Comparison of the responses of peritoneal macrophages from Japanese flounder (*Paralichthys olivaceus*) against high-virulent and low-virulent strains of *Edwardsiella tarda*

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Abstract

In vivo infection studies in Japanese flounder (*Paralichthys olivaceus*) demonstrated that the number of viable cells of the virulent strain (NUF251) of *Edwardsiella tarda* increased gradually in kidney and hepato-pancreas after intraperitoneal injection, but the low virulent strain (NUF194) did not. To gain insight into the virulence factor of *E. tarda*, in vitro responses of Japanese flounder (*Paralichthys olivaceus*) peritoneal macrophages to these strains were compared in terms of phagocytosis, bactericidal activity, and reactive oxygen species (ROS) generation as measured by chemiluminescence (CL) responses. Microscopic observation revealed that these two strains of *E. tarda* were phagocytosed by the peritoneal macrophages, and there was no significant difference in the mean numbers of ingested bacteria per macrophage between these strains. A gradual increase in the number of viable cells of the highly virulent strain within macrophages was observed during 9 h post-phagocytosis, whereas no significant replication of the low virulent strain within macrophages was detected. These results suggest that the virulent strain of *E. tarda* has an ability to survive and replicate within macrophages, while the low virulent strain has no such ability. When the peritoneal macrophages were exposed to the opsonized low virulent *E. tarda* strain, a rapid increase in CL response was induced. However, the highly virulent strain caused only background level of CL response. By the subsequent stimulation with phorbol myristate acetate, the macrophages exposed to the virulent *E. tarda* strain showed extremely higher CL response than that of the one exposed to the low virulent *E. tarda* strain. These results suggest that the virulent *E.*

tarda prevents the activation of ROS generation system during the bacterial phagocytosis, and such system is still capable to respond to other stimulation. The virulent strain significantly reduced the CL response induced by xanthine/xanthine oxidase system, while the low virulent strain had almost no effect. Furthermore, the virulent strain showed greater resistibility to H_2O_2 than the low virulent strain. Our results suggest that the virulent strain of *E. tarda* is highly resistant to ROS, and such ability might allow the organism to survive and multiply within phagocytes, and may serve to disseminate *E. tarda* throughout the host during in vivo infection.

1. Introduction

Edwardsiella tarda, a Gram-negative bacterium, is the etiological agent of several diseases of marine and freshwater fish. Edwardsiellosis caused by this bacterium has been responsible for significant losses in fish culture industry, particularly in Japanese eel (*Anguilla japonica*), channel catfish (*Ictalurus punctatus*), and Japanese flounder (*Paralichthys olivaceus*), and these infectious diseases are often associated with poor water quality and stress [1]. The disease signs may include small cutaneous lesions that can develop into necrotic abscesses, distended abdomen and swollen anus due to the accumulation of ascitic fluid, pigment loss, enlarged kidney, and abscesses on internal organs [1]. In addition to fish, *E. tarda* has been found to infect other species including amphibians [2], reptiles [3], birds [4], and mammals including humans [5]. In spite of the importance of *E. tarda* as a fish pathogen and the increasing significance of the disease, little is known about the pathogenesis of *E. tarda* infection. Some potential virulence factors of this bacterium have been suggested, namely dermatotoxin [6], hemolysis [7], ability to invade epithelial cells [8], and capability of surviving in phagocytes [9].

Phagocytosis, a fundamental defense mechanism in most animal species including fish, is mediated by phagocytic cells such as neutrophils, monocytes and macrophages. It is well-known that the antibacterial defense mechanism of these cells is mediated by the production of reactive oxygen species (ROS) [10]. Since the production of ROS has been correlated with killing bacteria by phagocytic cells in fish and humans [10], the ability of *E. tarda* to survive within phagocytic cells and even replication may serve to disseminate the bacteria throughout the host. Previous study demonstrated that a strain of *E. tarda* which is highly virulent to eel induced no significant level of ROS generation by eel neutrophils as measured by luminol-enhanced chemiluminescence, a technique commonly used to measure ROS generation by phagocytic cells, whereas *E. coli* and *V. anguillarum* induced potent CL in the same assay system [11]. The underlying mechanism for this is still unclear, but it seems that this finding may be related to the ability of *E. tarda* to survive inside host phagocytic cells as a virulence factor described above. Similar to *E. tarda*, it has been reported that the ability to survive inside host macrophages is an essential virulence requirement for *Salmonella* [12], and S*almonella phoP* mutants that cannot survive in macrophages are avirulent [13].

To gain further insight into the virulence factor of *E. tarda*, in this study, we examined the early responses of Japanese flounder peritoneal macrophages against two strains of *E. tarda* with different virulence potential. The results indicated that the highly virulence strain has the ability to resist to ROS, and is capable to survive and multiply within macrophages, whereas the low virulent stain has no such abilities.

