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Studies on the Pathogenicity and Serological Properties of the Fish Pathogen
Tenacibaculum maritimum

By

Tanvir Rahman

A Thesis submitted to Nagasaki University for the degree of Doctor of Philosophy

Graduate School of Fisheries Science and Environmental Studies
Nagasaki University, Japan

December, 2014
Studies on the Pathogenicity and Serological Properties of the Fish Pathogen
*Tenacibaculum maritimum*

By

Tanvir Rahman

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December, 2014
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microliters</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>cm²</td>
<td>Centimeter square</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>d</td>
<td>Day(s)</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DDBJ</td>
<td>DNA data bank of Japan</td>
</tr>
<tr>
<td>DW</td>
<td>Distilled water</td>
</tr>
<tr>
<td>ECP</td>
<td>Extracellular product</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamino tetra-acetic acid</td>
</tr>
<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Lethal dose 50%</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>N</td>
<td>Normal</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>NOTG</td>
<td>n-Octyl-β-D-thioglycoside</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>Second(s)</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tris buffered saline plus Tween-20</td>
</tr>
<tr>
<td>TYS</td>
<td>Tryptone yeast seawater</td>
</tr>
<tr>
<td>wk</td>
<td>Week</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
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The Author
Abstract

*Tenacibaculum maritimum* is a Gram-negative, gliding marine bacterium that causes tenacibaculosis, an ulcerative disease of marine fish around the world. The pathology of the disease has mainly been associated with characteristic gross lesions on the body surface of fish such as ulcers, necroses, eroded mouth, frayed fins and tail rot, and sometimes necroses on the gills and eyes. Despite the significance of *T. maritimum* in Japanese aquaculture, especially on Japanese flounder *Paralichthys olivaceus*, relatively little is known about its pathogenicity, and no vaccine is still available to prevent the disease. The present study is planned to carry out a detailed study on the serological characterization of *T. maritimum* and pathogenicity assessment on Japanese flounder.

First of all, a non-gliding strain of *T. maritimum* was characterized. Although the bacterium usually forms rhizoid colonies on agar media, we isolated *T. maritimum* that formed slightly yellowish round compact colonies together with the usual rhizoid colonies on an agar plate from a puffer fish *Takifugu rubripes* suffering from tenacibaculum, and studied the biological and serological characteristics of a representative isolate of the compact colony phenotype, designated strain NUF1129. It revealed that the strain was non-gliding and avirulent in Japanese flounder in immersion challenge test, reduced in adhesion to glass wall in shaking broth culture and to the body surface of flounder and lacked a cell-surface antigen, designated antigen X, common among gliding strains in gel immunodiffusion tests using sonicated cell extracts as antigens. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of sonicated cell extracts showed different polypeptide banding patterns between NUF1129 and gliding strains. Like gliding strains, NUF1129 exhibited both chondroitinase and gelatinase activities, which are potential virulence factors of the bacterium. These results suggest that some cell-surface components related to gliding and adhesion ability are implicated in the virulence of *T. maritimum*.

The second experiment was conducted to isolate antigen X from the pathogenic strain, *T. maritimum* NUF1128. The partial purification of antigen X was succeeded by hydrophobic interaction chromatography. PAGE analysis of partially purified antigen X showed distinctive
expression of a larger molecular weight single protein band and it was detected as immunogenic by western blot. SDS-PAGE study revealed that antigen X is a high molecular weight protein consists of two polypeptide chains.

In the third experiment, a pathogenicity test was conducted using Japanese flounder after abraded the skin with cotton swabs or steel blades or cut off the tips of dorsal fin with scissors. Pretreated fish were immersed for 30 min in seawater containing $10^6$ CFU/mL of cultured NUF1128 or NUF1129 cells and reared for 7 d. To investigate the kinetics of infection, two groups of fish abraded with cotton swabs or steel blades were challenged as above, and the viable counts of \textit{T. maritimum} from the abraded skin were assessed at 30 min, 2 h, 6 h, 24 h and 48 h post infection. The skin tissues were examined for the bacterial proliferation by immunohistochemistry. The pathogenicity test resulted in 100% mortality of fish pretreated with steel blades and scissors and challenged with NUF1128. NUF1129 was unable to induce infection regardless of treatments applied. Infection kinetics and immunohistochemical studies revealed that NUF1128 adhered more readily than NUF1129 to dermal connective tissues which were exposed by abrasion with blades and proliferated mainly in the dermal and muscular connective tissues.

The fourth experiment was undertaken to find out the serological relationship among \textit{T. maritimum} strains isolated from diseased Japanese fish. Twenty-five \textit{T. maritimum} strains isolated from diseased Japanese flounder, tiger puffer (including the compact colony forming non-gliding avirulent strain, NUF1129), red sea bream, black sea bream and Dover sole, three strains of different species under \textit{Tenacibaculum} genus and an unidentified gliding bacterium were tested. Two antisera were raised against \textit{T. maritimum} NUF684 (from diseased Japanese flounder) and NUF1081 (from diseased tiger puffer) by immunizing rabbits with formalin-killed cells (FKC, formalin 0.5%). Agglutination titrations of the antisera were performed using microtiter plates against FKC and heat-killed (boiled for 1 h) cells (HKC). Microtiter agglutination tests revealed that titers of anti-\textit{T. maritimum} NUF684 serum against \textit{T. maritimum} strains were 8,192-131,072 for FKC and 16-256 for HKC and those of anti-\textit{T. maritimum} NUF1081 serum were 8,192-131,072 for FKC but 32-512 for HKC. The FKC (16-32) and HKC (8-32) of NUF1129 showed similar titers for both antisera due to lack of heat-labile antigens on
its cell surface. Low titers were observed in both antisera against FKC (<4-128) and HKC (<4-32) of other species under Tenacibaculum genus. Anti-NUF684 serum absorbed with NUF1081-FKC or anti-NUF1081 serum absorbed with NUF684-FKC showed significant reduction of titers against FKC of T. maritimum strains due to sharing common heat-labile surface antigens. Difference in titers of absorbed antisera against HKC of T. maritimum strains indicated the existence of different O-serogroups.

A distinctive expression of a 19.6 kDa protein was reported previously to distinguish T. maritimum gliding strains from the non-gliding avirulent strain, NUF1129. To determine the role of 19.6 kDa in the pathogenesis of tenacibaculosis, the 19.6 kDa protein was isolated from the polytron homogenized aliquots of the pathogenic strain, NUF1128 and extracted from the ultracentrifuged precipitation of the outer membrane components using n-octyl-β-D-thioglucoside (NOTG). Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the α-chymotrypsin digested 19.6 kDa polypeptide revealed several bands from which the 16.8 kDa band was sequenced and yielded a 16 N-terminal amino acid sequence, SQVSVGGQDADTVFT. Primers derived from this sequence were used in an inverse PCR strategy to clone the full-length gene from T. maritimum NUF1128 DNA. An open reading frame (ORF) of 588 bp encoded a 196 amino acid protein having a molecular mass of 19 kDa. The predicted amino acid sequence was identical to the hypothetical protein of T. maritimum by homology search with specific hits on the OMP_b-brl (outer membrane protein beta barrel domain) under the superfamily of OM_channels. The role of 19.6 kDa in bacterial pathogenesis remains to be elucidated.

Overall, the cell-surface components related to gliding and adhesion ability are implicated in the virulence of T. maritimum. The isolated partially purified surface antigen is an immunogenic high molecular weight protein which consists of two polypeptide chains. The gliding strain was found highly pathogenic for Japanese flounder, adhered more readily to dermal connective tissues through the abraded points and proliferated exponentially associated with mortalities. Due to presence of common heat-labile antigens on the cell surface of T. maritimum strains, a common FKC vaccine can be developed to prevent tenacibaculosis. The investigated 19.6 kDa band is a hypothetical outer membrane protein of T. maritimum that is predicted to be involved in putative channel which demands further clarification.
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General Introduction
First outbreaks of tenacibaculosis occurred in red sea bream *Pagrus major* and black sea bream *Acanthopagrus schlegeli* cultured in Japan (Masumura and Wakabayashi, 1977). The bacterium was isolated and the authors announced their intention to make a separate formal proposal of the name "*Flexibacter marinus*" (Hikida *et al*., 1979). Phenotypical characteristics were extensively studied (Wakabayashi *et al*., 1984; Baxa *et al*., 1986) and the eventual formal proposal was a taxon called *Flexibacter maritimus* (Wakabayashi *et al*., 1986, Holmes 1992). Reichenbach (1989) listed the pathogen as *Cytophaga marina*, but the priority of the name *Flexibacter maritimus* was later recognized (Holmes, 1992) and DNA investigations further demonstrated the homogeneity of this taxon (Bernardet *et al*., 1990, 1994). The genus *Tenacibaculum* now belongs to the family *Flavobacteriaceae*, phylum *Bacteroidetes* and *T. maritimum* (Suzuki *et al*., 2001) is an exclusively marine species (Bernardet *et al*., 1996). It is a Gram-negative filamentous gliding bacterium that causes ulcerative disease with massive mortalities in cultures of marine fish.

Marine tenacibaculosis is widely distributed in cultured and wild fish in Europe, Japan, North America and Australia (McVicar and White, 1979, Wakabayashi *et al*., 1986; Devesa *et al*., 1989; Pazos *et al*., 1993; Chen *et al*., 1995; Handlinger *et al*., 1997; Ostland *et al*., 1999; Santos *et al*., 1999). In Japan, *T. maritimum* was isolated from the body surface, skin lesions, or kidney of several fish species other than sea breams, such as Japanese flounder *Paralichthys olivaceus*, leather jacket *Aluterus monoceros*, rock bream *Oplegnathus fasciatus*, plaice *Cleisthenes pinetorum herzensteini*, puffer *Takifugu rubripes*, and yellowtail *Seriola quinqueradiata* (Baxa *et al*., 1986, 1987, 1988b; Wakabayashi *et al*., 1986) from spring to early summer and sometimes in cold season (Kusuda and Kawai, 1998). Other susceptible species include Dover sole *Solea solea* in Scotland (Bernardet *et al*., 1990), turbot *Scophthalmus maximus* in Spain (Alsina and Blanch,
1993), sea bass *Dicentrarchus labrax* in France (Bernardet *et al.*, 1994), Atlantic salmon *Salmo salar* in Australia (Soltani and Burke, 1994) and Pacific sardine *Sardinops sagax* in the USA (Chen *et al.*, 1995). *T. maritimum* directly attacks the body surface of fish (Magariños *et al.*, 1995), causing lesions such as ulcer, necrosis, eroded mouth, frayed fins and tail-rot (Campbell and Buswell, 1982; Devesa *et al.*, 1989). As these lesions favor the entrance of other bacteria such as *Vibrio* spp. (Kimura and Kusuda, 1983) and saprophytic organisms such as ciliated protozoans (McVicar and White 1979; Devesa *et al.*, 1989), *T. maritimum* thus often appears in mixed infections.

