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Title

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

Authors

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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.

40 **Introduction**

41 Paralytic shellfish poisoning (PSP) is a serious problem in the marine bivalve aquaculture industries
42 and gives bad effects on marine lives through food webs in coastal ecosystems of the world [1]. PSP
43 incidents have shown globally increasing trends of scale and frequency [2]. *Alexandrium tamarense*
44 (Lebour) Balech (Dinophyceae) is an infamous species of PSP occurrences. *A. tamarense* widely
45 distributes in the world especially in cold water areas. However, at present we have no feasible prevention
46 measures against PSP occurrences, and it is urgently needed to establish practical methods.

47 Another environmental problem given by phytoplankton in coastal waters is red tide. Noxious red
48 tides have caused mass mortalities of cultured marine lives such as fishes and bivalves accompanying
49 huge amounts of fisheries damages. Consequently, studies on protective measures are seriously needed.
50 Chemical and physical countermeasures, such as spraying copper sulfate and clay scattering to aggregate
51 and sink red tide algae, are considered to give bad effects on coastal ecosystems, because chemical agents
52 would cause serious secondary pollution accompanying mortalities of other organisms and resulting in
53 changes of marine food webs.

54 In general, bacteria play an important role in nutrient regeneration and energy transformation in
55 marine ecosystems [3]. However, in recent years, biological countermeasures to employ bacteria have
56 gathered attentions as environment-friendly strategies in the marine environments [4-8]. Rather many
57 algicidal bacteria were isolated from coastal waters so far such as *Cytophaga* sp. J18/M01 against the
58 fish-killing raphidophyte *Chattonella antiqua* [4, 5], and *Flavobacterium* sp. 5N-3 against the harmful
59 dinoflagellate *Gymnodinium nagasakiense* (currently *Karenia mikimotoi*) [6]. Algicidal bacteria showed
60 an increase particularly at the late phase of red tides in seawater [7, 8]. These bacteria are expected to
61 control these red tide causing microalgae. In addition to algicidal activity, other algal-bacterial
62 interactions are reported such as changing the dominant algal species [7−10], growth-promotion [11],
63 growth-inhibition [12], promoting cyst formation [13], controling the cell toxicity [14] and inducing
64 sexual reproduction of diatoms [15], etc.

65 The seagrass beds have an important function in coastal ecosystems to maintain biodiversity, to
66 provide feeding, housing and spawning grounds for marine lives [16]. The seagrass meadows are known
67 to be hot spots for carbon burial and nutrient cycling in the ocean [17, 18]. As an interesting feature, the
68 seagrasses *Zostera marina* Linnaeus and *Z. noltii* Hornemann exhibit a growth-inhibiting activity against
69 phytoplankton through allelopathy [19, 20]. Since highly diverse microorganisms possessing various
70 activities live in seagrass beds, it is expected that there exist some kinds of algicidal and/or
71 growth-inhibiting bacteria against phytoplankton. Algicidal bacteria against the red-tide flagellates were
72 actually found to distribute with high densities in the biofilm on the blades of the seagrass *Z. marina* [21].
73 Therefore, it is expected that seagrasses are favorite habitats of algicidal bacteria, and they have a
74 potential ability to kill red tide phytoplankton. We consequently inferred that algicidal bacteria in
75 association with seagrasses have a killing and/or growth-inhibiting ability against toxic dinoflagellates,
76 and seagrasses contribute to the reduction of frequency and scale in occurrences of toxic blooms. In this
77 study, we succeeded in isolating bacterial strains possessing markedly strong activities of growth
78 inhibition against the toxic dinoflagellate *Alexandrium tamarense* from the seagrass *Z. marina*, and here
79 we report some characteristics of growth inhibiting activities of these bacteria.
80
81 **Materials and Methods**
82 **Algal cultures**
83 Microalgal species used in this study were presented in Table 1. They were all axenic and maintained
84 in the modified SWM-3 medium prepared with natural seawater [22, 23]. Incubations were made at 15 or
85 20 ºC depending on species and under light intensities about 100 to 120 μmol photons m^{-2} s^{-1} with a 14 h
86 light: 10 h dark photo-cycle. The light conditions for incubation were identical throughout this study.
87
88 **Sampling**
89 Samples of seagrass (*Z. marina*) were collected on 15 October 2009 at a seagrass bed in Usujiri

