

Possible involvement of hemolytic activity in the contact-dependent lethal effects of the dinoflagellate *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*

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Abstract

We investigated the effects of two strains (SUO-1 and FUK) of the dinoflagellate *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*. The SUO-1 strain was highly toxic to rotifers, whereas the FUK strain was less toxic. After 10-hour incubations, the survivorship of rotifers exposed to SUO-1 and FUK was 20% and 95%, respectively. Both the cell-free culture supernatant and the ruptured cell suspension prepared from these strains were not toxic to rotifers. Furthermore, when direct contact between *K. mikimotoi* and rotifers was interrupted with a cell-impermeable membrane (3- μ m pores), the toxicity of both the SUO-1 and FUK strains of *K. mikimotoi* to rotifers were completely inhibited even after a 24-h exposure. Cell suspensions of SUO-1 showed hemolytic activity toward horse erythrocytes, but the FUK strain did not. The cell-free supernatant and the ruptured cell suspension of SUO-1 showed no significant hemolytic activity. These results suggest that this highly toxic strain of *K. mikimotoi* causes lethality in rotifers by direct contact in which live cell-mediated hemolytic activity might be a contributing factor.

Keywords: *Brachionus plicatilis*; Cell contact; Hemolytic activity; *Karenia mikimotoi*; Reactive oxygen species; Toxic effect

1. Introduction

Harmful algal blooms (HABs) are increasing in frequency, magnitude, and duration worldwide. Among nearly 100 toxic or harmful species of marine phytoplankton, dinoflagellates are well known as major HAB-causing species. *Karenia mikimotoi* (formerly *Gyrodinium aureolum*, *G. cf. aureolum*, *G. type-'65*, *G. nagasakiense*, and *G. mikimotoi*) is a common dinoflagellate that causes red tide in many coastal waters. HABs due to this species have been reported in Western Japanese waters (Honjo, 1994; Yoshimatsu, 2008), the North Atlantic (Gentien, 1998; Davidson et al., 2009), and other coastal areas (Lu and Hodgkiss, 2004; Sun et al., 2007), and are frequently associated with severe damage to wild fish, aquaculture fish, and shellfish.

Previous studies have demonstrated that *K. mikimotoi* produces various toxic agents, including low molecular weight, hemolytic toxins (Arzul et al., 1994; Parrish et al., 1998; Fossat et al., 1999; Sola et al., 1999; Jenkinson and Arzul, 2001; Neely and Campbell, 2006; Mooney et al., 2007), cytotoxic polyethers (Satake et al., 2002, 2005), and reactive oxygen species (ROS) (Yamasaki et al., 2004; Gentien et al., 2007). Matsuyama (1999) showed that *G. mikimotoi* strongly inhibited the filtration rate of bivalves. Sellem et al. (2000) demonstrated that the 18:5n3 fatty acid produced by *G. cf. mikimotoi* delayed or inhibited the first cleavage of sea urchin (*Paracentrotus lividus*) eggs and produced abnormalities in their embryonic development. Mitchell and Rodger

(2007) reported that an algal bloom of *K. mikimotoi* that occurred during the summer of 2005 in Ireland was associated with mortalities of both fish and shellfish. They also reported histopathological changes in the gills, gastrointestinal tracts, and livers of fish and shellfish killed by *K. mikimotoi*. Despite the toxicity potential of *K. mikimotoi*, the exact mechanism of toxicological action remains unclear.

Herbivorous zooplankton, such as rotifers and copepods, have been previously used to elucidate toxic mechanisms of HAB species (Wang et al., 2005; Zhenxing et al., 2006; Estrada et al., 2008). For instance, previous studies have demonstrated that several dinoflagellates had lethal effects on *Brachionus plicatilis* (Abe and Hirayama, 1979; Kim et al., 2000).

To gain insight into the toxic mechanism of *K. mikimotoi*, we examined the effects of two *K. mikimotoi* strains, SUO-1 and FUK, on the rotifer *B. plicatilis* under various experimental conditions. The hemolytic activity of these *K. mikimotoi* strains toward various mammalian erythrocytes was also examined.

2. Materials and methods

2.1. Algal species and rotifer cultures

Two strains of *K. mikimotoi* were isolated from the Fukuoka Bay (FUK), Japan in

2004 and Suo Nada (SUO-1), Japan in 2006. *Chattonella marina*, which is known to produce high levels of ROS (Oda et al., 1997), was generously provided by Kagoshima Prefectural Fisheries Experimental Station, Japan. These clonal strains were maintained at 26°C in 200-mL flasks containing 100 mL of modified seawater medium (SWM-3) at a salinity of 25 (Yamasaki et al., 2007). In addition, cultures were kept under a 12:12-h photoperiod using a cool-white fluorescent lamp ($200 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$). The modified SWM-3 contained a Tris-HCl buffer system and was autoclaved for 15 min at 121°C before use. Culture cell numbers were counted microscopically using a hemocytometer (Erma Inc., Tokyo, Japan). The rotifer *B. plicatilis* was provided by Dr. A. Hagiwara (Faculty of Fisheries, Nagasaki University, Japan) and was cultured with *Nannochloropsis oculata* as described previously (Kim et al., 2000).

