Extracellular products from virulent strain of *Edwardsiella tarda* stimulate mouse macrophages (RAW264.7) to produce nitric oxide (NO) and tumor necrosis factor (TNF)-α

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Abstract

We have previously found that high virulent strain (NUF251) of *Edwardsiella tarda,* but not low virulent strain (NUF194), was able to survive and multiply within Japanese flounder (*Paralichthys olivaceus*) peritoneal macrophages. Further studies demonstrated that NUF251 induced much higher levels of NO and TNF-α productions than NUF194 in both Japanese flounder peritoneal macrophages and mouse macrophage cell line RAW264.7. In this study, we examined the effects of extracellular products (ECP) from two strains of *E. tarda* on RAW264.7 cells in terms of the induction of NO and TNF-α. ECP from NUF251 stimulated RAW264.7 cells to induce NO production in a concentration-dependent manner. The activity of NUF251-ECP completely disappeared by heat-treatment (at 100°C for 5 min), but it could not be removed by dialysis. Polymyxin B, an endotoxin inhibitor, had no effect on NUF251-ECP-induced NO production. These results suggest that active agents in NUF251-ECP responsible for NO induction may be heat-labile high molecular weight substances rather than the cell wall-derived endotoxin like substances. Since NO synthase (NOS) inhibitor, L-NAME, suppressed NUF251-ECP-induced NO production, inducible NO synthase (iNOS) in RAW264.7 cells may be a main source of NO. Furthermore, NUF251-ECP-induced high level of TNF-α secretion from RAW264.7 cells. Both NO and TNF-α productions induced by NUF251-ECP were significantly blocked by a JNK inhibitor. In contrast to NUF251-ECP, no significant activities of NUF194-ECP to induce NO and TNF-α productions were detected. SDS-PAGE and subsequent proteomic analysis of ECP from both strains suggested that

NUF251-specific protein, which has sequence homology with flagellin, is present in NUF251-ECP as a main component. Our results suggest that the high virulent strain (NUF251) of *E. tarda* may specifically produce an exotoxin capable of inducing high levels of NO and TNF-α from macrophages through the activation of JNK system, and most probable candidate for such exotoxin might be a flagellin-like protein.

Keywords: *Edwardsiella tarda*; macrophages; NO; TNF-α; MAP kinase; flagellin

1. Introduction

Edwardsiella tarda, a Gram-negative bacterium, has been reported to cause serious infectious diseases in several species of freshwater and marine fishes [1-4]. In addition to fish species, *E. tarda* can also become pathogenic bacteria in other vertebrates including amphibians [5], reptiles [6], birds [7], mammals, and humans [8].

Biochemical properties or virulence factors such as dermatotoxin [9], hemolysis [10], ability to invade epithelial cells [11], and capability of surviving in phagocytes [12], have been proposed to be responsible for the pathogenesis of *E. tarda* so far, but the exact pathogenic mechanism of *E. tarda* is still controversial.

Regarding the pathogenic mechanisms of bacterial infection, several lines of evidence have pointed out that extracellular products (ECP) from pathogenic bacteria were involved in the virulence of the bacteria. Lallier *et al*. reported that a toxic factor present in the ECP of *Aeromonas hydrophila* caused fish mortality [13]. Magarinos *et al*. reported that the ECP from *Pasteurella piscicida*, which was strongly toxic to fish, plays an important role in the pathogenesis of pasteurellosis [14]. In addition to these reports, streptococcal pyrogenic exotoxins A and C produced by group A streptococci have been well characterized to play a pivotal role in the pathogenesis of streptococcal toxic shock syndrome through the induction of inducible nitric oxide synthase (iNOS) and TNF [15].

Similar to these findings, it has been reported that certain extracellular products or exotoxins are also involved in the virulence of *E. tarda*. A toxin with

molecular weight of 37 kDa found in both extracellular and intracellular fractions of *E. tarda* was demonstrated to be lethal to Japanese eel and Japanese flounder [16,17]. Exotoxins specifically produced by virulent strain of *E. tarda* was found to cause the dermatotoxicity in rabbits [9,18].

In general, the innate immune host defense mechanisms can be activated during pathogenic bacterial infections. In most cases, including fishes and mammals, these responses are mediated by macrophages through the production of various mediators such as nitric oxide (NO), reactive oxygen species (ROS), and proinflammatory cytokines (e.g. TNF-α). NO and TNF-α are generally considered to be important mediators in the protective immune response system. However, their overproduction can also cause toxic or harmful effects on the host itself as well [19-24]. Although the exact underlying mechanism is still unclear, our recent comparative studies between high virulent strain (NUF251) and low virulent strain (NUF194) of *E. tarda* suggested that virulent strain NUF251 may have an ability to prevent ROS generation by macrophages and survive within macrophages [25]. More interestingly, we have found that NUF251 tended to induce higher levels of NO and TNF-α productions as compared to NUF194 in both Japanese flounder peritoneal macrophages and mouse macrophage cell line RAW264.7 [24].

Based on these findings, it is conceivable that certain extracellular products or toxins of NUF251 may involve in the overproduction of NO and TNF-α by macrophages. To gain insight into this point, in this study, we examined the effects of ECP prepared from high virulent (NUF251) and low virulent (NUF194) strains of *E.*

tarda on mouse macrophage cell line RAW264.7 cells with respect to induction of NO and TNF-α productions. Our results suggested that virulent strain NUF251 may specifically produce exotoxin capable of inducing NO and TNF-α production, which has sequence homology with flagellin, a major structural component of bacterial flagella.

2. Materials and methods

2.1. Materials

Lipopolysaccharide (*Escherichia coli* 026:B6; LPS) and polymyxin B were purchased from Sigma (MO, USA). NG-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase, was purchased from Dojin Chemical Laboratories (Kumamoto, Japan). PD98059, SB202190 and SP600125, which are specific inhibitors of extracellular-regulated kinase (ERK), p38 MAP kinase and c-jun NH_2-terminal kinase (JNK), respectively, were purchased from Calbiochem (LaJolla, CA, USA).

2.2. 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 virulent and pathogenic to Japanese eel and Japanese flounder,

whereas strain NUF194 is found to be relatively low virulent [16,26]. These strains were stored in nutrient medium with 10% glycerol at −80°C, and subcultured on nutrient agar medium at 25°C for 24 h prior to the onset of experiments. The cultured bacterial cells on agar medium were harvested in Dulbecco's modified phosphate-buffered saline (PBS) and washed twice with PBS by centrifugation (9,000 × g, 1 min at 4°C). The colony forming units (CFU) was determined as previously reported [25].