90 Fishing Port in Hakodate, Hokkaido, Japan (41°56.10' N, 140°56.58' E). Seagrass leaves were taken in a
91 sterilized bottle (500 ml) using forceps and brought back to the laboratory of Hokkaido University kept in
92 a cooler box.
93 Sterilized sea water (200 ml) was added to the bottle containing the *Z. marina* sample and the bottle
94 was shaken 500 times by hand to obtain its easily-detaching biofilm. The seawater with the suspended
95 biofilm was used for enumerating algicidal and/or growth-inhibiting bacteria described in detail in 2.3.
96
97 **Isolation of the growth-inhibiting bacteria active against *A. tamarense***
98 The growth-inhibiting bacteria against *A. tamarense* were enumerated using the MPN method [24, 25].
99 The cultures of *A. tamarense* at the late logarithmetic phase were diluted with the SWM-3 culture
100 medium to 3.3 x 10^3 cells ml^{-1}, and 0.5-ml aliquots were added to the wells of 48-well microplates. The
101 biofilm sample in seawater was filtered through Nuclepore filter (pore size of 1.0 μm) and diluted
102 decimally with sterilized seawater. Aliquot of volume 0.1 ml of each diluted sample was inoculated into
103 each well of the 48-well microplates, containing the 0.5-ml *A. tamarense* culture. The assay cultures in
104 the microplates were incubated under the same conditions described above, and the growth inhibition
105 and/or survival of the diniflagellate in each well was assessed daily with an inverted microscope for two
106 weeks. The wells in which *A. tamarense* cells lost the swimming ability, sank to the bottom of wells,
107 showed roundish form without thecal plates, and were broken were scored as "positive". Sterilized
108 seawater was inoculated into five wells with assay cultures as controls. From the "positive" wells, 0.5-ml
109 aliquots were added to the culture of *A. tamarense* in the wells (6.0 x 10^3 cells ml^{-1}), and the activity of
110 growth-inhibition was twice confirmed. Aliquots of 0.1 ml "positive" culture were spread onto the $ST10^{-1}$
111 agar medium [26] and incubated at a temperature of 20 ºC under the dark conditions for two weeks to
112 form colonies. Individual bacterial colonies of the total 23 strains were isolated, grown in the $ST10^{-1}$
113 liquid medium, and frozen at -30 ºC until the experiments.
114

115 **Screening**

116 For screening the growth-inhibiting bacteria, frozen clones were thawed and grown again in the
117 $ST10^{-1}$ liquid medium to reach cell densities of about 10^8 cells ml^{-1}. An aliquot of each appropriately
118 diluted bacterial culture was inoculated with the densities of about 10^4 cells ml^{-1} in to 4-ml cultures of *A.*
119 *tamarense* (10^2 cells ml^{-1}) in glass tubes (diameter of 13 mm). The growth and/or growth-inhibition of *A.*
120 *tamarense* were monitored by *in vivo* fluorescence using a fluorometer (10-AU Fluorometer, Turner
121 Designs, Inc.). Determinations of fluorescence were made after the agitation of culture tubes using a
122 vortex mixer. Control was set by the inoculation of sterilized seawater to *A. tamarense* culture in tubes. As
123 a result, two strains of E8 and E9 were obtained as growth-inhibiting bacteria against *A. tamarense*.
124
125 **Molecular analysis of bacteria**

126 The isolated 23 clones of bacteria were grown in the $ST10^{-1}$ liquid medium, and bacterial cells in 200
127 µl-culture were collected with centrifugation (2000 x g for 5 min) followed by twice washings with the
128 PBS buffer. After removing the supernatant from sample, DNA was extracted using the Chelex method
129 [27]. The 16S rRNA gene was amplified by PCR by using of the primers 8F and 1492R following the
130 conditions of 2x PCR buffer 10 µl, 2 mM of dNTP 4 µl, 10 pM of each primer 0.5µl, template 1µl,
131 Milli-Q 3.6µl and KOD FX Neo (TOYOBO, Osaka, Japan). The initial denaturizing period of 3 min was
132 followed by 35 cycles at 94 ºC for 30 sec, 56 ºC for 30 sec, 72 ºC for 2 min, and the final extension time
133 (72 ºC) was 7 min. PCR products were checked using 1% agarose gel electrophoresis. To purify DNA
134 template strands, PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA)
135 following the instruction manual. The cycle sequencing samples were purified by ethanol precipitation.
136 Sequencing was conducted using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The
137 obtained sequences were assembled using Chromas PRO (Technelysium Pty Ltd, Tewantin, Australia).

138 Phylogenetic and molecular evolutionary analyses for obtained sequences were conducted using
139 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. 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 ºC under the above light conditions. Growth and/or survival of *A. tamarense* 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. tamarense* 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. tamarense* culture (6.0×10^3 cells ml^{-1}) in 300 ml flasks. The bacterium attacked and partially killed *A. tamarense* 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. tamarense* culture was inoculated (3.0×10^3 cells ml^{-1}) with

concentrations of culture filtrates of 50 and 80%. Incubations were made at 15 ºC 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 x 10^3 cells ml^{-1}); three harmful raphidophytes *Chattonella antiqua* (initial density of 2.0 x 10^3 cells ml^{-1}), *Fibrocapsa japonica* (initial density of 2.2 x 10^3 cells ml^{-1}) and *Heterosigma akashiwo* (initial density of 2.7 x 10^3 cells ml^{-1}), and a centric diatom *Chaetoceros mitra* (initial density of 2.2 x 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 x 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 x 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 ºC 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).