2.2. Preparation of cell-free culture supernatant and ultrasonic-ruptured cell suspension of *K. mikimotoi*

Cell-free culture supernatant was obtained from 1 mL of each cell suspension in the late exponential growth phase, which was isolated by centrifugation at 5,000 x g for 5 min at 4°C. Cell densities of the FUK and SUO-1 strains were $4\text{-}5 \times 10^4$ cells mL⁻¹ and $8\text{-}10 \times 10^4$ cells mL⁻¹, respectively. The ruptured cell suspension was prepared by ultrasonic treatment of 1 mL of each cell suspension in a bath-type sonicator (XL2020,

Wakenyaku Co., Ltd., Kyoto, Japan) for 60 s at 20°C. Microscopic observation confirmed that all cells were ruptured by this treatment. The cell-free culture supernatant and ruptured cell suspension were immediately used for the rotifer toxicity test (Section 2.3.) and the hemolytic assay (Section 2.8.).

2.3. Rotifer toxicity test

This test was conducted in 48-well plates (Becton-Dickinson Co., LTD., Franklin Lakes, NJ, USA). Each well contained 10 individual rotifers in 100 μL of modified SWM-3 added to 900 μL of each *K. mikimotoi* cell suspension (2×10^4 cells mL^{-1}). As a negative control, 10 individual rotifers were cultured in 1 mL of the modified SWM-3 alone. The number of dead rotifers were counted every two hours with stereomicroscopic observation over the course of a 24-h incubation. At the beginning (0 h) and end (24 h) of the test, the number of *K. mikimotoi* cells was also counted microscopically. Three wells were used per treatment.

2.4. Effects of growth phase on the toxicity of *K. mikimotoi* to *B. plicatilis*

Cells of the FUK and SUO-1 strains were inoculated at a density of 5×10^2 cells mL^{-1} in 200-mL glass flasks ($n = 3$) containing 100 mL of modified SWM-3. Cell

numbers in 3-mL aliquots of suspension were counted microscopically with a hemocytometer following incubations for 2 d (early exponential phase), 5 d (exponential phase), 8 d (late exponential phase), as well as 11 and 14 d (stationary phase). These five subsamples were labeled in sequence as Phase A to E and were then used for rotifer toxicity testing as described above (see Section 2.3). In this experiment, three wells were used for both experimental and control treatments.

2.5. Rotifer toxicity under interrupted contact

To interrupt the direct contact between *K. mikimotoi* cells and *B. plicatilis*, 12-well plates with BD Falcon Cell Culture Inserts (353292; Becton-Dickinson Co., Ltd., Franklin Lakes, NJ, USA) were used as previously described (Yamasaki et al. 2007). This insert plate has a membrane filter (3.0- μm pore size), which allows soluble materials to transfer between two compartments. *K. mikimotoi* cell suspensions (3 mL) in late exponential growth phases (FUK and SUO-1 cell density of $4\text{-}5 \times 10^4$ cells mL^{-1} and $8\text{-}10 \times 10^4$ cells mL^{-1} , respectively) were added to the well outer chambers, while 10 individual rotifers in 1 mL of modified SWM-3 were added to the inner chamber. After 24-hour incubations, the number of live rotifers was counted with a stereomicroscope. Three wells were used per assay.

2.6. Measurement of superoxide anion (O_2^-)

Several lines of evidence suggest that some HAB species, including *K. mikimotoi*, produce ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$), and that these ROS might be involved in HAB-linked fish mortalities (Oda et al., 1992; Ishimatsu et al., 1996; Kim et al., 2007, 2009). To examine whether the FUK and SUO-1 strains generate ROS, chemiluminescence analysis using L-012 (Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan) as a superoxide-specific, chemiluminescent probe was undertaken as described previously (Kadomura et al., 2006; Kim et al., 2009). Chemiluminescent responses were recorded for *Chattonella marina* (2×10^4 cells mL^{-1}) that are known to produce ROS and the two strains of *K. mikimotoi* (FUK and SUO-1 cell density of $4-5 \times 10^4$ cells mL^{-1} and $8-10 \times 10^4$ cells mL^{-1} , respectively). Chemiluminescence was measured for 30 s using a Bio-Orbit Luminometer (1254-001, Bio-Orbit Oy, Turku, Finland) with the addition of L-012 to each cell suspension. Reaction mixtures consisted of 145 μL of each flagellate cell suspension, 50 μL of L-012 (10 mM), and 5 μL of superoxide dismutase (SOD: Cu, Zn-SOD) solution (final 100 U mL^{-1}) or 5 μL of modified SWM-3 medium. Chemiluminescent responses of modified SWM-3 alone were included as background measures. All chemiluminescence measurements were conducted in triplicate at 26°C using 1.5-mL microtube cuvettes.

2.7. Rotifer toxicity in the presence of SOD and catalase

To examine the potential for ROS involvement in *K. mikimotoi* lethality to rotifers, toxicity tests were conducted in the presence of SOD (100 U mL^{-1}), which catalyzes the conversion of superoxide to hydrogen peroxide, and catalase (final 500 U mL^{-1}), which then catalyzes the decomposition of hydrogen peroxide to water and oxygen. The test was also conducted in the presence of both enzymes. Culture conditions for these tests were the same as those described above for the rotifer toxicity tests (Section 2.3). Three wells were used per treatment.

