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Investigation of the algicidal exudate produced by *Shewanella* sp. IRI-160 and its effect on dinoflagellates

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ABSTRACT

The bacterium, *Shewanella* sp. IRI-160, was previously shown to have negative effects on the growth of dinoflagellates, while having no negative effects on other classes of phytoplankton tested (Hare et al., 2005). In this study, we investigated the mode of algicidal activity for *Shewanella* sp. IRI-160 and found that the bacterium secretes a bioactive compound. The optimum temperature for production of the algicidal compound by this bacterium was at 30 °C. Bacteria-free filtrate of medium containing the algicide (designated IRI-160AA) was stable at temperatures ranging from −80 °C to 121 °C, and could be stored at room temperature for at least three weeks with no loss in activity. Algicidal activity was eluted in the aqueous portion after C18 extraction, suggesting that the active compound is likely polar and water-soluble. The activity of IRI-160AA was examined on a broad range of dinoflagellates (*Karlodinium veneficum*, *Karenia brevis*, *Gyrodinium instriatum*, *Cochlodinium polykrikoides*, *Heterocapsa triquetra*, *Prorocentrum minimum*, *Alexandrium tamarense* and *Oxyrrhis marina*) and three species from other classes of algae as controls (*Dunaliella tertiolecta*, *Rhodomonas* sp. and *Thalassiosira pseudonana*). Algicidal activity was observed for each dinoflagellate and little to no negative effect was observed on chlorophyte and cryptophyte cultures, while a slight (non-significant) stimulatory effect was observed on the diatom culture exposed to the algicide. Finally, the effect of the algicide at different growth stages was investigated for *K. veneficum* and *G. instriatum*. IRI-160AA exhibited a significantly greater effect during logarithmic growth compared to stationary phase, suggesting a potential application of the algicide for prevention and control of harmful dinoflagellate blooms in the future.

1. Introduction

Bacteria in association with harmful algal blooms (HABs) may play a role in regulating or terminating algal blooms in the natural environment (Doucette, 1995; Doucette et al., 1998; reviewed by Mayali and Doucette, 2002). In an effort to develop short-term solutions for controlling HABs, laboratory studies have evaluated isolated bacteria or bacteria-derived algicidal compounds for their effectiveness in controlling algal growth (Lovejoy et al., 1998; Mayali and Doucette, 2002; Skerratt et al., 2002; Hare et al., 2005; Roth et al., 2007, 2008a; Kim et al., 2008). Results of these studies demonstrate that algicidal bacteria may exhibit a broad range of specificity and may use a range of mechanisms to mediate algicidal effects on targeted algal species. Several studies, for instance, have described algicidal bacteria that are only effective on a specific genus or species of algae (Fukami et al., 1992; Doucette et al., 1999), while others target a broader range of algal classes (Imai et al., 1995; Lovejoy et al., 1998; Kang et al., 2008; Roth et al., 2008a). The mode of activity can also vary such that direct cell-to-cell contact is necessary for algicidal activity in some bacterial species (Kim et al., 1998; Roth, 2005; Roth et al., 2007, 2008b), while other algicidal bacteria have been found to secrete bioactive compounds with varying effects on the target algal species (Lovejoy et al., 1998; Doucette et al., 1999; Lee et al., 2000; Skerratt et al., 2002; Kang et al., 2008; Kim et al., 2008; Mayali et al., 2008). For example, Roth et al. (2007) found that direct contact with the dinoflagellate *Karenia brevis* was required for algicidal activity of *Flavobacteriaceae* strain S03, which induced cell lysis after 24 h exposure. In contrast, cell-free filtrate from cultures of bacterium *Micrococcus luteus* strain SY-13 caused loss of motility and cell lysis in the dinoflagellate *Cochlodinium polykrikoides* (Kim et al., 2008) and the cell-free filtrate from an algicidal bacterium "strain-Y" resulted in dissociation of chains, swelling and lysis of *Gymnodinium catenum* within 2–4 h after inoculation (Lovejoy et al., 1998).

Differences in the effectiveness of bacterial algicides have also been noted for algal growth stages, suggesting specific interactions with metabolic pathways or cellular processes associated with growth or senescence of algal cultures. Kang et al. (2008) discovered

Abbreviations: AA, algicidal agent; IRI, Indian River Inlet; DIB, Delaware Inland Bays; HSD, honestly significant difference; LC–MS, liquid chromatography–mass spectrometry; HABs, harmful algal blooms; NCMA, National Center for Marine Algae and Microbiota.

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that lag and logarithmic growth stages of the diatom, *Stephanodiscus hantzschii*, and the dinoflagellate *Peridinium bipes*, were more sensitive to bacterial algicides than cells in stationary phase. Conversely, the algicidal bacterium, *Cytophaga* strain 41-DBG2, had a greater effect on cultures of *K. brevis* in late logarithmic and stationary phase compared to early-logarithmic growth, suggesting a growth stage effect or that the bacterium had a higher requirement for dissolved organic matter or greater cell density (Mayali and Doucette, 2002). Knowledge of the stage-specific activity of algicides may have important implications in their potential application. Algicides targeting early growth stages may be more valuable in the prevention of HABs while those targeting later growth stages may be more effective for mitigation of HABs already underway.