The morphological, physiological and biochemical characteristics used in the identification of *T. maritimum* have been detailed by several authors (Hikida *et al.*, 1979, Baxa *et al.*, 1986, Wakabayashi *et al.*, 1986, Bernardet and Grimont 1989, Bernardet *et al.*, 1990, 1994, Alsina and Blanch 1993, Pazos *et al.*, 1993, Soltani and Burke 1994, Chen *et al.* 1995, Ostland *et al.*, 1999, Suzuki *et al.*, 2001, Avendaño- Herrera *et al.* 2004). These phenotypic results indicate that *T. maritimum* constitutes a homogeneous species. *T. maritimum* is a filamentous bacterium 0.5 \( \mu \text{m} \) wide by 2 to 30 \( \mu \text{m} \) long; however, occasionally cells up to 100 \( \mu \text{m} \) in length can be observed. In older liquid and solid cultures, cells appear shorter and tend to become spherical (approximately 0.5 \( \mu \text{m} \)). Spheroplast-like forms are rarely observed even after prolonged incubation of the *T. maritimum* cells. Gliding motility on wet surfaces is a general feature of all isolates. The bacterium is mesophilic and can grow at temperatures from 15 to 34\(^\circ\)C, with an optimum growth temperature of 30\(^\circ\)C. On solid media, *T. maritimum* colonies absorb Congo red but the cells do not contain cell wall-associated flexirubin-type pigment. Different types of colonies, varying in their rhizoid aspect and adherence, may coexist on the same agar plate (Sorongon *et al.* 1991). *T. maritimum* produces enzymes that degrade casein, tyrosine and tributyrin, but it does not
hydrolyse agar, carboxymethyl cellulose, cellulose, starch, esculin or chitin. Variable results have been reported for gelatin hydrolysis, hydrogen sulfide production and nitrate reductions.

The first serological studies described by Wakabayashi et al. (1984) and Pazos et al. (1993), based on slide agglutination assays, reported antigenic homogeneity of *T. maritimum*, regardless of their origin and source of isolation. Further studies by Ostland et al. (1999) demonstrated antigenic differences among *T. maritimum* isolates, suggesting that this microorganism may not be as homogeneous as previously thought. Dot blot assays and immunoblot analysis of lipopolysaccharides (LPS) revealed the existence of antigenic diversity in *T. maritimum* and demonstrated that at least three major O-serogroups seemingly related to the host species (Avendaño-Herrera et al., 2004, 2005b). Thus, the majority of *T. maritimum* isolated from sole *Solea senegalensis* in the northwest of Spain and all gilthead sea bream isolates belonged to serotype O1, while all strains isolated from sole *Solea senegalensis* in Portugal and southern Spain constituted serotype O3. Strains isolated from turbot belonged to serotype O2. However, this serological scheme could certainly be extended if further studies, including more strains of *T. maritimum* isolated from different hosts and/or geographical origins, are conducted. The analysis of total and outer membrane proteins revealed that all strains had common bands which are antigenically related (Avendaño-Herrera et al., 2006c).

Despite the significance of *T. maritimum* in the aquaculture industry, relatively little is known about the pathogenicity of this bacterium. Different challenge methods of infection have been attempted to reproduce the disease. The challenge test by subcutaneous injection induced tenacibaculosis in the previous studies using black sea bream (Baxa et al., 1987), Dover sole *Solea solea* (Campbell and Buswell, 1982), Senegalese sole *Solea senegalensis* (Faiļde et al., 2014) and turbot *Psetta maxima* (Faiļde et al., 2013). Wakabayashi, et al. (1984) and Baxa et al.
(1987) demonstrated that immersion challenge was not a reliable method of inducing the disease unless the skin was previously scarified or abraded. However, other authors have reproduced the disease using prolonged immersion of fish (Avendaño-Herrera et al., 2006a) or high inoculum doses (van Gelderen, et al., 2011). Recently, immersion challenge (Nishioka et al., 2009; Rahman et al., 2014) or immersion and dilution method (Yamamoto et al., 2010) was succeed to reproduce experimental infection in Japanese flounder. To date, many of the studies on disease transmission support the hypothesis that *T. maritimum* is an opportunistic pathogen that primarily causes extensive skin damage and gill abrasion with subsequent systemic infection (Avendaño-Herrera et al., 2006c).

Japanese flounder is a major coastal commercial fish. The total catch of the flounder is stable between 6,500 and 8,400 tonnes in the last decade. It is also one of the major target species of stock enhancement in Japan. A total of 30 million juveniles are released every year. Economic return rate (amount of catch/ cost of stocking) can increase to 2.5 in the case appropriate releasing techniques are applied (Yamashita and Kurita, 2007). Economical losses due to diseases in cultured flounder become a major problem. Sometimes natural outbreaks of tenacibaculosis occur a few weeks after transferring fish from hatchery tanks to inshore net cages (McVicar and White 1979, Wakabayashi et al., 1984) and hence, fish farms have to face financial losses. Considering overall, the present study was conducted to carry out a detailed and in-depth scientific study on the tenacibaculosis of Japanese flounder, with special attention to the serological properties and pathogenicity of *T. maritimum*. A preliminary research was conducted on the characterization of a non-gliding strain of *T. maritimum* isolated from a diseased puffer fish (chapter 1) to find out biological and serological characteristics as well as virulence in fish in comparison with those of typical *T. maritimum* strains. With respect to study *T. maritimum*, a
surface exposed antigen of *T. maritimum* was isolated for characterization (chapter 2). To investigate the pathogenicity of *T. maritimum* on Japanese flounder followed by disease progression, a third study was conducted on the kinetics of infection of a gliding and non-gliding strain of *T. maritimum* on the abraded skin of flounder (chapter 3). To reveal the serological variation among Japanese isolates, another serological study was conducted on *T. maritimum* strains isolated from Japanese flounder and puffer fish (chapter 4). Lastly, a 19.6 kDa outer membrane protein, common in gliding strains, was isolated from the pathogenic strain NUF1128 to clarify its role in the pathogenesis of tenacibaculosis (chapter 5).
Chapter 1

Biological and Serological Characterization of a Non-gliding

Strain of *Tenacibaculum maritimum* Isolated from a

Diseased Puffer Fish *Takifugu rubripes*
1.1. Introduction

Marine Tenacibaculosis is a serious bacterial disease affecting a great variety of cultured marine fish species (Toranzo et al., 2005). Although studies have been done on the capacity of adhesion and hydrophobicity (Sorongon et al., 1991), adherence to fish skin and mucus (Magariños et al., 1995), toxic activity of extracellular products (Baxa et al., 1988a; Van Gelderen et al., 2009), capsular material (Avendaño-Herrera et al., 2006c) and iron-uptake mechanisms (Avendaño-Herrera et al., 2005a), actual virulence mechanisms of this bacterium are still not clear. Association of virulence with colony morphology has been found in many pathogenic bacteria (Simpson et al., 1987; Van der Woude and Bäumler, 2004).

Bacterial gliding motility is defined as the movement of a non-flagellated cell in the direction of its long axis on a surface (Henrichsen, 1972). This type of motility is common within the phylum *Bacteroidetes*, of which *T. maritimum* is a member. As a result of the movement, *T. maritimum* usually produces flat rhizoid colonies with uneven edges, which are adherent to the agar medium (Pazos et al., 1996). During routine laboratory diagnosis in 2010, slightly yellowish round compact colonies grew together with the usual rhizoid colonies of *T. maritimum* on an agar plate inoculated with a lesion specimen of puffer fish *Takifugu rubripes* suffering from tenacibaculosis. A representative isolate of the compact colony phenotype, designated strain NUF1129, was identified as *T. maritimum* by the species-specific PCR (Toyama et al., 1996) and 16S rRNA and *gyrB* gene sequencing (Suzuki et al., 1999). The aim of the present study was to investigate the biological and serological characteristics as well as virulence in fish in comparison with those of typical *T. maritimum* strains including NUF1128, which was isolated on the same agar plate where NUF1129 was collected, for uncovering the virulence mechanisms
of *T. maritimum*.

1.2. Materials and Methods

1.2.1. Bacterial strains

Fourteen *T. maritimum* strains including NUF1129 derived from diseased fish were used in this study (Table 1.1). Like other strains, NUF1129 was identified as *T. maritimum* by the species-specific PCR (Toyama *et al*., 1996) and exhibited approximately 99.8% and 98.7% identity for the 16S rRNA (size of polynucleotide analyzed = 1,406 bp) and gyrB (1,059 bp) gene sequences with those of *T. maritimum* NBRC15946 = ATCC43398T (NR_113825) and ATCC43398T (AB034229), respectively. The partial sequences of the 16S rRNA and gyrB genes determined were deposited in the DDBJ/EMBL/GenBank databases under accession numbers from AB979246 to AB979249 for the 16S rRNA gene of NUF1128 and NUF1129 and gyrB gene of NUF1128 and NUF1129, respectively. The strains were routinely cultured on TYS agar medium consisted of 0.3% Bacto™ Tryptone (Difco), 0.2% Bacto™ Yeast Extracts (Difco) and 1.5% agar (Wako), pH 7.4-7.6, in filtered seawater for 24 h at 27°C. For broth culture, medium was prepared with the above composition except 1.5% agar. The stock cultures were kept at -80°C, and the main stocks were maintained in liquid nitrogen.

1.2.2 Observation of motility
Gliding motility was assessed by the plate technique (Perry, 1973), in which a few glass beads (φ 0.1 mm) were deposited on the surface of TYS agar at the part of bacterial growth margins, and a coverslip was placed over this area. A gentle finger press on the coverslip creates a liquid (extruded from agar) graded area, which allows the bacterial cells to move freely. Under a light microscope (×400), the gliding movement was observed clearly by cessation of Brownian movement.

### 1.2.3. Virulence test

*T. maritimum* NUF1128 and NUF1129 were cultured in TYS broth at 27°C for 24 h with shaking at 120 rpm. Bacterial cells grown were harvested by centrifugation (9,000 × g, 10 min), washed and resuspended in sterile PBS. Tenfold serial dilutions of the bacterial suspension were prepared, and five fish per dilution were used for immersion challenge and subcutaneous injection. Juvenile Japanese flounder (mean body weight 51.2±11.8 g) were stocked separately per dilution into 30-L rectangular aquaria equipped with continuous flow of filtered seawater and aeration. Challenge doses of NUF1128 and NUF1129 employed for immersion challenge were ranged from 7.9×10⁴ to 7.9×10⁷ CFU/mL and 2.8×10⁵ to 2.8×10⁸ CFU/mL, respectively. Control fish were immersed in seawater without the bacterial suspension. After immersion for 30 min fish were transferred to the experimental aquaria. For subcutaneous injection, inoculation doses of NUF1128 and NUF1129 were 4.0×10³ to 4.0×10⁶ CFU/fish and 1.4×10⁴ to 1.4×10⁷ CFU/fish, respectively. Another five fish were injected with PBS for control. The fish received no feed for 15 days of the experiment, and the water temperature ranged from 21.0 to 22.5°C during the experimental period. Gross pathological changes and moribundness were checked daily. Samples
from the external lesions, i.e. the skin, fins, gills and mouth, and kidney of dead fish were
directly streaked onto TYS agar supplemented with kanamycin at 100 µg/mL and incubated at
27°C for 2 days to confirm that *T. maritimum* was the cause of mortality. LD<sub>50</sub> value was
calculated using the method described by Reed and Muench (1938).

