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Title

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

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Keywords

toxic blooms, *Alexandrium tamarense*, algicidal bacteria, seagrass, *Zostera marina*, mitigation, prevention

Abstract

The seagrasses are known to have allelopathic activity to reduce the growth of phytoplankton. We found growth-inhibiting bacteria (strains E8 and E9) from *Zostera marina* possessing strong activities against the toxic dinoflagellate *Alexandrium tamarense*. The strain E9 markedly inhibited the growth of *A. tamarense* even with the initial inoculum size as few 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*. The SSU rDNA sequencing analyses 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 isolated from the same seagrass sample, revealed no growth-inhibiting activity. The strain E4-2 interestingly showed the same sequences with E8 and E9 (100%) and the strain E10 matched the 99.80% similarity with the E8 and E9. The growth-inhibiting bacteria against the toxic dinoflagellate *Alexandrium tamarense* associated with seagrass, such as *Flavobacterium* spp. E8 and E9, are possible to repress the shelfish poisoning besides allelopathic activity of seagrass itself.

Introduction

Paralytic shellfish poisoning (PSP) is a serious problem in the marine bivalve aquaculture industries and gives bad effects on marine lives through 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 of PSP occurrences. *A. tamarense* widely distributes in the world especially in cold water areas. However, at present we have no feasible prevention measures against PSP occurrences, and it is urgently needed to establish practical methods.

Another environmental problem given by phytoplankton in coastal waters is red tide. Noxious red tides have caused mass mortalities of cultured marine lives such as fishes and bivalves accompanying huge amounts of fisheries damages. Consequently, studies on protective measures are seriously needed. Chemical and physical countermeasures, such as spraying copper sulfate and clay scattering to aggregate and sink red tide algae, are considered to give bad effects on coastal ecosystems, because chemical agents would cause serious secondary pollution accompanying mortalities of other organisms and resulting in changes of 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 to employ bacteria have gathered attentions as environment-friendly strategies in the marine environments [4-8]. Rather many algicidal bacteria were isolated from coastal waters so far such as *Cytophaga* sp. J18/M01 against the fish-killing raphidophyte *Chattonella antiqua* [4, 5], and *Flavobacterium* sp. 5N-3 against the harmful dinoflagellate *Gymnodinium nagasakiense* (currently *Karenia mikimotoi*) [6]. Algicidal bacteria showed an increase particularly at the late phase of red tides in seawater [7, 8]. These bacteria are expected to control these red tide causing microalgae. In addition to algicidal activity, other algal-bacterial interactions are reported such as changing the dominant algal species [7–10], growth-promotion [11], growth-inhibition [12], promoting cyst formation [13], controlling the cell toxicity [14] and inducing sexual reproduction of diatoms [15], etc.

The seagrass beds have an important function in coastal ecosystems to maintain biodiversity, to provide feeding, housing and spawning grounds for marine lives [16]. The 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 a 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 some kinds of algicidal and/or growth-inhibiting bacteria against phytoplankton. Algicidal bacteria against the red-tide flagellates were actually found to distribute with high densities in the biofilm on the blades of the seagrass *Z. marina* [21]. Therefore, it is expected that seagrasses are favorite habitats of 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 contribute to the reduction of frequency and scale in occurrences of toxic blooms. In this study, we succeeded in isolating bacterial strains possessing markedly strong activities of growth inhibition against the toxic dinoflagellate *Alexandrium tamarense* from the seagrass *Z. marina*, and here we report some characteristics of growth inhibiting activities of these bacteria.

Materials and Methods

Algal cultures

Microalgal species used in this study were presented in Table 1. They were all axenic and maintained in the modified SWM-3 medium prepared with natural seawater [22, 23]. Incubations were made at 15 or 20 °C depending on species and under light intensities about 100 to 120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a 14 h light: 10 h dark photo-cycle. The light conditions for incubation were identical throughout this study.

Sampling

Samples of seagrass (*Z. marina*) were collected on 15 October 2009 at 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 kept 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 its easily-detaching biofilm. The seawater with the suspended biofilm was used for enumerating algicidal and/or growth-inhibiting bacteria described in detail in 2.3.

