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Environment

The seagrass *Zostera marina* harbors growth-inhibiting bacteria against the toxic dinoflagellate *Alexandrium tamarense*

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Abstract Seagrasses are known to have allelopathic activity to reduce growth of phytoplankton. We found growth-inhibiting bacteria (strains E8 and E9) from *Zostera marina* possessing strong activity against the toxic dinoflagellate *Alexandrium tamarense*. Strain E9 markedly inhibited growth of *A. tamarense* even with initial inoculum size as small as 2.9 cells ml^{-1}. This bacterium also had growth-inhibiting effects on the red-tide raphidophytes *Chattonella antiqua* and *Heterosigma akashiwo*, the dinoflagellate *Heterocapsa circularisquama*, and the diatom *Chaetoceros mitra*. Small subunit (SSU) ribosomal DNA (rDNA) sequencing analysis demonstrated that the most probable affiliation of these strains was *Flavobacteriaceae*, and proved that another inhibitory bacterial strain (E8) was the same species as strain E9. Two other bacterial strains (E4-2 and E10), showing different colony color and isolated from the same seagrass sample, revealed no growth-inhibiting activity. Interestingly, strain E4-2 showed the same sequences as E8 and E9 (100 %), and strain E10 matched E8 and E9 with 99.80 % similarity. Growth-inhibiting bacteria against the toxic dinoflagellate *Alexandrium tamarense* associated with seagrass, such as *Flavobacterium* spp. E8 and E9, are able to repress shellfish poisoning besides the allelopathic activity of seagrass itself.

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Introduction

Paralytic shellfish poisoning (PSP) is a serious problem in the marine bivalve aquaculture industry, having negative effects on marine species throughout food webs in coastal ecosystems of the world [1]. PSP incidents have shown globally increasing trends of scale and frequency [2]. *Alexandrium tamarense* (Lebour) Balech (Dinophyceae) is an infamous species involved in PSP occurrences. *A. tamarense* is widely distributed in the world, especially in cold water areas. However, at present, we have no feasible prevention measures against PSP occurrences, and establishment of practical methods is urgently needed.

Another environmental problem resulting from phytoplankton in coastal waters is red tide. Noxious red tides have caused mass mortalities of cultured marine species such as fishes and bivalves, accompanied by huge amounts of damage to fisheries. Consequently, studies on protective measures are seriously needed. Chemical and physical countermeasures, such as spraying copper sulfate and scattering clay to aggregate and sink red-tide algae, are considered to have negative effects on coastal ecosystems, because chemical agents would cause serious secondary pollution accompanied by mortality of other organisms and resulting in changes to marine food webs.

In general, bacteria play an important role in nutrient regeneration and energy transformation in marine ecosystems [3]. However, in recent years, biological countermeasures employing bacteria have attracted attention as environmentally friendly strategies for use in marine

environments [4–8]. Quite a number of algicidal bacteria have been isolated from coastal waters so far, such as *Cytophaga* sp. J18/M01 against the fish-killing raphidophyte *Chattonella antiqua* [4, 5] and *Flavobacterium* sp. 5 N-3 against the harmful dinoflagellate *Gymnodinium nagasakiense* (currently *Karenia mikimotoi*) [6]. Algicidal bacteria show an increase particularly at the late phase of red tides in sea water [7, 8]. These bacteria are expected to control these red-tide-causing microalgae. In addition to algicidal activity, other algal–bacterial interactions have been reported, such as change of the dominant algal species [7–10], growth promotion [11], growth inhibition [12], promotion of cyst formation [13], control of cell toxicity [14], induction of sexual reproduction of diatoms [15], etc.

Seagrass beds have an important function in coastal ecosystems to maintain biodiversity and provide feeding, housing, and spawning grounds for marine species [16]. Seagrass meadows are known to be hot spots for carbon burial and nutrient cycling in the ocean [17, 18]. As an interesting feature, the seagrasses *Zostera marina* Linnaeus and *Z. noltii* Hornemann exhibit growth-inhibiting activity against phytoplankton through allelopathy [19, 20]. Since highly diverse microorganisms possessing various activities live in seagrass beds, it is expected that there exist various kinds of algicidal and/or growth-inhibiting bacteria against phytoplankton. Algicidal bacteria against red-tide flagellates were actually found to be distributed with high density in the biofilm on blades of the seagrass *Z. marina* [21]. Therefore, it is expected that seagrasses are favorite habitats for algicidal bacteria, and they have a potential ability to kill red-tide phytoplankton. We consequently inferred that algicidal bacteria in association with seagrasses have a killing and/or growth-inhibiting ability against toxic dinoflagellates, and seagrasses can contribute to reduce the frequency and scale of toxic bloom occurrences. In this study, we succeeded in isolating from the seagrass *Z. marina* bacterial strains possessing markedly strong growth-inhibition activity against the toxic dinoflagellate *Alexandrium tamarense*, and herein we report some characteristics of the growth-inhibiting activity of these bacteria.