2.3. Preparation of ECP from E. tarda strains

The ECP from *E. tarda* strains was prepared as previously reported [17,27]. Briefly, NUF251 and NUF194 were cultured on nutrient agar medium at 25°C for 96 h, and these bacterial cells were harvested by washing with PBS. The cell suspensions were centrifuged at 12,000 × g for 20 min. The supernatant was filtered through 0.22-μm pore membrane, and stored at −80°C until use. The protein concentration of each ECP sample was determined by a BCA protein assay kit (Pierce, Rockford, IL, USA).

2.4. Mammalian cell cultures

RAW264.7 (mouse macrophage) and Vero (African green monkey kidney) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). RAW264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM)

supplemented with 10% fetal bovine serum (FBS), penicillin G (100 IU/mL), and streptomycin (100 μg/mL) in a humidified atmosphere with 5% CO_2 and 95% air as described previously [28]. Vero cells were cultured in α-minimum essential medium (α-MEM) supplemented with 10% FBS, 10 μg/ml each of adenosine, guanosine, cytidine, and thymidine, penicillin (100 IU/ml), and streptomycin (100 μg/mL). For cytotoxic assay, the cells were subcultured in 96-well plates after the treatment with 0.1% trypsin-0.05% EDTA in PBS.

2.5. *Nitrite assay for estimation of NO*

The evaluation of NO production was performed according to the method described previously [29]. Briefly, adherent RAW264.7 cells in 96-well plates (2×10^4 cells/well) in serum-free DMEM were treated with varying concentrations of ECP samples at 37°C. After 24 h incubation, the level of NO in the culture medium was determined by Griess method, which is an assay for spectrophotometric quantification of nitrite, a stable reaction product of NO with molecular oxygen, using Griess reagent (3 mM sulfanilic acid, 30 μM N-1-naphtyl-ethlenediamine dihydrochloride, 25% glacial acetic acid). After the incubation for 20 min with Griess reagent at room temperature, the absorbance of the medium at 550 nm was measured using a microplate reader (Thermo Electron). The nitrite concentrations were estimated from a reference to a standard curve for serial twofold dilution of sodium nitrite.

2.6. Dialysis- and heat-treatment of ECP

ECP samples were put into the BioDesign dialysis tube (BioDesign Inc.) with a molecular mass exclusion size of 8000 MWCO, and dialyzed against PBS for 24 h at 4°C. The samples inside dialysis tube were collected and sterilized by filtration through 0.22-μm pore membrane. To examine heat-stability of ECP, ECP samples were treated at 100°C for 5 min.

2.7. Enzyme-linked immunosorbent assay (ELISA) for TNF-α

The levels of TNF-α in culture supernatants following ECP treatment were measured by sandwich ELISA as described previously [24,30]. Briefly, the 96-well Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated with anti-mouse TNF-α monoclonal antibody (R&D Systems, Minneapolis, MN) in PBS. After blocking with 4% bovine serum albumin (BSA) in PBS, 100 μL of each culture supernatant was added to the well in triplicate and incubated at room temperature for 2 h, and then exposed to anti-mouse TNF-α polyclonal antibody (R&D Systems, Minneapolis, MN) at room temperature for 2 h. The plate was developed by peroxidase-conjugated antibodies/substrate system (TMB microwell peroxidase substrate system; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). Color development was stopped by the addition of 1 N phosphoric acid and the optical density was measured at 450 nm with a microplate reader (Thermo Electron). The TNF-α concentration was estimated

from a reference to a standard curve for serial twofold dilution of recombinant mouse TNF-α (R&D Systems, Minneapolis, MN).

2.8. Cytotoxic, hemolytic, and protease activities of ECP

Cytotoxicities of ECP to RAW264.7 and Vero cells were measured by the cell counting kit-8 (WST-8) (Dojin Laboratories, Kumamoto, Japan). In brief, RAW264.7 or Vero cells in a 96-well plate (2×10^4 cells/well) were incubated with varying concentrations of ECP from NUF251 or NUF194 in the serum-free DMEM (for RAW264.7 cells) or α-MEM (for Vero cells) for 24 h. The viabilities of the treated cells were then measured by WST-8 assay according to the manufacturer's protocol.

Hemolytic activities of ECP of *E. tarda* toward mouse and rabbit erythrocytes were measured as described previously [31]. The protease activities of the ECP were measured by casein zymography as described previously [32].

2.9. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of ECP

SDS-PAGE analysis of ECP was performed according to the method reported by Laemmli with slight modification [33]. ECP from NUF251 and NUF194 were concentrated by a centrifugal concentrator (Taitec VC-360) equipped with a freeze trap (Taitec VA-300). The concentrated ECP samples were mixed with equal volume of

SDS-PAGE sample buffer (final 62.5 mM Tris-hydrochloride pH 6.8, 10% glycerol, 5% 2-mercaptoethanol and 2% SDS). After heating at 100°C for 5 min, 40 μg of protein of each sample was subjected to SDS-PAGE (12% gel). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250.

2.10. Determination of N-terminal amino acid sequences of the proteins separated by SDS-PAGE

To determine the N-terminal amino acid sequence, the proteins separated in SDS gels were transferred to a polyvinylidene difluoride (PVDF) membrane in transfer buffer (10 mM CAPS, 10% methanol, pH11). The band of interest was isolated, and analyzed with a PPSQ-33A automated protein/peptide sequencer (Shimadzu, Kyoto, Japan) according to the manufacturer's instructions. A similarity search of amino acid sequence was performed using the BLAST program on the National Center for Biotechnology Information (NCBI) protein database.

2.11. Statistics

Statistical analysis was conducted by the Student’s t-test and Tukey’s multiple comparison *post hoc* analysis following one-way ANOVA. The criterion of significance was set at $p < 0.05$. All results are expressed as mean ± SD.

3. Results

3.1. NO levels in RAW264.7 cells treated with ECP from E. tarda strain NUF251 and NUF194

To compare the effects of ECP from two strains of *E. tarda* on RAW264.7 cells, varying concentrations of ECP from strain NUF251 or NUF194 of *E. tarda* were added to the adherent RAW264.7 cells. After the incubation for 24 h, the culture medium was withdrawn to detect the NO by Griess method. As shown in Fig.1, the ECP from the high virulent strain of *E. tarda* (NUF251) induced NO production in a concentration-dependent manner, and more than 20 μM of NO was induced at 100 ng/mL. However, the ECP from low virulent strain of *E. tarda* (NUF194) caused no significant level of NO production even at 100 ng/mL (Fig. 1). The NO in the control culture medium without ECP was undetectable (data not shown). These results suggest that virulent strain NUF251 may specifically secrete substances capable of inducing NO production from macrophages.