190 Controls (no addition of bacteria) showed continuous increase of *A. tamarense* cells until the end of
191 the culture experiment (day 20). On the other hand, the growth of *A. tamarense* was inhibited by the all
192 additions of the bacterial strain with eight different cell densities (2.9×10^0–2.9×10^7 cells ml^{-1}).
193 The growth-inhibiting effects of the strain E9 against *A. tamarense* were observed under a light
194 microscope (Fig. 2). A normal *A. tamarense* cell is shown in Fig. 2a. When the bacterial strain E9 was
195 added to *A. tamarense* culture, the swimming activities of *A. tamarense* cells were inhibited and the thecal
196 plates were often detached from the cell (Fig. 2b). Spherical cells, presumably the temporary cyst formed
197 from vegetative cells against the stress by bacterial addition (Fig. 2c), were frequently observed on the
198 day 3 and thereafter. Eventually, disrupted *A. tamarense* cells were frequently observed in the culture with
199 bacteria (Fig. 2d).
200
201 **Growth-inhibiting activity of the bacterial culture filtrate**
202 Growth-inhibiting activity of the bacterial culture filtrate against *Alexandrium tamarense* was
203 examined using the culture of the bacterium strain E9.
204 The tubes of control (no addition of filtrates) showed a continuous increase during the experiment
205 period (Fig. 3). In the case of the additions of bacterial culture filtrate with 50% and 80% concentrations
206 to *A. tamarense,* the dinoflagellate revealed a growth-inhibition until the day 4 to 6. The growth of *A.*
207 *tamarense* appeared to recover the growth after the day 6.
208
209 **Effects of the bacterium E9 on the growths of other phytoplankton species**
210 The growth-inhibiting range of the bacterium strain E9 was examined using other five marine
211 phytoplankton species, i.e., the three fish-killing raphidophytes *Chattonella antiqua*, *Fibrocapsa japonica*
212 and *Heterosigma akashiwo*, the bivalve-killing dinoflagellate *Heterocapsa circularisquama*, and the
213 diatom *Chaetoceros mitra.*
214 The dinoflagellate *Heterocapsa circularisquama* revealed a growth inhibition by the bacterium E9

215 (Fig.4a), and the all cells lost motility and sank to the bottom of experimental tubes. The diatom
216 *Chaetoceros mitra* with the addition of the bacterium E9 showed almost the same growth pattern until the
217 day 13 as the control (no addition of the bacterial cells, Fig. 4b). However, the diatom growth was
218 inhibited by the bacterium thereafter. The raphidophyte *Chattonella antiqua* also revealed the growth
219 inhibition by the bacterium E9 (Fig. 4c). The cells tended to sink to the bottom of tubes. In the case of *F.*
220 *japonica,* the effects of the bacterium E9 was not apparent as compared with the tubes of control (no
221 addition of bacteria, Fig. 4d). *H. akashiwo* showed a similar pattern of the growth as the experiment of the
222 diatom *Chaetoceros mitra* (Fig. 4e), and the growth of *H. akashiwo* was inhibited after the day 14.
223
224 **Identification of the growth-inhibiting bacteria E8 and E9**
225 The two strains E8 and E9 of growth-inhibiting bacteria isolated from the leaf of the seagrass *Z.*
226 *marina* were identified according to the molecular analyses, and the analysis showed that these two
227 strains belonged to the same clade in the group of Flavobacteriaceae (Fig. 5). And further, other two
228 bacterial strains (E4-2 and E10) possessing no growth-inhibiting activity made the same clade in the
229 phylogenetic tree (Fig. 5). The growth-inhibiting bacterial strains E8 and E9 had the completely same 16S
230 rRNA gene sequence as that of the strain E4-2 possessing no ability of growth-inhibiting activity against
231 *A. tamarense* (Table 2). The strain E10 showed the difference of only 2 bp of 1485 bp in the sequence
232 data among four strains. The bootstrap values of these four bacterial strains were 100 and 99 for NJ and
233 ML trees. A distinct difference among these bacterial strains was a color of the colonies. The
234 growth-inhibiting strains E8 and E9 were yellowish ivory, and the non active strains E4-2 and E10 were
235 white. Therefore we can conclude that this clade formed one species. A relatively close species of
236 algicidal bacteria was *Flavobacterium* sp. strain 5N-3 [6] and the 16S rRNA gene sequence homology
237 with the strain E9 was 97.64%, and the number of different base sequence was 35. The 16S rRNA gene
238 sequence homology of Flavobacteriaceae bacterium strain LPK5 [36] and E9 with 94.07%, and the
239 number of different base sequence was 76.