Materials and methods

Algal cultures

The microalgal species used in this study are presented in Table 1. They were all axenic and maintained in modified SWM-3 medium prepared with natural sea water [22, 23]. Incubation was carried out at 15 or 20 °C depending on species, under light intensity of about 100–120 μmol photons m^{-2} s^{-1} with a 14 h light:10 h dark photocycle. The light conditions for incubation were identical throughout this study.

Sampling

Samples of seagrass (*Z. marina*) were collected on 15 October 2009 from a seagrass bed in Usujiri Fishing Port in Hakodate, Hokkaido, Japan (41°56.10′N, 140°56.58′E). Seagrass leaves were taken in a sterilized bottle (500 ml) using forceps and brought back to the laboratory of Hokkaido University in a cooler box.

Sterilized sea water (200 ml) was added to the bottle containing the *Z. marina* sample, and the bottle was shaken 500 times by hand to obtain an easily detaching biofilm. The sea water with the suspended biofilm was used for enumerating algicidal and/or growth-inhibiting bacteria as described in detail in "Isolation of growth-inhibiting bacteria active against *A. tamarense*" section.

Isolation of growth-inhibiting bacteria active against *A. tamarense*

Growth-inhibiting bacteria against *A. tamarense* were enumerated using the most probable number (MPN)

Table 1 Species of phytoplankton used in the present study and the temperature conditions for experiments

Class and species	Strain name	Locality of origin	Isolator	Temperature (°C)
Dinophyceae				
Alexandrium tamarense		Osaka Bay	K. Yamamoto	15
Heterocapsa circularisquama		Uranouchi Inlet	T. Uchida	20
Bacillariophyceae				
Chaetoceros mitra		Bering Sea	K. I. Ishii	15
Raphidophyceae				
Chattonella antiqua	NIES-1	Harima-Nada	NIES	20
Fibrocapsa japonica		Harima-Nada	I. Imai	20
Heterosigma akashiwo	IWA	Bingo-Nada	H. Iwasaki	20

All cultures were kept under light intensity of 100–120 μmol photons m^{-2} s^{-1} and 14 h L:10 h D light–dark cycle

NIES National Institute for Environmental Studies

method [24, 25]. The cultures of *A. tamarense* at the late logarithmic phase were diluted with SWM-3 culture medium to 3.3×10^3 cells ml^{-1}, and 0.5-ml aliquots were added to the wells of 48-well microplates. The biofilm sample in sea water was filtered through Nuclepore filter (pore size 1.0 μm) and diluted decimally with sterilized sea water. An aliquot of volume 0.1 ml of each diluted sample was inoculated into each well of the 48-well microplates, containing the 0.5-ml *A. tamarense* culture. The assay cultures in the microplates were incubated under the same conditions described above, and the growth inhibition and/or survival of the dinoflagellate in each well was assessed daily with an inverted microscope for two weeks. The wells in which *A. tamarense* cells lost swimming ability, sank to the bottom of wells, showed roundish form without thecal plates, and were broken were scored as "positive." Sterilized sea water was inoculated into five wells with assay cultures as controls. From the "positive" wells, 0.5-ml aliquots were added to the culture of *A. tamarense* in the wells (6.0×10^3 cells ml^{-1}), and the activity of growth inhibition was twice confirmed. Aliquots of 0.1 ml "positive" culture were spread onto $ST10^{-1}$ agar medium [26] and incubated at temperature of 20 °C under dark conditions for two weeks to form colonies. Individual bacterial colonies of the total 23 strains were isolated, grown in $ST10^{-1}$ liquid medium, and frozen at −30 °C until the experiments.

Screening

To screen the growth-inhibiting bacteria, frozen clones were thawed and grown again in $ST10^{-1}$ liquid medium to reach cell density of about 10^8 cells ml^{-1}. An aliquot of each appropriately diluted bacterial culture was inoculated at density of about 10^4 cells ml^{-1} into 4-ml cultures of *A. tamarense* (10^2 cells ml^{-1}) in glass tubes (diameter 13 mm). The growth and/or growth inhibition of *A. tamarense* was monitored by in vivo fluorescence using a fluorometer (10-AU; Turner Designs, Inc.). Determinations of fluorescence were made after agitation of culture tubes using a vortex mixer. Control was set by inoculation of sterilized sea water into *A. tamarense* culture in tubes. As a result, two strains (E8 and E9) were obtained as growth-inhibiting bacteria against *A. tamarense*.

Molecular analysis of bacteria

The isolated 23 clones of bacteria were grown in $ST10^{-1}$ liquid medium, and bacterial cells in 200-μl culture were collected by centrifugation ($2000 \times g$ for 5 min) followed by twice washing with phosphate-buffered saline (PBS) buffer. After removing the supernatant from the sample, DNA was extracted using the Chelex method [27]. The 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using the primers 8F and 1492R following the conditions of 10 μl 2× PCR buffer, 4 μl 2 mM deoxyribonucleotide triphosphates (dNTP), 0.5 μl of 10 pM of each primer, 1 μl template, 3.6 μl Milli-Q, and KOD FX Neo (Toyobo, Osaka, Japan). The initial denaturizing period of 3 min was followed by 35 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min, and the final extension time (72 °C) was 7 min. PCR products were checked using 1 % agarose gel electrophoresis. To purify DNA template strands, PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) following the instruction manual. The cycle sequencing samples were purified by ethanol precipitation. Sequencing was conducted using an ABI PRISM 3130xl genetic analyzer (Applied Biosystems). The obtained sequences were assembled using Chromas PRO (Technelysium Pty Ltd, Tewantin, Australia).