2. Materials and methods

2.1. Chemicals

L012, a highly sensitive ROS specific chemiluminescence probe, and superoxide dismutase (Cu, Zn-SOD) (3800 units/mg of protein, from bovine erythrocytes) were purchased from Wako Pure Chemical Industry, Co., Ltd. (Osaka, Japan). Other chemicals were of the highest grade commercially available.

2.2. Fish

Uninfected healthy Japanese flounder *Paralichthys olivaceus* were obtained from local commercial farms (Nagasaki, Japan). The mean body length and weight of the fish were 31 ± 2 cm and 319 ± 40 g, respectively. The fish were kept in a flow-through system (1000 L tank), and were fed flounder pelleted food daily.

2.3. Bacterial strains

Two strains of *E. tarda* (NUF251 and NUF194) isolated from Japanese flounder and eel pond water, respectively were used in this study. Strain NUF251 belonging to serotype A, is known to be highly virulence and pathogenic to Japanese eel and Japanese flounder, whereas strain NUF194 is found to be relatively low virulence [14, 15]. These strains were stored in nutrient medium with 10% glycerol at −80℃, and subcultured on nutrient agar medium at 27℃ for 24 h prior to the onset of experiments. The cultured bacterial cells were harvested in Dulbecco's modified phosphate-buffered saline (PBS) and washed twice with PBS by centrifugation (9,000 x g , 1 min , at 4℃). The number of colony forming units (CFU) was determined by plating 10-fold serial dilutions on agar plates, and the bacterial suspension was diluted with PBS to the desired concentration.

*2.4. Opsonization of bacteri*a

Equal volume of bacterial cell suspension in PBS and 50% of freshly prepared normal serum from Japanese flounder in PBS were mixed. After incubation at 25℃ for 30 min, the bacterial cells were diluted with PBS to appropriate cell density.

2.5. Preparation of peritoneal macrophages

The fish were anaesthetized with 0.02% of 2-phenoxy ethanol, and 5 ml of PBS containing 40 unit/ml of heparin were then injected through the peritoneal wall at the midline using 5 ml syringe attached with a 25 G needle. Using the same syringe system, peritoneal fluid was gently withdrawn from the specimen. This procedure was repeated twice and the harvested cell suspensions were pooled in siliconized centrifuge tubes on ice. The cells were washed with PBS by centrifugation (200 x g, 10 min at 4℃). The final cell pellet was resuspended in a small amount of PBS, and was layered over 5 ml Percoll in 7 ml polycarbonate centrifuge tube (density: 1.075 g/ml) in which continuous density gradient was preformed by centrifugation (20,000 x g, 30 min at 20℃), and centrifuged at 490 x g for 60 min at 20℃. Macrophage-enriched fraction was harvested from the gradient by syringe, and the cells were washed three times with PBS by centrifugation (200 x g, 10 min at 4℃). The final cell pellet was resuspented in RPMI1640 medium supplemented with 10% of FCS, and the viability of the cells was confirmed by staining with 0.5% trypan blue. The cell number was counted by a haemocytometer, and cell suspensions were diluted with the medium to appropriate concentration. The cells were cultured in CO_2 incubator in a 5% CO_2 atmosphere at 25℃.

2.6. Bacterial infection by intraperitoneal injection

The fish were anaesthetized with 0.02% of 2-phenoxy ethanol, and were then injected intraperitoneally with $10^{3.9}$ CFU of each strain of *E. tarda* per 100 g fish body weight. Control groups were injected PBS alone. After recovery from anesthesia, each groups of fish was transferred to a 1000 L rearing tank in the flow-through system and maintained for 7 days. After 1, 2, 3, and 7 days, three fish from each group were sampled and the hepato-pancreas and kidney were aseptically removed from each fish. The isolated each organ was homogenized with a polytorone homogenizer. To measure the number of viable bacteria in each organ, the homogenized samples were serially diluted in PBS, plated on nutrient agar medium, and incubated at 27℃ for 24 h.

2.7. Phogocytic assays

The phagocytic bactericidal activities of peritoneal macrophages against two strains of *E. tard*a were evaluated by following procedure. To macrophage cell suspension (final 1 x 10^6 cells/ml) in RPMI1640 medium supplemented with 10% FCS, each of *E. tarda* strain (final 5 x10^5 CFU/ml) was added. After 1 h incubation at 25℃, gentamicin (final 50 μg/ml) was added to the cell mixture and incubated further 1 h at 25℃ to kill any remaining extracellular bacteria. The cells were washed three times with PBS to remove gentamicin, and then 1 ml sterile distilled water was added to lyse the cells, and the number of viable intracellular bacteria was quantified by colony formation assay. To visualize the internalized bacteria, the macrophage cell suspension incubated with each strain of *E. tarda*, and subsequent treatment with gentamicin as described above was washed three times with PBS, and stained using Giemsa staining solution. The average number of the bacteria per macrophage was determined by counting the number of bacteria associated with macrophages, using light microscopy at a magnification of

1000x.