3.2. Effects of dialysis- and heat-treatment on the NO-inducing activity of the ECP of NUF251 in RAW264.7cells

To characterize the substances in the ECP of NUF251 responsible for the induction of NO production from RAW264.7 cells, the ECP from NUF251 (100 ng/mL) was

dialyzed against PBS, and examined its NO-inducing activity. As shown in Table. 1, only a slight decrease in the activity was observed after dialysis. On the other hand, the activity of NUF251-ECP (100 ng/mL) was almost completely disappeared after heat-treatment at 100°C for 5 min. These results suggest that the substances in the ECP from NUF251 responsible for NO induction may be high molecular weight heat-labile substances.

3.3. Effects of polymyxin B and L-NAME on NUF251-ECP-induced NO production

To ascertain whether or not the cell wall derived endotoxin like substances are involved in the NO-inducing activity of NUF251-ECP, we examined the effect of polymyxin B, an endotoxin inhibitor. In the presence of polymyxin B, no significant decrease in the NO-inducing activity of NUF251-ECP was observed, while polymyxin B showed potent inhibitory effect on LPS-induced NO production (Fig. 2a).

In macrophages, iNOS is a well-known NO producing enzyme system [20,21]. Therefore, we examined the effect of L-NAME, an NO synthase inhibitor, on the NO level in RAW264.7 cells stimulated with NUF251-ECP. As shown in Fig. 2b, L-NAME strongly inhibited the NUF251-ECP-induced NO production as well as the inhibition of LPS-induced NO production.

3.4. Effects of MAP kinase inhibitors on NUF251-ECP-induced NO production

MAP kinase systems have been reported to be involved in the intracellular signal transduction leading to NO production in macrophages [29,34,35]. Since three major MAP kinases such as ERK, p38 and JNK are known to involve in the signaling [36], we examined the effects of three specific inhibitors for ERK, p38, and JNK, namely PD98059 [37], SB202190 [38], and SP600125 [39] on NUF251-ECP-induced NO production. As shown in Fig. 3, only the JNK inhibitor, SP600125 (10 μM) showed inhibitory effect on the ECP-induced NO production from RAW264.7 cells, whereas other inhibitors had no effect at 10 μM.

3.5. Secretion of TNF-α from RAW264.7 cells treated the ECP from NUF251 and NUF194

Since our previous studies demonstrated that live cells of NUF251 potently induced TNF-α secretion from RAW264.7cells as well as NO, we next examined the effects of ECP from NUF251 and NUF194 on RAW264.7 cells in terms of TNF-α secretion. After 24 h incubation with varying concentrations of ECP from each strain of *E. tarda*, the levels of TNF-α in the culture medium was determined by ELISA method. As shown in Fig. 4a, the ECP from NUF251 induced TNF-α secretion in a concentration-dependent manner, and nearly 100 ng/mL of TNF-α was induced at 100 ng/mL. However, no significant TNF-α inducing activity was observed in the ECP from NUF194 (Fig. 4a).

As did in NO-inducing activities, we investigated whether MAP kinases are involved in the TNF-α production. As shown in Fig. 4b, among the three specific inhibitors, JNK inhibitor showed the greatest inhibitory effect on the NUF251-ECP-induced TNF-α production (Fig. 4b).

3.6. Cytotoxic, hemolytic, and protease activities in the ECP from NUF251 and NUF194

To gain insight into the potential biological activities of the ECP from two strains of *E. tarda*, we examined cytotoxicities of the ECP towards RAW264.7 and Vero cells. Since Vero cells were often used to detect the cytotoxicity of bacterial exotoxin as sensitive cell line, we selected this cell line in addition to RAW264.7 cells to estimate the cytotoxic potential of ECP from *E. tarda*. As shown in Fig. 5, no significant cytotoxicities of the ECP from both NUF251 and NUF194 on RAW264.7 and Vero cells were observed up to 1000 ng/mL. Neither hemolytic activity against mouse and rabbit erythrocytes nor casein degradation activity was detected in the ECP from both NUF251 and NUF194 (data not shown).

3.7. SDS-PAGE analysis of ECP from NUF251 and NUF194

To analyze protein profiles of ECP, NUF251-ECP and NUF194-ECP were subjected to SDS-PAGE. As shown in Fig. 6, NUF251-ECP sample gave a main band with molecular mass of approximately 45 kDa, which could not be detected in the

NUF194-ECP sample. In NUF194-ECP sample, a main band with molecular mass of approximately 39 kDa and several minor bands were observed. These results suggest that the 45 kDa protein in NUF251-ECP may be a NUF251-specific protein.

3.8. Sequence analysis of the isolated major protein of NUF251-ECP

The 45 kDa protein specifically detected in NUF251-ECP was isolated and analyzed by N-terminal Edman protein sequencing. The N-terminal amino acid sequence was determined to be AQVINTNSLSLMAQNNLNKS, which matched with five bacterial proteins: *Edwardsiella tarda* flagellin (NCBI protein accession number AAN52540), *Serratia marcescens* flagellin (BAA06987), *Yersinia bercovieri* ATCC 43970 flagellin (ZP_04626839), *Escherichia coli* flagellin (CAI56313) and *Salmonella enterica* subsp. *enterica* serovar *Choleraesuis* flagellin (ADC35412), using the NCBI protein database. Flagellin is a principal component of bacterial flagella. The result suggests that the main component of NUF251-ECP may be flagellin-like protein (Fig. 7).