240

241 **Discussion**

242 Kim et al. (2009) [29] described that about 50% isolates of growth-inhibiting bacteria belonged to the
243 group of CFB (Cytophaga/Flavobacterium/Bacteroidetes), and about 45% to that of the *γ-Proteobacteria*.
244 Members of the family Flavobacteriaceae have often been reported to be algicidal bacteria against red tide
245 algae. For example, *Flavobacterium* sp. strain 5N-3 showed the 16S rRNA gene sequence homology of
246 97.64% with the strain E9, and this bacterium was isolated from a water sample of a bloom by the
247 dinoflagellate *Karenia mikimotoi* and the strain 5N-3 showed growth-inhibitory effects against *K.*
248 *mikimotoi* [6, 30]. Another strain belonging to Flavobacteriaceae, the strain LPK5 was reported to reveal
249 motility-inhibiting activity against the dinoflagellate *Lingulodinium polyedrum* [31, 32]. We isolated two
250 strains (E8 and E9) of growth-inhibiting bacteria against *A. tamarense*, and they showed the same
251 growth-inhibiting activity against the examined phytoplankton species. Both strains belonged to the
252 family Flavobacteriaceae, and the results of the 16S rRNA gene sequence analyses proved these strains to
253 be the closely resembling species with the DNA homology of 100%. Interestingly, two bacterial strains
254 E4-2 and E10 were isolated from the same seagrass sample and they possess no growth-inhibiting activity
255 against *A. tamarense,* despite the fact that 16S rRNA gene sequence homologies of E9 and E4-2 was
256 100%, and that of E9 and E10 was 99.80%, respectively. This is the first report in marine bacteria that the
257 same species showed conflicting activities on algicial effect. It is needed to analyze whole genome
258 analyses of these bacterial strains in the future in order to understand which genome controls the
259 production of algicidal matters.

260 The previous studies on algicidal bacteria against *A. tamarense* [33, 34, 35, 36] and *A. catenella* [37]
261 described that these bacterial strains inhibited the toxic dinoflagellates with the addition of initial density
262 of as many as 10^8~10^{10} cells ml^{-1}. On the other hand, in the present study, the growth of *A. tamarense* was
263 markedly inhibited by the bacterium strain E9 even the initial inoculums size of 2.9 cells ml^{-1} (Fig. 1),
264 demonstrating the significantly strong growth-inhibiting activity of this bacterial strain. The activity of

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

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

273 Growth-inhibiting activities of the culture filtrate of the bacterial strain E9 were observed against *A.*
274 *tamarense* to some extent (Fig.3). This result suggests that the bacterium E9 produced some matters
275 which inhibit an increase of *A. tamarense*. However, the growth-inhibiting activity disappeared after six
276 days of the experiments (Fig. 3). It was confirmed that the marine bacterium *Pseudoalteromonas* sp.
277 strain A28 was able to produce an extracellular serine protease against the diatom *Skeletonema costatum*
278 strain NIES-324 [41]. It was reported that the bacterial strain DHQ25 made indirect attack against *A.*
279 *tamarense* and produced algicidal proteins with molecular weight of 14.5 kDa [42]. *A. tamarense* has a
280 resistant ability to algicidal bacteria of direct attack type, because *A. tamarense* swims and has the thecal
281 plates, and these characteristics work as protecting measures to direct attack bacteria. Consequently, the
282 algicidal bacteria of indirect attack type (producing algicidal matters) probably work more effectively
283 than algicidal bacteria of direct attack type.

284 The algicidal bacteria against raphidophytes such as *Chattonella* spp. [4, 5, 10, 43, 44, 45] and
285 *Heterosigma akashiwo* [5, 7, 8, 43, 46] were members of the genera *Alteromonas*, *Cytophaga*. and
286 *Pseudoalteromonas.* Studies on algicidal bacteria against diatoms have been relatively fewer than those
287 against harmful phytoflagellates. *Cytophaga* sp. strain J18/M01 [5] was able to kill four diatoms
288 (*Skeletonema costatum, Ditylum brightwellii, Chaetoceros didymus,* and *Thalassiosira* sp.). *Alteromonas*
289 sp. strains S, D, R had an ability to kill two diatoms (*Ditylum brightwellii* and *Chaetoceros didymus*), and

290 *Alteromonas* sp. strain K killed *Chaetoceros didymius* [43]. The bacterial strain K12 exerted algicidal
291 activities against nine diatoms including the species of Centrales and Pennales [15]. The diatoms show a
292 wide variety in morphology, cell size, and life form pattern which includes planktonic, benthic, and
293 periphytic forms. Therefore, the tolerance of diatoms to algicidal bacteria probably differs depending on
294 their taxonomy and bacterial attack pattern.

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

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

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

314 The seagrass beds have rapidly been disappearing at a rate of 110 km^2 yr^{-1} in the world since 1980,

315 and 29% of the initial areas disappeared since 1879, when seagrass areas were first approximately
316 determined [52]. On the other hand, the scale and frequency of occurrences of harmful algal blooms were
317 increasing globally [2]. There is a report that the large scale decline of seagrass beds accompanied
318 increasing frequencies of the toxic blooms of the dinoflagellate *A. minutum* Halim in the Mediterranean
319 coast [53].