1.2.4. Adherence to fish body

A portion of the upper side of ten juvenile Japanese flounder (mean body weight 36.4±8.5 g)
was abraded with sterilized cotton swabs following the method of Miwa and Nakayasu (2005).
Immediately after abrasion, five fish per strain were immersed for 30 min in seawater containing
5.5×10<sup>6</sup> CFU/mL of NUF1128 or 2.7×10<sup>7</sup> CFU/mL of NUF1129 cells cultured as above and then
reared in a 30-L aquarium with continuous water flow for another 30 min. The water temperature
was around 25°C. After fish were anesthetized using 2-phenoxyethanol, a piece of skin, about 1
cm<sup>2</sup>, was cut out from the abraded and non-abraded regions of each fish and homogenized with 2
mL of sterile PBS. The homogenate was serially diluted using sterile PBS, and 100 µL of each
dilution was spread onto TYS agar supplemented with kanamycin at 100 µg/mL and incubated at
27°C for 2 days. Lastly, colony forming units per cm<sup>2</sup> were calculated.

1.2.5. Preparation of sonicated cell extracts

The *T. maritimum* strains were cultured on TYS agar plates at 27°C for 24 h. The cells grown
were washed off the plate with autoclaved seawater and collected by centrifugation. The pelleted
bacterial cells were washed three times and resuspended in PBS at a concentration of 0.2 g wet
weight/mL, and sonicated at a rate of 5 min/mL using a Microson™ XL-2000 Ultrasonic Cell Disruptor (Misonix). After centrifugation at 15,000 ×g for 30 min, the supernatant was sterilized by passing through a 0.45 µm sterile filter unit (Advantec) and kept at –20°C until use.

1.2.6. Serological characterization

Preparation of rabbit antiserum: *T. maritimum* NUF1081 was cultured in TYS broth at 27°C for 24 h. The cells grown were inactivated by 0.5% formalin for 2 days at room temperature. Formalin-killed cells (FKC) were then washed twice with PBS, resuspended in PBS containing 0.02% NaN₃ at a concentration of 100 mg wet weight/mL and stored at 4°C. One milliliter of the diluted suspension containing 50 mg FKC was emulsified with an equal volume of Freund’s complete adjuvant (Wako) and injected subcutaneously to a Japanese white rabbit twice with a 2-wk interval. Two weeks after the second injection the rabbit received an intraperitoneal injection of 50 mg FKC without adjuvant, and 3-wk after the third injection the total blood was collected. The antiserum was heated at 56°C for 30 min and stored at –20°C.

Absorption of antiserum by FKC: One milliliter of the rabbit anti-*T. maritimum* NUF1081 serum was incubated with 100 mg of *T. maritimum* NUF1081 FKC for 2 h at room temperature and overnight at 4°C. The absorption procedure was repeated twice.

Adsorbed antibody: The FKC used in antiserum absorption was washed three times with PBS, and antibodies adsorbed to the FKC were eluted by treating with glycine-HCl (pH 3.0) at room temperature. Immediately after the treatment, pH of the eluate was raised to neutral with 1 M
Tris. The collected antibodies (aAb) were used as the antibodies specific for the cell-surface antigens.

**Gel immunodiffusion:** Gel immunodiffusion was performed with 1% agarose (Bio-Rad) in PBS on glass slides. The reservoirs were cut using a 7-well cutter. The reactants were added to wells, and the slides were allowed to stand for 24 h in a moist chamber at room temperature. In this study, precipitation reactions of the sonicated cell extracts of *T. maritimum* strains were studied with the anti-*T. maritimum* NUF1081 serum (AS), the antiserum absorbed with NUF1081 FKC (aAS) and the adsorbed antibodies (aAb).

**1.2.7. SDS-PAGE analysis of sonicated cell extracts**

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to Laemmli (1970) using 4% (w/v) stacking and 12.5% (w/v) separating gels in a mini-slab electrophoresis apparatus AE-6530 (Atto). Sonicated cell extracts of the strains were used as samples. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Wako).

**1.2.8. Chondroitinase and gelatinase activity**

Chondroitinase activity was detected based on the method described by Teska (1993) using TYS agar supplemented with 0.2% chondroitin sulfate C sodium salt (Wako). The bacterial strains were grown on the medium at 27°C for 3 days, and the agar plate was flooded with 2 mL of 4% bovine serum albumin (Sigma-Aldrich) and 5 mL of 1 N HCl. The clear zones around the
bacterial growth indicate positive results. Gelatinase activity was tested by culturing the strains on TYS agar supplemented with 0.1% gelatin at 27°C for 24 h. The plate was flooded with 15% HgCl₂ in 2.4 N HCl. The appearance of clear zones around the colonies indicates positive results. As the *Edwardsiella tarda* isolates from human were unable to degrade gelatin and chondroitin sulfate (Waltman *et al.*, 1986), *E. tarda* strain NUF1100 (isolated from human) was used as a negative control in both studies.

**1.3. Results**

**1.3.1. Biological and morphological characteristics**

Single cells of the strain NUF1128 were slender rods (Fig. 1.1a) and more or less similar to the average length of other rhizoid strains (4.4 ± 0.9 to 5.7 ± 1.3 µm). On the other hand, cells of the strain NUF1129 were similar in shape (Fig. 1.1b) but the shortest (2.4 ± 0.5 µm) in length among the strains studied (Table 1.1). No bacterial movement was exhibited by NUF1129 (non-gliding), whereas the other strains showed various degrees of gliding movements. The gliding strain NUF1128 produced yellow centered with greenish glistening, flat and rhizoid with irregularly spreading-edged colonies (Fig. 1.1c), but colonies of the non-gliding strain NUF1129 appeared as slightly yellowish, round, convex ones on TYS agar plates (Fig. 1.1d). Differences in the bacterial adherence to glass wall were observed after 24 h of rotary shaking of TYS broth culture: NUF1128 showed adherence to glass wall with forming bacterial aggregations (Fig. 1.2a), while NUF1129 was found as non-adherent with homogenous bacterial suspension (Fig. 1.2b).
1.3.2. Virulence

The virulence test using Japanese flounder revealed that the strain NUF1128 was virulent (LD$_{50}$=10$^{6.0}$ CFU/mL) in immersion challenge but non-pathogenic (LD$_{50}$>10$^{7.1}$ CFU/fish) in subcutaneous injection (Table 1.2). Fish in control groups did not die and show any pathological signs. The most apparent pathological changes observed in the infected fish were lesions (i.e., ulcers and necrosis) on the body surface, frayed fins and tail rot. *T. maritimum* was re-isolated from the lesions but not from the kidneys of dead fish on kanamycin enriched TYS agar plates. On the other hand, the non-gliding strain NUF1129 was avirulent in the both challenges. None of the fish died or showed any signs of disease within the experimental period (Table 1.2).

1.3.3. Adherence to body surface

The results of the *in vivo* adhesion assay are shown in Table 1.3. For all fishes, NUF1129 exhibited less ability to adhere to the non-abraded body surface than that of NUF1128. No difference in adherence between NUF1128 and NUF1129 was observed on the abraded region.

1.3.4. Serological characteristics

Using anti-*T. maritimum* NUF1081 serum, the microtiter agglutination test resulted in low titer (2$^4$) with NUF1129 FKC, although both NUF1128 and NUF1081 FKCs exhibited high titer reactions (2$^{16}$). Three distinct precipitin lines presumably derived from three antigens, designated
as antigens X, Y and Z, were observed primarily in gel immunodiffusion test using anti-\textit{T. maritimum} NUF1081 serum and sonicated cell extracts of the \textit{T. maritimum} strains. The lines derived from antigens Y and Z were observed in all the strains, but the line of antigen X was absent in NUF1126 and NUF1129 (Fig. 1.3a). All the three antigens were supposed to be protein in nature since heat treatment (55°C, 15 min) of the sonicated cell extracts resulted in disappearance of the precipitin lines (data not shown). Immunodiffusion test using the absorbed antiserum (aAS) and adsorbed antibodies (aAb) demonstrated that antigen Z was probably not exposed on the cell-surface since the antigen formed a precipitin line with aAS but not with aAb, while antigens X and Y were supposed to be cell-surface since they formed lines with aAb but not with aAS (Fig. 1.3b).

\textit{1.3.5. Polypeptide profile}

SDS-PAGE separation of the sonicated cell extracts revealed that all the \textit{T. maritimum} strains shared a considerable number of common polypeptide bands between 14.4 and 111.4 kDa. The polypeptide profile of NUF1129 also showed the similarities, however, there was distinctive expression of the bands at 35.8 and 16.9 kDa but visibly less expression of the 19.6 kDa band (Fig. 1.4).

\textit{1.3.6. Chondroitinase and gelatinase activities}
All the *T. maritimum* strains including NUF1129 showed positive for chodroitinase and gelatinase activities (Table 1.1). No clear zone around the colony was observed in the case of negative control.

### 1.4. Discussion

Here we described, for the first time, a compact colony phenotype of *T. maritimum* from a naturally infected fish. The isolate such as the strain NUF1129 was supposed to be mutated in the lesion of the diseased fish or in the natural environment before entering into the lesion. It is not surprising that the isolate of the compact colony phenotype lacks gliding motility, because the rhizoid colony morphology is a result of the gliding movement of growing cells (Henrichsen, 1972). It was reported that several surface proteins that are unique to the phylum *Bacteroidetes* involved in gliding motility (Braun *et al.*, 2005; Nelson *et al.*, 2008). Cells of NUF1129 were non-gliding probably due to changes in cell-surface components and ultimately appeared as the compact colony morphology. Compared with usual strains, the colonies of NUF1129 were easy to remove from TYS agar plates and the strain produced homogeneous suspension in shaken broth culture without visible adherence to glass wall. These characteristics may also come from the changes in the cell-surface components.

The average length of the single cells of all the strains remained within the range (0.5 μm wide by 2 to 30 μm long) mentioned by Avendaño-Herrera *et al.* (2006c), although the non-gliding strain NUF1129 exhibited the shortest cell size. The cell length of NUF1129 was always shorter than gliding strains even after changing the culture conditions (*i.e.*, temperature, culture duration etc.) (data not shown). Functional decrease in the nutrient uptake may occur in NUF1129 cells.
since it is known that some bacterial species reduced their cell size in nutrient-poor medium (Chien et al., 2012). Shortened cell size of non-motile mutants is also evidenced from *Cytophaga johnsonae* (Chang et al., 1984).