4. Discussion

In aquacultural industry in Japan, Edwardsiellosis caused by *E. tarda* is one of the most serious infectious diseases, and sometimes provokes mass mortality in cultured fish especially to Japanese eel (*Anguilla japonica*) and Japanese flounder (*Paralichthys olivaceus*). It is also known that *E. tarda* causes diarrhea for humans probably due to

direct contact with or consumption of the infected fish. In the comparative studies of virulent and avirulent strain of *E. tarda*, it was found that only the virulent strain, but not the avirulent strain, was able to enter fish body, multiply inside various internal organs, and finally caused fish death [11]. In accordance with these findings, our previous study demonstrated that the high virulent strain of *E. tarda* (NUF251) could multiply within the hepato-pancreas and kidney of Japanese flounder by overcoming the fish defense system after infection, but low virulent strain (NUF194) could not [25]. Although the exact pathogenic mechanism remained to be clarified yet, several possible virulence factors of this bacterium such as lethal proteinaceous exotoxin with protease activity, dermatotoxin, hemolysin, siderophore, and resistance to phagocytes have been proposed [16]. Regarding the resistance mechanisms to phagocytes, we have found that the responses of peritoneal macrophages of Japanese flounder (*P. olivaceus*) against high virulent strain (NUF251) and low virulent strain (NUF194) of *E. tarda* were different [25], and NUF251 has an ability to prevent ROS generation by macrophages and survive inside macrophages. More recent our studies also demonstrated that virulent strain NUF251 caused faster induction of NO release and much higher level of TNF-α production than NUF194 from Japanese flounder peritoneal macrophages. The more potent ability of NUF251 to induce NO and TNF-α productions as compared to NUF194 were also observed in RAW264.7 cells [24].

In general, NO and TNF-α play defensive roles against invading bacterial pathogens, and being thought as essential elements of antimicrobial immunity during inflammation. Cooperation between reactive oxygen and nitrogen intermediates in

killing of pathogenic bacteria by activated macrophages has been reported as major defense mechanism in murine model [23]. It has been reported that TNF-α plays protective roles against *Mycobaterium tuberculosis* in mice [19]. In addition to the defensive roles or beneficial effects in host, NO and TNF-α are also known to behave as toxic and injurious factors toward host tissues or cells when they were overproduced exceeding the critical levels. For instance, during sepsis shock or acute inflammation, NO and TNF-α can be causative factors for circulatory and multiple organ failure [40-42].

Although the underlying mechanisms is still unknown, several lines of evidence suggest that bacterial products including exotoxins possess cytokine-inducing activities and that the host proinflammatory cytokine response may contribute to the pathogenesis [43]. For example, pneumolysin, a protein exotoxin produced by *Streptococcus pneumoniae*, was found to be an important pneumococcal virulence factor, and it has shown that pneumolysin exerts potent ability to induce the production of NO and various inflammatory cytokines [21]. This study also proposed that pneumolysin-induced production of large amounts of NO might be responsible for hypotension, a critical pathogenic feature of the septic shock [21]. Excessive amount of TNF-α secretion has also been suggested to lead to lethal damage to the host during the inflammation. Furthermore, TNF has been identified as a common mediator of hepatocellular apoptosis and liver injury in experimental mouse models [44]. In the case of acute hepatotoxicity caused by *Pseudomonas aeruginosa* exotoxin A in mice, it has been shown that neutralization of TNF by anti-TNF antibody prevented liver injury [45].

These findings evoke the notion that inflammatory mediators such as NO and TNF-α produced by macrophages in response to invading bacteria can cause serious detrimental effects on the host apart from the beneficial effects. Therefore, we would like to propose that the potent ability of virulent *E. tarda* (NUF251) to induce NO and TNF-α production from macrophages is one of the important virulence factors.

During the recent decades, several studies focusing on the involvement of bacterial extracellular products in the pathogenic mechanism of bacterial infectious diseases including *E. tarda*-induced diseases have been conducted [9,14,16-18,46,47]. Therefore, in this study, we compared the effects of ECP from high and low virulent strains of *E. tarda* on macrophage cell line RAW264.7 in terms of NO production, and found only NUF251-ECP, but not NUF194-ECP, induced high level of NO production. These results suggest that NUF251-ECP may contain a potent NO-inducer which might be a factor responsible for the pathogenesis of *E. tarda*. There are several reports supporting the notion. Stanley *et al*. observed the differences between the extracellular products from virulent and avirulent strains of *E. ictaluri*, which might be related to the pathogenesis of *E. ictaluri* [48]. In other fish bacterial pathogens, the biological activities present in the ECP were responsible for the development and severity of these bacterial diseases [14,49-53].

Although we could not obtain the active agent in purified form in the ECP from NUF251 in this study, heat- and dialysis-treatment analysis suggested that NO-inducer in the ECP might be a heat-labile high molecular weight substance. Furthermore, no significant inhibitory effect of polymyxin B on NUF251-ECP-induced NO production

was observed in contrast to its complete blockade of LPS-induced NO production. These results suggest that the potent activity of NUF251-ECP to induce NO production is not likely to be due to endoxotoxin or LPS like cell wall derived compounds. Therefore, it is possible to speculate that the main active agent in the NUF251-ECP may be a novel proteinaceous exotoxin. This notion may be also supported by previous findings that *E. tarda* produces high molecular weight proteinaceous exotoxins [14,17,18,52].

Since L-NAME, an NO synthase inhibitor, significantly inhibited the NUF251-ECP-induced NO production, NO is mainly produced from L-arginine by iNOS in RAW264.7 cells [20,21].

In addition to NO, we also observed that NUF251-ECP, but not NUF194-ECP, induced high level of TNF-α production from RAW264.7 cells consistent with previous studies [24]. To gain insight into the intracellular signaling pathway leading to the NO and TNF-α productions caused by NUF251-ECP in RAW264.7cells, we examined the effects of three specific MAP kinase inhibitors on NUF251-ECP-induced NO and TNF-α productions. In fact, many reports have demonstrated that MAP kinase family such as ERK, p38 and JNK are involved in the production of NO and TNF-α by activated macrophages [54-57]. Among the three specific inhibitors, only JNK inhibitor showed significant inhibitory effect on both NO and TNF-α production induced by NUF251-ECP, suggesting that JNK plays an important role in the intracellular signal transduction leading to both NO and TNF-α productions in NUF251-ECP-treated RAW264.7 cells. Regarding the involvement of MAP kinase systems, it has been

reported that ERK and p38 signaling pathways are mainly involved in LPS-induced TNF-α production [58]. Thus, it seems likely that the exotoxin-like agents present in NUF251-ECP may take specific intracellular pathway distinct from that of endotoxin to induce eventual NO and TNF-α productions.