2.8. Chemiluminescence assay

The generation of reactive oxygen species (ROS) by peritoneal macrophages was measured by the chemiluminescence (CL) assay. The assays were performed in a TR717 microplate luminometer (Applied Biosystems Foster City, CA), using 96-well white plates at 25℃. L012 was used as a highly sensitive ROS specific chemiluminescence probe. L012 was dissolved in ultrapure water to give a concentration of 10 mM, and was stored in a small aliquots at -30℃. Prior to use, the L012 stock solution was thawed and diluted in PBS to a final concentration of 100 μM. The assay mixtures in CL assays consisted of, in order of addition, 80 μl of macrophage suspension (final 10^6 cells/ml), 10 μl of opsonized each strain of bacterial suspension (final 10^6 cells/ml) or control PBS, and finally 10 μl of L012 (final 10 μM). The plates were placed in the luminometer, and CL value of each well was recorded for 60 min at 30 sec intervals. To examine the effect of phorbol myristate acetate on the CL responses of the macrophages exposed to each bacteria, 10 μl of phorbol myristate acetate (1 μg/ml) solution in PBS was added to each well after 40 min recording of CL responses by the same way as described above, and then continued to record the CL responses for additional 80 min.

2.9. Effect of E. tarda strains on CL response induced by xanthine/xanthine oxidase system

To compare the scavenging abilities of *E. tarda* strains to ROS, xanthine/xanthine oxidase system was applied as a source of ROS. Ninety μl of reaction mixture containing each strain of bacteria (final 10^4 CFU/ml), xanthine (final 5 mM)), and L012 (final 10 μM) was put into each well of a 96-well white plate. To initiate the enzymatic reaction, 10 μl xanthine oxidase (final 0.02 U/ml) was added to each well, and then the CL intensity of each well was recorded for 30 min.

2.10. Susceptibility of E. tarda strains to H_2O_2

The susceptibility of the low and highly virulent stains of *E. tarda* to H_2O_2 was examined. Ten μl of each of the bacterila suspensions containing 10^8 CFU/ml were mixed with 1 ml of 0.01% H_2O_2 in PBS. After incubation at 25℃ for varying time of periods, 10 μl of the aliquot of the reaction mixture was withdrawn from each reaction mixture, and the number of CFU at each incubation interval was counted by plating on nutrient agar medium in triplicate.

2.11. Statistics

The statistical significance of the data was determined by two-tailed Student's *t* test, and $p<0.05$ was considered statistically significant throughout the study.

3. Results

3.1. Experimental E. tarda infection in Japanese flounder

To confirm the differences in the virulent potential between two strains of *E. tarda* (NUF194 and NUF251), live cells of each strain were intraperitoneally inoculated into Japanese flounder, and viable bacterial cell counts in hepato-pancreas and kidney of each infected fish were investigated up to 7 days post-infection. As shown in Fig. 1, gradual increase in viable cell counts in strain NUF251 in both organs were observed. At 24 h post-infection, significant level of viable bacteria of strain NUF251 was already detected in the kidney, while appearance of viable bacteria in the hepato-pancreas was delayed and undetectable until 48 h post-infection. On the other hand, no viable bacteria were detected in both organs from the fish infected with strain NUF194 during 7 days experimental interval. These results clearly indicate that stain NUF251 has greater invasive potency against Japanese flounder than strain NUF194, and confirm that strain NUF251 of *E. tarda* is highly virulent strain, while strain NUF194 is almost avirulent.

3.2. Phagocytosis of E. tarda by Japanese flounder macrophages

The phagocytotic activities of Japanese flounder macrophages toward the highly virulent and low virulent strains of *E. tarda* were estimated by direct microscopic observation. As shown in Fig. 2A, these two strains were ingested into the macrophages.

The mean number of bacteria of the virulent strain (NUF251) per macrophage evaluated by microscopic observation of Giemsa-stained macrophages was slightly higher than that of the low virulent strain (NUF194), but the difference was not statistically significant (Fig. 2B).

3.3. Intracellular replication of E. tarda in Japanese flounder macrophages

We compared the level of intracellular replication of two strains of *E. tarda* in Japanese flounder macrophages. As shown in Fig. 3, the number of viable cells gradually increased during 9 h incubation for the virulent strain (NUF251), whereas no significant increase in the number of viable cells of the low virulent stain (NUF194) was detected. These results suggest that the virulent strain is capable to survive and multiply within Japanese flounder macrophages, but the low virulent one had no such ability.