Although both NUF1128 and NUF1129 have been isolated from puffer fish, we used Japanese flounder in the virulence test, because our past preliminary experiment using puffer fish exhibited its low susceptibility to *T. maritimum* challenge compared to Japanese flounder. In this study, the gliding strain NUF1128 was shown to be virulent in Japanese flounder by immersion route, which is consistent with the previous experiment conducted with Japanese flounder (Nishioka et al., 2009). On the contrary, the non-gliding strain NUF1129 was avirulent, causing no signs of infection or mortalities. Although many factors are thought to be involved in the virulence, changes in the cell-surface components of NUF1129 that related to gliding and adhesion ability can be implicated in the non-pathogenic feature of the strain.

The challenge test by subcutaneous injection worked unsuccessfully even for NUF1128, although the previous studies using black sea bream (Baxa et al., 1987), Dover sole (Campbell and Buswell, 1982) and Senegalese sole (Failde et al., 2014) were succeeded. The difference in fish species and bacterial strains used may influence the results. Considering that the primary sites of *T. maritimum* infection are body surfaces such as the head, mouth, fins and flanks (reviewed by Bernardet, 1998) and that lesions occurred on the skin surface followed by progressing into deeper layers (so called ‘outside-in’ skin lesion) (Vilar et al., 2012), the conditions within the subcutaneous region of flounder probably did not support the growth and proliferation of the bacteria.
Adhesion of pathogens to host tissues is an important step for bacterial infections. In tenacibaculosis, the skin is thought to be a portal of entry for the pathogen (Bernardet, 1998), and *T. maritimum* adhered strongly to the skin mucus of turbot, sea bream and sea bass (Magariños *et al.*, 1995). Difference in the viable counts on the intact skin between NUF1128 and NUF1129 (Table 1.3) indicated that NUF1128 attached more readily to the flounder mucus. On the other hand, the viable counts on the abraded region were at the same level between NUF1128 and NUF1129. Although adhesion mechanism is still unknown, the mechanisms implicated in adhesion to the mucus and underlying skin tissues may be different. Further virulence study using abraded and non-abraded fish will be helpful to establish the fact.

Unlike the other *T. maritimum* strains, NUF1129 seemed to lack or less express a cell-surface antigen (antigen X) (Fig. 1.3a). Interestingly, NUF1126 also lacked this antigen (Fig. 1.3a). NUF1126 exhibited gliding motility and formed rhizoid colonies, but was less adhesive to glass wall as NUF1129 (Table 1.1). These observations suggest that antigen X is related to the adhesion ability of the bacteria.

In SDS-PAGE analysis of the sonicated cell extracts, distinctive band pattern was observed in NUF1129: over expression of 35.8 and 16.9 kDa and less expression of 19.6 kDa polypeptides. However, no band commonly lacked in both NUF1129 and NUF1126, which can be candidate(s) for the antigen X, could not be identified. A set of cell surface proteins required for gliding motility of the *Bacteroidetes* group acts also as a protein secretion system (Nelson *et al.*, 2008; Sato *et al.*, 2010; Rhodes *et al.*, 2011). It is thinkable that two polypeptides of 35.8 and 16.9 kDa were accumulated in the non-gliding NUF1129 cells due to the disruption of the system (Rhodes *et al.*, 2011). On the other hand, the 19.6 kDa polypeptide seemed to be less expressed in NUF1129 as well as several other strains used in this study compared with the virulent strain.
NUF1128. Further studies are necessary to elucidate whether these polypeptides are virulence factors of *T. maritimum* or not.

Both chondroitinase and protease are supposed to be virulence factors of gliding bacterial pathogens (Suomalainen *et al.*, 2006; Dalsgaard, 1993). In the present study, chondroitinase and gelatinase activities were detected in all the *T. maritimum* strains including the avirulent strain NUF1129 (Table 1.1). However, when the enzyme activities of the sonicated cell extracts were assessed, a very large clear zone due to chondroitin degradation was observed on the test agar for NUF1129 compared with NUF1128 (data not shown). Therefore, the enzyme might be accumulated in the bacterial cells due to the disruption of the protein secretion system (Rhodes *et al.*, 2011), and reduced secretion of the enzyme might be related to the avirulence of the strain.

Considering the pleiotropic effects and multifactorial pathogenesis of *T. maritimum* it is presumed that some cell surface components that differ between NUF1129 and other usual *T. maritimum* strains may be related to the gliding motility resulting in rhizoid colony morphology and adherence, and ultimately influence the virulence in fish. To confirm this, further studies are necessary to characterize the interesting cell-surface components.
Fig. 1.1. Photomicrographs of *T. maritimum* NUF1128 (a) and NUF1129 (b) cells grown at 27°C for 24 h in TYS broth and colonies of NUF1128 (c) and NUF1129 (d) grown at 27°C for 36 h on TYS agar plates. Note; NUF1129 cells were shorter than NUF1128 cells, and NUF1128 exhibited rhizoid colonies while NUF1129 produced round compact ones.
Fig. 1.2. Adhesiveness of *T. maritimum* NUF1128 (a) and NUF1129 (b) to glass wall of conical flasks grown at 27°C for 24 h with shaking in TYS broth. Note; NUF1128 was adhesive and showed aggregated growth, whereas NUF1129 was non-adhesive and produced homogenous suspension.
Fig. 1.3. Gel immunodiffusion analysis of cellular antigens of *T. maritimum* strains with rabbit anti serum (a) and with absorbed antiserum and adsorbed antibodies (b). AS, anti-NUF1081 FKC serum; aAS, anti-NUF1081 FKC serum absorbed with NUF1081 FKC; aAb, antibodies adsorbed to NUF1081 FKC; 1–13 and C, sonicated cell extracts of *T. maritimum* strains (1, NUF433; 2, NUF434; 3, NUF492; 4, NUF493; 5, NUF684; 6, NUF685; 7, NUF686; 8, NUF1035; 9, NUF1126; 10, NUF1127; 11, NUF1128; 12, NUF1129; 13, NS110; C, NUF1081 as control); X–Z, antigen X–Z.
Fig. 1.4. SDS-PAGE analysis of whole cell proteins of *T. maritimum* strains stained with Coomassie Brilliant Blue R-250. M, molecular weight markers (Bio-Rad).
Table 1.1. Sources of *T. maritimum* strains with their biological and morphological characteristics

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host fish</th>
<th>Location and year of isolation</th>
<th>Colony type</th>
<th>Gliding motility</th>
<th>Adhesion to glass wall**</th>
<th>Cell length (Mean ± SD) (µm)</th>
<th>Enzyme activity</th>
<th>Chondroitinase</th>
<th>Gelatinase</th>
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<tr>
<td>NUF433</td>
<td>Japanese flounder</td>
<td>Nagasaki/1989</td>
<td>Rhizoid</td>
<td>+</td>
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<td>±</td>
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</table>

NUF = Nagasaki University Fisheries, NS= Nagasaki-ken Suisan-shikenjo (Nagasaki Prefectural Institute of Fisheries).

* NUF1081 was used for preparing the rabbit antiserum against formalin-killed cells used in this study; **+, adherent; ±, weakly adherent; -, non-adherent.
Table 1.2. Virulence of *T. maritimum* NUF1128 and NUF1129 studied in Japanese flounder

<table>
<thead>
<tr>
<th>Strain</th>
<th>Challenge method</th>
<th>Challenge dose (CFU/mL or fish)</th>
<th>No. of dead fish during the periods of (n=5)</th>
<th>Mortality (%)</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (CFU/mL or fish)</th>
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<td></td>
<td></td>
<td>0-1 d</td>
<td>2-3 d</td>
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<td>Immersion</td>
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<td>3</td>
<td>2</td>
<td>-</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>injection</td>
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<td>0</td>
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<td></td>
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<td>1.4×10&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
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<td>1.4×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0</td>
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</tr>
</tbody>
</table>
**Table 1.3.** Adhesion of *T. maritimum* NUF1128 and NUF1129 to the body surface of Japanese flounder*

<table>
<thead>
<tr>
<th>Fish no.</th>
<th>Viable count of NUF1128 (CFU/cm²)</th>
<th>Viable count of NUF1129 (CFU/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-abraded region</td>
<td>Abraded region</td>
</tr>
<tr>
<td>1</td>
<td>2.6×10³</td>
<td>3.4×10³</td>
</tr>
<tr>
<td>2</td>
<td>6.2×10²</td>
<td>1.3×10³</td>
</tr>
<tr>
<td>3</td>
<td>3.4×10²</td>
<td>1.8×10³</td>
</tr>
<tr>
<td>4</td>
<td>4.4×10²</td>
<td>6.8×10²</td>
</tr>
<tr>
<td>5</td>
<td>3.8×10²</td>
<td>1.8×10²</td>
</tr>
</tbody>
</table>

*Fish were partially abraded and immersed in seawater containing 5.5×10⁶ CFU/mL of NUF1128 or 2.7×10⁷ CFU/mL of NUF1129 cells for 30 min. Viable counts of *T. maritimum* on the skin were assessed after reared in aquaria with continuous water flow for another 30 min.
Chapter 2

Isolation of a Surface Exposed Antigen (Antigen X) of

*Tenacibaculum maritimum*
2.1. Introduction

Bacterial surface structures play a major role in the initiation of infection, i.e., in the adherence of bacterial cells to epithelial or endothelial cells of the host, and components of the bacterial surface are involved either directly or indirectly in host injury. Moreover, the inflammatory process and fundamental aspects of host response to bacterial invasion depends upon the ability of the host to recognize bacterial surface components (Peterson and Quie, 1981). In medical situations, the surface components of bacterial cells are major determinants of virulence for many pathogens. In animals, they may be used to colonize tissues and resist phagocytosis and immune responses, and to induce inflammation, complement activation and harmful immune responses. Surface-associated components also play a key role in bacterial physiology and pathogenesis and are the major targets for vaccine development.

*Tenacibaculum maritimum*, a Gram-negative and filamentous bacterium, has been described as the aetiological agent of tenacibaculosis in marine fish. The infection route of this microorganism is by direct attack on the body surface of fishes (Magariños *et al.*, 1995), causing lesions such as ulcers, necrosis, eroded mouths, frayed fins and tail-rot (Campbell and Buswell, 1982, Devesa *et al.*, 1989). While some studies are devoted to reveal the existence of antigenic diversity in *T. maritimum* based on the analysis of lipopolysaccharides (LPS) (Avendaño-Herrera *et al.*, 2004, 2005b), surface proteins are attracting increasing interest for studies of virulence mechanism and pathogenesis. Our previous study demonstrated that three distinct precipitin lines presumably derived from three antigens (protein in nature), i.e., antigens X, Y and Z, were observed primarily in gel immunodiffusion test using anti-*T. maritimum* NUF1081 serum and sonicated cell extracts of the *T. maritimum* strains. Precipitation line X was also observed from
the ECP of the usual *T. maritimum* strains (data not shown) in gel immunodiffusion test. The lines derived from antigens Y and Z were observed in all the strains, but that from antigen X was absent in the non-gliding strain NUF1129. From the findings it is thought that antigen X is related to the gliding, adhesion ability and virulence of *T. maritimum* (Rahman et al., 2014). The present study aims to isolate the surface antigen X from a virulent, gliding strain, *T. maritimum* NUF1128.