Isolation of the growth-inhibiting bacteria active against *A. tamarens*

The growth-inhibiting bacteria against *A. tamarens* were enumerated using the MPN method [24, 25]. The cultures of *A. tamarens* at the late logarithmic phase were diluted with the 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 seawater was filtered through Nuclepore filter (pore size of 1.0 µm) and diluted decimally with sterilized seawater. 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. tamarens* 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. tamarens* cells lost the swimming ability, sank to the bottom of wells, showed roundish form without thecal plates, and were broken were scored as “positive”. Sterilized seawater 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. tamarens* 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 the ST10⁻¹ agar medium [26] and incubated at a temperature of 20 °C under the dark conditions for two weeks to form colonies. Individual bacterial colonies of the total 23 strains were isolated, grown in the ST10⁻¹ liquid medium, and frozen at -30 °C until the experiments.

Screening

For screening the growth-inhibiting bacteria, frozen clones were thawed and grown again in the ST10⁻¹ liquid medium to reach cell densities of about 10⁸ cells ml⁻¹. An aliquot of each appropriately diluted bacterial culture was inoculated with the densities of about 10⁴ cells ml⁻¹ in to 4-ml cultures of *A. tamarensis* (10² cells ml⁻¹) in glass tubes (diameter of 13 mm). The growth and/or growth-inhibition of *A. tamarensis* were monitored by *in vivo* fluorescence using a fluorometer (10-AU Fluorometer, Turner Designs, Inc.). Determinations of fluorescence were made after the agitation of culture tubes using a vortex mixer. Control was set by the inoculation of sterilized seawater to *A. tamarensis* culture in tubes. As a result, two strains of E8 and E9 were obtained as growth-inhibiting bacteria against *A. tamarensis*.

Molecular analysis of bacteria

The isolated 23 clones of bacteria were grown in the ST10⁻¹ liquid medium, and bacterial cells in 200 µl-culture were collected with centrifugation (2000 x g for 5 min) followed by twice washings with the PBS buffer. After removing the supernatant from sample, DNA was extracted using the Chelex method [27]. The 16S rRNA gene was amplified by PCR by using of the primers 8F and 1492R following the conditions of 2x PCR buffer 10 µl, 2 mM of dNTP 4 µl, 10 pM of each primer 0.5µl, template 1µl, Milli-Q 3.6µl and KOD FX Neo (TOYOBO, Osaka, Japan). The initial denaturizing period of 3 min was followed by 35 cycles at 94 °C for 30 sec, 56 °C for 30 sec, 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 computer program [28]. Alignments were checked manually. Maximumlikelihood (ML) tree

was calculated using the software with the best fits model by BIC (Bayesian Information Criterion) 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 was 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 bacterium strain E9 was used for the following culture experiments. The bacterial clone was grown in the 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. tamarens* (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. tamarens* were monitored with a fluorometer, and all culture experiments with tubes were done using the fluorometer for monitoring the algal growth and/or survival.

Growth-inhibiting activity of bacterial culture filtrate

The effects of the bacterial culture filtrate were examined targeting *A. tamarens* by the bacterium strain E9. The bacterium was grown in ST10⁻¹ liquid culture medium, and inoculated at the initial concentration of 2.0×10^4 cells ml⁻¹ into *A. tamarens* culture (6.0×10^3 cells ml⁻¹) in 300 ml flasks. The bacterium attacked and partially killed *A. tamarens* for 3 days, and the reached cell density was 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 in which *A. tamarens* culture was inoculated (3.0×10^3 cells ml⁻¹) with

concentrations of culture filtrates of 50 and 80%. Incubations were made at 15 °C under the same light conditions mentioned before.

Growth-inhibiting ability against other phytoplankton species

The growth-inhibiting range of the bacterium strain E9 was examined with co-culture experiments using the following five species of marine phytoplankton other than *A. tamarense*: the bivalve-killing dinoflagellate *Heterocapsa circularisquama* (initial density of 1.5×10^3 cells ml⁻¹); three harmful raphidophytes *Chattonella antiqua* (initial density of 2.0×10^3 cells ml⁻¹), *Fibrocapsa japonica* (initial density of 2.2×10^3 cells ml⁻¹) and *Heterosigma akashiwo* (initial density of 2.7×10^3 cells ml⁻¹), and a centric diatom *Chaetoceros mitra* (initial density of 2.2×10^2 cells ml⁻¹). Each algal species was grown in the modified SWM-3 medium and 4.5 ml aliquots were inoculated into four replicate tubes. The bacterium strain E9 was grown in the liquid ST10⁻¹ medium (final yield of 2.8×10^8 cells ml⁻¹), and the bacterial culture was diluted with sterilized sea water and 0.5-ml aliquots were added to the tubes (obtained density of 2.8×10^4 cells ml⁻¹) in which algal cells were inoculated. Sterilized sea water was added to the four algal tubes as a control. Incubations were made at 20 °C under the above light conditions. The growth of phytoplankton in tubes was measured with the fluorometer. The monitorings of the growth were 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 the leaf of the seagrass *Z. marina*. The growth-inhibiting activity against *A. tamarense* was tested with different initial bacterial cell densities of the bacterium strain E9 (Fig. 1).