2.8. Hemolytic assay

Horse, rabbit, sheep, and cattle blood were obtained from Nippon Bio-Test Laboratories (Tokyo, Japan) and were used within seven days of receipt. These erythrocytes were washed three times with phosphate-buffered saline (PBS) and adjusted to a final concentration of 4% (v/v) in modified SWM-3. Duplicate 50- μL aliquots of serial two-fold dilutions of intact cell suspension, cell-free culture supernatant, or ruptured cell suspension of each *K. mikimotoi* strain using modified SWM-3 were added to round-bottom 96-well plates (Becton-Dickinson, New Jersey, USA). Each well contained the same 4% (v/v) suspension of erythrocytes in modified

SWM-3; the well plates were gently shaken. After incubation for 5 h at 26°C under illumination from a fluorescent lamp ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$), the plates were centrifuged at $900 \times g$ for 5 min. Aliquots (70 μL) of supernatant were withdrawn from the wells and transferred to flat-bottom 96-well plates (Becton-Dickinson, New Jersey, USA). Released hemoglobin was determined by measuring absorbance at 560 nm using a microplate reader (MPR-A4i, TOSOH Co., LTD., Tokyo, Japan). Negative controls (zero hemolysis) and positive controls (100% hemolysis) were included using erythrocytes suspended in modified SWM-3 alone and in modified SWM-3 containing 1% v/v Triton X-100, respectively.

For the hemolytic kinetic analysis of *K. mikimotoi* strains (FUK and SUO-1 cell density of $4\text{-}5 \times 10^4$ cells mL^{-1} and $8\text{-}10 \times 10^4$ cells mL^{-1} , respectively) toward sheep erythrocytes, duplicate aliquots of 1 mL of each intact cell suspension in modified SWM-3 was added to a 24-well plate (Becton-Dickinson, New Jersey, USA). The same volume of 4% (v/v) suspension of erythrocytes in modified SWM-3 was added to each well, and the plate was gently shaken. Every 20 or 60 minutes, 100- μL aliquots of the assay mixture were withdrawn and centrifuged at $900 \times g$ for 5 min. Aliquots (70 μL) of the resulting supernatant were then transferred to flat-bottom 96-well plates, and the released hemoglobin was determined by measuring absorbance at 560 nm using a microplate reader (MPR-A4i, TOSOH Co., LTD., Tokyo, Japan).

2.9. Statistical analysis

Experimental data (Figs. 1A and 2B) were analyzed by one-way analysis of variance (ANOVA) using a Dunnett's test (Dunnett, 1955). This statistical analysis was conducted with the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS, Inc., Chicago, IL, USA). Sample differences were considered significant at $p < 0.05$.

3. Results

3.1. Toxic effect of two *K. mikimotoi* strains on rotifer *B. plicatilis*

Rotifer toxicity testing was conducted with two strains of *K. mikimotoi* (SUO-1 and FUK) at the same cell density (2×10^4 cells mL⁻¹). As shown in Fig. 1A, both strains of *K. mikimotoi* were significantly ($p < 0.05$) lethal to rotifers, although the potency of the strains were quite different. The toxicity of the SUO-1 strain was greater than that of the FUK strain, and all the rotifers exposed by the two strains died after a 16-h exposure. After exposure to SUO-1, rotifer stress was immediately apparent and their movement declined within 1 h of exposure (Data not shown). Dead rotifers with partial morphological changes, especially to the corona (Fig. 1B) located at the anterior end of

two ciliated rings, started to appear after exposures of 6 h. Relative to control rotifers (Fig. 1B), damaged coronas with excessive secretion of mucus (Fig. 1C) and abnormal foam-like structures (Fig. 1D) in the corona were observed in some rotifers exposed to the SUO-1 strain. The FUK strain was only weakly toxic to the rotifers in comparison to SUO-1, and about 40% of the rotifers were viable after the 24-h exposure (Fig. 1A). During the exposure, no decrease in viable *K. mikimotoi* cells was observed, indicating that these cells were not preyed upon by the rotifers (Data not shown).

Tests were conducted at different growth phases of *K. mikimotoi* (Fig. 2A) to ascertain whether the growth stage of *K. mikimotoi* altered its toxic effect on *B. plicatilis*. After a 12-h exposure, strain SUO-1 was significantly ($p < 0.05$) lethal to the rotifers, with lethality being dependent on cell density rather than growth phase (Fig. 2B). At early growth phases with low cell density (2-5 day cultures), the SUO-1 strain showed partial toxicity to rotifers, with full toxicity not reached until the nearly maximum cell density at 8 d or later. No significant toxicity of the FUK strain was detected throughout its growth even at the final growth phase when cell densities are high (Fig. 2B).

The cell-free culture supernatants and ruptured cell suspensions of both *K. mikimotoi* strains were not toxic to *B. plicatilis* (Table 1). Furthermore, when direct contact of *K. mikimotoi* cells and rotifers was interrupted with a cell-impermeable membrane, the toxic effects of both strains completely disappeared (Table 1) even though the 3.0- μm pore size of the membrane is sufficient to allow large molecular

weight compounds to transfer from *K. mikimotoi* to the rotifers.