2.2. Materials and Methods

2.2.1. Ammonium sulfate precipitation of extracellular products (ECP)

*T. maritimum* strain NUF1128 was cultured using cellophane overlayed technique on TYS agar at 27°C for 24 h. The cultures were washed off the cellophane sheets with PBS and centrifuged at 12,000 × g for 15 min at 4°C. The resulting supernatant, designated ECP, was filter-sterilized by passing through a 0.2 μm sterile filter unit (Advantec). After ammonium sulfate (Wako) was gently mixed to the ECP to obtain 30% saturation at room temperature the precipitate was collected by centrifugation (12,000 × g for 15 min at 4°C), and (NH₄)₂SO₄ was added further to the supernatant to obtain 40% saturation. The process was repeated to achieve 50% and 60% saturation. The precipitate obtained at each saturation was dissolved in a small volume of distilled water (DW) and dialyzed against phosphate buffered saline (PBS), pH 7.2 overnight.

2.2.2. Hydrophobic chromatography
The ECP sample precipitated at 30% saturation of (NH₄)₂SO₄ was dialyzed against 0.01 M Na₂HPO₄/ 0.8 M (NH₄)₂SO₄, pH 6.9 and centrifuged at 14,000 × g for 15 min at 4°C, and the supernatant was applied to a column (1.1 cm diam. × 5 cm) of Phenyl-Sepharose High Performance™ (GE Healthcare) that had been equilibrated with 200 mL of 0.01 M Na₂HPO₄/ 0.8 M (NH₄)₂SO₄, pH 6.9. After the application, the column was washed with the same buffer for the first 10 fractions. From the fraction no. 11 until no. 100 adsorbed materials were eluted with a linear gradient of 0.01 M Na₂HPO₄/ 0.8 M (NH₄)₂SO₄, pH 6.9 to DW. Lastly, chromatography was continued up to several fractions using only DW. Fractions (5 mL) collected were analyzed by spectrophotometer (280 nm) and assayed for the presence of *T. maritimum* surface antigen X by gel immunodiffusion test.

2.2.3. Polyacrylamide gel electrophoresis (PAGE) and western blot analysis

The fractions containing the partially purified antigen X were separated by polyacrylamide gel electrophoresis (PAGE) using 4% (w/v) stacking and 10% (w/v) separating gels, transferred to a PVDF membrane, and subjected to immunoreactions. Non-specific binding sites of antibody were blocked with 3% gelatin in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.5; TBS) for 1 h. The blocked PVDF membrane was incubated with rabbit anti-*T. maritimum* NUF1081 serum diluted 1:1,000 in 1% gelatin Tween TBS (0.05% Tween 20 in TBS; TTBS) for 1 h at room temperature, followed by incubating with goat anti-rabbit antibody HRP conjugate (Bio-Rad) diluted 1:3,000 in 1% gelatin-TTBS and incubating for 1 h at room temperature. Specific bindings of the HRP conjugate were detected by incubating with a mixture of 20 mg
diaminobenzidine tetrahydrochloride (DAB) and 0.1 mL of 5% hydrogen peroxide in 100 mL of 50 mM Tris-HCl, pH 7.6, for 2 to 3 min.

2.2.4. SDS-PAGE analysis of isolated protein

SDS-PAGE was carried out according to Laemmli (1970) using 4% (w/v) stacking and 10% (w/v) separating gels in a mini-slab electrophoresis apparatus AE-6530 (Atto). Chromatographed fractions were used as samples. Gels were stained with Silver stain (Ez Stain, Atto).

2.2.5. Gel immunodiffusion

Gel immunodiffusion was performed following the method described previously (chapter 1) with 1% agarose (Bio-Rad) in PBS on glass slides. The reservoirs were cut using a 7-well cutter. The reactants were added to wells, and the slides were allowed to stand for 24 h in a moist chamber at room temperature. In this study, gel immunodiffusion test was performed firstly to identify antigenic line X from the samples of different fractionated peaks. To confirm the partial purification of antigen X, all the chromatographed samples (fraction nos. 44-54), related to the peak of antigen X, were mixed, concentrated by filtration with an ultrafilter (molecular cut off 10,000 Da) (Advantec) and subjected to gel immunodiffusion test. Different concentrations of the partially purified antigen X were added to wells to react with rabbit anti-\textit{T. maritimum} NUF1081 serum (AS).

2.3. Results
2.3.1. Chromatography

The adsorption of the protein of the ECP to the mildly hydrophobic adsorbent phenyl-Sepharose, was investigated in the present study. It was found that 0.8 M of (NH4)2SO4 in combination of the distilled water, an effective means of recovering adsorbed material, and a linear gradient (NH4)2SO4 (0.8-0.0 M) in aqueous buffer resolved adsorbed protein from *T. maritimum* ECP (Fig. 2.1). A total of 106 fractions were collected which consisted of three large peaks and numerous sub-peaks. Samples from different fractions were taken for gel immunodiffusion test using the anti-*T. maritimum* 1081 serum and found presence of antigen X in the fraction no. 49.

2.3.2. PAGE, western blot and SDS-PAGE analysis

Protein profile study by normal polyacrylamide gel electrophoresis (PAGE) of partially purified antigen X showed distinctive expression of a larger molecular weight protein band (Fig. 2.2a) with silver stain. Further analysis by western blot indicated that the partially purified protein is immunogenic (Fig. 2.2b). Moreover, SDS-PAGE separation of the partially purified protein revealed two distinctive expressions of polypeptide bands at 100 kDa and more than 250 kDa along with some minor bands between them (Fig. 2.3)

2.3.3. Gel immunodiffusion test
Precipitation line X was identified from the chromatographed fraction no. 49. In later study by combining fractions related to the peak of antigen X, a single precipitation line is observed up to a concentration of 1/8 and thus partial purification of antigen X is confirmed (Fig. 2.4).

2.4. Discussion

The adsorption of a surface exposed antigen of *T. maritimum* to the mildly hydrophobic adsorbent phenyl-Sepharose was conducted for its isolation. Hydrophobic interactions are known to increase with increasing ionic strength (Rosengren et al., 1975; Pahlman et al., 1977). The minimum ionic strength at which the antigen X could be adsorbed to phenyl-Sepharose was therefore established as 0.8 M (NH₄)₂SO₄ (pH 6.9).

Adsorbed materials are recovered by disruption of their hydrophobic bonding with the matrix (von Hippel and Schleich 1969) and may be resolved by altering the elution conditions to give selective desorption based on the differing strengths of their hydrophobic interactions. In the present study, 0.8 M (NH₄)₂SO₄, functioned by distilled water, successfully recovered the adsorbed material, and a linear gradient of (0.8-0.1) M (NH₄)₂SO₄ in aqueous buffer resolved adsorbed proteins which is slightly hydrophobic in nature and hence, this method proved a better mean of resolving partially purified surface antigen X.

Analysis of the *T. maritimum* surface antigen X peak as eluted from phenyl-Sepharose, by polyacrylamide-gel electrophoresis (PAGE) gave a single protein band. Further analysis by SDS-PAGE exhibited two distinctive bands: one is 100 kDa and another is apparently higher molecular weight than 250 kDa due to the disruption of the tertiary structure of *T. maritimum* surface antigen X. Thus, it is confirmed the antigen X consists of two single polypeptide chains.
It can be concluded that the partial purification of *T. maritinum* surface exposed antigen X was succeeded by hydrophobic interaction chromatography with phenyl-Sepharose. The antigen is a high molecular weight protein consists of two polypeptide chains.
Fig. 2.1. Phenyl-Sepharose chromatography of ECP prepared from \textit{T. maritimum} strain NUF1128. Bound proteins were eluted with a linear gradient (…….) of (NH$_4$)$_2$SO$_4$ (0.8 M – 0.0 M) in 0.01 M Na$_2$HPO$_4$, pH 6.9. Peak (○) of the fraction no. 49 showed the existence of \textit{T. maritimum} surface antigen X in a gel immunodiffusion test.
Fig. 2.2. Normal polyacrylamide gel electrophoresis (a) and western blot analysis (b) of fraction proteins (fraction nos. 47-50) of the partially purified *T. maritimum* surface antigen X. PAGE gel (10% gel) stained with silver stain (Atto, Japan), showed high molecular weight single protein band that was further detected as immunogenic by western blotting. M, molecular weight marker (Bio-Rad).
**Fig. 2.3.** Silver stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis (10% gel) of the partially fractions of phenyl-Sepharose chromatography. M, molecular weight marker (Bio-Rad); other lanes, chromatographed fraction nos. 44-54.
Fig. 2.4. Gel immunodiffusion analysis of partially purified surface exposed antigen X of *T. maritimum*. A single precipitation line was seen up to 1/8 dilution of the precipitation. Wells, 2-fold dilution of antigen X (1/1-1/32). AS, rabbit anti-NUF1081 FKC serum.
Chapter 3

Kinetics of Infection of a Gliding and Non-gliding Strain of

Tenacibaculum maritimum on the Abraded Skin of

Japanese Flounder
3.1. Introduction

*Tenacibaculum maritimum* (formerly *Flexibacter maritimus*), a Gram-negative and filamentous bacterium, has been described as the aetiological agent of tenacibaculosis in marine fish, which causes heavy losses to the aquaculture industry worldwide (Santos *et al*., 1999; Toranzo *et al*., 2005). In Japan, the disease breaks out frequently in juvenile and young flounder *Paralichthys olivaceus*, sea breams *Pagrus major* and *Acanthopagrus schlegeli*, yellowtail *Seriola quinqueradiata* and other marine fishes from spring to early summer and sometimes in the cold season (Kusuda and Kawai, 1998). Generally, the affected fish have eroded and hemorrhagic mouth, ulcerative skin lesions, frayed fins and tail rot. Although the disease has a great impact in aquaculture, relatively little is known about its pathogenesis and mode of infection (Avendaño-Herrera *et al*., 2006b). *T. maritimum* is a ubiquitous and opportunistic pathogen (Avendaño-Herrera *et al*., 2006c), able to withstand the bactericidal action of the skin mucus (Magariños *et al*., 1995), which may serve as a reservoir and source of nutrients for the bacterium. For this reason, the bacterium can be detected as part of the autochthonous populations in the skin of fish (Avendaño-Herrera *et al*., 2006c), even if no evident lesions are present (Piñeiro-Vidal *et al*., 2008). Thus, the primary sites of *T. maritimum* infection are considered as body surfaces (Bernardet, 1998), specially the skin (Failde *et al*., 2014). However, the mechanism by which the pathogen enters inside the host is not clear.