3.4. ROS generation by macrophages induced by E. tarda

To study ROS production by macrophages following incubation with *E. tarda*, the Japanese flounder peritoneal macrophages were incubated with each strain of *E. tarda*, and the production of ROS was investigated by chemiluminescence assay. As shown in Fig. 4, strain NUF194, a low virulent *E. tarda* induced significant CL response with a peak time of 7 min after the addition of bacterial cells to the macrophages. In contrast to strain NUF194, highly virulent strain NUF251 induced only slight CL response with nearly background level. These results suggest that the virulent strain of *E. tarda* may have the ability to prevent the activation process of ROS generation system or direct inhibition of such system. To clarify this point, the macrophage cell suspension exposed to each strain of *E. tarda* was further stimulated with phorbol myristate acetate, a potent chemical stimulant to induce ROS generation of phagocytic cells. As shown in Fig. 5, the macrophages exposed to the virulent strain induced extremely higher CL response to the stimulation by phorbol myristate acetate than that of the macrophages exposed to the low virulent strain. Probably, most part of ROS generation system in the macrophages exposed to the virulent strain remains inactive form that may be still capable to respond to the second stimulation with phorbol myristate acetate, while the macrophages exposed to the low virulent strain were already fully activated after exposure to the bacteria, and showed only slight CL response to the second stimulation.

3.5. Effects of E. tarda strains on the CL response induced by xanthine/xanthine oxidase *system*

One possible strategy of pathogenic bacteria to survive within phagocytes is acquisition of antioxidant or radical scavenging system such as catalase or superoxide dismutase. To ascertain whether or not this is the case for the virulent *E. tarda* strain, we examined the scavenging activities of *E. tarda* strains to O_2^- generated by xanthine/xanthine oxidase system. As shown in Fig. 6, the virulent strain reduced the

CL response, whereas the low virulent strain had almost no effect.

3.6. Comparison of susceptibility of E. tarda strains to H_2O_2

Time course of the viabilities of *E. tarda* strains after exposure to H_2O_2 were shown in Fig. 7. The low virulent strain was killed more strongly over the 3 h incubation period than the highly virulent strain. These results suggest that the highly virulent strain may have extremely higher ROS-neutralizing system as compared to the low virulent strain.

4. Discussion

E. tarda is one of the major bacterial pathogens in freshwater and marine fish, and natural *E. tarda* infection has been recorded predominantly in Japanese eel, Japanese flounder, and channel catfish [16, 17], but also in many other fish species [18]. Although pathogenesis of *E. tarda* is considered to be multifactorial, and several potential virulence factors have been reported so far [6-9], the details of the pathogenic mechanism is still controversial. Comparative studies of virulent and avirulent strains have demonstrated that only the virulent strain could enter fish and multiply inside various internal organs, and caused eventual fish death, even though both virulent and avirulent strains had the similar ability to invade cultured cells in vitro [19]. In the investigation of the pathogenic characteristics of 35 *Edwardsiella* strains, it was found that pathogenic potency of *E. tarda* did not correlate with plasmid content, chemotactic mobility, serum resistance, or expression of selected enzyme activities [20]. Based on these findings, it seems likely that some virulent *E. tarda* strains acquire the specific strategy to overcome fish defense system against pathogens. In general, humoral and cellular innate immune systems are the two major defense mechanisms against invading bacterial in fish. Thus the acquisition of resistance to these defense mechanisms by pathogens leads to severe infection. For instance, it has been shown that virulent fish pathogens such as *Aeromonas hydrophila* [21, 22] and *Renibacterium salmoninarum* [23] are resistant to serum- and phagocyte-mediated killing. In the present study, the experimental infection of Japanese flounder with a low and a highly virulent *E. tarda* strains was conducted. In naturally infected fish and experimentally infected Japanese flounder and channel catfish, it has been reported that significant pathological changes in the kidney and liver were observed, most often in the form of abscesses and necrotic lesions [24]. Therefore, we examined viable bacterial cell counts in kidney and hepato-pancreas that are supposed to be the major target organs of *E. tarda* in the infected fish. The infection kinetics showed that only the highly virulent strain showed a sequential increase in the number of viable bacterial cells within both organs, whereas the levels of low virulent strain within these organs were less than the detectable limit throughout the postinfection period. Thus it is obvious that strain NUF251 is highly virulent *E. tarda* as compared to strain NUF194 that may be even avirulent, and strain NUF251 is capable to multiply within the internal organs by overcoming Japanese flounder defense system. To further evaluate the escape mechanism of strain NUF251 from fish defense system, in vitro analysis with isolated fish macrophages was done in which we investigated the ability of these bacterial strains to survive and multiply within macrophages. The assay to determine the number of intracellular viable cells of the virulent and the low virulent *E. tarda* strains revealed that only the virulent strain NUF251 could replicate within macrophages. Similar to *E. tarda*, it has been reported that the ability to survive inside host macrophages is an essential virulence requirement for *Salmonella* [12], and *Salmonella phoP* mutants are avirulent due to their inability to survive in macrophages [13].