3.2. ROS levels of *K. mikimotoi* and toxicity to *B. plicatilis* in the presence of SOD and catalase

Chemiluminescence was used to determine ROS levels in the FUK and SUO-1 strains of *K. mikimotoi*. After the addition of the L-012 probe, chemiluminescence emissions were immediately measured for 30 s. As shown in Fig. 3A, no significant responses were observed in either *K. mikimotoi* strain, whereas the *C. marina* cell suspension (positive control) induced rapid and potent chemiluminescence under the same conditions (Fig. 3A). These results suggest that the FUK and SUO-1 strains of *K. mikimotoi* might not be able to produce ROS. Furthermore, neither SOD (final concentration 100 U mL⁻¹) nor catalase (final concentration 500 U mL⁻¹), alone or in a mixture, influenced the toxicity of *K. mikimotoi* to *B. plicatilis* (Fig. 3B).

3.3. Hemolytic activity of *K. mikimotoi* on horse erythrocytes

Previous studies have reported that *K. mikimotoi* causes hemolysis of horse erythrocytes (Arzul et al., 1994; Parrish et al., 1998; Fossat et al., 1999; Sola et al., 1999; Jenkinson and Arzul, 2001; Mooney et al., 2007). Thus, we examined hemolytic

activity of the FUK and SUO-1 strains of *K. mikimotoi* against horse erythrocytes. Intact cell suspensions of the SUO-1 strain showed hemolytic activity in a cell density-dependent manner (Figs. 4 and 5). However, no significant hemolytic activity of the FUK strain was observed up to the highest cell density (5×10^4 cells mL⁻¹). In addition, no hemolytic activities were detected in the cell-free supernatants or ruptured cell suspensions of either *K. mikimotoi* strain (Fig. 4).

3.4. Hemolytic activity of K. mikimotoi on erythrocytes from other mammalian species

Hemolytic activities of the FUK and SUO-1 strains of *K. mikimotoi* toward other mammalian erythrocytes were examined. As shown in Fig. 5, intact cell suspensions of the SUO-1 strain were strongly hemolytic to sheep, rabbit, and cattle erythrocytes in a cell density-dependent manner. No hemolytic activity was observed in the FUK strain. Furthermore, time-course analysis revealed that sheep erythrocytes were highly sensitive to the SUO-1 strain with hemolysis induced immediately after exposure (Fig. 6).

4. Discussion

HABs due to *K. mikimotoi* are often associated with mass fish and shellfish

mortalities (Honjo, 1994; Yamaguchi, 1994). Several studies have proposed hypotheses for the toxic mechanism of action of *K. mikimotoi*. However, the toxicity mechanism of *K. mikimotoi* varies among exposed species and depends on the dinoflagellate strain tested. Thus, the precise toxicity mechanism of *K. mikimotoi* is still controversial. In the present study, we compared the toxic effect of the FUK and SUO-1 strains of *K. mikimotoi* on *B. plicatilis*. Our results indicate major differences in the potential toxicity of these two strains of *K. mikimotoi* to *B. plicatilis*. All the rotifers died after a 16-h exposure to the SUO-1 strain, whereas 70% of the rotifers exposed to the FUK strain were still alive (Fig. 1A). The growth rate of SUO-1 was slightly higher than that of the FUK strain, and the maximum cell densities of SUO-1 and FUK were $4\text{--}5 \times 10^4$ cells mL^{-1} and $8\text{--}10 \times 10^4$ cells mL^{-1} , respectively (Fig. 2A). Although the underlying reason for the difference in toxicity among the two *K. mikimotoi* strains is unclear, some bioactive potential that could then relate to toxicity on rotifer might vary across the SUO-1 and FUK strains.

Regarding the observed interactions between zooplankton and dinoflagellates, it has been reported that the growth of the tintinnid species, *Favella ehrenbergii* was inhibited by *Alexandrium tamarense* and *Gyrodinium aureolum* (Hansen, 1989; 1995). In these dinoflagellates, a certain toxic substance released from the algal cells has been suggested to cause their toxicity. Conversely, in the case of *Heterocapsa circularisquama*, the involvement of protein-like toxic substances located on the cell

surface are linked to toxicity in exposed bivalves (Matsuyama et al., 1997). This toxic mechanism of *H. circularisquama* is also observed in *B. plicatilis* (Kim et al., 2000). Because *H. circularisquama*-induced toxicity to rotifers was not observed in the culture supernatant of *H. circularisquama* (Kim et al., 2000), it has been proposed that the substances causing the toxicity may not be secreted from the algal cells. However, the toxic effect may immediately disappear once the toxic substance is released from the cells due to its instability. Consistent with these findings, no toxicity was observed among rotifers exposed to the cell-free culture supernatant of *K. mikimotoi* (Table 1). Furthermore, ultrasonic-ruptured cell suspensions of *K. mikimotoi*, even for the more potent SUO-1 strain, were non-toxic to *B. plicatilis* (Table 1). These results suggest that the integrity of living cells is essential for the SUO-1 strain to be lethal to rotifers. Another interesting finding regarding the relationship between zooplankton and dinoflagellate is that the tintinnid ciliate *Favella taraikaensis* was killed upon exposure to *H. circularisquama* in a cell density-dependent manner (Kamiyama and Arima, 1997; Kamiyama, 1997). Specifically, frequent contact of living *H. circularisquama* with the cytoplasm surrounding the oral plug of *F. taraikaensis*, and subsequent morphological changes of *F. taraikaensis*, were observed at high algal cell concentrations. Conversely, *F. taraikaensis* actively fed *H. circularisquama* grew only at low concentrations of this alga (Kamiyama and Arima, 1997). Based on these findings, these authors proposed that *H. circularisquama* causes a physiological disorder in *F. taraikaensis* via direct algal

cell contact and upon repeated exposures to certain toxic substance located on the algal cell surface. Our results may support such a zooplankton-killing mechanism of dinoflagellate toxicity, which may also be applicable to the rotifer-killing mechanism of *K. mikimotoi*. The direct attack of *K. mikimotoi* cells is probably a key mechanism causing the lethality observed in rotifers. It is unlikely that soluble toxins released from *K. mikimotoi* cells are responsible for the observed toxicity.