Phylogenetic and molecular evolutionary analyses for obtained sequences were conducted using MEGA 5 software [28]. Alignments were checked manually. The maximum-likelihood (ML) tree was calculated using the software with the best-fit model using Bayesian information criterion (BIC) scores, and the substitution nucleotide matrix parameters were calculated by the software. One thousand bootstraps were generated. Neighbor-joining (NJ) analyses were performed using the same model as for the ML. Bootstrapping values for the NJ tree were also generated using 1000 replicates. All positions containing gaps and missing data were eliminated. The nucleotide sequences of 16S rDNA for 4 isolates were deposited in the DDBJ/EMBL/GenBank databases with accession numbers AB819155 and AB819394 to AB819396.

Inoculation size and growth-inhibiting activity

The growth-inhibiting bacterial strain E9 was used for the following culture experiments. The bacterial clone was grown in $ST10^{-1}$ liquid medium, and diluted serially with sterilized sea water. Aliquots of 0.5-ml diluted culture were inoculated into four replicate tubes in which axenic cells of *A. tamarense* (3.6×10^2 cells ml^{-1}, 4.5 ml) were contained. The initial concentrations of bacteria were 2.9×10^0–10^7 cells ml^{-1} with eight decimal degrees. Four replicate tubes were set for each bacterial cell density condition. Incubations were kept at 15 °C under the above light conditions. Growth and/or survival of *A. tamarense* was monitored by fluorometer, and all culture experiments with tubes were done using the fluorometer for monitoring algal growth and/or survival.

Growth-inhibiting activity of bacterial culture filtrate

The effects of bacterial culture filtrate were examined by targeting *A. tamarense* with the bacterial strain E9. The bacterium was grown in $ST10^{-1}$ liquid culture medium, and inoculated at initial concentration of 2.0×10^4 cells ml^{-1} into *A. tamarense* culture (6.0×10^3 cells ml^{-1}) in 300-ml flasks. The bacterium attacked and partially killed *A. tamarense* for 3 days, reaching cell density of 1.1×10^8 cells ml^{-1}. The attacked cultures were filtered with 0.1-μm-pore sterilized Nuclepore filter, and were added to four replicate tubes into which *A. tamarense* culture was inoculated (3.0×10^3 cells ml^{-1}) with concentrations of culture filtrate of 50 and 80 %. Incubations were carried out at 15 °C under the same light conditions mentioned above.

Growth-inhibiting ability against other phytoplankton species

The growth-inhibiting range of bacterial strain E9 was examined by coculture experiments using the following five species of marine phytoplankton other than *A. tamarense*: the bivalve-killing dinoflagellate *Heterocapsa circularisquama* (initial density 1.5×10^3 cells ml^{-1}), three harmful raphidophytes *Chattonella antiqua* (initial density 2.0×10^3 cells ml^{-1}), *Fibrocapsa japonica* (initial density 2.2×10^3 cells ml^{-1}), and *Heterosigma akashiwo* (initial density 2.7×10^3 cells ml^{-1}), and a centric diatom *Chaetoceros mitra* (initial density 2.2×10^2 cells ml^{-1}). Each algal species was grown in modified SWM-3 medium, and 4.5-ml aliquots were inoculated into four replicate tubes. Bacterial strain E9 was grown in $ST10^{-1}$ liquid medium (final yield 2.8×10^8 cells ml^{-1}); the bacterial culture was diluted with sterilized sea water, and 0.5-ml aliquots were added to the tubes (obtained density 2.8×10^4 cells ml^{-1}) into which algal cells were inoculated. Sterilized sea water was added to the four algal tubes as control. Incubations were carried out at 20 °C under the above light conditions. The growth of phytoplankton in tubes was measured by fluorometer. Monitoring of growth was continued until the fluorescence of each control tube of each species showed peak fluorescence value.

Results

Isolation of growth-inhibiting bacteria

Two bacterial strains (E8 and E9) possessing remarkable growth-inhibiting activity against *Alexandrium tamarense* were obtained from the biofilm on leaves of the seagrass *Z. marina*. The growth-inhibiting activity against *A. tamarense* was tested with different initial bacterial cell densities of bacterial strain E9 (Fig. 1).

Controls (no addition of bacteria) showed continuous increase of *A. tamarense* cells until the end of the culture experiment (day 20). On the other hand, growth of *A. tamarense* was inhibited by all the additions of the bacterial strain with eight different cell densities (2.9×10^0 to 2.9×10^7 cells ml^{-1}).