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

The growth-inhibiting effects of the strain E9 against *A. tamarens* were observed under a light microscope (Fig. 2). A normal *A. tamarens* cell is shown in Fig. 2a. When the bacterial strain E9 was added to *A. tamarens* culture, the swimming activities of *A. tamarens* 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 the day 3 and thereafter. Eventually, disrupted *A. tamarens* cells were frequently observed in the culture with bacteria (Fig. 2d).

Growth-inhibiting activity of the bacterial culture filtrate

Growth-inhibiting activity of the bacterial culture filtrate against *Alexandrium tamarens* was examined using the culture of the bacterium strain E9.

The tubes of control (no addition of filtrates) showed a continuous increase during the experiment period (Fig. 3). In the case of the additions of bacterial culture filtrate with 50% and 80% concentrations to *A. tamarens*, the dinoflagellate revealed a growth-inhibition until the day 4 to 6. The growth of *A. tamarens* appeared to recover the growth after the day 6.

Effects of the bacterium E9 on the growths of other phytoplankton species

The growth-inhibiting range of the bacterium strain E9 was examined using other five marine phytoplankton species, i.e., the 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* revealed a growth inhibition by the bacterium E9

(Fig.4a), and the all cells lost motility and sank to the bottom of experimental tubes. The diatom *Chaetoceros mitra* with the addition of the bacterium E9 showed almost the same growth pattern until the day 13 as the control (no addition of the bacterial cells, Fig. 4b). However, the diatom growth was inhibited by the bacterium thereafter. The raphidophyte *Chattonella antiqua* also revealed the growth inhibition by the bacterium E9 (Fig. 4c). The cells tended to sink to the bottom of tubes. In the case of *F. japonica*, the effects of the bacterium E9 was not apparent as compared with the tubes of control (no addition of bacteria, Fig. 4d). *H. akashiwo* showed a similar pattern of the growth as the experiment of the diatom *Chaetoceros mitra* (Fig. 4e), and the growth of *H. akashiwo* was inhibited after the day 14.

Identification of the growth-inhibiting bacteria E8 and E9

The two strains E8 and E9 of growth-inhibiting bacteria isolated from the leaf 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). And further, other two bacterial strains (E4-2 and E10) possessing no growth-inhibiting activity made the same clade in the phylogenetic tree (Fig. 5). The growth-inhibiting bacterial strains E8 and E9 had the completely same 16S rRNA gene sequence as that of the strain E4-2 possessing no ability of growth-inhibiting activity against *A. tamarense* (Table 2). The strain E10 showed the difference of only 2 bp of 1485 bp in the sequence data among 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 a color of the colonies. The growth-inhibiting strains E8 and E9 were yellowish ivory, and the non active 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 5N-3 [6] and the 16S rRNA gene sequence homology with the strain E9 was 97.64%, and the number of different base sequence was 35. The 16S rRNA gene sequence homology of Flavobacteriaceae bacterium strain LPK5 [36] and E9 with 94.07%, and the number of different base sequence was 76.

Discussion

Kim et al. (2009) [29] described that about 50% isolates of growth-inhibiting bacteria belonged to the group of CFB (Cytophaga/Flavobacterium/Bacteroidetes), and about 45% to that of the γ -Proteobacteria. Members of the family Flavobacteriaceae have often been reported to be algicidal bacteria against red tide algae. For example, *Flavobacterium* sp. strain 5N-3 showed the 16S rRNA gene sequence homology of 97.64% with the strain E9, and this bacterium was isolated from a water sample of a bloom by the dinoflagellate *Karenia mikimotoi* and the strain 5N-3 showed growth-inhibitory effects against *K. mikimotoi* [6, 30]. Another strain belonging to Flavobacteriaceae, the strain LPK5 was reported to reveal motility-inhibiting activity against the dinoflagellate *Lingulodinium polyedrum* [31, 32]. We isolated two strains (E8 and E9) of growth-inhibiting bacteria against *A. tamarensis*, and they showed the same growth-inhibiting activity against the examined phytoplankton species. Both strains belonged to the family Flavobacteriaceae, and the results of the 16S rRNA gene sequence analyses proved these strains to be the closely resembling species with the DNA homology of 100%. Interestingly, two bacterial strains E4-2 and E10 were isolated from the same seagrass sample and they possess no growth-inhibiting activity against *A. tamarensis*, despite the fact that 16S rRNA gene sequence homologies of E9 and E4-2 was 100%, and that of E9 and E10 was 99.80%, respectively. This is the first report in marine bacteria that the same species showed conflicting activities on algicidal effect. It is needed to analyze whole genome analyses of these bacterial strains in the future in order to understand which genome controls the production of algicidal matters.