In phagocytes such as macrophages and neutrophils, the generation of ROS through respiratory burst is one of the major bactericidal mechanisms. This oxygen-dependent bactericidal mechanism has been demonstrated in phagocytes of many different fish species [22, 23, 25, 26]. In mammalian phagocytes, it has been well documented that the generation of ROS occurs via a membrane-bound flavocytochtome *b*558, consisting two phagocytic oxidase (phox) components (gp91phox and p22phox) and four cytosolic components (p40phox, p47phox, p67phox, and a GTP-binding Rac protein). During stimulation, the cytosolic components translocate to the site of gp91phox/p22phox on the phagosomal membrane to form a functional enzyme complex which generates superoxide anion (O_2^-) by catalyzing electron transfer from NADPH to molecular oxygen [27]. Several lines of evidence suggest that similar enzymatic system is responsible for the ROS generation in fish phagocytes [28].

In the present study, we found that the virulent *E. tarda* strain induced only a trace level of ROS production by Japanese flounder peritoneal macrophages in vitro as measured by chemiluminescence (CL) response analysis, whereas low virulent strain induced ROS production with significantly higher level. Similar to our virulent *E. tarda* strain, several bacteria are known to have the ability to escape the phagocyte respiratory burst, resulting in successful intracellular survival [25, 29]. In contrast, it has been reported that higher production of ROS in rainbow trout macrophages was induced by the highly virulent strain of *Flavobacterium psychrophilum* than by the low virulent strain, even though the virulent strain was more resistant to macrophage-mediated killing activity than low virulent strain. Therefore, it appears that the strategy to survive inside macrophage is depending on bacterial species, and they might evolve indigenous specific ability to overcome the phagocyte-mediated defense system. In principle, there are two effective mechanisms for resisting or avoiding ROS-mediated phagocytic microbicidal activity: Namely, (i) blocking of the elicitation of respiratory burst or inhibition of the activity itself, or (ii) neutralization of the ROS produced during the respiratory burst. In fact, regarding the possibility (i), *Edwardsiella ictaluri* LPS has been reported to suppress CL response of catfish neutrophils to opsonized zymosan [30]. When the macrophages exposed to the virulent strain of *E. tarda* were further stimulated with phorbol myristate acetate, extremely high CL response was induced. Therefore, it is considered that the ROS generation system in the macrophages exposed to the virulent strain remained inactive form that is still capable to respond to other stimulation to convert to active form. In other word, the virulent strain of *E. tarda* may prevent the activation process of ROS generation system in the macrophages rather than the irreversible inhibition of the activity itself.

On the other hand, some bacteria are known to resist ROS by producing SOD or catalase [31-33] that may be related to the notion (ii) described above. To ascertain whether or not the virulent *E. tarda* strain (NUF251) has such neutralizing activities against ROS with greater level than the low virulent strain (NUF194), we investigated the effects of the *E. tarda* strains on the CL response caused by xanthine/xanthine oxidase system in which O_2^- is continuously produced as a byproduct of the enzyme reaction. Although the virulent strain apparently reduced the CL response, the low virulent strain had almost no effect. To further clarify the possible presence of neutralizing activity against ROS in the virulent strain, the cytotoxic effects of H_2O_2 on the strains were examined. The results clearly indicated that there is significant difference in the susceptibility to H_2O_2 between two strains, and the virulent strain showed more stronger resistance to H_2O_2-mediated killing than the low virulent strain. These results suggest that the inability of the virulent strain to induce potent CL response by fish macrophages is due to the scavenging or neutralizing ROS produced during phagocytosis in addition to the prevention of activation process of ROS generation system. Such abilities of the virulent strain may allow the organism to survive and multiply within macrophages. Similar to the virulent strain of *E. tarda* observed in this study, *Aeromonas salmonicida* and *Photobacterium damselae* subsp. *piscicida* are shown to resist oxidative killing by producing SOD [31, 32]. More recent study has also demonstrated that the expression of catalase in *E. tarda* is required for resistance to H_2O_2 and phagocyte-mediated killing [9]. The presence or enhanced expression of one of such defense mechanisms in the highly virulent strain may account for higher resistance to killing by ROS, and may serve to disseminate *E. tarda* in the host during in vivo infection. Hence, resistance to bacterial killing by the macrophages may be an important virulent factor of *E. tarda*.

In conclusion, the present study demonstrated that the virulent *E. tarda* has multiple defense systems against ROS produced during phagocytosis to survive and multiply within fish macrophages. Such ability may serve as a major virulence factor of *E. tarda*.

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Figure legends

Fig. 1. Time course analysis of viable cell counts of intraperitoneally injected highly virulent (NUF251) (■) and low virulent (NUF194) (□) *E tarda* strains in the hepato-pancreas (A) and the kidney (B) of Japanese flounder. At the indicated period of the time, three fish each were sampled, and hepato-pancreas (A) and kidney (B) were dissected and homogenized, and number of viable bacteria was determined by colony formation assay. Each point represents an average of triplicate measurements. Each bar represents standard deviation. a: not detectable (less than detectable limit).