The growth-inhibiting effects of strain E9 against *A. tamarense* were observed under a light microscope (Fig. 2). A normal *A. tamarense* cell is shown in Fig. 2a. When bacterial strain E9 was added to *A. tamarense* culture, the swimming activities of *A. tamarense* cells were inhibited and the thecal plates were often detached from the cell (Fig. 2b). Spherical cells, presumably the temporary cyst formed from vegetative cells against the stress by bacterial addition (Fig. 2c), were frequently observed on day 3 and thereafter. Eventually, disrupted *A. tamarense* cells were frequently observed in the culture with bacteria (Fig. 2d).

Growth-inhibiting activity of bacterial culture filtrate

The growth-inhibiting activity of the bacterial culture filtrate against *Alexandrium tamarense* was examined using culture with bacterial strain E9.

The control tubes (no addition of filtrate) showed continuous increase during the experimental period (Fig. 3). In the case of additions of bacterial culture filtrate with 50 and 80 % concentrations to *A. tamarense*, the dinoflagellate exhibited growth inhibition until day 4–6. Growth of *A. tamarense* appeared to recover after day 6.

Effects of bacterium E9 on growth of other phytoplankton species

The growth-inhibiting range of bacterial strain E9 was examined using five other marine phytoplankton species, i.e., three fish-killing raphidophytes *Chattonella antiqua*, *Fibrocapsa japonica*, and *Heterosigma akashiwo*, the bivalve-killing dinoflagellate *Heterocapsa circularisquama*, and the diatom *Chaetoceros mitra*.

The dinoflagellate *Heterocapsa circularisquama* exhibited growth inhibition by the bacterium E9 (Fig. 4a), and all cells lost motility and sank to the bottom of experimental tubes. The diatom *Chaetoceros mitra* with addition of bacterium E9 showed almost the same growth pattern until day 13 as the control (no addition of bacterial cells, Fig. 4b). However, growth of the diatom was inhibited by the bacterium thereafter. The raphidophyte *Chattonella antiqua* also exhibited growth inhibition by bacterium E9 (Fig. 4c); the cells tended to sink to the bottom of tubes. In the case of *F. japonica*, the effect of bacterium E9 was not

