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Generation of Reactive Oxygen Species by Raphidophycean Phytoplankton

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***Chattonella marina*, a raphidophycean flagellate, is one of the most toxic red tide phytoplankton and causes severe damage to fish farming. Recent studies demonstrated that *Chattonella* sp. generates superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$), which may be responsible for the toxicity of *C. marina*. In this study, we found that other raphidophycean flagellates such as *Heterosigma akashiwo*, *Olisthodiscus luteus*, and *Fibrocapsa japonica* also produce O_2^- and H_2O_2 under normal growth condition. Among the flagellate species tested, *Chattonella* has the highest rates of production of O_2^- and H_2O_2 as compared on the basis of cell number. This seems to be partly due to differences in their cell sizes, since *Chattonella* is larger than other flagellate species. The generation of O_2^- by these flagellate species was also confirmed by a chemiluminescence assay by using 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA). All these raphidophycean flagellates inhibited the proliferation of a marine bacterium, *Vibrio alginolyticus*, in a flagellates/bacteria co-culture system, and their toxic effects were suppressed by the addition of superoxide dismutase (SOD) or catalase. Our results suggest that the generation of reactive oxygen species is a common feature of raphidophycean flagellates.**

Key words: red tide plankton; Raphidophyceae; *Chattonella marina*; reactive oxygen species; toxicity

Several species of marine plankton have been identified as the causative organisms of red tide.[1] In Japan, *Chattonella* sp. is one of the most frequently appearing noxious red tide phytoplankton and is highly toxic to fish, especially to yellowtail, *Seriola quinqueradiata*. In the Seto Inland Sea and the coastal areas of Kyushu, blooming of *Chattonella* sp. has repeatedly caused massive mortalities of cultured fish resulting in immense economic losses. Thus, red tide of *Chattonella* sp. is still one of the most serious aquacultural problems. Although the precise mechanism of the toxic action of *Chattonella* sp. remains unclear, suffocation is generally supposed to be the direct cause of the fish death by this flagellate species. Recent studies[2–4] demonstrated that a decrease in oxygen partial pressure of arterial blood is the earliest physiological disturbance observed in fish after exposure to *C. marina*. In addition, physiological and histological studies of fish exposed to *C. marina* suggested that the blockade of respiratory water flow through the gill lamellae caused by excessive mucus interferes with O_2 transfer, resulting in asphyxia.[5,6] Recently we and other groups have found that *Chattonella* sp. generates reactive oxygen species (ROS) such as O_2^- and H_2O_2.[7–14] Furthermore, our previous studies using electron spin resonance (ESR) spectroscopy with the spin traps 5,5-dimethyl-1-pyrroline-*N*-oxide and *N*-*t*-butyl-α-phenylnitrone showed that *C. marina* generates hydroxyl radical ($\cdot OH$) which is known as the most toxic reactive oxygen species.[14] Since harmful effects of oxygen radicals generated in various biological systems have been well documented,[15–19] these results suggest that ROS generated by *Chattonella* sp. is responsible for gill tissue injury, which eventually causes fish death. Consistent with this hypothesis, our previous experiments indicated that *C. marina* had a ROS-mediated toxic effect on a marine bacterium, *Vibrio alginolyticus*.[12]

Chattonella sp. is classified in the class Raphidophyceae of the Division Chrysophyta. The raphidophyceaen algae (Chloromonadophyceae) are small golden-brown flagellates which contain fucoxanthin as a common pigment. Although the taxonomy of this algal class is still controversial, *Heterosigma* sp., *Fibrocapsa* sp., and *Olisthodiscus* sp. are also classified in Raphidophyceae. Yang *et al.*[20] have shown that *H. carterae* has an oxygen-radical-mediated toxic effect on rainbow trout, *Oncorhynchus mykiss*.

This study was therefore undertaken to ascertain whether or not the generation of ROS is a common feature of raphidophycean flagellates. The results demonstrate that all raphidophycean flagellate species tested produce O_2^- and H_2O_2 to different extents.