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

325

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331

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469

470

471 **Figure caption**

472

473 Fig. 1. Effects of the growth-inhibiting bacterium strain E9 with different inoculum sizes on
474 *Alexandrium tamarense* in the modified SWM-3 medium. Initial cell density of *A. tamarense* was 3.6 x
475 10^2 cells ml^{-1}. Initial bacterial densities were (a) 2.9 x 10^7 cells ml^{-1}, (b) 2.9 x 10^6 cells ml^{-1}, (c) 2.9 x 10^5
476 cells ml^{-1}, (d) 2.9 x 10^4 cells ml^{-1}, (e) 2.9 x 10^3 cells ml^{-1}, (f) 2.9 x 10^2 cells ml^{-1}, (g) 2.9 x 10^1 cells ml^{-1},
477 (h) 2.9 x 10^0 cells ml^{-1}. Control (open circle) indicates the growth of *A. tamarense* with no addition of the
478 bacterial cells.

479

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

484

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

490

491 Fig. 4. Effects of the bacterium strain E9 on the growth and/or survival of the dinoflagellate
492 *Heterocapsa circularisquama* (a) the diatom *Chaetoceros mitra* (b), the raphidophytes *Chattonella*
493 *antiqua* (c), *Fibrocapsa japonica* (d) and *Heterosigma akashiwo* (e). Added bacterial cell density was 1 x
494 10^4 cells ml^{-1}. Control was no addition of bacterium.

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 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 μmol photons m^{-2} s^{-1} and 14h L: 10h D light-dark cycle.

Class and species	Strain name	Origin of locality	Isolater	Temperature (℃)
Dinophyceae				
Alexandrium tamarense		Osaka Bay	K. Yamamoto	15
Heterocapsa circularisquama		Uranouchi Inset	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

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. *Flavobacterium* sp. 5N-3	35	35	35	37	-	92.52
6. Flavobacteriaceae bacterium LPK5	76	76	76	78	112	-