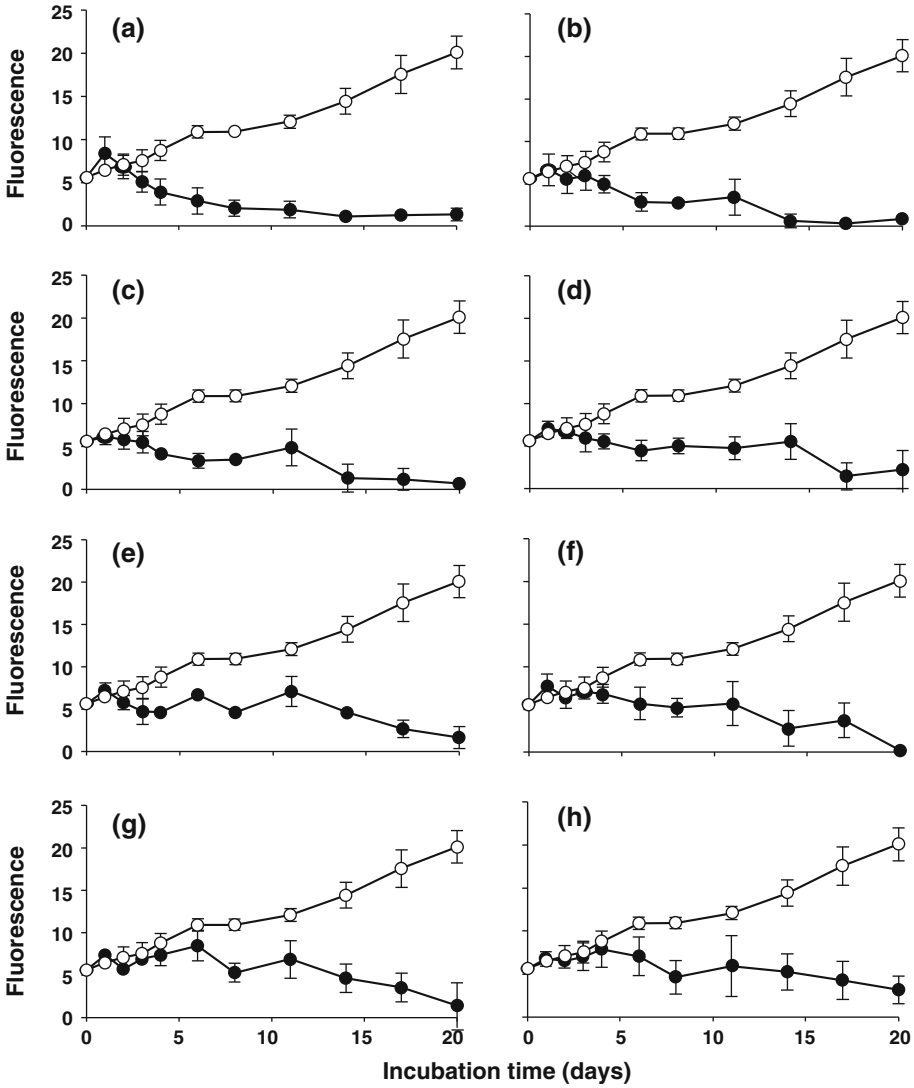

Fig. 1 Effects of growth-inhibiting bacterial strain E9 with different inoculum sizes on *Alexandrium tamarense* in modified SWM-3 medium. The initial cell density of *A. tamarense* was 3.6×10^2 cells ml^{-1}. Initial bacterial densities were **a** 2.9×10^7 cells ml^{-1}, **b** 2.9×10^6 cells ml^{-1}, **c** 2.9×10^5 cells ml^{-1}, **d** 2.9×10^4 cells ml^{-1}, **e** 2.9×10^3 cells ml^{-1}, **f** 2.9×10^2 cells ml^{-1}, **g** 2.9×10^1 cells ml^{-1}, and **h** 2.9×10^0 cells ml^{-1}. Control (*open circle*) indicates growth of *A. tamarense* with no addition of bacterial cells

apparent compared with the control tubes (no addition of bacteria, Fig. 4d). *H. akashiwo* showed a similar growth pattern as in the experiment with the diatom *Chaetoceros mitra* (Fig. 4e), and the growth of *H. akashiwo* was inhibited after day 14.

Identification of growth-inhibiting bacteria E8 and E9

The two strains (E8 and E9) of growth-inhibiting bacteria isolated from leaves of the seagrass *Z. marina* were identified according to the molecular analyses, and the analysis showed that these two strains belonged to the same clade in the group of *Flavobacteriaceae* (Fig. 5). Furthermore, another two bacterial strains (E4-2 and E10) possessing no growth-inhibiting activity were in the same clade in the phylogenetic tree (Fig. 5). The growth-inhibiting bacterial strains E8 and E9 had completely the same 16S rRNA gene sequence as that of strain E4-2 possessing no ability for growth-inhibiting activity against *A. tamarense* (Table 2). Strain E10 showed a difference of only 2 bp of 1485 bp in the sequence data among the four strains. The bootstrap values of these four bacterial strains were 100 and 99 for NJ and ML trees. A distinct difference among these bacterial strains was the color of their colonies. Growth-inhibiting strains E8 and E9 were yellowish ivory, whereas the nonactive strains E4-2 and E10 were white. Therefore, we can conclude that this clade formed one species. A relatively close species of algicidal bacteria was *Flavobacterium* sp. strain 5 N-3 [6], and the 16S rRNA gene sequence homology with strain E9 was 97.64 % and the number of bases differing in the sequence was 35. The 16S rRNA gene sequence homology between *Flavobacteriaceae* bacterium strain LPK5 [36] and E9 was 94.07 %, and the number of bases differing in the sequence was 76.

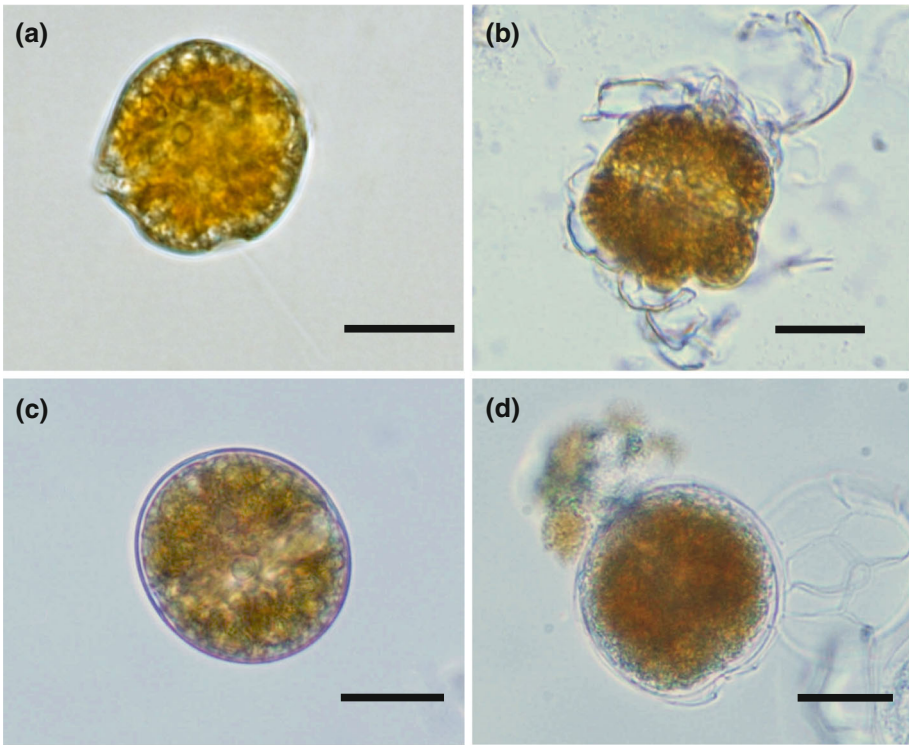

Fig. 2 Effects of bacterial strain E9 on morphology of *Alexandrium tamarense*: **a** control, no addition of bacterial cells; **b** *A. tamarense* cell with detached thecal plates after 3 days of incubation; **c** a spheral cell, presumably temporary cyst, after three days; **d** disrupted cell releasing cell contents, observed on day 7 after addition of bacterial cells. *Scale bar* 20 μm