Interestingly, NUF251-ECP and NUF194-ECP showed quite different protein band patterns on SDS-PAGE analysis (Fig. 6), and it was found that there was a NUF251-specific main band with molecular mass of approximately 45 kDa, which was not detected in NUF194-ECP (Fig. 6). Subsequent N-terminal sequence analysis of the protein revealed that the NUF251-specific protein has sequence homology with flagellins of several bacteria including *E. tarda*, suggesting that the NUF251-specific protein is flagellin-like protein. Similar to our results, a comparative proteomic analysis of extracellular proteins of 6 virulent strains including NUF251 and 8 avirulent strains of *E. tarda* has shown that flagellin is one of the major virulent strain-specific proteins of *E. tarda*, which was not detected in avirulent *E. tarda* strains tested [59]. Furthermore, it has been shown that flagellins derived from Gram-negative bacterial pathogens such as *Salmonella enteritides* and *Pseudomonas aeruginosa* are potent inducers of cytokine and nitric oxide production by mammalian monocytes and other cell types [60-62]. Based on the previous studies, it is considered that flagellin signals via toll-like receptor 5 (TLR5), whereas LPS signals via TLR4 in mammalian system [63,64]. It has been reported that rainbow trout (*Onchorhynchus mikiss*) and pufferfish (*Fugu rubripes*) have two TLR5 isoforms, membrane and soluble forms [65], and latter form seems to be fish-specific and is not found in humans. In addition, it has been

reported that fish are highly sensitive to flagellin, which induces endotoxin-like response in fish [66]. These findings suggest that flagellin-like protein in NUF251-ECP is the most probable candidate for the causative factor responsible for the potent induction of NO and TNF-α production from RAW264.7 cells. Further studies are required to clarify this point as well as the identification and purification of the active agent with especially focused on flagellin in NUF251-ECP. The detail analysis of the effects of NUF251-ECP on Japanese flounder peritoneal macrophages *in vitro* as well as the effects of NUF251-ECP on fish immune system *in vivo* through the direct injection of NUF251-ECP into fish may provide further insight into the virulence mechanism of *E. tarda*

Regarding extracellular products produced by *E. tarda*, hemolytic, cytotoxic, and protease activities have been reported so far. However, none of these activities were detected in ECP from both NUF251 and NUF194. Therefore, it is expected that the agent with potent NO and TNF-α inducing activity present in NUF251-ECP may be a novel exotoxin with unique action mechanism.

In conclusion, our results suggest that the high virulent strain (NUF251) of *E. tarda* specifically produces an exotoxin capable of inducing NO and TNF-α production from macrophages through activation of JNK system. This exotoxin, which is probably flagellin-like protein, may be an important virulence factor responsible for the pathogenesis of *E. tarda*. This finding may also provide important clue for the development of effective vaccination programs with particularly focused on flagellin protein as an antigen.

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Figure legends

Fig. 1. NO levels in RAW264.7 cells treated with ECP from different strains of *E. tarda*. RAW264.7 cells (2×10^4 cells/well in 96-well plates) were treated with indicated concentration of ECP from high virulent strain NUF251 and low virulent strain NUF194 of *E. tarda* in serum-free DMEM at 37°C. After incubation for 24 h, the NO levels in the culture medium of the cells were determined as described in the text. Each point represents an average of triplicate measurements. All data represent means ± SD. *Significant differences from values with NUF194-ECP: $P<0.05$.

Fig. 2. Effects of polymyxin B and L-NAME on NUF251-ECP-induced NO production in RAW264.7 cells. RAW264.7 cells (2×10^4 cells/well in 96-well plates) were pre-incubated with indicated concentrations of polymyxin B (a) or L-NAME (b) in serum-free DMEM at 37°C for 10 min, followed by the addition of 100 ng/mL NUF251-ECP or 10 ng/mL LPS. After 24 h incubation, the NO levels in the culture medium were evaluated as described in the text. Each point represents an average of triplicate measurements. All data represent means ± SD.

Fig. 3. Effects of MAP kinase inhibitors on NUF251-ECP-induced NO production in RAW264.7 cells. RAW264.7 cells (2×10^4 cells/well in 96-well plates) were pre-incubated with 10 μM of ERK (PD98059), p38 (SB202190), or JNK (SP600125) MAP kinase inhibitor in serum-free DMEM at 37°C for 1 h, followed by the addition of NUF251-ECP (100 ng/mL). After 24 h incubation, the NO levels in the culture medium

were evaluated as described in the text. Each point represents an average of triplicate measurements. Each point represents an average of triplicate measurements. All data represent means ± SD. *Significant differences from values with others: $P<0.05$.

Fig. 4. TNF-α production by RAW264.7 cells treated with ECP. (a) RAW264.7 cells (2×10^4 cells/well in 96-well plates) were incubated with indicated concentrations of ECP from NUF251 or NUF194 in serum-free DMEM at 37°C. After 24 h incubation, TNF-α levels in culture medium of the cells were estimated by ELISA method. (b) RAW264.7 cells were preincubated with 10 μM of ERK (PD98059), p38 (SB202190), or JNK (SP600125) MAP kinase inhibitor in serum-free DMEM at 37°C for 1 h, followed by the addition of NUF251-ECP (100 ng/mL). After 24 h incubation, the level of TNF-α in the culture medium was estimated. Each point represents an average of triplicate measurements. All data represent means ± SD. *Significant differences from values with NUF194-ECP (a) or NUF251-ECP (b): $P<0.05$.

Fig. 5. Cytotoxicities of ECP from NUF251 and NUF194 on RAW264.7 and Vero cells. To adherent RAW264.7 (a) or Vero cells (b) in 96-well plates (2×10^4 cells/well) in serum-free DMEM or α-MEM, respectively, various concentrations of ECP from NUF251 or NUF194 were added. After 24 h incubation 37°C, the cell viabilities were measured as described in the text. Each point represents an average of triplicate measurements. All data represent means ± SD.

Fig. 6. SDS-PAGE analysis of NUF251-ECP and NUF194-ECP. Electrophoresis was carried out using 12% polyacrylamide gel. Lane 1, molecular weight markers; lane 2, NUF251-ECP; lane 3, NUF194-ECP.

Fig. 7. Alignment of N-terminal amino acid sequence of NUF251-ECP protein and other bacterial flagellin proteins: *Edwardsiella tarda* flagellin (NCBI protein accession number AAN52540), *Serratia marcescens* flagellin (BAA06987), *Yersinia bercovieri* ATCC 43970 flagellin (ZP_04626839), *Escherichia coli* flagellin (CAI56313) and *Salmonella enterica* subsp. *enterica* serovar *Choleraesuis* flagellin (ADC35412), using the NCBI protein database. Amino acid residues matched with those of NUF251-ECP protein are indicated as boxed.

Table 1. Effects of dialysis- and heat-treatments on the NUF251-ECP-induced NO production in RAW264.7 cells.