The previous studies on algicidal bacteria against *A. tamarensis* [33, 34, 35, 36] and *A. catenella* [37] described that these bacterial strains inhibited the toxic dinoflagellates with the addition of initial density of as many as $10^8 \sim 10^{10}$ cells ml^{-1} . On the other hand, in the present study, the growth of *A. tamarensis* was markedly inhibited by the bacterium strain E9 even the 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

the strain E9 was significantly higher than that of the previously reported bacteria [33-36].

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

Growth-inhibiting activities of the culture filtrate of the bacterial strain E9 were observed against *A. tamarens* to some extent (Fig.3). This result suggests that the bacterium E9 produced some matters which inhibit an increase of *A. tamarens*. However, the 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 the diatom *Skeletonema costatum* strain NIES-324 [41]. It was reported that the bacterial strain DHQ25 made indirect attack against *A. tamarens* and produced algicidal proteins with molecular weight of 14.5 kDa [42]. *A. tamarens* has a resistant ability to algicidal bacteria of direct attack type, because *A. tamarens* swims and has the thecal plates, and these characteristics work as protecting measures to direct attack bacteria. Consequently, the algicidal bacteria of indirect attack type (producing algicidal matters) probably work more effectively than algicidal bacteria of direct attack type.

The algicidal bacteria against raphidophytes such as *Chattonella* spp. [4, 5, 10, 43, 44, 45] and *Heterosigma akashiwo* [5, 7, 8, 43, 46] were members of the genera *Alteromonas*, *Cytophaga*. and *Pseudoalteromonas*. Studies on algicidal bacteria against diatoms have been relatively fewer than 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, R had an 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 activities against nine diatoms including the species of Centrales and Pennales [15]. The 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 bacterial attack pattern.

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

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

The seagrasses such as *Z. marina* and *Z. noltii* has an ability to inhibit the growth of phytoplankton by allelopathy [19, 20, 51]. For example, the growth of phytoplankton delayed by addition of *Z. noltii* [20]. The extract made of the leaves of *Z. marina* and *Z. noltii* reduced photosynthetic activity of *A. catenella* (Whedon et Kofoid) Balech [19]. However, the present study newly demonstrated that *Z. marina* has an ability to inhibit the growth of the toxic dinoflagellate *A. tamarensis* severely by virtue of associated algicidal bacteria. It is the future investigation to evaluate which is more important to reduce phytoplankton growth, allelopathy or algicidal bacteria.

The seagrass beds have rapidly been disappearing at a rate of 110 km² yr⁻¹ in the world since 1980,

and 29% of the initial areas 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 were increasing globally [2]. There is a report that the large scale decline of seagrass beds accompanied increasing frequencies of the toxic blooms of the dinoflagellate *A. minutum* Halim in the Mediterranean coast [53].

When the cells of phytoplankton were killed by algicidal bacteria, marine organic matters derived from 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 matters, and hence function of microbial loop, and more extensive studies are needed on these processes in the future.

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Figure caption

Fig. 1. Effects of the growth-inhibiting bacterium strain E9 with different inoculum sizes on *Alexandrium tamarense* in the modified SWM-3 medium. 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, (h) 2.9×10^0 cells ml⁻¹. Control (open circle) indicates the growth of *A. tamarense* with no addition of the bacterial cells.

Fig. 2. Effects of the bacterial strain E9 on the morphology of *Alexandrium tamarense*: (a) Control, no addition of bacterial cells; (b) An *A. tamarense* cell with detached thecal plates after three days of incubation; (c) A spherical cell, presumably temporary cyst after three days; (d) A disrupting cell releasing cell contents observed on the day 7 after the addition of the bacterial cells. Scale bar is 20 µm.

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

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

495

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

Table 1. Species of phytoplankton used in the present study and the temperature conditions for experiments. All cultures were kept under the light intensities of 100-120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 14h L: 10h D light-dark cycle.