Materials and Methods

Materials. Superoxide dismutase (Cu, Zn-SOD) (3800 units/mg of protein, from bovine erythrocytes), catalase (5900 units/mg of protein), cytochrome *c* (from horse heart), and horseradish peroxidase (100 units/mg of protein) were purchased from Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan. 2-Methyl-6-(*p*-methoxyphenyl)3,7-dihydroimidazo-[1,2-*a*]pyrazin-3-one (MCLA) was obtained from Tokyo Kasei Kogyo, Co., Ltd., Tokyo, Japan. Other chemicals were of the highest grade commercially available.

Plankton culture. Chattonella marina (strains 78 and 85) and *Fibrocapsa japonica* were generously provided by Kagoshima Prefectural Fisheries Experimental Station, Japan. Another strain of *C. marina* (strain 309) isolated in the Seto Inland Sea was from the Akashiwo Research Institute of Kagawa Prefecture. *C. antiqua*, isolated in Nagasaki (Chijiwa Bay) in 1990 by Dr. A. Ishimatsu (Nomo Fisheries Station, Nagasaki University) was also used. *Heterosigma akashiwo* NIES-6 and *Olisthodiscus luteus*

Abbreviations: ESM medium, Erd-Schreiber modified medium; SOD, superoxide dismutase; ESR, electron spin resonance; ROS, reactive oxygen species; MCLA, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one.

NIES-15 were from the National Institute for Environmental Studies, Environmental Agency, Japan. These flagellates were cultured at 26°C in ESM medium (pH 8.2) under 3000 lx illumination with a cycle of 12 h light and 12 h dark.[21] ESM medium was prepared according to the composition reported originally but without soil extract, *i.e.*, 120 mg of $NaNO_3$, 5 mg of K_2HPO_4, 0.1 mg of vitamin B_1, 0.01 mg of vitamin B_{12}, 0.001 mg of biotin, 0.26 mg of EDTA-Fe^{3+}, 0.33 mg of EDTA-Mn^{2+}, and 1 g of tris(hydroxymethyl)aminomethane were dissolved in 1 liter of seawater and the pH was adjusted to 8.2, followed by autoclaving (121°C, 15 min). Under these conditions, maximum cell concentration of 5×10^4 cells/ml for *Chattonella* sp., and 6×10^5 cells/ml for the other species were routinely attained. Unless otherwise noted, flagellates in exponential growing phase were used throughout the experiments. All cultivation was done using sterilized instruments. Cells were counted with a hemocytometer. The cellular protein content of each flagellate was measured by the method of Lowry *et al.*[22] with bovine serum albumin as a standard.

Measurement of superoxide anion. Generation of superoxide anion (O_2^-) by flagellates was measured spectroscopically on the basis of SOD-inhibitable reduction of cytochrome *c*. After the addition of cytochrome *c* (final concentration, 50 μM) to the flagellate suspension in ESM medium, reduction of cytochrome *c* during the first 1 min of incubation was measured as an increase in the difference in absorbance between 550 and 540 nm with a spectrophotometer (Beckman DU-40, Beckman Instruments, Inc., Fullerton, CA) in the presence or absence of 100 units/ml of SOD at 26°C. The reading was converted to nmol of cytochrome *c* reduced using a molar absorbance coefficient of 19.1 $mM^{-1}cm^{-1}$[23] after subtracting the 0 time value in the presence of 100 units/ml of SOD as a background value.

Measurement of H_2O_2. Detection of H_2O_2 in the flagellate cell suspension was done by the scopoletin method[24] at 26°C. After the addition of scopoletin (final concentration, 1 μM) and horseradish peroxidase (final concentration, 20 units/ml) to the flagellate cell suspension in ESM medium, a decrease in fluorescence intensity during the first min of incubation was measured with a fluorescence spectrophotometer (Hitachi Model 650-60) at an excitation wavelength of 350 nm and an emission wavelength of 460 nm in the presence or absence of 500 units/ml catalase. The catalase-inhibitable decrease of fluorescence intensity was considered to reflect actual H_2O_2. The concentration of H_2O_2 was estimated by using a standard curve of H_2O_2 in cell-free ESM medium. Under the assay conditions, the decrease of fluorescence intensity was proportional to the concentration of H_2O_2.