Several lines of evidence suggest that *K. mikimotoi* produces ROS under normal growth conditions (Gentien, 1998; Yamasaki et al., 2004), and these ROS have been proposed to be involved in fish-kills associated with *C. marina*, *Heterosigma akashiwo*, and other raphidophyceae flagellates (Ishimatsu et al., 1996; Oda et al., 1997; Yang et al., 1995). However, a chemiluminescence analysis failed to detect ROS production in either SUO-1 or FUK strains of *K. mikimotoi* (Fig. 3A). In addition, the presence of SOD, catalase, or even both enzymes together, had no effect on reducing the toxicity of *K. mikimotoi* (SUO-1) to rotifers (Fig. 3B). Thus, it seems unlikely that ROS are involved in the lethal effects of the SUO-1 and the FUK strains on rotifers.

Interestingly, there seemed to be a positive correlation between the toxicity of *K. mikimotoi* to *B. plicatilis* and its hemolytic activity. The SUO-1 strain was more toxic to rotifers and induced potent hemolytic activity, whereas the less toxic FUK strain showed no hemolytic activity (Figs. 1 and 5). Furthermore, the cell-free supernatant and the ruptured cell suspension of the SUO-1 strain, which were incapable of killing

rotifers, did not show any hemolytic activity (Fig. 4). These results suggest that the live cell-mediated hemolytic activity might be linked with the toxic effects on rotifers. Further studies are required to clarify how hemolytic activity is involved in the eventual rotifer mortality caused by the SUO-1 strain. The rotifer corona may be especially sensitive to the SUO-1 strain due to a toxic agent located on the cell surface that is responsible for hemolytic activity. Thus, the gradual accumulation of damage to rotifers caused by live SUO-1 cells may eventually lead to their mortality. Morphological changes, concomitant with the secretion of mucous-like substances (Fig. 1C) and the formation of foam-like structures (Fig. 1D), observed around rotifer coronas exposed to SUO-1 live cells suggest that the corona is sensitive to attack by SUO-1 cells. It is possible that certain toxic agents located on the SUO-1 cell surface may cause membrane damage leading to impaired membrane permeability. Such an agent could also induce hemolysis upon contact with erythrocyte membranes.

Previous studies in our laboratory have demonstrated that hemolytic activity of *H. circularisquama* is species-specific, with cattle and sheep erythrocytes being less sensitive to this toxic dinoflagellate than highly susceptible rabbit and guinea pig erythrocytes (Kim et al., 2002). Based on this and other findings, it has been hypothesized that *H. circularisquama* may act on erythrocyte membranes through specific attack on ion channels rather than via simple membrane damage (Oda et al., 2001). In contrast to *H. circularisquama*, species-specific sensitivities in hemolytic

activity were not observed for the SUO-1 strain of *K. mikimotoi*; sheep and rabbit erythrocytes, which have different intracellular sodium/potassium (Na/K) ion balances, showed the same sensitivity to the SUO-1 strain (Fig. 5). Furthermore, the SUO-1 strain induced the maximum level of hemolysis without long lag times (Fig. 6) that are often observed in hemolysis caused by disorders of ion balance (Harbermann et al., 1981). Although further studies are required to identify the hemolytic mechanism of *K. mikimotoi* and the responsible hemolytic agents, these findings suggest that the hemolytic mechanism of *K. mikimotoi* is distinct from that of *H. circularisquama*. It appears that a hemolytic agent on the cell surface of *K. mikimotoi* may attack erythrocyte membranes through direct cell-to-cell contact, which in turn damages membrane structures.

5. Conclusions

We found that the lethal effects of intact cell suspensions of two strains of *K. mikimotoi* to the rotifer *B. plicatilis* were quite different. The more toxic SUO-1 strain showed strong hemolytic activity, while the less toxic FUK strain showed almost no hemolytic activity. Our results suggest that membrane damage of sensitive areas of rotifers, such as the corona, caused by cell-mediated attacks might be responsible for rotifer mortality.

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Figure captions

Fig. 1. Effects of the SUO-1 and FUK strains of *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*. **(A)** The viability of rotifers after exposure to SUO-1 (▲), FUK (■), or medium alone as control (●). Points indicate the mean of triplicate measurements, and the bars indicate \pm standard deviation (% SD). An asterisk denotes a significant difference from controls ($p < 0.05$). **(B)** Living normal rotifers. **(C)** Rotifers with morphological changes concomitant with discharges of mucous-like substances after 5-h exposures to the SUO-1 strain. Arrowhead (a) indicates mucous-like substances. **(D)** Rotifers with morphological changes concomitant with the formation of foam-like structures after 8-h exposures to the SUO-1 strain. Arrowhead (b) indicates the foam-like structures.