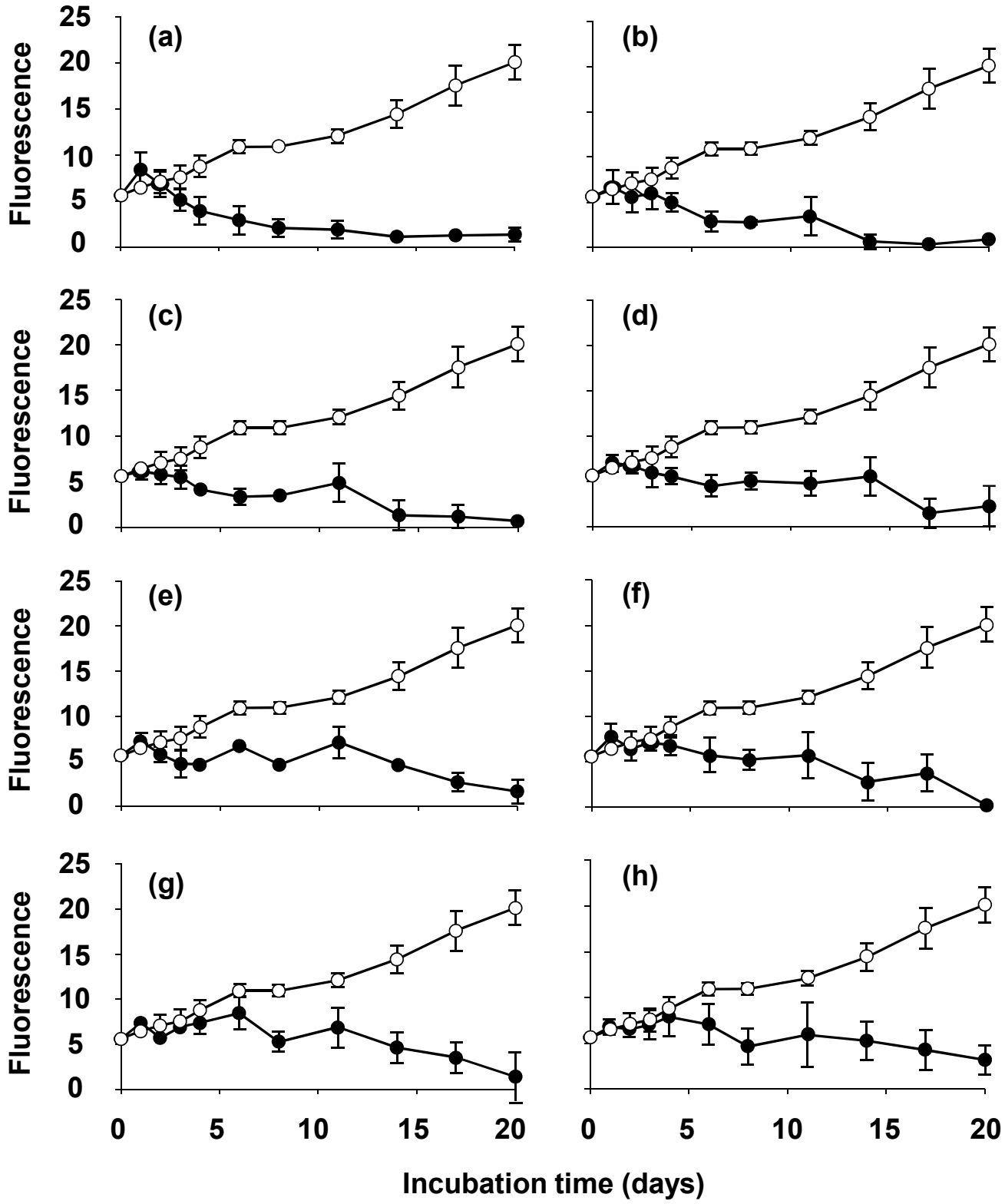


Fig. 1 (Onishi et al.)

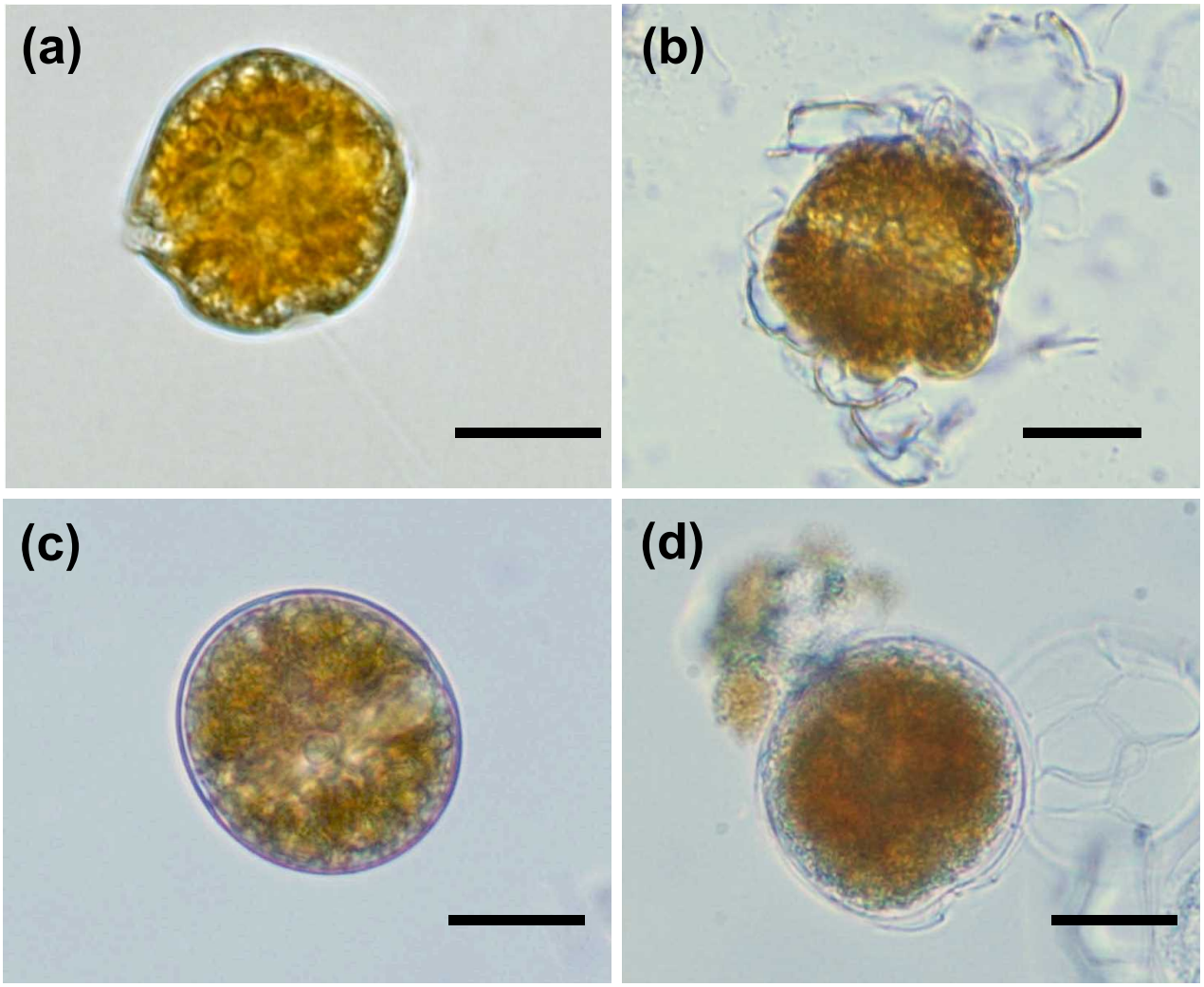
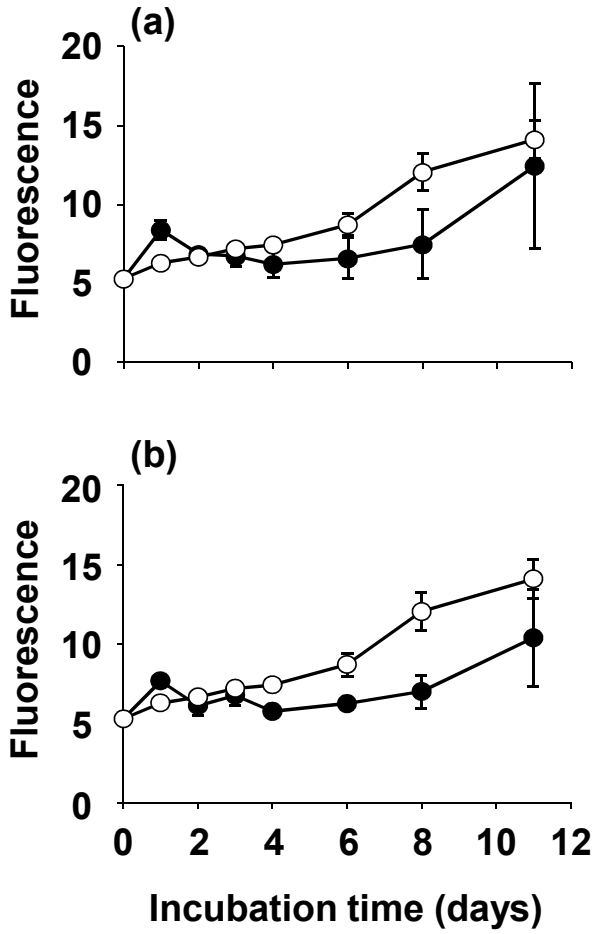