Different challenge methods have been attempted to reproduce the disease. In the previous studies challenge tests by subcutaneous injection induced tenacibaculosis using black sea bream *Acanthopagrus schlegeli* (Baxa *et al*., 1987), Dover sole *Solea solea* (Campbell and Buswell, 1982), Senegalese sole *Solea senegalensis* (Failde *et al*., 2014) and turbot *Psetta maxima* (Failde
et al., 2013), although our previous study showed that subcutaneous challenge failed to induce the infection in Japanese flounder (Rahman et al., 2014). Wakabayashi et al. (1984) and Baxa et al. (1987) demonstrated that immersion challenge was not a reliable method of inducing the disease unless the skin was previously scarified or abraded. However, other authors have succeeded to reproduce the disease using prolonged immersion of fish (Avendaño-Herrera et al., 2006a) or high inoculum doses (van Gelderen et al., 2011). Recent studies demonstrated that immersion challenge (Nishioka et al., 2009; Rahman et al., 2014) or immersion and dilution method (Yamamoto et al., 2010) was successful in reproducing the disease in Japanese flounder.

Conventional infection kinetic studies require quantification of the bacterial population from infected organs by culturing bacteria (Kusuda and Ishihara, 1981), and in some cases histopathological observation is also accompanied with it. They allow us to understand the organs and tissues that are invaded by a pathogen and the time course of the disease progression. In the present study immersion challenges were carried out to investigate the adhesion and proliferation of *T. maritimum* on the abraded skin of Japanese flounder, using *T. maritimum* NUF1128, a usual gliding strain, and NUF1129, a non-gliding and avirulent strain, by numeration and immunohistochemical detection of the bacteria.

3.2. Materials and Methods

3.2.1. Bacteria

*T. maritimum* strains NUF1128 and NUF1129 used in this study were derived from a naturally infected puffer fish *Takifugu rubripes* in 2010 (Rahman et al., 2014). The strain NUF1129 is non-gliding, less adhesive and avirulent to Japanese flounder (Rahman et al., 2014). The bacteria
were grown on TYS agar plates consisted of 0.3% Bacto™ Tryptone (Difco), 0.2% Bacto™ Yeast Extracts (Difco) and 1.5% agar (Wako), pH 7.4-7.6, in filtered seawater at 27°C. The stock cultures were kept at -80°C, and the main stocks were maintained in liquid nitrogen.

3.2.2. Pathogenicity test

*T. maritimum* NUF1128 and NUF1129 were cultured in TYS broth (agar was omitted from TYS agar) at 27°C for 24 h with shaking at 120 rpm. Bacterial cells grown were harvested by centrifugation (9,000 × g, 10 min) and resuspended in sterile TYS broth. Juvenile Japanese flounder (average body weight 26.8 ± 5.1 g) were reared in a 200 L tank for 2 d prior to the experiment to heal the skin injury caused by netting. Four pretreatments i.e., skin abrasion by cotton swabs (a portion of dorsal skin was rubbed with a cotton swab, Fig. 3.1a) (Miwa and Nakayasu, 2005) and blades (a portion of dorsal skin was scratched with a blade, Fig. 3.1b), fin clipping (a tip of the dorsal fin was clipped with dissection scissors) and no treatment were employed on forty anesthetized fishes (five fish/ treatment/ strain) before immersion challenge. Upon pretreatment 2-phenoxyethanol diluted with water was directly added to the rearing tank, and inactive fish was picked up with a hand and treated. Immediately after pretreatment fish were kept in a 100-L tank, to which the bacterial suspension of NUF1128 or NUF1129 was added. Challenge doses employed were 2.0×10⁶ CFU/mL and 1.3×10⁶ CFU/mL, respectively. Non-abraded five fish were immersed in seawater without the bacterial suspension for control. After immersion for 30 min, fish were transferred to rectangular aquaria (five fish of each experimental group/ aquaria) equipped with continuous flow of filtered seawater and aeration. The fish received no feed for 7 d of the experimental period. The water temperature ranged from
22 to 23.5°C during the experimental period. Gross pathological changes and moribundness were checked daily.

3.2.3. Infection kinetics of T. maritimum in the skin

3.2.3.1. Immersion challenge

Experiments on the infection kinetics of T. maritimum NUF1128 and NUF1129 were conducted using cotton swab-abraded and steel blade-abraded fish. Juvenile flounder fish (average body weight 38.2 ± 9.9 g) were stocked into two 200-L tanks 2 d before challenge for cotton swab abrasion. Another group of juvenile flounder fishes (average body weight 42.6 ± 8.0 g) were stocked separately as above for steel blade abrasion. The methods for abrasion and challenge were the same as those employed in pathogenicity test. The challenge doses of NUF1128 and NUF1129 were 2.6x10^7 CFU/mL and 1.5x10^7 CFU/mL for swab-abraded group and 1.8x10^6 CFU/mL and 1.7x10^6 CFU/mL for blade-abraded group, respectively. For the control, 10 fish/ abrasion pre-treatment were immersed in water without bacterial suspension and kept in a 30-L aquarium.

3.2.3.2. Enumeration of T. maritimum in tissue samples

Enumerations of T. maritimum in tissue samples of cotton swab-abraded and blade-abraded fish were conducted separately. Five fish from NUF1128 and NUF1129 challenged groups were sampled randomly from each 200-L tank at 30 min, 2 h, 6 h, 24 h and 48 h post infection for counting viable bacteria in the tissues of challenged fish. Approximately 1 cm² of skin at the
Abraded parts were dissected and ground by glass homogenizers containing 2 mL of PBS to make homogenates, from which serial tenfold dilutions were made. One hundred microliters of each dilution was plated on TYS agar containing 100 µg/mL kanamycin and incubated at 27°C for 2 d. Bacterial counts of the skin were expressed as colony forming units (CFU) per cm². Some confusing colonies were sub cultured on TYS agar and subjected to agglutination test with rabbit anti-\textit{T. maritimum} NUF1081 serum for confirmation. Due to severe infection and mortality in blade-abraded NUF1128-challenged group, the number of sampled fish at the last sampling was insufficient. The water temperature during the sampling periods of cotton swabs-abrasion and blade-abrasion experiments ranged from 22.5 to 23.5°C and 23 to 24.5°C, respectively. No feed was given to the fish during the experimental.

### 3.2.3.3. Histopathology and Immunohistochemistry

For each abrasion pre-treatment, two challenged fish from each 200-L tank were sampled randomly at 30 min, 2 h, 6 h, 24 h and 48 h post infection and were subjected to histological and immunohistochemical examination. Skin samples from the swab-abraded fishes or skin with underlying muscle, spleen, kidney and liver from the steel blade-abraded fishes were collected followed by fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (3 µm in thickness) were stained with haematoxylin and eosin (H-E) for light microscopic observation.

For Immunohistochemical detection of \textit{T. maritimum}, sections were incubated with 0.3% hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidase activity in tissues and treated with 3% gelatin in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.5;
TBS) for 2 h. The blocked sections were incubated with rabbit anti-
*T. maritimum* NUF1081 serum diluted 1:10,000 in 1% gelatin-Tween-TBS (0.05% Tween 20 in TBS; TTBS) for 1 h at room temperature, washed with TTBS and then incubated with goat anti-rabbit antibody HRP conjugate (Bio-Rad) diluted 1:3,000 in 1% gelatin-TTBS for 1 h at room temperature. Specific bindings of HRP conjugate were detected by incubating with a mixture of 20 mg diaminobenzidine tetrahydrochloride (DAB, Wako) and 0.1 mL of 5% hydrogen peroxide in 100 mL of 50 mM Tris-HCl, pH 7.6, for 2 to 3 min. Sections were counter-stained with hematoxylin for 30 sec. *T. maritimum* was detected as brown color in a section. Rabbit anti-*Lactococcus garvieae* NUF1019 serum was used for negative control.

### 3.3. Results

**3.3.1. Pathogenicity test**

Results of the pathogenicity test are shown in Table 3.1. The pathogenicity test using the strain NUF1128 resulted in 100% mortality of immersion challenged fish abraded with blades and fin-clipped with scissors before challenge and 20% mortality of non-treated fish accompanied with visible lesions of skin and fin necroses (Fig. 3.2a, b). In both cases, the lesions started from the abraded sites, spread towards the adjacent area and gradually turned into hemorrhagic skin. Fish died first within 24 h post challenge and all deaths occurred within 3 d in the blade-abraded and fin-clipped groups. On the contrary, the strain NUF1129 was non-pathogenic failed to induce any sign of infection or disease to experimental fish regardless of treatments applied. In the case of cotton swabs abrasion on fish skin, no fish either from NUF1128-challenged group or
NUF1129-challenged group became infected or died. Cotton swabs abrasion sign on the skin of live fish was found to be recovered (Fig. 3.2c) at the end of experiment. No fish died in the control group.

### 3.3.2. Infection kinetics of *T. maritimum*

**Cotton swab abrasion:** The abrasion made in this way was quite difficult to see with the naked eyes (Fig. 3.1a). After 30 min of immersion challenge, slightly higher adherences at the abraded sites were observed in the NUF1128-challenged fish ($10^3$-$10^4$ CFU/cm$^2$) (Fig. 3.3a) than NUF1129-challenged fish ($10^2$ CFU/cm$^2$) (Fig. 3.3b). Bacterial populations of NUF1128 on the skin was found at $10^3$ CFU/cm$^2$ level from 2 h to 48 h, while those of NUF1129 remained at $10^2$ CFU/cm$^2$ level upto 24 h but declined under detection limit at 48 h. No mortalities were observed in NUF1128 or NUF1129 challenged fishes during the experimental period.

**Steel blade abrasion:** Steel blade abrasion caused parts of the abraded sites looked whitish (Fig. 3.1b) due to the lost of epidermis. The kinetics of NUF1128 (Fig. 3.4a) and NUF1129 (Fig. 3.4b) in the skin were different. After 30 min of immersion challenges, the viable counts of NUF1128 ($10^4$ CFU/cm$^2$) were more at abraded sites than those of NUF1129 ($10^2$ CFU/cm$^2$). Larger bacterial population ($10^5$-$10^6$ CFU/cm$^2$) were detected in the cases of NUF1128-challenged fish at 6 h post-infection (Fig. 3.4a), became largest ($10^7$ CFU/cm$^2$) at 24 h associated with mortalities due to exponential proliferation of the bacteria in the fish body. At 48 h, slightly declined bacterial number ($10^4$ CFU/cm$^2$) was revealed from only one alive fish. On the contrary,
in the NUF1129-challenged group (Fig. 3.4b), the bacterial population declined gradually. No mortalities were observed in this group.

3.3.4. Histopathology and Immunohistochemistry

**Cotton swab abrasion:** Histological observations revealed that the outer layers of the epidermis of the abraded dorsal skin were partially or fully removed. Very few parts of the stratum spongiosum (loose connective tissues) of dermis were exposed due to abrasion and mostly covered with scales. Stratum spongiosum (dense connective tissues) of dermis remained intact (Fig. 3.5a). In the tissue sampled immediately after bath challenge, no bacterium was observed on the dermal connective tissues at the artificial abrasion on the dorsal skin of NUF1128-challenged group of fish (Fig. 3.5b). No bacteria were also found on the non abraded sites or normal skin. At 24 h, the abraded parts were found to be healed by the regeneration of epidermis (Fig. 3.5c) without any visible existence of bacteria either in the dermis or on the epidermis (Fig. 3.5d).