Fig. 2. Effects of the SUO-1 and FUK strains of *Karenia mikimotoi* growth phase on the toxicity to the rotifer *Brachionus plicatilis*. **(A)** Growth curves of strain FUK and SUO-1. **(B)** Toxic effect of *K. mikimotoi* at each growth phase to rotifers. Viable rotifers were counted after a 12-h exposure to intact cell suspensions of the SUO-1 and FUK strains of *K. mikimotoi* at different growth phases and different cell densities. Data are means \pm % SD of triplicate measurements. An asterisk denotes significant differences from controls ($p < 0.05$).

Fig. 3. ROS levels in the SUO-1 and FUK strains of *Karenia mikimotoi*, and the toxic effect of these strains on the rotifer *Brachionus plicatilis* in the presence of SOD and catalase. (A) L-012-dependent chemiluminescence responses of the SUO-1 and FUK strains as well as *Chattonella marina*. Algal cells of FUK (●,○), SUO-1 (▲,△), and *C. marina* (■,□) were subjected to chemiluminescence analysis in the presence (○,△, □) or absence (●,▲,■) of SOD (final 100 U mL⁻¹). Background luminescence (◇) in modified SWM-3 medium alone. Each point represents the average of triplicate measurements. (B) Survivorship of rotifers exposed to the SUO-1 and FUK strains of *K. mikimotoi* under various conditions. Ten individual rotifers were exposed to *K. mikimotoi* cells (FUK: 5 x 10⁴ cells mL⁻¹, SUO-1: 8 x 10⁴ cells mL⁻¹) in the absence or presence of catalase (final 500 U mL⁻¹), superoxide dismutase (final 100 U mL⁻¹), or both enzymes. After a 24-h exposure, the number of viable rotifers was counted. Data are means ± % SD of triplicate measurements.

Fig. 4. Hemolytic activity of the SUO-1 and FUK strain of *Karenia mikimotoi*. Intact cell suspension, cell-free culture supernatant, and ultrasonic ruptured cell suspension of *K. mikimotoi* were mixed with horse erythrocytes and incubated for 5 h at 26°C under a 12:12-h photoperiod. The extent of hemolysis was measured as described in the text. Each point represents an average of duplicate measurements.

Fig. 5. Hemolytic activity of the SUO-1 and FUK strains of *Karenia mikimotoi* toward horse, sheep, rabbit, and cattle erythrocytes. Various concentrations of intact cell suspensions of the SUO-1 and FUK strains were mixed with horse, sheep, rabbit, or cattle erythrocytes and incubated for 5 h at 26°C in the light. The extent of hemolysis was measured as described in the text. Each point represents an average of duplicate measurements.

Fig. 6. Time-course analysis of hemolytic activity of the SUO-1 and FUK strains of *Karenia mikimotoi* toward sheep erythrocytes. Intact cell suspensions from the SUO-1 or FUK strains (FUK: 5×10^4 cells mL⁻¹, SUO-1: 8×10^4 cells mL⁻¹) were mixed with sheep erythrocytes and incubated for the indicated periods of times at 26°C. The extent of hemolysis was measured as described in the text. Each point represents an average of duplicate measurements.

Table 1

Viability of the rotifer *Brachionus plicatilis* under various exposure conditions.

	Viability of rotifer (%)	
	SUO-1	FUK
Intact cell suspension	0	43
Non-direct contact	100	100
Cell-free supernatant	100	100
Ruptured-cell suspension	100	100