Fig. 2. (Onishi et al.)



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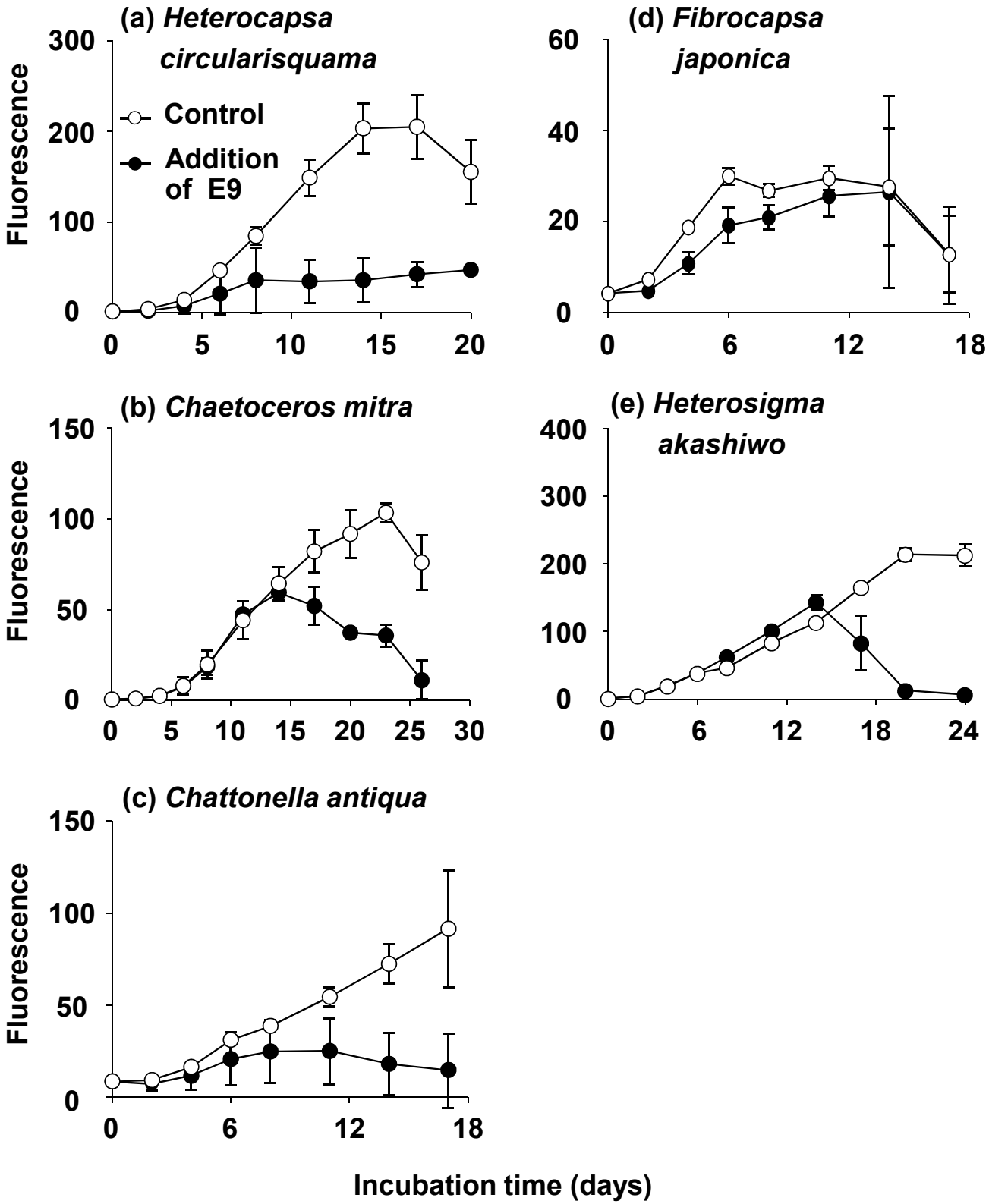