Hare et al. (2005) described an algicidal bacterium, *Shewanella* sp. IRI-160, isolated from the Indian River Inlet (IRI) in the Delaware Inland Bays (DIB), DE, USA. When introduced into culture, the bacterium had a negative effect on the growth of three dinoflagellates (*Pfiesteria piscicida*, *Prorocentrum minimum* and *Gyrodinium uncatenum*), while having either no effect or a slight stimulatory effect on the growth of other phytoplankton species tested. The negative effects on dinoflagellate growth were observed several days after inoculation, suggesting that algicidal activity may be more effective at later growth stages and/or may be due to the accumulation of a secreted bioactive compound in the medium. Molecular analysis of the bacterial population in these cultures showed the abundance of *Shewanella* sp. IRI-160 was reduced over time compared to other bacteria in the culture (Hare et al., 2005). Moreover, the mode of algicidal activity was unclear, and it was not determined if direct cell-to-cell contact was required for activity or if the activity was due to a bioactive compound secreted by the bacterium.

In this study, we conducted a detailed investigation of the algicidal activity of *Shewanella* sp. IRI-160. Our results demonstrate that algicidal activity is due to a secreted compound, designated IRI-160AA (for "algicidal agent"). We also examined the production of the algicide by the bacterium under different incubation temperatures, the thermal stability of IRI-160AA, and the effect of the algicide on a broad range of dinoflagellate species. Additionally, we evaluated the growth stage-specific activity of IRI-160AA on two HAB dinoflagellates (*Karlodinium veneficum* and *Gyrodinium instriatum*) during early, middle and late logarithmic growth and at stationary phase.

2. Materials and methods

2.1. Phytoplankton culture maintenance

Phytoplankton cultures used for this study are listed in Table 1, along with culture media, salinity and incubation temperatures for each species. Cultures were maintained at a 12:12 light:dark illumination regimen at approximately 185 μmol photons m^{-2} s^{-1} light intensity, and all experiments were performed on batch cultures unless otherwise noted. *Oxyrrhis marina* was fed *Dunaliella tertiolecta* every 2–3 days. *D. tertiolecta* was frozen at −80 °C for 30 min before addition to *O. marina* culture to prevent growth of *D. tertiolecta*. None of the cultures were axenic.

Experiments described below were conducted with an $N = 3$ or 4 and, except where noted, changes in cell density were monitored daily by *in vivo* Chl *a* fluorescence with a hand-held Aquafluor Fluorometer (Turner Designs, Sunnyvale, CA). Fluorescence has been shown in other studies to be a good proxy for algal biomass under nutrient replete/logarithmic growth conditions (*e.g.* Brand et al., 1981; Brand, 1985; Doblin et al., 1999; Gustavs et al., 2009; Maldonado and Price, 2001; Wood et al., 2005). All experiments were conducted using logarithmic cells except for the growth phase experiments where cell counts were performed to ensure that fluorescence was an accurate measure of algal abundance. Specific growth rates (μ) were calculated as follows:

$$\mu = \frac{\ln(N_2/N_1)}{t_2 - t_1}$$

where N_1 and N_2 are the average values of *in vivo* Chl *a* flourescence at times t_1 and t_2 (Guillard, 1973).

2.2. Bacterial culture and filtrate

Shewanella sp. IRI-160 was cultured on modified LM plates (Sambrook et al., 1989; Luria Bertani medium, 20 g L^{-1} NaCl, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 15 g L^{-1} agar) with sterile techniques. Plates were incubated at room temperature for 2–3 days, until colonies formed. A single colony was transferred to 100 mL of sterile liquid LM medium (Sambrook et al., 1989) and incubated overnight at 25 °C on an orbital shaker (Forma Scientific, Marietta, OH) at 100 rpm. For long-term storage, bacterial culture was mixed with an equal volume of freezing medium (Sambrook et al., 1989; 65% glycerol, 0.1 M $MgSO_4$, and 25 mM Tris–Cl; pH 8.0) and frozen at −80 °C. Cells were harvested from liquid culture by centrifugation for 5 min at 2760 × *g*. The LM broth was decanted and *Shewanella* sp. IRI-160 cells were washed by re-suspending in F/2 medium, at a salinity of 20. Cells were centrifuged, and again re-suspended in F/2 medium. Unless otherwise noted below, the *Shewanella* sp. cell suspension was incubated for 1 week at 30 °C then centrifuged for 10 min at 3975 × *g* and filtered through a 0.2 μm Steriflip Filter Unit equipped with a polyethersulfone (PES) filter

Table 1
Phytoplankton culture maintenance.

Species	Family	Source	Media	Sal. (psu)	Temp. (°C)
Karlodinium veneficum	Kareniaceae	NCMA 2936	[a]F/2 (−Si)	20	25
Karenia brevis	Kareniaceae	NCMA 2281	L1 (−Si)	32	25
Gyrodinium instriatum	Gymnodiniaceae	NCMA 2935	F/2 (−Si)	20	25
Cochlodinium polykrikoides	Gymnodiniaceae	CPPB17	[b]GSe (−Si)	30	18
Heterocapsa triquetra	Heterocapsaceae	NCMA 448	F/2 (−Si)	32	18
Prorocentrum minimum	Prorocentraceae	NCMA 2233	F/2 (−Si)	20	25
Alexandrium tamarense	Gonyaulacaceae	NCMA 1493	F/2 (−Si)	30	18
Oxyrrhis marina	Oxyrrhinaceae	NCMA 1739	F/50 (−Si)	32	18
Dunaliella tertiolecta	Dunaliellaceae	NCMA 1320	L1 (−Si)	32	18
Rhodomonas sp.	Cryptophyceae	NCMA 757	F/2 (−Si)	20	25
Thalassiosira pseudonana	Thalassiosiraceae	NCMA 1335	F/2 (+Si)	32	18

NCMA, National Center for Marine Algae and Microbiota.
C. polykrikoides was provided by Christopher Gobler at Stony Brook University.
[a] Guillard (1975).
[b] Tang and Gobler (2010).