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Selective growth promotion of bloom-forming raphidophyte *Heterosigma akashiwo* by a marine bacterial strain

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ABSTRACT

Algal bloom is typically caused by aberrant propagation of a single species, resulting in its predomination in the local population. While environmental factors including temperature and eutrophication are linked to bloom, the precise mechanism of its formation process is still obscure. Here, we isolated a bacterial strain that promotes growth of *Heterosigma akashiwo*, a *Raphidophyceae* that causes harmful algal blooms. Based on 16S rRNA gene sequence, the strain was identified as *Altererythrobacter ishigakiensis*, a member of the class *Alphaproteobacteria*. When added to culture, this strain facilitated growth of *H. akashiwo* and increased its cell culture yield significantly. Importantly, this strain did not affect the growth of other raphidophytes, *Chattonella ovate* and *C. antiqua*, indicating that it promotes growth of *H. akashiwo* in a species-specific manner. We also found that, in co-culture, *H. akashiwo* suppressed the growth of *C. ovate*. When *A. ishigakiensis* was added to the mixed culture, *H. akashiwo* growth was facilitated while *C. ovate* propagation was markedly suppressed, indicating that the presence of the bacterium enhances the dominance of *H. akashiwo* over *C. ovate*. This is the first example of selective growth promotion of *H. akashiwo* by a marine bacterium, and may exemplify importance of symbiotic bacterium on algal bloom forming process in general.

1. Introduction

Several species of unicellular algae are known to form blooms in aquatic environments, thereby exerting a powerful influence on their surrounding ecosystem. Harmful algal blooms (HAB) are formed by noxious species and negatively impact the ecosystem, and are thus of particular interest in an environmental and industrial context. Bloom status is determined by the speed of algal propagation and disappearance. The propagation rate of bloom-forming algae has been shown to be affected by many environmental factors, including temperature, salinity, eutrophic condition, carbon dioxide concentration, and light intensity (Anderson et al., 2002; Backer and McGillicuddy, 2006; Eppley, 1972; Hallegraeff, 1993; Heisler et al., 2008; Maso and Garces, 2006; Raven and Geider, 1988; Smayda, 1997; Smith and Schindler, 2009). In addition, interactions with other organisms such as predator zooplanktons or other grazers (Demir et al., 2008; Graham and Strom, 2010; McManus et al., 2007; Tillmann, 2004; Xie et al., 2008), viruses (Lang et al., 2009; Lawrence, 2008; Nagasaki, 2008; Short, 2012; Suttle, 2007; Wommack and Colwell, 2000), and marine bacteria (Amin et al., 2012; Doucette et al., 1998; Mayali and Azam, 2004) are known to play pivotal roles in algal population control in nature. Particularly, studies on the interactions between phytoplanktons and marine bacteria have revealed both synergistic and parasitic interactions, resulting in propagation and fatality, respectively, of the algae (Amin et al., 2012; Buchan et al., 2014). Several reports have described the existence of a characteristic marine bacterial assemblage associated with a particular algal species (Barlaan et al., 2007; Burkholder et al., 2007; Ferrier et al., 2002; Grossart et al., 2005). Furthermore, one study showed that different algal species harbor different bacterial assemblages with distinctive populations (Sapp et al., 2007), implying that certain bacterial species preferably associate with specific algal species. To improve our understanding of bloom formation in nature, the implications of such interactions between algae and bacteria should be investigated.

H. akashiwo is a noxious raphidophyte species that often forms HAB during the summer, particularly in thalassic sea of the Pacific-rim area, including North and South America, Eastern Asia, Oceania, and Northern Atlantic area (Black et al., 1991; Chang et al., 1990; Honjo, 1993; Lackey and Lackey, 1963; Mackenzie, 1991; O'Halloran et al., 2006; Park, 1991; Rensel et al., 1989; Rojas

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de Mendiola, 1979; Taylor, 1993; Throndsen, 1969; Tseng et al., 1993). While several reports have described marine bacteria that kill *H. akashiwo* (Cho, 2012; Imai et al., 2001; Kim et al., 2007, 2009a, 1998, 2009b; Liu et al., 2008b; Lovejoy et al., 1998; Park et al., 2010; Skerratt et al., 2002; Tarutani et al., 2000; Yoshinaga et al., 1998), only one article has reported on a bacterial strain that facilitates *H. akashiwo* propagation (Liu et al., 2008a). This strain facilitated the growth of a wide variety of algal species including raphidophytes, diatoms, cryptophytes, and chlorophytes by a mechanism yet to be clarified (Liu et al., 2008a).

To gain more information about *H. akashiwo*-bacteria interactions, we isolated bacteria associated with laboratory-maintained *H. akashiwo* cultures. Here, we found that the bacterial strain *Altererythrobacter ishigakiensis* YF1 facilitates *H. akashiwo* propagation, though it did not alter the growth rates of two other raphidophytes, *Chattonella ovate* and *C. antiqua*. This is the first study that demonstrates selective raphidophyte growth facilitation by a marine bacterial strain.