Fig. 4. (Onishi et al.)

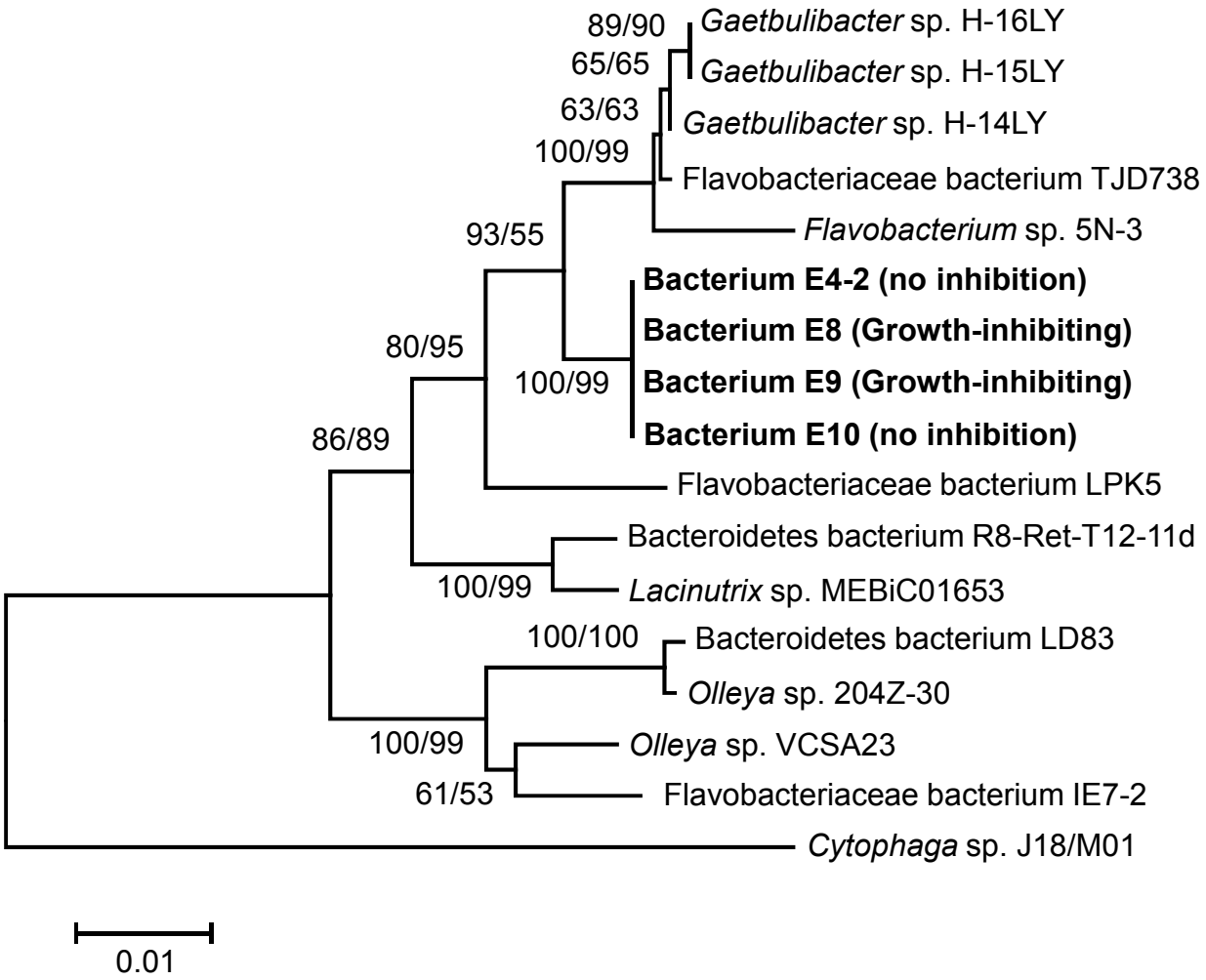

Fig. 5. (Onishi et al.)