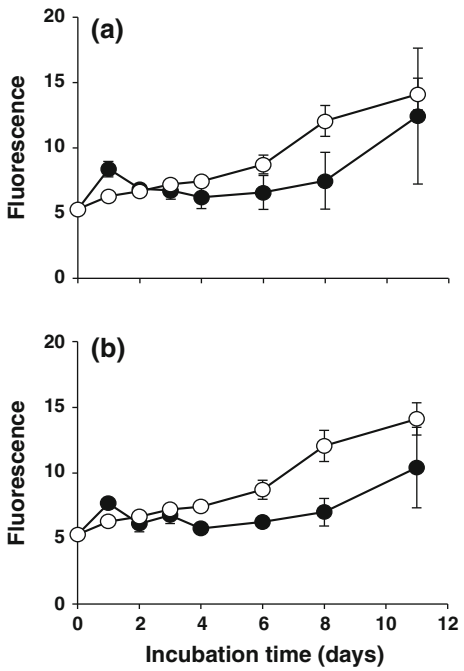

Fig. 3 Effects of culture filtrate on growth and/or survival of *A. tamarense* with culture filtrate concentration of **a** 50 % and **b** 80 %. Control (*open circle*) indicates growth of *A. tamarense* with no addition of culture filtrate. Culture filtrates were prepared by coculture of *A. tamarense* and bacterial strain E9 for 3 days for killing and growth inhibition. Cells of bacterium and alga were eliminated by filtration with 0.1-μm-pore filter before the experiment

Discussion

Kim et al. [29] described that about 50 % of isolates of growth-inhibiting bacteria belonged to the *Cytophaga/Flavobacterium/Bacteroides* (CFB) group and about 45 % to that of the *γ-Proteobacteria*. Members of the family *Flavobacteriaceae* have often been reported to be algicidal against red-tide algae; For example, *Flavobacterium* sp. strain 5 N-3 showed 16S rRNA gene sequence homology of 97.64 % with strain E9; this bacterium was isolated from a water sample from a bloom of the dinoflagellate *Karenia mikimotoi*, and strain 5 N-3 showed growth-inhibitory effects against *K. mikimotoi* [6, 30]. Another strain belonging to *Flavobacteriaceae*, strain LPK5, was reported to exhibit motility-inhibiting activity against the dinoflagellate *Lingulodinium polyedrum* [31, 32]. We isolated two strains (E8 and E9) of growth-inhibiting bacteria against *A. tamarense*, and they showed the same growth-inhibiting activity against the examined phytoplankton species. Both strains belonged to the family *Flavobacteriaceae*, and the results of 16S rRNA gene sequence analysis proved these strains to be closely resembling species with DNA homology of 100 %. Interestingly, two other bacterial strains (E4-2 and E10) were isolated from the same seagrass sample but possessed no growth-inhibiting activity against *A. tamarense*, despite the fact that the 16S rRNA gene sequence homology of E9 and E4-2 was 100 % and that of E9 and