Class and species	Strain name	Origin of locality	Isolater	Temperature (°C)
Dinophyceae				
<i>Alexandrium tamarense</i>		Osaka Bay	K. Yamamoto	15
<i>Heterocapsa circularisquama</i>		Uranouchi Inset	T. Uchida	20
Bacillariophyceae				
<i>Chaetoceros mitra</i>		Bering Sea	K.I. Ishii	15
Raphidophyceae				
<i>Chattonella antiqua</i>	NIES-1	Harima-Nada	*NIES	20
<i>Fibrocapsa japonica</i>		Harima-Nada	I. Imai	20
<i>Heterosigma akashiwo</i>	IWA	Bingo-Nada	H. Iwasaki	20

Table 2. The sequence similarity (%; upper half) and the number of different base sequence (under half) among four isolated bacterial strains and closely-related algicidal species, Flavobacteriaceae bacterium LPK5 and *Flavobacterium* sp. 5N-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. <i>Flavobacterium</i> sp. 5N-3	35	35	35	37	-	92.52
6. Flavobacteriaceae bacterium LPK5	76	76	76	78	112	-

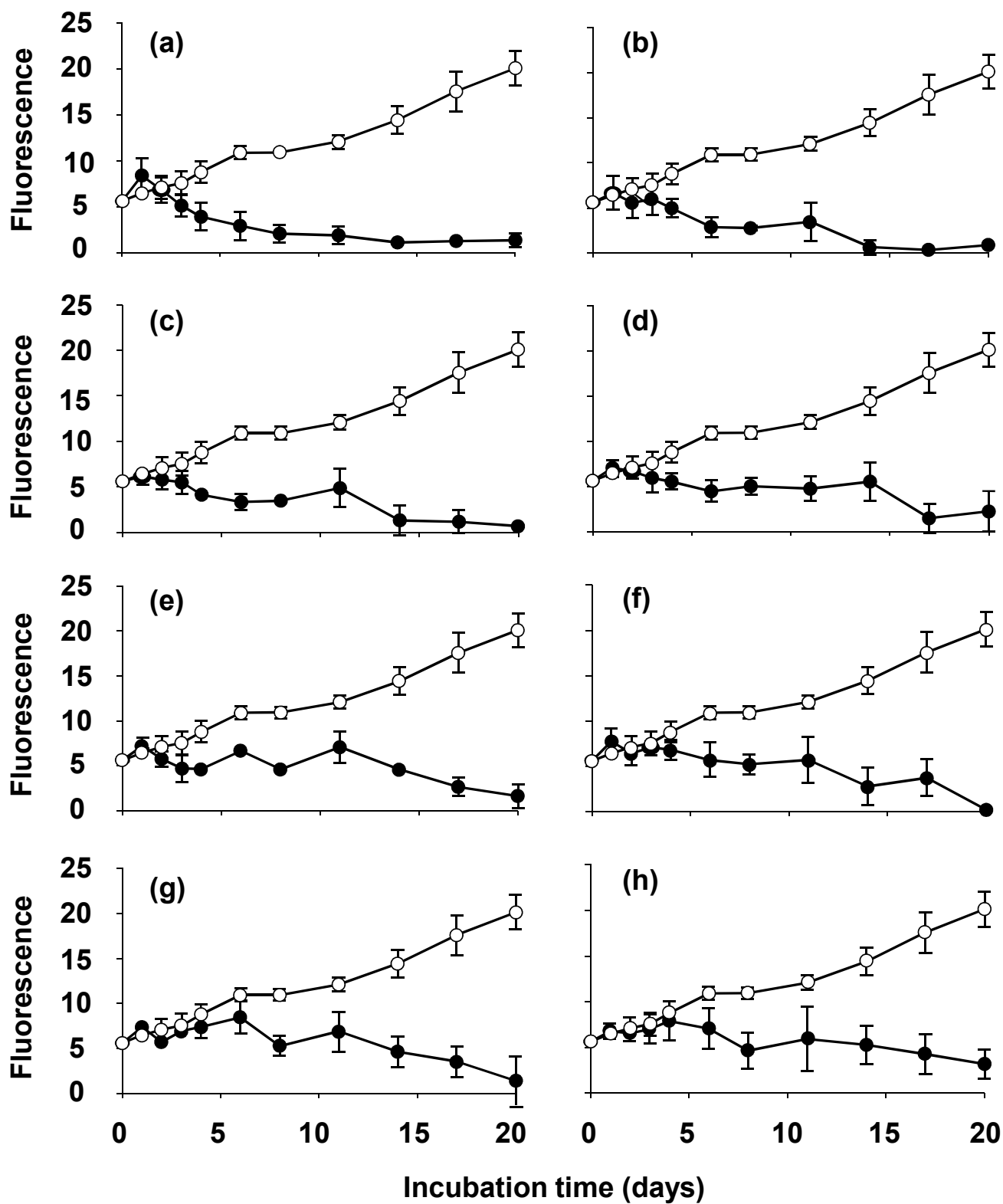


Fig. 1 (Onishi et al.)