Samples	Level of NO_2^- (μM) [a]
Control	0.6 ± 0.3
NUF251-ECP	15.9 ± 0.8
Dialyzed NUF251-ECP	14.3 ± 1.6
Heat-treated NUF251-ECP	0.2 ± 0.2

[a] NUF251-ECP was dialyzed against PBS for 24 h at 4°C. The heat-treatment was conducted at 100°C for 5min. After the addition of each sample (100 ng/mL), RAW264.7 cells (2×10^4 cells/well in 96-well plates) were incubated at 37°C for 24 h. The NO levels in the culture medium of the cells were determined as described in the text. The control indicated culture medium without ECP. Each point represents an average of triplicate measurements. All data represent means ± SD.

Fig. 1.

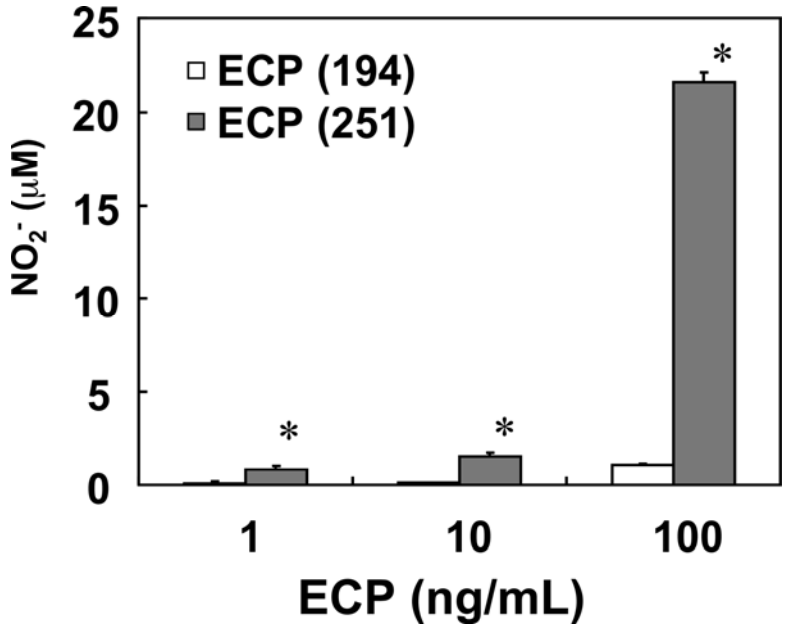


Fig. 2.

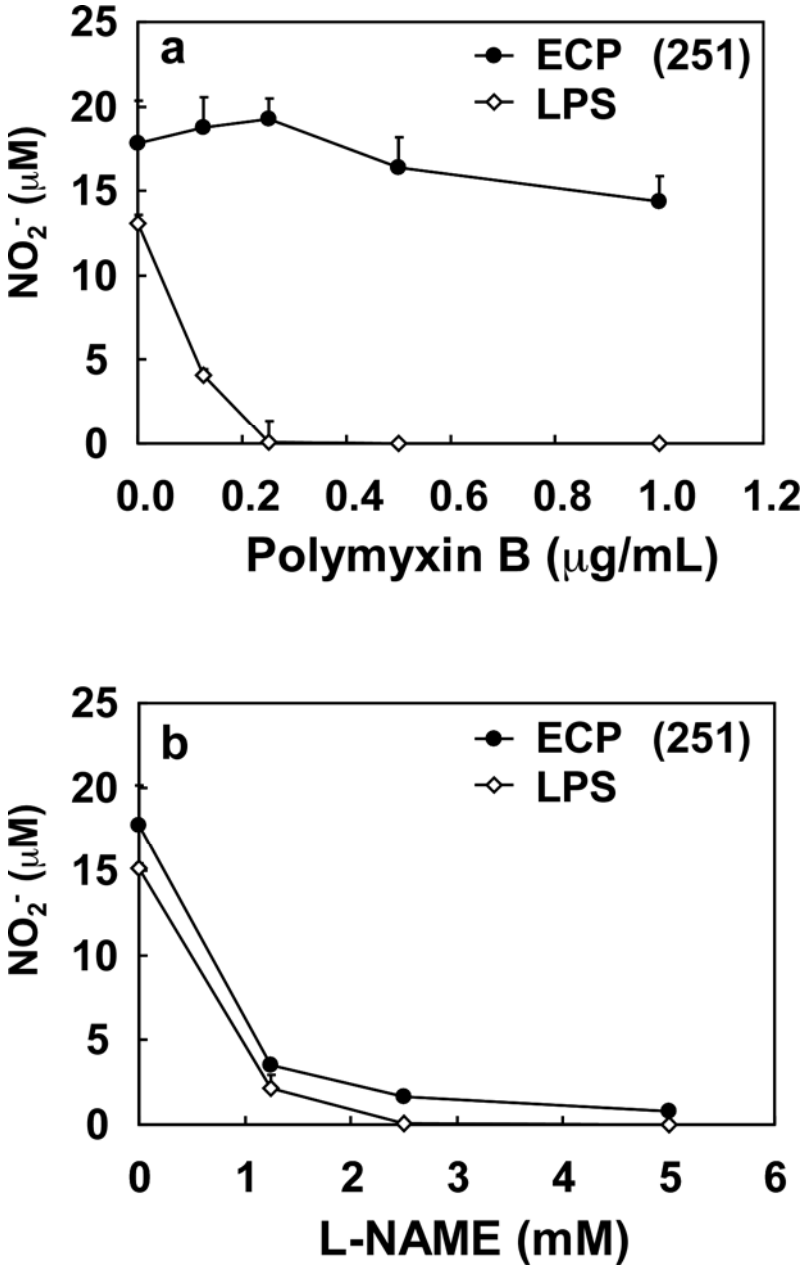

a
ECP (251)
LPS
NO_2^- (μM)
25
20
15
10
5
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Polymyxin B (μg/mL)
b
ECP (251)
LPS
NO_2^- (μM)
25
20
15
10
5
0
0
1
2
3
4
5
6
L-NAME (mM)

Fig. 3.