Chemiluminescence assay. In the chemiluminescence analysis for the detection of superoxide produced by flagellates, we used 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA) as a superoxide-specific chemiluminescent probe as previously described.[25] MCLA was dissolved in distilled water and stored at −80°C until use. After the addition of MCLA to a flagellate cell suspension, the chemiluminescence response was recorded immediately with a lumiphotometer TD-4000 (Labo Science).

Growth inhibition of marine bacteria in flagellate culture. Vibrio alginolyticus was isolated from seaweed and maintained in our laboratory. After being cultured overnight at 31°C with rotational shaking at 100 rpm in ESM medium containing 2% glucose, the bacteria were diluted to a concentration of 3 to 5×10^6 cells/ml with ESM medium. The bacteria were added to the flagellate cell suspension at ratios of 0.4 to 5.0 bacteria per flagellate, then incubated at 26°C under flagellate culture conditions in 2.0 ml total assay mixture. After 8 h incubation, a 10 μl sample of cell suspension (plankton/bacteria co-culture) was withdrawn for enumeration of viable bacteria. Suitable dilutions of these samples were inoculated in triplicate onto Muellar Hinton II agar plates and incubated at 32°C for 24 h before colony counting.

Results

Generation of reactive oxygen species by raphidophycean flagellates

In our previous experiments, we had found that the generation of O_2^- and H_2O_2 by *C. marina* reaches a plateau within a few minutes of incubation.[12,14] Therefore, we measured the rate of generation of O_2^- and H_2O_2 by each flagellate during the initial 1 min. As shown in Fig. 1, it was found that all these raphidophycean flagellate species generate both O_2^- and H_2O_2 to different extents. Among the flagellates tested, *C. marina* (strain 85) showed the highest rate of production of O_2^- and H_2O_2. Although the production rates of O_2^- and H_2O_2 were quite different among the strains tested, all strains of *Chattonella* including *C. antiqua* produced O_2^- and H_2O_2 at higher rates than those of other species, compared on the basis of cell number (Fig. 1). This is partly due to differences in their cell sizes, since *Chattonella* is larger than the others (Fig. 2). Reflecting their different cell sizes, the cellular protein contents of *C. marina* (strain 85), *H. akashiwo*, *O. luteus*, and *F. japonica* were 14.55, 2.07, 1.96, and 1.75 μg/10^4 cells, respectively. Among the *Chattonella* strains tested, no significant differences in cellular protein contents were seen. Based on the data of Fig. 1, the production rates of O_2^- of *C. marina* (strain 85), *H. akashiwo*, *O. luteus*, and *F. japonica* were calculated to be 0.041, 0.019, 0.019, and 0.017 nmol/μg cell protein/min, respectively. The production rate of H_2O_2 of *C. marina* (strain 85) was 0.11 nmol/μg cell protein/min which was also 2–4-fold higher than those of other raphidophycean flagellate species.

Although the details of the mechanism of the production of ROS by *C. marina* are still unclear, our previous observations have demonstrated that the amount of H_2O_2 significantly increased after the disruption of flagellate cells by sonication or filtration through a GF/C glass filter as compared to the H_2O_2 level detected in intact flagellate cell suspensions.[13] These findings suggest that *C. marina* has a compartment in which H_2O_2 accumulated at high concentrations and from which a small amount of H_2O_2 is gradually released during normal growth. To ascertain whether or not this is the case for other raphidophycean flagellate species, we measured H_2O_2 levels in the cell-free filtrates which were obtained by the filtration of each flagellate cell suspension through a GF/C glass filter. As shown in the Table, the level of H_2O_2 detected in each filtrate was 2–6 fold higher than that of the intact cell suspension.