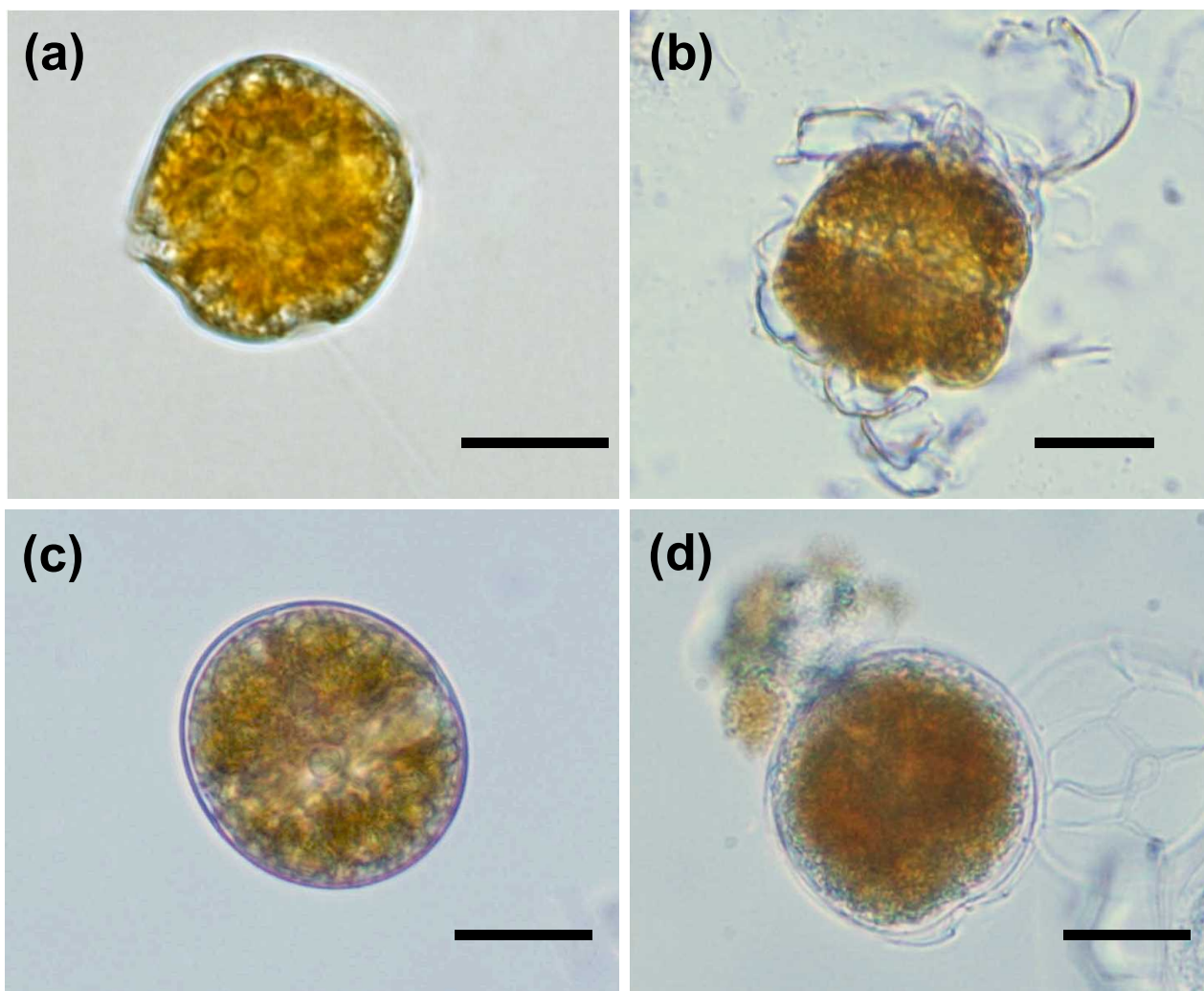


Fig. 2. (Onishi et al.)