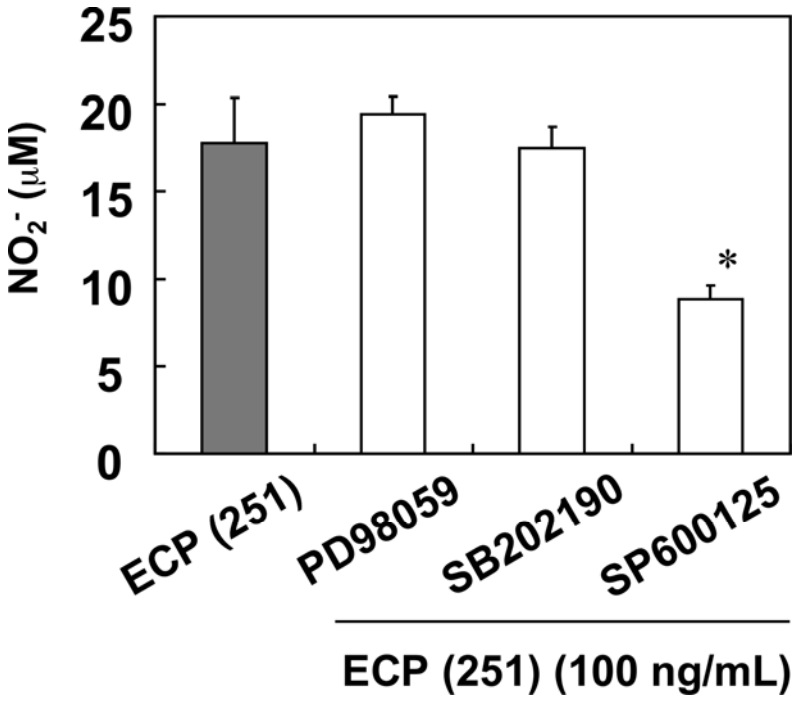

25
20
15
10
5
0
NO_2^- (μM)
*
ECP (251)
PD98059
SB202190
SP600125
ECP (251) (100 ng/mL)

Fig. 4.

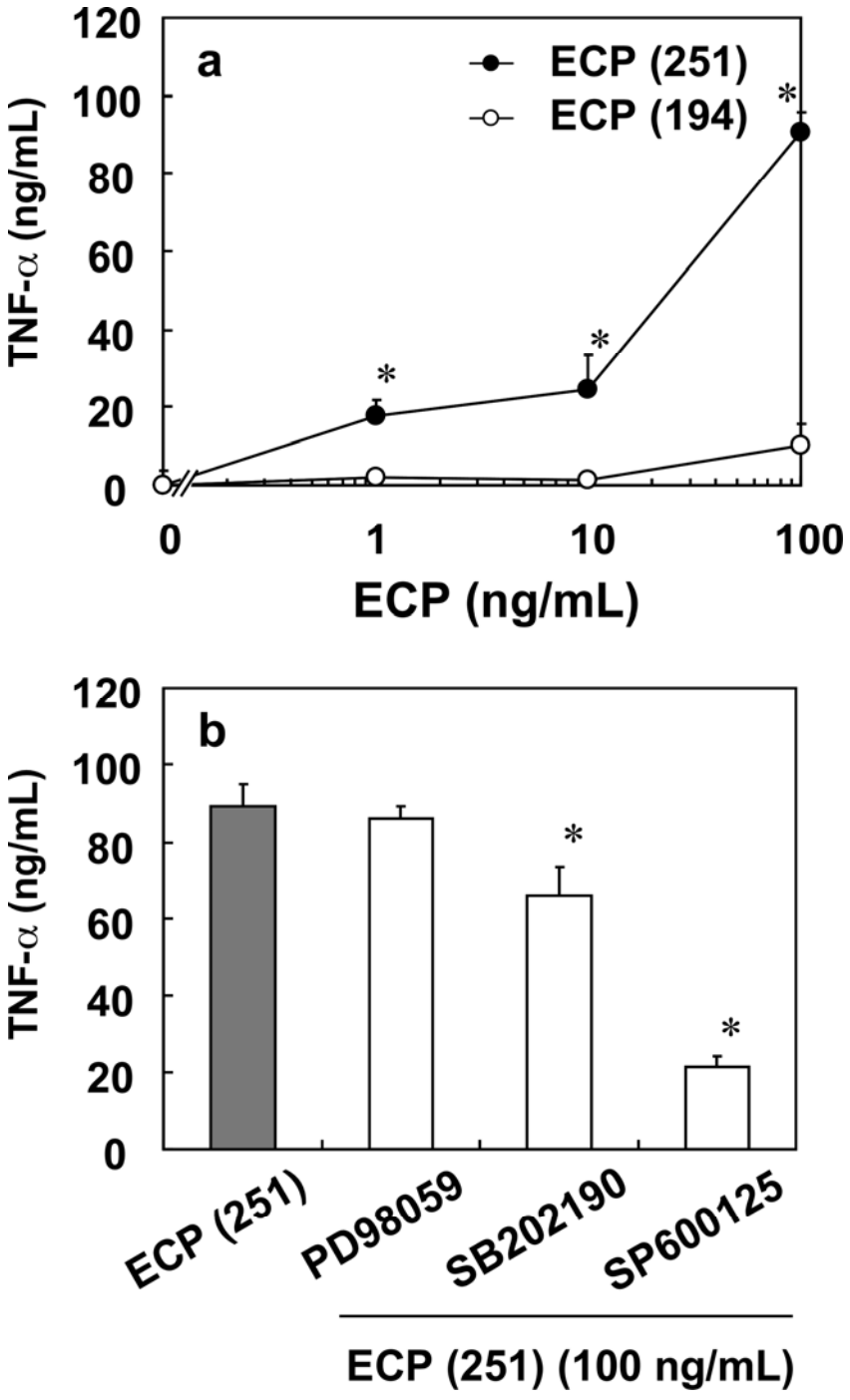

a
ECP (251)
ECP (194)
TNF-α (ng/mL)
ECP (ng/mL)
0
20
40
60
80
100
120
0
1
10
100
*
b
TNF-α (ng/mL)
ECP (251)
PD98059
SB202190
SP600125
ECP (251) (100 ng/mL)

Fig. 5.