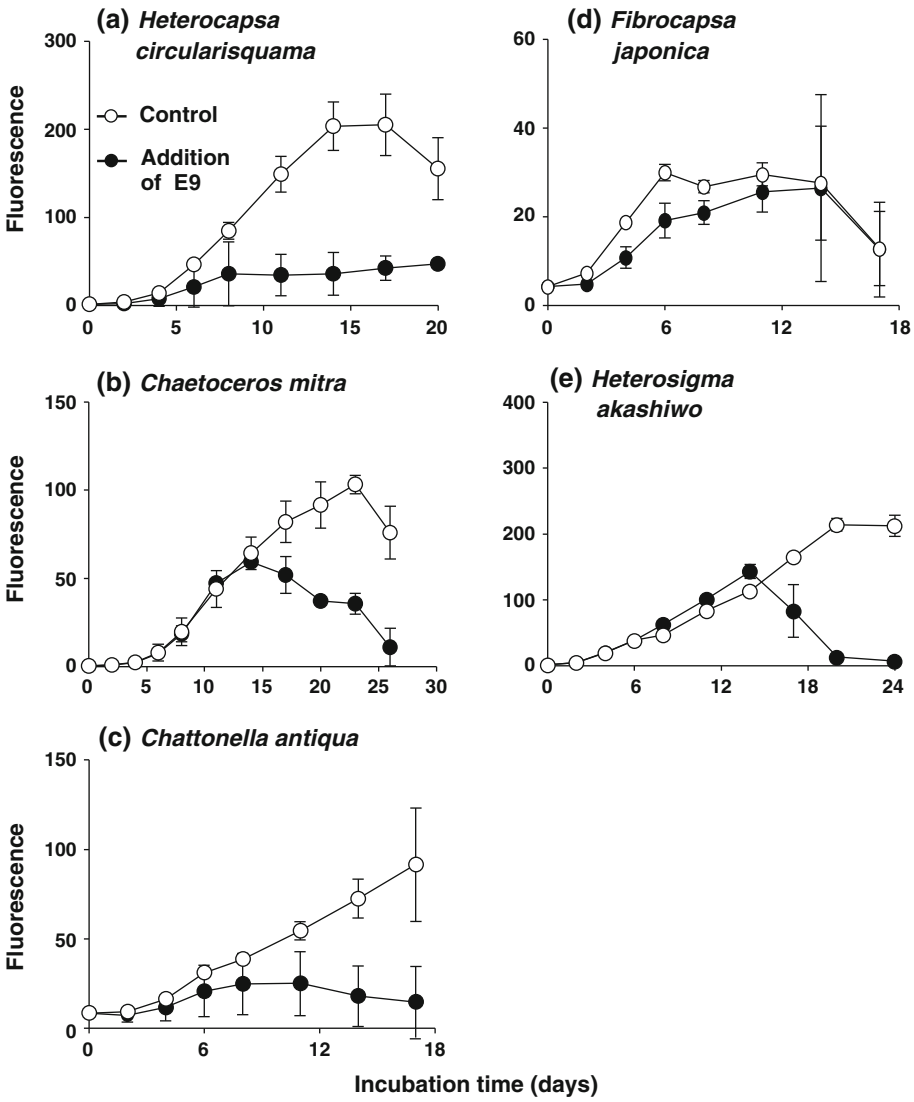

Fig. 4 Effects of bacterial strain E9 on growth and/or survival of the dinoflagellate *Heterocapsa circularisquama* (**a**), the diatom *Chaetoceros mitra* (**b**), and the raphidophytes *Chattonella antiqua* (**c**), *Fibrocapsa japonica* (**d**), and *Heterosigma akashiwo* (**e**). The added bacterial cell density was 1×10^4 cells ml^{-1}. Control was no addition of bacterium

E10 was 99.80 %. This is the first report in marine bacteria that the same species can show conflicting activity in terms of algicidal effect. Whole-genome analyses of these bacterial strains are necessary in the future to understand which genome controls the production of algicidal material.

Previous studies on algicidal bacteria against *A. tamarense* [33–36] and *A. catenella* [37] described that these bacterial strains inhibited the toxic dinoflagellates with the addition of initial densities as high as 10^8–10^{10} cells ml^{-1}. On the other hand, in the present study, growth of *A. tamarense* was markedly inhibited by bacterial strain E9 even for initial inoculum size of 2.9 cells ml^{-1} (Fig. 1), demonstrating the significantly strong growth-inhibiting activity of this bacterial strain. The activity of strain E9 was significantly higher than that of previously reported bacteria [33–36].

Although growth of *A. tamarense* was inhibited by bacterium E9, some cells survived at the end of culture experiments (Fig. 1). We observed spherical cells of *A. tamarense* showing the same morphology as temporary cysts at the bottom of experimental tubes (Fig. 2). Temporary cyst formation was induced when some kinds of bacteria were added to bloom-forming dinoflagellates such as *Heterocapsa circularisquama*, *Lingulodinium polyedrum*, and *Karenia brevis* [31, 32, 38, 39]. Dinoflagellates usually produce temporary cysts due to some types of physical and/or chemical stresses [40]. Algicidal bacteria are evaluated to be a strong stress to dinoflagellates such as *A. tamarense* (Fig. 2c).

Growth-inhibiting activity of culture filtrate of bacterial strain E9 was observed against *A. tamarense* to some extent (Fig. 3). This result suggests that bacterium E9 produces some material which inhibits increase of *A. tamarense*. However, this growth-inhibiting activity disappeared after six days of the experiments (Fig. 3). It was confirmed that the marine bacterium *Pseudoalteromonas* sp. strain A28 was able to produce an extracellular serine protease against

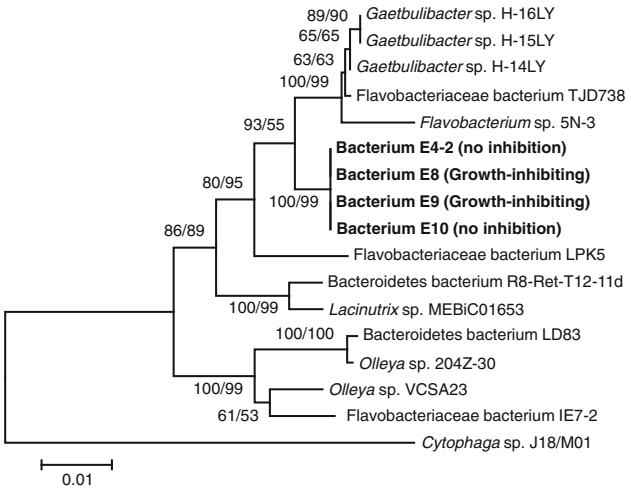

Fig. 5 Phylogenetic tree including the growth-inhibiting bacteria E8 and E9 and two closely related bacterial strains (E10 and E4-2) based on 16S rRNA gene sequences. The tree was constructed using neighbor-joining method and maximum-likelihood method (NJ/ML)