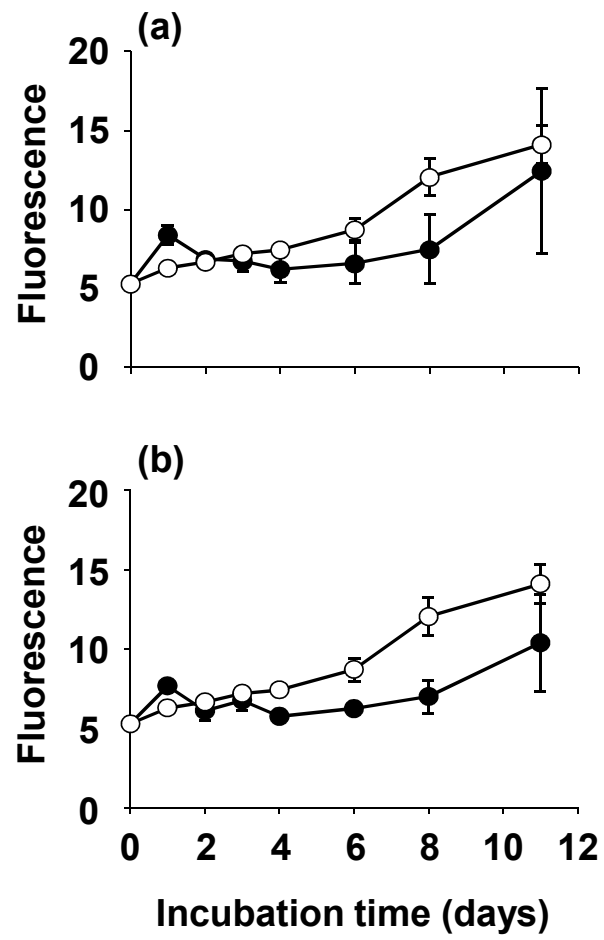


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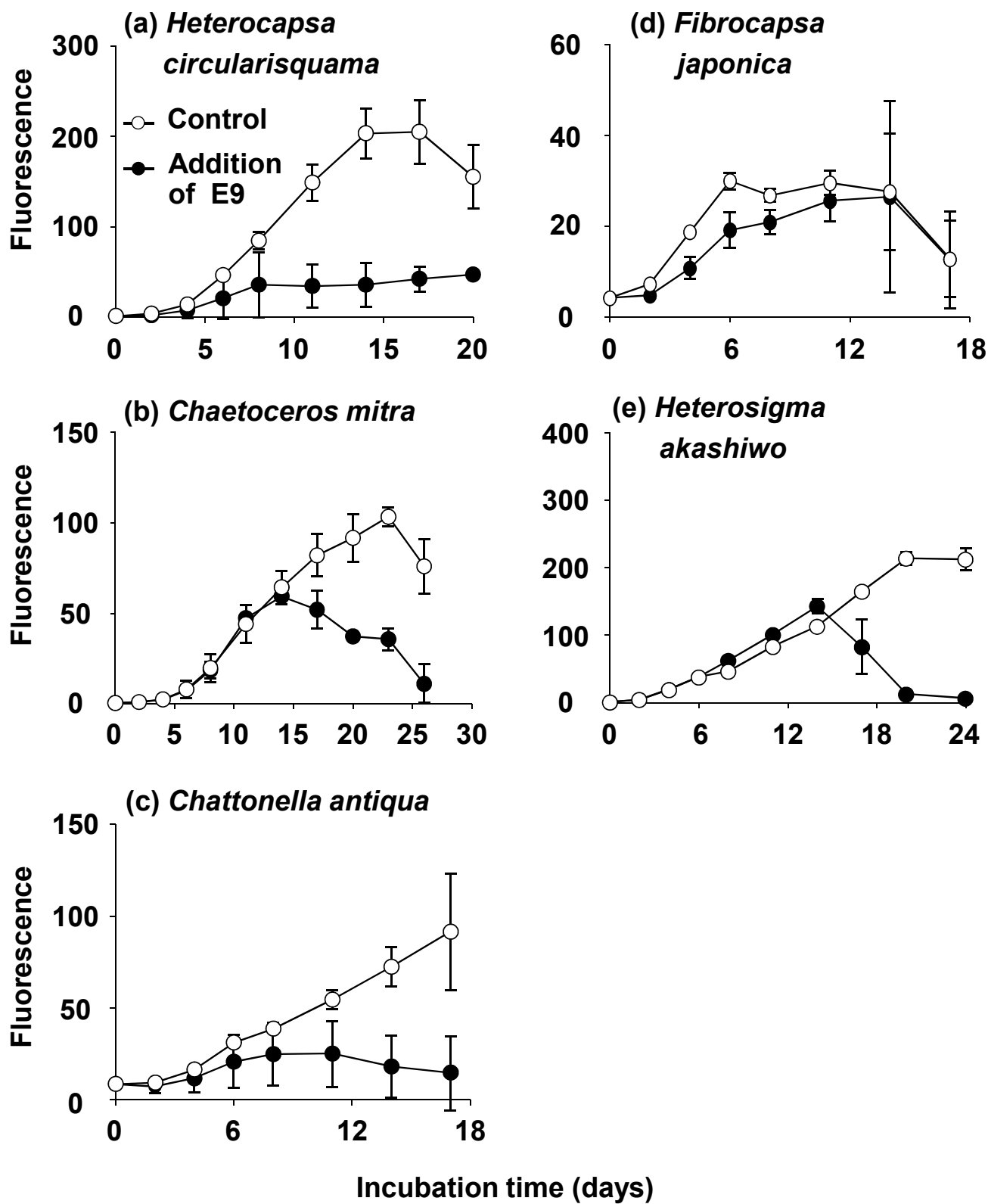