Fig. 2. Phagocytic ingestion of *E. tarda* strains by Japanese flounder macrophages. (A) After 1 h incubation of Japanese flounder peritoneal macrophages with opsonized highly virulent (a) or low virulent strain (b) of *E. tarda*, cells were treated with gentamicin (final 50 μg/ml) for 1 h at 25℃ followed by fixing with 100% methanol and staining with Giemsa staining solution, and then observed microscopically. Each bar indicates 10 μm. (B) To determine the mean number of ingested bacteria per macrophage, numbers of bacteria of more than 10 macrophages were counted. The difference in the mean number of bacteria per macrophage between two strains was not statistically significant (P>0.05).

Fig. 3. Intracellular multiplication of the highly virulent (NUF251) (●) and the low virulent (NUF194) (○) of *E. tarda* strains in Japanese flounder peritoneal macrophages. Macrophages were incubated with each strain of bacteria with bacteria/macrophage of

1:2. After the incubation for the indicated period of the time, cells were treated with gentamicin (final 50 μg/ml) for 1 h, and then lysed with sterile distilled water. The number of viable bacteria in each cell lysate was determined by colony formation assay. Each point represents an average of triplicate measurements. Each bar represents standard deviation. Asterisks indicate significant differences between two stains (* $P<0.05$).

Fig. 4. Chemiluminescent responses of Japanese flounder peritoneal macrophages to opsonized highly virulent (NUF251) (●) and low virulent (NUF194) (○) *E tarda* strains. Macrophages (final 10^6 cells/ml) were exposed to opsonized each strain of bacteria (final 10^6 CFU/ml). After addition of L012 (final 10 μM) with equipped injector, the chemiluminescent responses were recorded immediately. (×): control CL response of macrophages without bacteria.

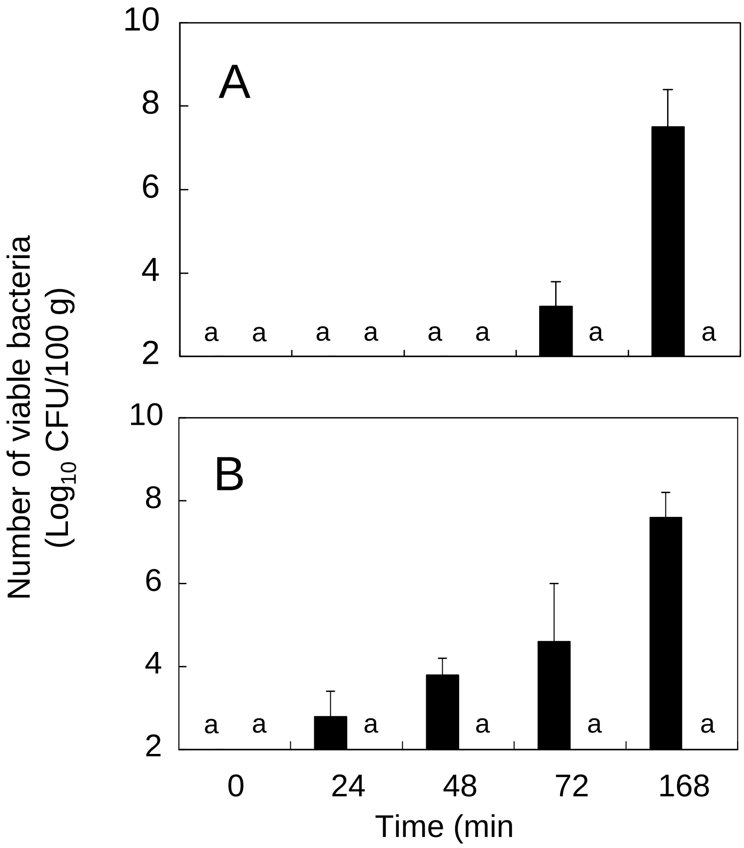
Fig. 5. Effects of phorbol myristate acetate on the chemiluminescent responses of Japanese flounder peritoneal macrophages exposed to opsonized highly virulent (NUF251) (●) and low virulent (NUF194) (○) *E tarda* strains. Macrophages (final 10^6 cells/ml) were exposed to opsonized each strain of bacteria (final 10^6 CFU/ml). After addition of L012 (final 10 μM) with equipped injector, the chemiluminescent responses were recorded immediately. After 40 min recording , phorbol myristate acetate (final 0.1 μg/ml) was added to each reaction mixture at the time indicated with arrow, and continued to record the CL responses. (×): control CL response of macrophages alone

without bacteria and phorbol myristate acetate.

Fig. 6. Effects of viable bacteria of highly virulent (NUF251) and low virulent (NUF194) *E tarda* strains on the chemiluminescent responses induced by xanthine/xanthine oxidase system. In the presence of *E. tarda* strain NUF251 (●) or NUF194 (○) (final 10^4 CFU/ml), the chemiluminescent responses induced by 5 mM of xanthine and 0.02 U/ml of xanthine oxidase were measured. (×): control chemiluminescent response without bacteria.