Fig. 1

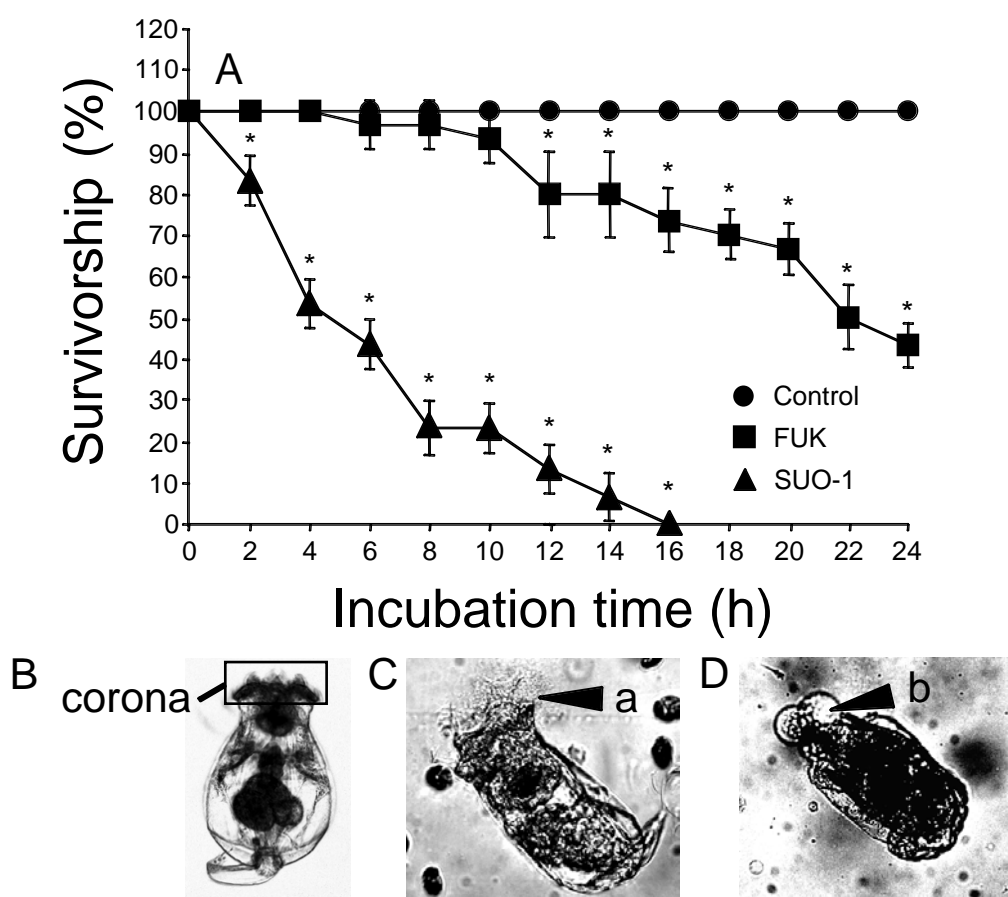


Fig. 2

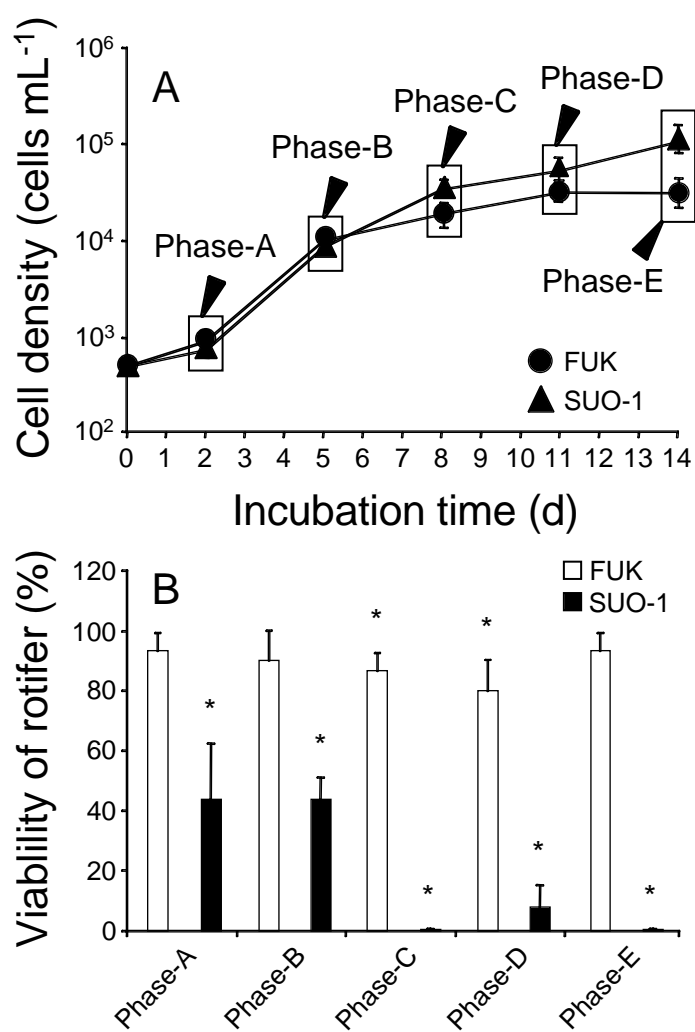


Fig. 3

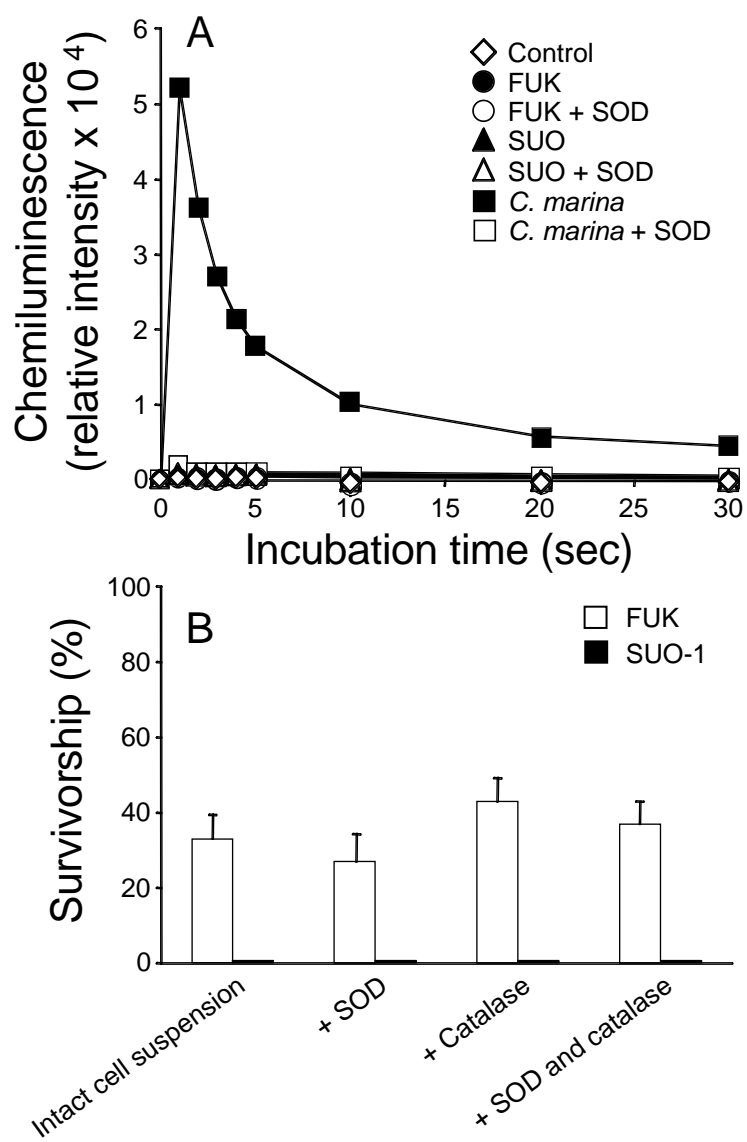