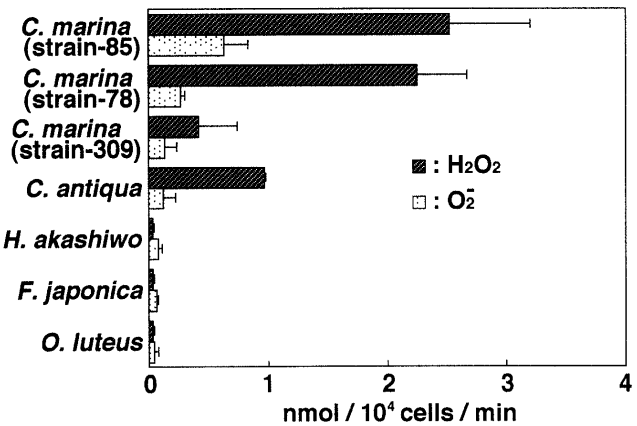

Fig. 1. Detection of O_2^- and H_2O_2 Produced by *C. marina*, *C. antiqua*, *H. akashiwo*, *O. luteus*, and *F. japonica*.

The generation of O_2^- (□) and H_2O_2 (▨) from each flagellate cell suspension during the first min of incubation was measured as described in Materials and Methods. The concentrations of flagellate cells used were $1.3–1.5 \times 10^4$ cells/ml for three different strains of *C. marina* (strain 85, 78, and 309) and *C. antiqua*, and $1.4–2.3 \times 10^5$ cells/ml for *H. akashiwo*, *O. luteus*, and *F. japonica*. The data represent the mean ± SE of three experiments.

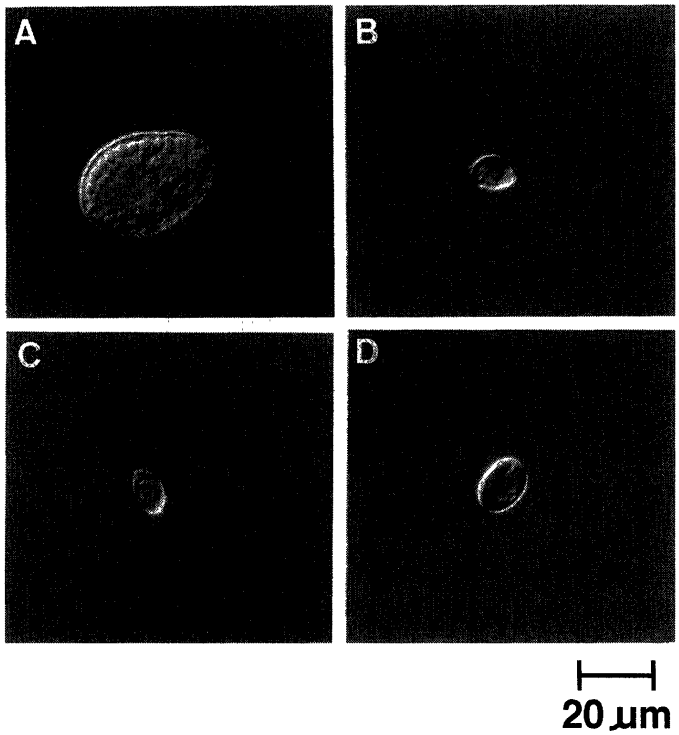

Fig. 2. Phase Contrast Micrographs of *C. marina* (strain 85) (A), *O. luteus* (B), *H. akashiwo* (C), and *F. japonica* (D).

Flagellate cells in exponentially growing phase were observed by microscope (Carl Zeiss Axiophot), and photographed in phase contrast.

Table The Concentrations of H_2O_2 in Intact Flagellate Cell Suspensions and Filtrates of Ruptured Flagellates

The concentrations of H_2O_2 in each flagellate cell suspensions and cell-free filtrates which were obtained by the filtration of each flagellate cell suspension through GF/C glass filter, were measured as described in Materials and Methods. The concentrations of flagellate cells used were 2.2×10^4 cells/ml for *C. marina* (strain 85), and $1.8–2.7 \times 10^5$ cells/ml for *H. akashiwo*, *f. japonica*, and *O. luteus*. Each value is the concentration of H_2O_2 detected during the first min of incubation and represents the mean±SE of triplicate measurements.