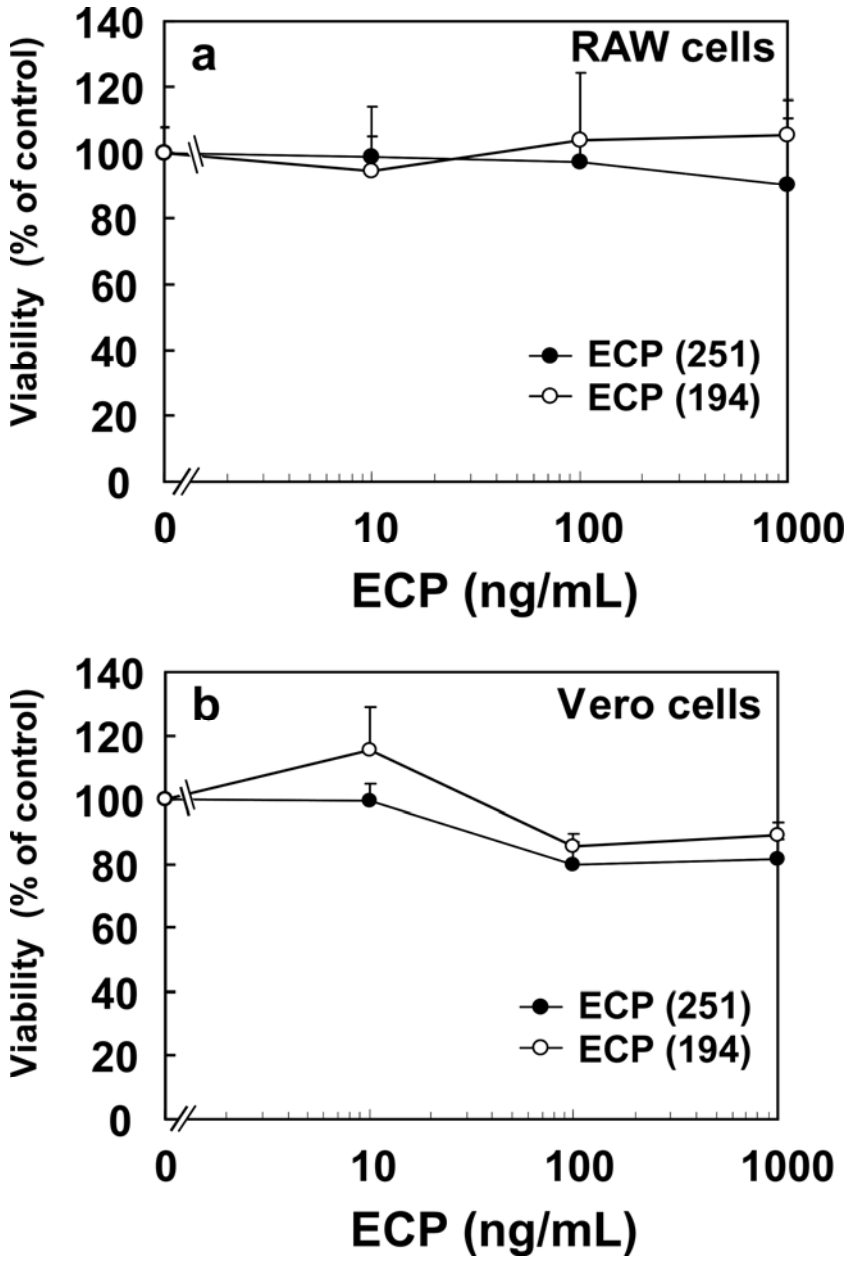

a
RAW cells
Viability (% of control)
140
120
100
80
60
40
20
0
ECP (251)
ECP (194)
0
10
100
1000
ECP (ng/mL)
b
Vero cells
Viability (% of control)
140
120
100
80
60
40
20
0
ECP (251)
ECP (194)
0
10
100
1000
ECP (ng/mL)

Fig. 6.

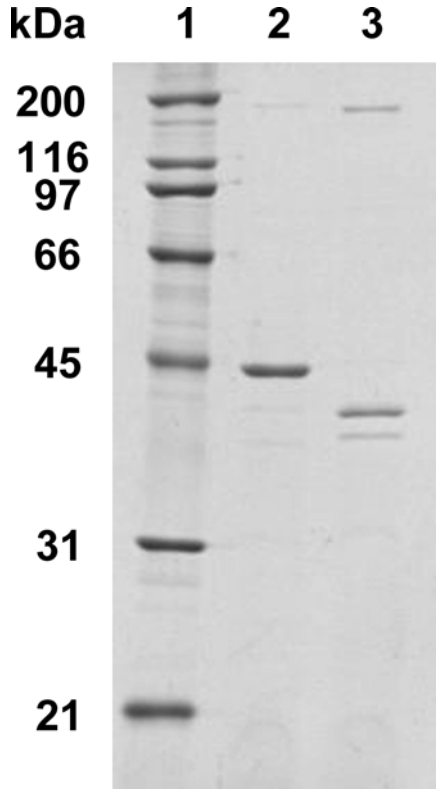
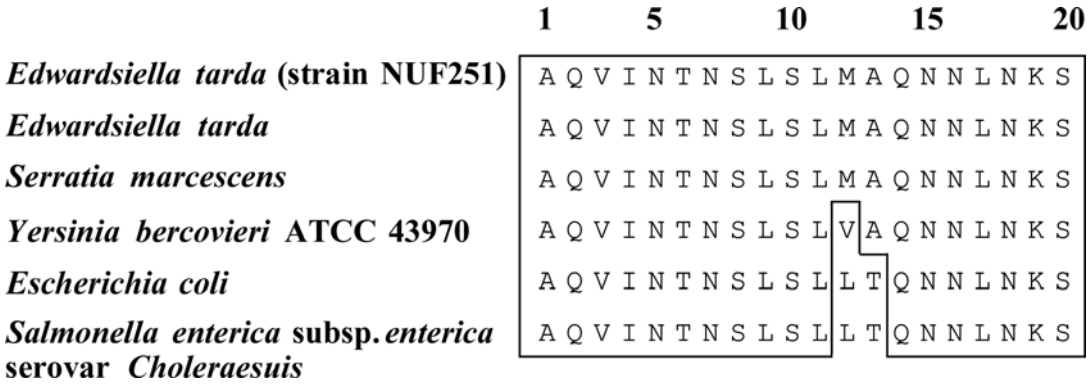


Fig. 7.


1 5 10 15 20
Edwardsiella tarda (strain NUF251) A Q V I N T N S L S L M A Q N N L N K S
Edwardsiella tarda A Q V I N T N S L S L M A Q N N L N K S
Serratia marcescens A Q V I N T N S L S L M A Q N N L N K S
Yersinia bercovieri ATCC 43970 A Q V I N T N S L S L V A Q N N L N K S
Escherichia coli A Q V I N T N S L S L L T Q N N L N K S
Salmonella enterica subsp. enterica serovar Choleraesuis A Q V I N T N S L S L L T Q N N L N K S