**Steel blade abrasion:** Epidermis was found to be missed completely by steel blades but both parts of dermis (i.e., stratum spongiosum and stratum compactum) were intact without the existence of scales at abrasion sites with no visible pathological changes (Fig. 3.6a). After 30 min of immersion challenge, some bacteria were found to adhere on the connective tissues of the exposed stratum spongiosum (Fig. 3.6b, c) of the skin samples of NUF1128-challenged group without any inflammatory response associated. No bacteria were found on the normal skin. Bacterial adherence was not observed in the case of NUF1129 challenged group.
At 6 h post challenge, no pathological change was observed (Fig. 3.7a). Bacterial proliferation was clearly visible in the samples of NUF1128-challenged group deeply in the dermal connective tissues (upto stratum compactum) (Fig. 3.7b, c) but not in the NUF1129-challenged group. An early inflammatory response was seen spreading adjacent to the damaged area.

At 24 h, hypodermis and muscle showed degeneration at various degrees of severity in the skin of NUF1128-challenged group. Separation of muscular fibers, hemorrhages, area of necrosis was extended through the connective tissue of hypodermis reaching and affecting other muscular packets (Fig. 3.8a). Inflammatory cells often accumulated around the infection sites, although in most cases the bacterial antigen was not observed in those cells or in the muscle fibers (Fig. 3.8b). *T. maritimum* was found to spread in the fish body by proliferation along the dermal connective tissues and perimysium (Fig. 3.8c, d, e, f). Pathological changes and bacterial multiplication was absent in the samples of NUF1129 treated group (Fig. 3.9a, b, c).

In the samples of internal organs (*i.e.* spleen, kidney and liver), no histopathological changes or *T. maritimum* antigens were evidenced in any specimen along the experiment.

### 3.4. Discussion

The present study provides the first description of the kinetics of infection of gliding (NUF1128) and non-gliding (NUF1129) strains of *T. maritimum* by enumeration and immunohistochemical detection in the experimentally infected Japanese flounder. Moderate doses of bacterial suspension (10^6 CFU/ mL) were used here with a view to determine the pathogenicity as well as to observe the gradual progression of tenacibaculosis in the infected fish that generally happens in the natural environment. The strain NUF1128 was highly pathogenic
resulting 100% mortality of fin-clipped and blade-abraded fish and 20% mortality of non-treated fish within 3 d. NUF1128 has previously been demonstrated as virulent ($LD_{50} = 10^{6.0}$ CFU/mL) to Japanese flounder by immersion route (Rahman et al., 2014). Fin-clipping and blade-abrasion were thought to create entry points for *T. maritimum* and caused infection leading to the death of flounder. On the other hand, abrasion with cotton swabs is thought to be insufficient to create enough portal of entry for the examined *T. maritimum* strains and, hence, no pathogenicity was observed. The avirulent strain NUF1129 lacked some cell-surface components common to ordinary *T. maritimum* strains (Rahman et al., 2014), which may also be responsible for the inability to produce any infection or disease in flounder regardless of blade-abrasion or fin-clipping.

*T. maritimum* cells exposed to the fish surface during immersion challenge were supposed to adhere to the abraded sites. In the case of NUF1128, the results of its first detection on the abraded skin immediately after challenge and proliferation at 6 h post challenge suggest that pathogenic strain of *T. maritimum* was spreading to the body rapidly from the sites of entry. At 24 h post challenge, higher viable counts obtained from the skin samples indicated that bacterial exponential multiplication occurred. The strain NUF1129 declined gradually from the blade-abraded sites instead of proliferation probably due to its less adhesive nature on the mucus and underlying skin (Rahman et al., 2014).

The bacterial entry into the body through healthy skin seemed improbable since no *T. maritimum* antigens were observed in the intact skin (data not shown). In this study, at the early stage of infection in the blade-abraded and NUF1128-challenged group, *T. maritimum* adhered to the stratum spongiosum of dermal connective tissue and proliferated along the stratum compactum. Regardless of the degenerative changes observed in the muscle and hypodermis of
infected flounder, the bacteria were only observed in the dermal connective tissues and adjacent hypodermis but not in the muscle. However, a moderate inflammatory response and hemorrhage extending into the adjacent healthy area was observed in the muscle below dermis. It is probable that once the bacteria reached the dermis and proliferate along the dermal and perimysial connective tissues they produce tissue-degradation enzymes including protease and chondroitinase (Rahman et al., 2014) which damage subjacent tissues causing pathological changes in flounder.

Red sea bream and black sea bream were experimentally infected with T. maritimum using smear or immersion infection and the bacteria were detected in the skin, gills, liver and spleen (Kimura and Kusuda 1983; Baxa et al., 1987). In addition, T. maritimum antigens were detected by immunohistochemistry in the internal organs in turbot inoculated by subcutaneous and intraperitoneal routes (Failde et al., 2013) and in Senegalese sole inoculated by subcutaneous route (Failde et al., 2014). Conversely, in the present study, T. maritimum was not detected from the internal organs i.e., spleen, kidney or liver, which is similar to the findings of natural outbreak in cultivated Senegalese sole (Vilar et al., 2012). Like the report of Yamamoto et al., (2010), our study suggest that T. maritimum is not detected from the internal organs as it affects principally the outer tissues of immersion-challenged Japanese flounder.

In summary, the gliding strain NUF1128 was found highly pathogenic for Japanese flounder when the body surface was injured. The kinetics of infection revealed strong adhesion of T. maritimum NUF1128 to the blade-abraded sites which leaded to the exponential proliferation associated with mortalities. Based on the pathological and immunohistochemical observations, it is strongly concluded that T. maritimum enters through the injured or abrasive sites of body surface where epidermis is absent, adheres to the dermal connective tissues, proliferates and thus
infection starts from the point of entry. The methods to determine the kinetics of infection can be used for further investigation on the pathogenesis of tenacibaculosis, and the present challenge method may be a successful means for the future evaluation of the efficacy of *T. maritimum* vaccines for Japanese flounder.
Fig. 3.1. The appearances (red circles) of the abraded skin of Japanese flounder. (a) Abrasion by a cotton swab and (b) abrasion by a steel blade.
Fig. 3.2. Gross lesions on the body surface of Japanese flounder 2 d after challenge with *T. maritimum* NUF1128. **Note:** (a) A large necrotic lesion (arrow) started from the site abraded by a blade; (b) a lesion started from the fin (arrow) clipped by scissors; (c) abrasion by a cotton swab did not cause a lesion on the skin (circled area).
Fig. 3.3. Viable counts of *T. maritimum* in the skin of flounder abraded by cotton swabs followed by immersion-challenged with (a) NUF1128 and (b) NUF1129 at doses of $2.6 \times 10^7$ CFU/mL and $1.5 \times 10^7$ CFU/mL, respectively. Five fish were sampled at each time point.
Fig. 3.4. Viable counts of *T. maritimum* in the skin of flounder abraded by blades followed by immersion-challenged with (a) NUF1128 and (b) NUF1129 at doses of $1.8 \times 10^6$ CFU/mL and $1.7 \times 10^6$ CFU/mL, respectively. Five fish were sampled at each time point.
Fig. 3.5. Japanese flounder pretreated with cotton swabs followed by challenged with *T. maritimum* NUF1128. Histological sections were stained with haematoxylin and eosin (H-E) (a, c) and by immunohistochemistry (b, d) against *T. maritimum*. (a) Abrasion on the dorsal skin immediately after exposure to the bacterium. The epidermis (E) is partially or superficially missed but dermis is intact, the bacterial antigen is not observed probably due to have very few space (▲) to adhere to stratum spongiosum (b) on the abraded epidermis or dermis (D), (c) healing of bacterial epidermis (E) with no evidence of bacterial antigens (d). S = scale; SS = stratum spongiosum; SC= stratum compactum.
Fig. 3.6. Histopathological and immunohistochemical observation of the abraded skin after 30 min of immersion challenge with *T. maritimum* NUF1128. **Note:** (a) Stratum spongiosum of dermis is exposed due to complete missing of epidermis (H-E stain); (b, c) adhesion of *T. maritimum* (arrow) to the stratum spongiosum; (c) a magnified image of black border in b. D=dermis; SS=stratum spongiosum; SC=stratum compactum.
Fig. 3.7. Histopathological and immunohistochemical observation of the abraded skin after 6 h of immersion challenge with *T. maritimum* NUF1128. **Note:** (a) Exposed stratum spongiosum of dermis due to skin abrasion; (b, c) proliferation of *T. maritimum* (arrows) along the dermal connective tissues (stratum spongiosum and stratum compactum), (c) a magnified image of black border in b. D = dermis; SS = stratum spongiosum; SC = stratum compactum.
Fig. 3.8. Histopathological and immunohistochamical observation of the abraded skin after 24 h of immersion challenge with *T. maritimum* NUF1128. **Note:** (a) Necrosis (*) in the epidermis (adjacent to abrasion) and hypodermis, hemorrhage (◄) and degeneration of muscle tissues (<) due to the infection by *T. maritimum* NUF1128. Severe inflammatory response located in the muscular layer (▷) (H-E stain); (b) widespread distribution of *T. maritimum* cells in the epidermis and dermis; (c) epidermal region with the presence of the bacteria (arrows) (a magnified image of green border in b); (d) *T. maritimum* was found in the hypodermal region (arrows) (a magnified image of blue border in b); (e) the perimysium and degenerative muscle fibers; (f) presence of *T. maritimum* in the perimysium (arrows). D=dermis; SS=stratum spongiosum; SC= stratum compactum; P=perimysium.
Fig. 3.9. Histopathological and immunohistochemical observation of the abraded skin after 30 min of immersion challenge with *T. maritimum* NUF1129. **Note:** (a) Dermis was found intact (H-E stain); (b) *T. maritimum* was not observed at the abraded site due to less adhesion of NUF1129; (c) a magnified image of black border in b. D=Dermis; SS = stratum spongiosum; SC= stratum compactum.
Table 3.1. Pathogenicity of *T. maritimum* NUF1128 and NUF1129 studied in Japanese flounder

<table>
<thead>
<tr>
<th>Strain</th>
<th>Challenge dose (CFU/mL)</th>
<th>Treatment</th>
<th>No. of dead fish during the periods (n=5)</th>
<th>Mortality (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0-1 d</td>
<td>2 d</td>
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<tr>
<td>NUF1128 (Gliding strain)</td>
<td>2.0×10^6</td>
<td>Swab abrasion</td>
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<td>0</td>
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<tr>
<td></td>
<td></td>
<td>Blade abrasion</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
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<td></td>
<td>Fin clipping</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not treated</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>NUF1129 (Non-gliding strain)</td>
<td>1.3×10^6</td>
<td>Swab abrasion</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blade abrasion</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fin clipping</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Not treated</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>Not challenged</td>
<td>Not treated</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Weight (g) of challenged fish (Ave. ± S.D), 26.8 ± 5.1 g
Water temperature (Ave. ± S.D), 22.5 ± 0.5ºC
Chapter 4

Serological Studies on *Tenacibaculum maritimum* Strains

Isolated from Japanese Flounder and Tiger Puffer Fish
4.1. Introduction

Tenacibaculosis caused by *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) (Suzuki *et al.*, 2001) is considered a potential limiting factor for the culture of economically important marine fish species (Piñeiro-Vidal *et al.*, 2007). The first serological studies described by Wakabayashi *et al.* (1984) and Pazos *et al.* (1993), based on slide agglutination assays, reported the antigenic homogeneity of *T. maritimum* regardless of their origin and source of isolation. Further study by Ostland *et al.* (1999) demonstrated antigenic differences among *T. maritimum* isolates, suggesting that this microorganism may not be as homogeneous as previously thought. Moreover, dot blot assays and immunoblot analysis of lipopolysaccharides (LPS) revealed the existence of antigenic diversity in *T. maritimum* and demonstrated that at least 3 major O-serogroups seemingly related to the host species can be detected (Avendaño-Herrera *et al.*, 2004, 2005b).