Flagellate species	Concentration of H_2O_2 (μM)	
	Intact cell suspension	Cell-free filtrate
Chattonella marina	1.06±0.25	6.55±0.23
Heterosigma akashiwo	0.71±0.17	1.69±0.06
Fibrocapsa japonica	0.80±0.01	3.55±0.02
Olisthodiscus luteus	0.59±0.01	1.55±0.30

Chemiluminescence responses of raphidophycean flagellates in the presence or absence of SOD

To further confirm the generation of O_2^- by these raphidophycean flagellate species, we examined the MCLA-dependent chemiluminescence response of flagellate cell suspensions. Each species kept in ESM medium was assayed immediately after the addition of MCLA (final concentration, 5 μM). As shown in Fig. 3, rapid chemiluminescence responses were observed in all these flagellate cell suspensions after addition of MCLA with a time lag of a few seconds. Similar patterns of luminescence responses were observed in these flagellate cell suspensions: peak activity was reached within 5 s, followed by a slight decline to plateau levels. These chemiluminescence responses were suppressed

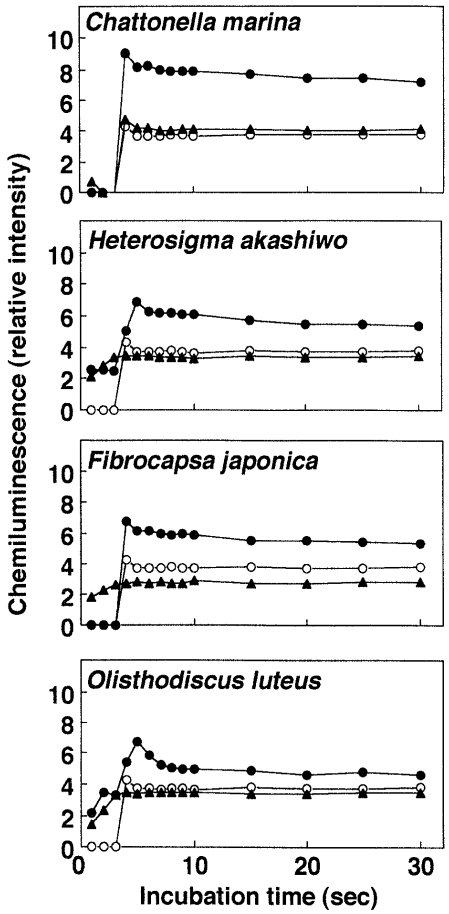

Fig. 3. MCLA-Dependent Chemiluminescence Responses of *C. marina* (strain 85), *H. akashiwo*, *O. luteus*, and *F. japonica* in the Absence or Presence of SOD (100 units/ml).

Immediately after the addition of MCLA (final concentration, 5 μM) to each flagellate cell suspension, the chemiluminescence response was recorded with a luminescence analyzer at 26°C in the absence (●) or presence (▲) of SOD (100 units/ml). The concentrations of flagellate cells used were 2×10^4 cells/ml for *C. marina*, and 2×10^5 cells/ml for *H. akashiwo*, *O. luteus*, and *F. japonica*. ○, ESM medium alone.

in the presence of 100 units/ml SOD to the blank level of ESM medium alone, suggesting that O_2^- mainly contributes to the chemiluminescence. Addition of catalase had no effect, indicating H_2O_2 did not contribute to this luminescence (data not shown).

Effects of raphidophycean flagellates on the growth of V. alginolyticus in ESM medium

Our previous observations have demonstrated that *C. marina* inhibited the proliferation of a marine bacterium, *Vibrio alginolyticus*, in flagellates/bacteria co-culture system.[12] The growth inhibition of bacteria caused by *C. marina* was completely abolished by the addition of catalase and SOD, suggesting that *C. marina* had a ROS-mediated toxic effect on *V. alginolyticus*. Thus, we examined the effects of other raphidophycean flagellates on the growth of *V. alginolyticus*. As shown in Fig. 4, the number of viable bacteria inoculated into *C. marina* suspension was decreased after 8 h of incubation as previously reported.[12] The toxic effect of *C. marina* on *V. alginolyticus* was inhibited by the addition of SOD (100 units/ml) or catalase (500 units/ml). Other raphidophycean flagellate species also inhibited the growth of *V. alginolyticus*, albeit they were less toxic than *C. marina* even at a 10-fold higher ratio of flagellates per bacteria than that of *C. marina*. The growth inhibitions of

