell volume. Photosynthetic health and capacity were determined from measurement of cellular chlorophyll *a* content and efficiency of photosystem II (F_v/F_m ratio). Autotrophic growth characterization included inorganic C-uptake capacities, from measurements of ^{13}C-uptake rates. Mixotrophy of *Alexandrium tamarense* cells was assayed from observations of digestive vacuoles. Toxin profiles were determined, taking into account high potency carbamate toxins (saxitoxin [STX], neosaxitoxin [NEO], gonyautoxins [GTX1-GTX6]), low potency N-sulfocarbamoyl toxins (C1–C4) and decarbamoyl analogues (dcSTX, dcGTX2, dcGTX3).

The experiment started with the initiation of "re-xenic cultures", axenic cells of CCMP 1598 inoculated with bacteria collected from a culture of CCMP 1493. One culture of each parent or donor clone (2L) in exponential phase was used for the creation of three re-xenic subclones. The culture of CCMP 1493 was lysed by vortexing with 0.5 mm silica–zirconium beads (BioSpec Products, Inc.). The resulting lysate was filtered through a 20 μm Nitex mesh to remove intact dinoflagellate cells. The filtrate was then centrifuged for 5 min at 3000 × *g* to remove large cell fragments, and finally filtered through a 5.0 μm nitex mesh to remove small cell fragments. Re-xenic cultures were created by adding the resulting bacterial filtrate (500 mL of filtrate of CCMP 1493 culture) to axenic cells of *Alexandrium tamarense* (500 mL of CCMP 1598 culture). Re-xenic subclones were maintained under the same conditions as the parent and donor clones of *A. tamarense* for 15 months, by transferring cells to fresh f/2 medium when cultures reached late-log phase and without bubbling but a gentle stirring every other day. Re-xenic cultures were grown in 1L of medium in Fernbach flasks. Only one 1-L flask was simultaneously maintained for each re-xenic subclone. Cell density of *A. tamarense* was monitored during growth of each subculture, to determine growth rate in exponential phase and maximal cell density at plateau. When it came time to make a larger set of measurements, three replicated flasks were created for each subclone: one culture was used for maintenance of the subclone and two cultures were sacrificed for measurements.

One of the three subclones was lost in less than a month, despite our best efforts to recover it. The other two subclones are designated as Re-xenic 1 and 2 in the present study. For both Re-xenic 1 and 2, three replicate cultures were created after nine months and 15 months of culture maintenance and, each time, two of the replicated flasks were used for characterization of their growth and toxin production. The same parameters were monitored in the Re-xenic and parent and donor clones (CCMP 1598 and 1493). These measurements were done during the exponential phase of growth, on cultures showing a cell density higher than 2500 cells mL^{-1}.

2.3. Microscopic observations

Cell abundances were determined in triplicate during the exponential growth of *Alexandrium tamarense* cultures, from samples preserved in Lugol's iodine. For each sample, a minimum

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