2. Materials and methods

2.1. Raphidophyte strains and media used for the experiments

H. akashiwo isolates, *Ha*Fk01 (isolated from Fukuoka Bay, Fukuoka prefecture, Japan), H93616 (isolated from Uranouchi Bay, Kochi Prefecture, Japan), *Ha*Tj01 (isolated from Tajiri Bay, Hiroshima Prefecture, Japan), *C. ovate Co*Tj23 (Tajiri Bay, Hiroshima Prefecture, Japan) and *C. antiqua Ca*Ag03 (Ago Bay, Mie Prefecture, Japan) were used for this study. All strains used in this study were originally established by isolating single algal cells from *H. akashiwo* or *Chattonella* bloom samples observed at the areas of origin, then passaged in growth media without antibiotics. Modified SWM3 medium(Yamasaki et al., 2007) was used for algal growth medium, and the culture was maintained in an environment-controlled chamber with a photoperiod (12 h of 100 $\mu mol\, m^{-2}\, s^{-1}$ light/12 h dark) at 25 °C.

The strains were rendered bacteria-free by being maintained in the growth medium supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), ampicillin (100 μg/mL), and kanamycin (60 μg/mL). To analyze whether any bacteria were present in the algal cultures, 100 μL samples of raphidophyte cultures, each containing 5×10^5-10^6/mL cells, were inoculated in commercial Difco™ Marine Broth 2216 (Becton Dickinson and Company, Franklin Lakes, NJ, USA) solidified with 0.2% bacto-agar by piercing into the media to a depth of at least 5 cm using long pipette tips (Cappuccino and Natalie Sherman, 2013). The media were incubated at 25–28 °C for two to three weeks, and confirmed the absence of microorganisms. We also conducted 16S rRNA gene amplification by PCR using primers Eu8f and Eu1492r (Devereux and Wilkinson, 2004), followed by cloning into pCR-Blunt II-TOPO plasmid (Life Technologies, Carlsbad, CA, USA) and sequencing of the amplicon in fifteen independent clones to detect traces of microorganisms in the treated culture. All sequenced amplicons were identified as *H. akashiwo* chloroplast 16S rRNA, confirming that the cultures were successfully rendered bacteria-free by our method. The bacteria-free strains were maintained with the four antibiotics thereafter.

2.2. Isolation of Altererythrobacter ishigakiensis from H. akashiwo strain H93616

Nonaxenic *H. akashiwo* cultures (100 μL) containing 5×10^4 cells were stab-pierced into soft-agar media containing 0.2% of bacto-agar and commercial Difco™ Marine Broth 2216. (Becton Dickinson and Company, Franklin Lakes, NJ, USA). The bacteria grown in the soft-agar media were streaked on Difco™ Marine Broth 2216 solidified with 2% agar, and incubated at 25 °C under aerobic conditions. When colonies appeared, a partial sequence of the 16S rRNA gene was amplified using primers Eu8f and Eu1492r by colony PCR using a standard protocol (Devereux and Wilkinson, 2004), and its sequence was determined using Big Dye v3.1 (Applied Biosystems) according to the manufacturer's instruction. The sequences were used for identification in the EZ-Taxon database (Chun et al., 2007; Kim et al., 2012).

2.3. Analyzing effect of bacterial strains on growth of H. akashiwo and Chattonella

The bacteria-free *H. akashiwo* and *Chattonella* strains were transferred to an antibiotic-free growth medium and propagated for three days at least twice to decrease the antibiotic concentrations to <1/1000 of the treatment concentrations. During the preculture period, *H. akashiwo* was allowed to propagate at least 8 generations (in cell number, to >500 times) to acclimate to the growth condition without antibiotics. The bacterial strains were precultured in Marine Broth, and each 600-μL bacterial culture adjusted to $OD_{600} = 0.01$ (total ~9×10^6 cells) was added to a 60-mL algal culture. For controls, the same volume of Marine Broth was added to equivalent cultures. *H. akashiwo*, *C. ovate*, and *C. antiqua* cells were innoculated at 100, 500 and 1000 cells/mL. These were the minimum innoculation densities that allowed stable growth of bacteria-free strains, thus adopted for the experiments.

For cell enumeration, a MoxiZ Mini Automated Cell Counter (E.I. Spectra, LLC, Hailey, ID, USA) was used according to the manufacturer's instructions. The algal species *H. akashiwo* and *Chattonella* are readily distinguishable on the counter because of the difference in their diameters, which are 8–12 μm and 18–30 μm, respectively; thus the two species could be enumerated independently in mixed culture. The bacterial cell density was enumerated by counting colony-forming units on solidified Marine broth. Specific growth rate was calculated according to the equation $k = \ln(N_2/N_1)/(t_2\text{-}t_1)$, where N is concentration of cells and t is the date when the cell density was measured. All experiments were continued until the cell population reach to

Table 1
Bacterial strains isolated from *H. akashiwo* .

Isolated strains	*Closest relatives	Identities (%)	$Accessions
YF1	*Altererythrobacter ishigakiensis* JPCCMB0017(T)	98.73	LC078994
AH2	*Winogradskyella poriferorum* UST030701-295(T)	100	LC078996
AH3	*Stappia indica* B106(T)	99.31	LC078997
AH4	*Spongiibacterium flavum* A11(T)	97.31	LC078998
AH5	*Alteromonas macleodii* DSM 6062(T)	99.79	LC078999

*The closest relatives were identified according to the identities of rRNA sequence.
$The NCBI accession numbers.