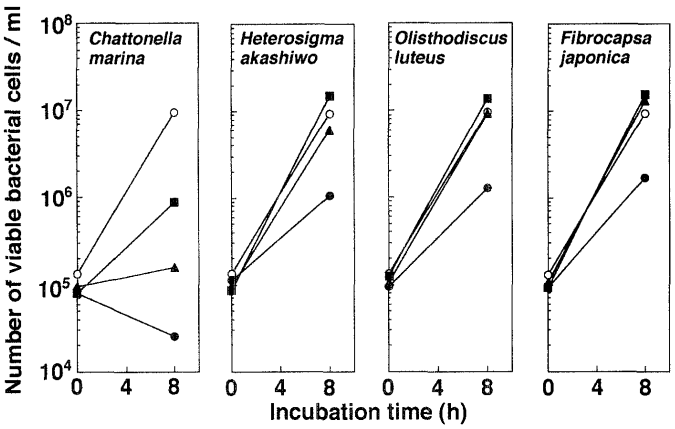

Fig. 4. Effects of *C. marina* (strain 85), *H. akashiwo*, *O. luteus*, and *F. japonica* on the Growth of *V. alginolyticus*.

Bacteria were added to each flagellate cell suspension at initial concentration of 0.8–1.3 × 10^5 cells/ml, and number of viable bacteria was measured after 8 h of incubation at 26 C in the absence (●) or presence of SOD (100 units/ml) (▲) or catalase (500 units/ml) (■) as described in Materials and Methods. The concentrations of plankton cells used were 2 × 10^4 cells/ml for *C. marina*, and 2 × 10^5 cells/ml for *H. akashiwo*, *O. luteus*, and *F. japonica*. ○, Bacteria alone in ESM medium.

bacteria induced by *H. akashiwo*, *F. japonica*, and *O. luteus* were also abolished by the addition of SOD or catalase. These results suggest that these three species also have a ROS-mediated toxic effect on *V. alginolyticus*.

Discussion

Reactive oxygen species (ROS) are produced in various biological systems,[15,26–28] particularly during respiratory burst activation of phagocytic cells.[16] In addition to animal cells, higher plant cells also produce O_2^- and H_2O_2 in several biological processes including photosynthesis in chloroplasts,[28] ferric reduction during iron uptake,[29] and defense mechanisms against pathogens.[30] ROS generated in various biological systems are known to be involved in oxidative damage to various cellular components.[15–19]

In this study, we found that all the raphidophycean flagellates tested produce O_2^- and H_2O_2 under the normal growth conditions, although the production rates of ROS by *H. akashiwo*, *O. luteus*, and *F. japonica* were considerably lower than those by *Chattonella*. Moreover, there was a large difference in the production rates of ROS among the strains of *Chattonella* (Fig. 1). The production of O_2^- by these flagellate species was also supported by a MCLA-dependent chemiluminescence assay (Fig. 3). In contrast to our results, Lee *et al.*[25] have reported that *H. akashiwo* did not induce chemiluminescence as assayed by using MCLA, while *C. marina* caused a marked luminescence. This discrepancy may be due to the lower concentration of cells they used, since chemiluminescence response depends on density of flagellate cells and *H. akashiwo* required more than 10-fold greater cell concentration (> 10^5 cells/ml) to produce a detectable luminescent response as compared to *C. marina*. Moreover, it has been reported that O_2^- and H_2O_2 were detected in *H. carterae* (formerly *H. akashiwo*) cell suspensions based on biochemical assay methods.[20]