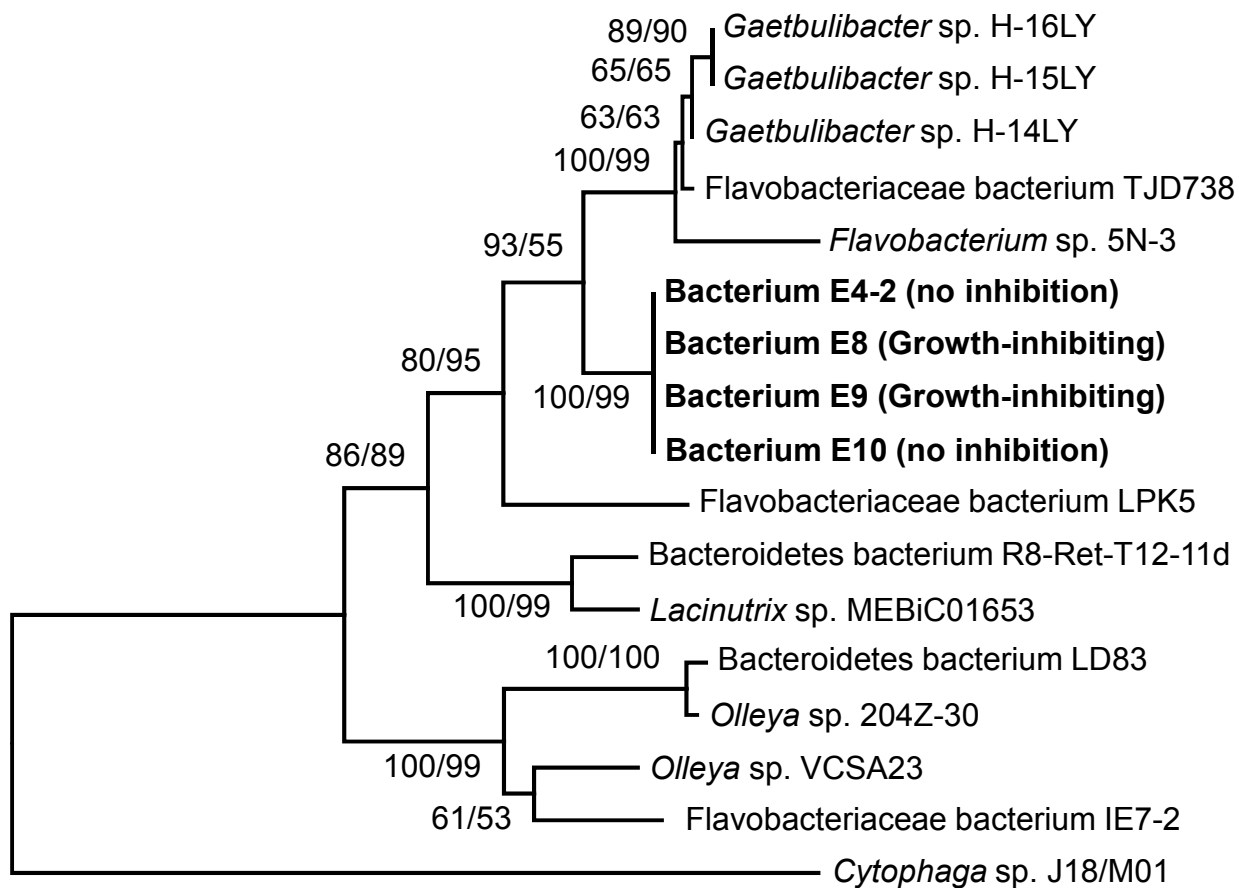


Fig. 4. (Onishi et al.)



0.01

Fig. 5. (Onishi et al.)