Table 2 Sequence similarity (%, upper half) and number of bases differing in the sequence (lower half) among four isolated bacterial strains and closely related algicidal species, *Flavobacteriaceae* bacterium LPK5 and *Flavobacterium* sp. 5 N-3

Bacterial strain	1	2	3	4	5	6
1. E9	–	100.00	100.00	99.80	97.64	94.07
2. E8	0	–	100.00	99.80	97.64	94.07
3. E4-2	0	0	–	99.80	97.64	94.07
4. E10	2	2	2	–	97.44	93.87
5. *Flavobacterium* sp. 5 N-3	35	35	35	37	–	92.52
6. *Flavobacteriaceae* bacterium LPK5	76	76	76	78	112	–

the diatom *Skeletonema costatum* strain NIES-324 [41]. It was reported that the bacterial strain DHQ25 made an indirect attack against *A. tamarense* and produced algicidal proteins with molecular weight of 14.5 kDa [42]. *A. tamarense* has the ability to resist direct attack by algicidal bacteria, because *A. tamarense* swims and has thecal plates, and these characteristics work as protective measures against direct attack by bacteria. Consequently, algicidal bacteria of indirect attack type (producing algicidal material) probably work more effectively than algicidal bacteria of direct attack type.

Algicidal bacteria against raphidophytes such as *Chattonella* spp. [4, 5, 10, 43–45] and *Heterosigma akashiwo* [5, 7, 8, 43, 46] were members of the genera *Alteromonas*, *Cytophaga*, and *Pseudoalteromonas*. There are relatively few studies on algicidal bacteria against diatoms compared with those against harmful phytoflagellates. *Cytophaga* sp. strain J18/M01 [5] was able to kill four diatoms (*Skeletonema costatum*, *Ditylum brightwellii*, *Chaetoceros didymus*, and *Thalassiosira* sp.). *Alteromonas* sp. strains S, D, and R had the ability to kill two diatoms (*Ditylum brightwellii* and *Chaetoceros didymus*), and *Alteromonas* sp. strain K killed *Chaetoceros didymus* [43]. The bacterial strain K12 exerted algicidal activity against nine diatoms, including the species of Centrales and Pennales [15]. Diatoms show a wide variety in morphology, cell size, and life form pattern, which includes planktonic, benthic, and periphytic forms. Therefore, the tolerance of diatoms to algicidal bacteria probably differs depending on their taxonomy and the bacterial attack pattern.

In the present study, bacteria possessing strong growth-inhibiting activity against *A. tamarense* were isolated from the biofilm on leaves of the seagrass *Z. marina*. Accordingly, it is considered that seagrass beds have potential to prevent occurrences of not only harmful red tides [21, 47] but also toxic dinoflagellate blooms by virtue of the existence of strong growth-inhibiting bacteria. As well as acting as nursery grounds for larvae of marine species, it is proposed that restoration of seagrass beds is important to maintain the health of the coastal sea. This is a kind of harmony between humankind and nature in conformity with the concept of "*Sato-Umi*" [48].

The ecosystem services value of seagrasses and seaweed beds (US \$19,004 ha^{-1} $year^{-1}$) is estimated to be high next to estuaries (US \$22,832 ha^{-1} $year^{-1}$) and floodplains (US \$19,580 ha^{-1} $year^{-1}$) [49]. Seagrass meadows additionally provide high-value ecosystem services such as supporting commercial fisheries worth as much as US \$3500 ha^{-1} $year^{-1}$ [50]. Thus, seagrass bed is one of the most productive ecosystems on Earth.

Seagrasses such as *Z. marina* and *Z. noltii* have the ability to inhibit growth of phytoplankton by allelopathy [19, 20, 51]; For example, growth of phytoplankton was delayed by addition of *Z. noltii* [20]. An extract made from leaves of *Z. marina* and *Z. noltii* reduced the photosynthetic activity of *A. catenella* (Whedon et Kofoid) Balech [19]. However, the present study newly demonstrates that *Z. marina* has the ability to inhibit growth of the toxic dinoflagellate *A. tamarense* severely by virtue of associated algicidal bacteria. Future work will evaluate whether allelopathy or algicidal bacteria are more important to reduce phytoplankton growth.

Seagrass beds have been rapidly disappearing at a rate of 110 km^2 $year^{-1}$ in the world since 1980, and 29 % of the initial area has disappeared since 1879, when seagrass areas were first approximately determined [52]. On the other hand, the scale and frequency of occurrences of harmful algal blooms have been increasing globally [2]. There is a report that large-scale decline of seagrass beds was accompanied by increasing frequency of toxic blooms of the dinoflagellate *A. minutum* Halim in the Mediterranean coast [53].

When phytoplankton cells are killed by algicidal bacteria, marine organic materials derived from the killed phytoplankton should be decomposed rapidly through the process of microbial loop. Consequently, seagrass beds are expected to be hot spots of microbial processes such as algicidal activity, decomposition of excessively generated organic material, and hence function of microbial loop; more extensive studies are needed on these processes in the future.

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