Fig. 7. Viability of highly virulent (NUF251) (●) and low virulent (NUF194) (○) *E tarda* strains after exposure to H_2O_2. Each bacterial strain (10^8 CFU/ml) was exposed to final 0.01% H_2O_2. After the indicated period of the time, the numbers of the viable cells were determined by colony formation assay. Each point represents an average of triplicate measurements. Each bar represents standard deviation. Asterisks indicate significant differences between two stains (*P<0.05).

Fig .1



A
B
Number of viable bacteria
(Log10 CFU/100 g)
10
8
6
4
2
a a a a a a a a
a a a a a a
0
24
48
72
168
Time (min

Fig.2

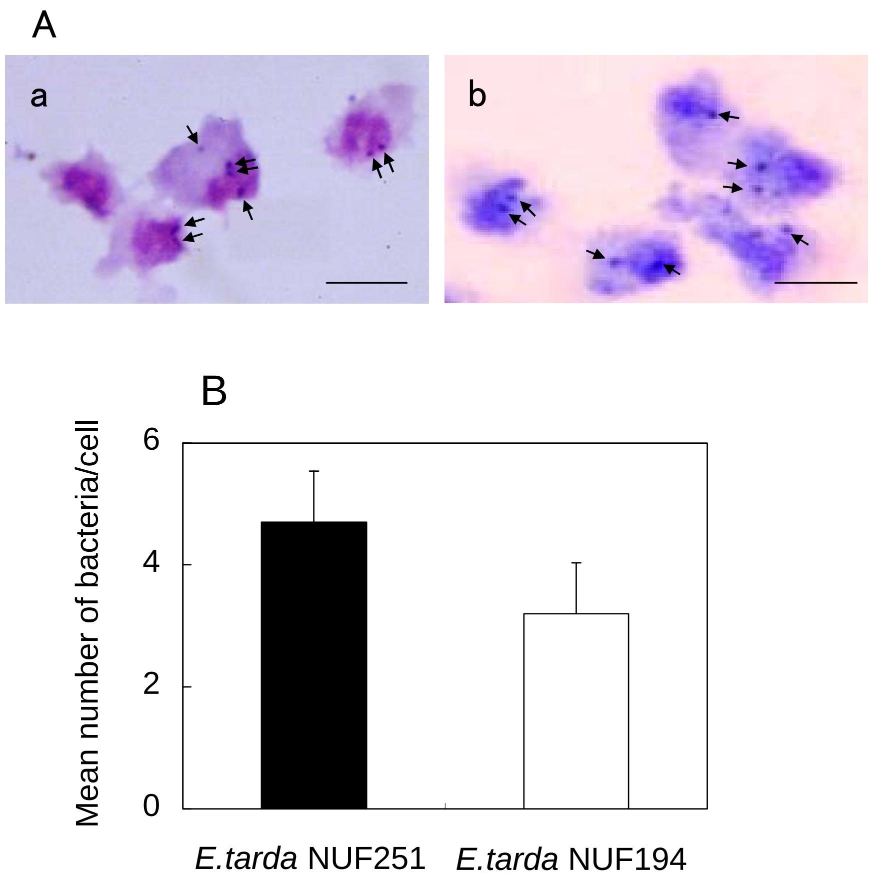

A
a
b
B
Mean number of bacteria/cell
6
4
2
0
E.tarda NUF251
E.tarda NUF194

Fig.3

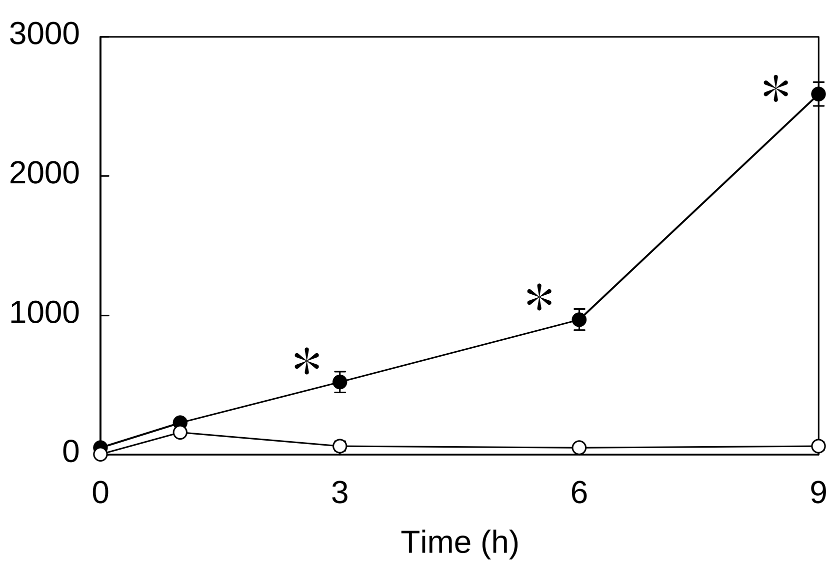
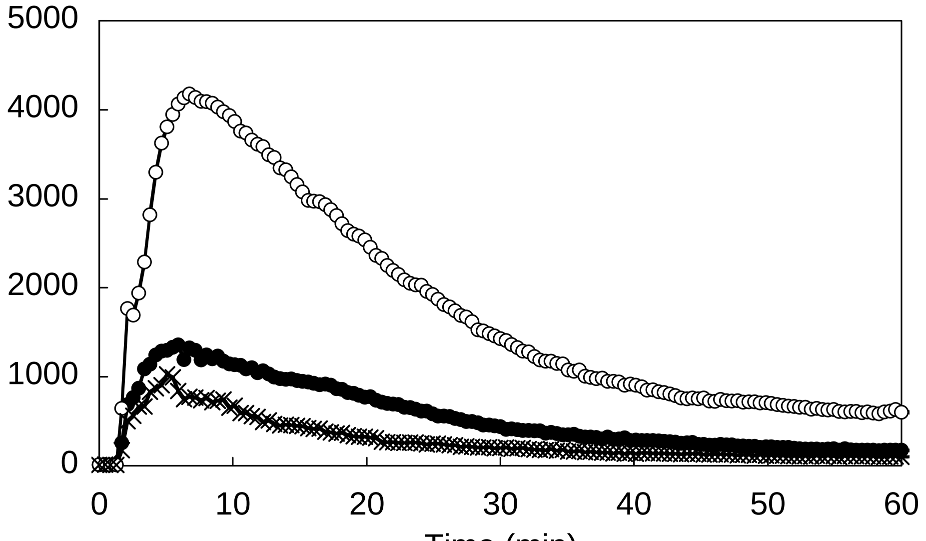