Among the flagellate species tested, *C. marina* (strain 85) showed the highest rate of production of O_2^- and H_2O_2. In agreement with these results, *C. marina* showed the most pronounced toxic effect on marine bacteria even at the lower ratio of flagellates per bacteria (Fig. 4). These results suggest that there is a correlation between flagellate's ability to produce ROS and toxicity to surrounding living organisms. This notion is supported by the recent finding that the strain of *C. marina* that is less toxic to yellowtail showed a much lower productivity of O_2^- than that of a more toxic strain.[31]

Our results also suggest that the production of ROS is one of the common characteristics of raphidophycean flagellates. Raphidophycean flagellates generally have several common basic structural features. First, two unequal, heterodynamic flagella arising from an apical depression: the forward flagellum bears two rows of fine tripartite hairs, while the trailing flagellum is smooth and lies close to the cell surface. Second, the cells contain numerous golden-brown chloroplasts in the peripheral cytoplasm. Third, they are naturally wall-less, and have therefore been called naked plankton. Since the taxonomy of this algal class is the subject of controversy, our finding may provide a new clue to clarify disputed taxonomic affinities based on cell morphology alone.

Although the detailed mechanism of the production of ROS by these flagellate species is still unclear, they may have specific metabolic or enzymatic systems that are responsible for the production of ROS. Since these flagellate species have photosynthetic ability, it may be speculated that these organisms have a mechanism of generation of ROS resembling that of plant cells rather than that of animal cells. Regarding this point, it has been reported that there are some NADH oxidation activities capable of generating O_2^- and H_2O_2 in plant plasma membranes.[32] Since this NADH oxidase activity was inhibited by an iron-specific chelator, Desferal, it has been speculated that the enzyme activity is strictly dependent on the presence of iron ions, which seem essential for the generation of ROS.[33] Similar to the NADH oxidase of radish plasma membrane, we have recently found that the generation of ROS by *C. marina* was also inhibited by Desferal.[34]

In a previous paper, we demonstrated that the exogenous H_2O_2 level in a *C. marina* cell suspension decreased with 30 min half-life, while H_2O_2 in ESM medium is stable for at least 5 h, suggesting the presence of a degradation system for H_2O_2 as well as a generation system in *C. marina*.[13] The fact that the generation of O_2^- and H_2O_2 by *C. marina* reaches a plateau level within a few minutes may also support the possibility of the presence of a scavenger system against ROS in *C. marina*. Since the increased level of H_2O_2 in ruptured *C. marina* cells rapidly decreased,[13] this scavenger system may still function after disruption of flagellate cells.

Although the physiological role of ROS generated by these flagellate species has not been established, our recent studies demonstrated that SOD and catalase strongly inhibited the growth of *C. marina*. Furthermore, morphological observation found that binary fission or cell division of *C. marina* was arrested in the presence of these enzymes. Therefore, ROS generated by *C. marina* may act as an autocrine growth factor.[35]

Regarding the toxic mechanisms of *Chattonella* sp. on fish, recent studies demonstrated that a decrease in oxygen partial pressure of arterial blood is the earliest physiological disturbance observed in fish after *Chattonella* sp. exposure.[2–4] Furthermore, several lines of evidence suggest that excessive mucus on the gill surface, which was probably induced by *Chattonella* sp., may interface with O_2

transfer, resulting in asphyxia.[5,6] It is noteworthy that the ·OH radical has been reported to induce mucus secretion.[36] In fact, our previous study showed that the hydroxyl radical (·OH) was also detected in *C. marina* cell suspensions by using ESR spectroscopy.[14] Thus, it is possible that ROS produced by *Chattonella* sp. may induce excessive mucus secretion on gill lamellae. Recently, Yang *et al.*[20] have reported that *H. carterae* has an oxygen-radical-mediated toxic effect on rainbow trout, *Oncorhynchus mykiss.*

In conclusion, our results demonstrated that all strains of *Chattonella* (three strains of *C. marina* and one strain of *C. antiqua*), *H. akashiwo*, *O. luteus*, and *F. japonica*, which belong to the class Raphidophyceae, generate O_2^- and H_2O_2 under normal culture conditions and inhibited the growth of a marine bacterium *V. alginolyticus*, as a common feature of raphidophycean flagellate species.

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