Fig. 4

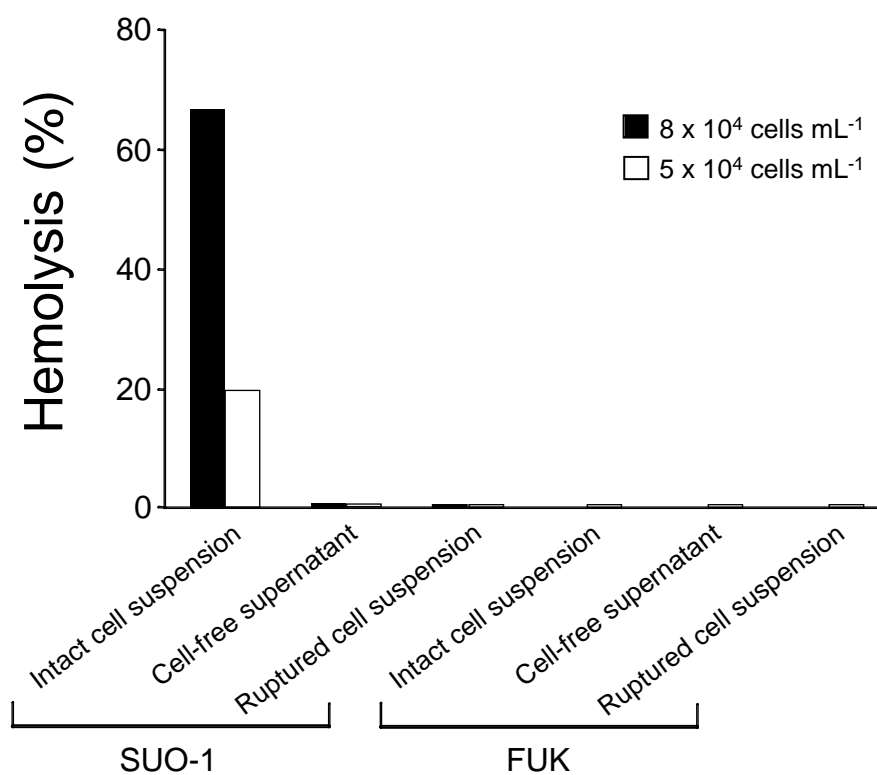


Fig. 5

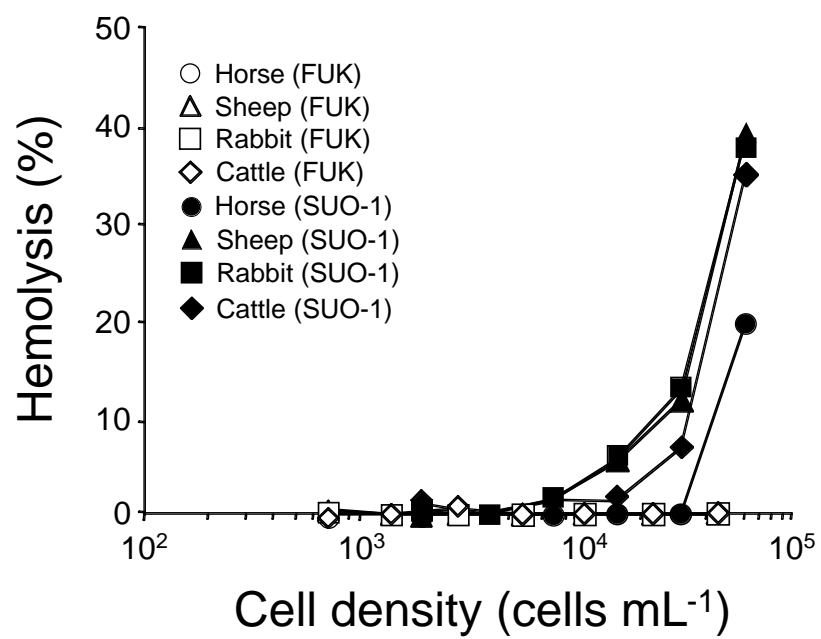


Fig. 6