Fig.4



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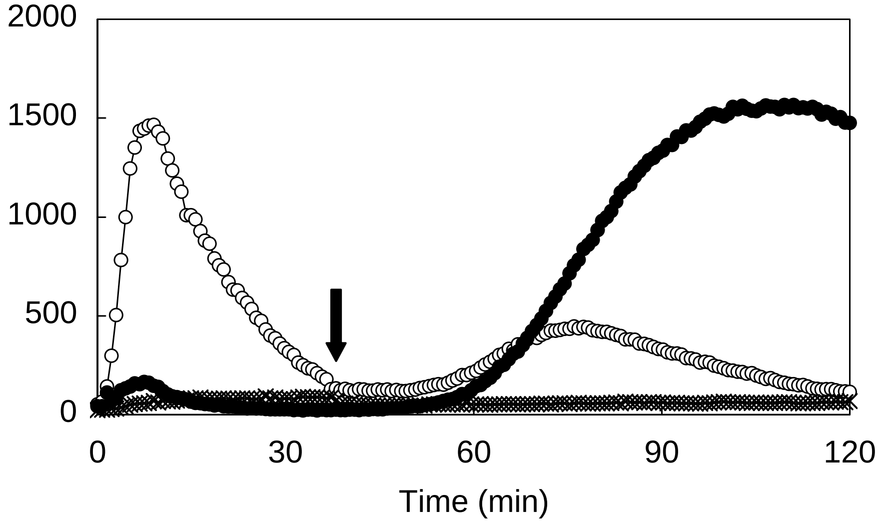

Fig.6

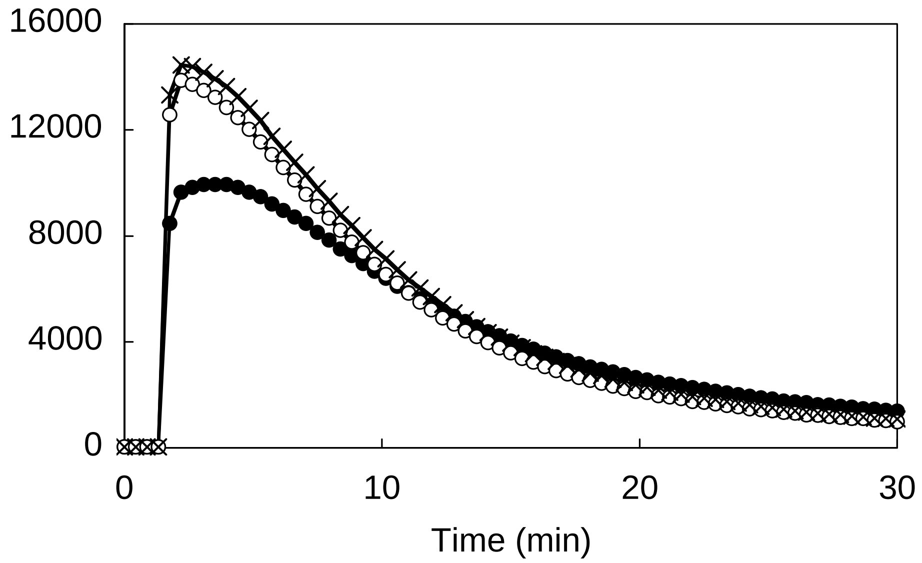

Fig. 7