After the first description of tenacibaculosis (Masumura and Wakabayashi, 1977), *T. maritimum* was further isolated from the body surface, skin lesions, or kidney of several fish species other than sea breams, such as Japanese flounder *Paralichthys olivaceus*, leather jacket *Aluterus monoceros*, rock bream *Oplegnathus fasciatus*, plaice *Cleisthenes pinetorum herzensteini*, puffer *Takifugu rubripes*, and yellowtail *Seriola quinqueradiata* (Baxa *et al.*, 1986, 1987; Wakabayashi *et al.*, 1986) in Japan. The existing situation is now demanding for further studies on the serological relationship(s) among *T. maritimum* strains isolated from different fishes not only for the diagnosis and epidemiological research, but also for development of vaccines. Therefore, the present work was undertaken to determine the agglutination titers of formalin-killed and heat-killed cells of the strains against rabbit anti-*T. maritimum* NUF684 and
NUF1081 sera for finding out the serological relationship(s) among Japanese *T. maritimum* strains isolated from Japanese fish.

4.2. Materials and Methods

4.2.1. Bacteria

Twenty-four *T. maritimum* strains (including three reference strains) isolated from diseased Japanese flounder, tiger puffer, red sea bream, black sea bream and Dover sole, three reference strains of different species under *Tenacibaculum* genus and an unidentified gliding bacterium were tested. These strains were isolated from diseased fish with typical signs of tenacibaculosis in Nagasaki, Oita and Kumamoto Prefectures. An unidentified gliding isolate from the diseased flounder of Kagawa prefecture, Japan was also included in the experiments. List of the strains, reference strains and their sources are summarized in Table 4.1.

4.2.2. Preparation of antigens

Strains of *T. maritimum* and other reference strains were grown on TYS agar at 27°C for 24 h except the reference strain, *T. ovolyticum* NBRC16947, which required 20°C for 24 h to grow on TYS agar. A portion of the cells harvested was killed by heating at 100°C for 1 h. Another part was killed by adding formalin to a final concentration of 0.5% and kept at room temperature for 48 h. Both heat-killed (HKC) and formalin-killed bacteria (FKC) were washed three times with PBS and suspended in PBS at 100 mg/mL and stored at 4°C.
4.2.3. Rabbit antisera

Anti-\textit{T. maritimum} NUF1081 serum was raised earlier against the FKC of \textit{T. maritimum} NUF1081 (isolated from a diseased puffer fish). Preparation technique was described in the chapter 1. Following similar method, another antiserum was raised against \textit{T. maritimum} NUF684 (isolated from a diseased Japanese flounder fish) by immunizing a rabbit with FKC.

4.2.4. Agglutination test

The microtiter technique was used to examine the serological relationship among the strains from Japanese flounder and tiger puffer fish and the representative strains from other fish (Table 4.1). Twenty-five microliters of each antigen and twofold serial dilutions of antiserum in PBS were mixed in a 96-well microtiter plate and incubated in a refrigerator overnight.

4.2.5. Absorption test

About 100 mg of FKC and HKC of NUF684 was added separately to 1 mL of anti-\textit{T. maritimum} NUF1081 serum. Similarly, 100 mg of FKC and HKC of NUF1081 was added separately to 1 mL of anti-\textit{T. maritimum} NUF684 serum. The cells and antisera were mixed and incubated in a refrigerator at 4°C overnight. The mixture was centrifuged and the resultant supernatant was designated as absorbed antiserum. The procedure was repeated until agglutination titer against the corresponding FKC and HKC became below the detection limit.
4.3. Results and Discussion

The agglutinin titer of antisera for the FKCs and HKCs are shown in Table 4.2. The results of microtitre agglutination tests using unabsorbed anti-*T. maritimum* NUF684 serum against *T. maritimum* strains were 8,192-131,072 for FKC and 16-256 for HKC and those of anti-*T. maritimum* NUF1081 serum were 8,192-131,072 for FKC but 32-512 for HKC. The results demonstrating the possibility to exist different serological groups among Japanese *T. maritimum* strains isolated from diseased fish. Titters from the FKCs and HKCs (<4) of both antisera indicated that the unidentified gliding isolate may not be *T. maritimum*. Low titers were observed in both antisera against FKC (<4-128) and HKC (<4-32) of other species under *Tenacibaculum* genus (Table 4.3).

Using anti-*T. maritimum* NUF684 serum absorbed with the FKC and HKC of NUF1081 (FKC and HKC) or anti-*T. maritimum* NUF1081 serum absorbed with the FKC and HKC of NUF684, some strains were found to lose agglutinability completely probably due to share similar serological group (Table 4.2). On the contrary, the above mentioned absorption test also showed moderate to high titters for both antisera regardless of their sources might due to the existence of different serological groups. Distinguished O-serogroups are designated as serogroup(s) A, B and C (Table 4.2 and 4.3).

After absorption with FKC and HKC of NUF684, anti-NUF1081 serum still agglutinated with the FKC and HKC of NUF1081 but the titer reduced to a great extent in the case of agglutination with FKC of NUF1081 (Table 4.2). Similarly, Anti-NUF684 serum after absorption with FKC and HKC of NUF1081 also agglutinated with the FKC and HKC of NUF684 but titer became significantly lower while agglutinated with FKC of NUF684. The results of cross-absorption
tests indicated that NUF684 and NUF1081, the representative strains of flounder and tiger puffer respectively, possessed common surface antigens (Table 4.4). The results of these agglutination reactions using absorbed antisera indicated the existence of common heat-labile surface antigens among the Japanese strains of *T. maritimum*.

Unabsorbed anti-*T. maritimum* NUF684 and anti-*T. maritimum* NUF1081 sera showed almost similar titers against the FKC (16-32) and HKC (8-32) of NUF1129 due to lack of heat-labile antigens on the cell surface of NUF1129. Actually, this is an exceptional strain of *T. maritimum* isolated from a diseased puffer fish with compact colony morphology and found to lack gliding motility and virulence probably due to change of its cell surface component (Rahman *et al.*, 2014) which might affect agglutinability and hence, not placed in any O-serogroup (Table 4.2).

Considering above results and discussion, it is opined that difference in titers of absorbed antisera against HKC of *T. maritimum* strains indicated the existence of different O-serogroups. Japanese strains of *T. maritimum* possess common heat-labile surface antigens that may pave the way to enable the possibility of usage a common vaccine for tenacibaculosis using FKC.
Table 4.1. *T. maritimum* strains and reference strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Location</th>
<th>Host fish</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUF433</td>
<td>Nagasaki</td>
<td>Japanese flounder</td>
<td>1989</td>
</tr>
<tr>
<td>NUF434</td>
<td>Nagasaki</td>
<td>Japanese flounder</td>
<td>1989</td>
</tr>
<tr>
<td>NUF492</td>
<td>Kumamoto</td>
<td>Japanese flounder</td>
<td>1991</td>
</tr>
<tr>
<td>NUF493</td>
<td>Kumamoto</td>
<td>Japanese flounder</td>
<td>1991</td>
</tr>
<tr>
<td>NUF684*</td>
<td>Nagasaki</td>
<td>Japanese flounder</td>
<td>1993</td>
</tr>
<tr>
<td>NUF685</td>
<td>Nagasaki</td>
<td>Japanese flounder</td>
<td>1993</td>
</tr>
<tr>
<td>NUF686</td>
<td>Nagasaki</td>
<td>Japanese flounder</td>
<td>1993</td>
</tr>
<tr>
<td>NUF1035</td>
<td>Nagasaki</td>
<td>Japanese flounder</td>
<td>2006</td>
</tr>
<tr>
<td>085562</td>
<td>Oita</td>
<td>Japanese flounder</td>
<td>2008</td>
</tr>
<tr>
<td>NUF1126</td>
<td>Nagasaki</td>
<td>Japanese flounder</td>
<td>2009</td>
</tr>
<tr>
<td>NUF1127</td>
<td>Nagasaki</td>
<td>Japanese flounder</td>
<td>2009</td>
</tr>
<tr>
<td>NUF1081*</td>
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<td>2009</td>
</tr>
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</tr>
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<td>2010</td>
</tr>
<tr>
<td>NUF1160</td>
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<td>2014</td>
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<tr>
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<td>Oita</td>
<td>Japanese flounder</td>
<td>2014</td>
</tr>
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<td>141102</td>
<td>Oita</td>
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<tr>
<td>141103</td>
<td>Oita</td>
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</tr>
<tr>
<td>141331</td>
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<td>2014</td>
</tr>
<tr>
<td>141332</td>
<td>Oita</td>
<td>Japanese flounder</td>
<td>2014</td>
</tr>
<tr>
<td>Unidentified gliding isolate</td>
<td>Kagawa</td>
<td>Japanese flounder</td>
<td>2014</td>
</tr>
</tbody>
</table>

Reference Strains

*T. maritimum*  
- NBRC110778\(^7\) Japan Red sea bream -  
*T. maritimum*  
- NBRC15991 UK Dover sole -  
*T. maritimum*  
- NBRC16015 Japan Black sea bream -  
*T. amylolyticum*  
- NBRC16310 Palau Green algae -  
*T. mesophilum*  
- NBRC16307 Japan Sponge -  
*T. ovolyticum*  
- NBRC16947 Norway Atlantic halibut (egg) -  

NUF = Nagasaki University Fisheries, NS= Nagasaki-ken Suisan-shikenjo (Nagasaki Prefectural Institute of Fisheries);  
* NUF684 and NUF1081 were used to prepare the rabbit antisera against formalin-killed cells used in this study.
Table 4.2. Serological reactions of *T. maritimum* strains against rabbit anti-*T. maritimum* sera

<table>
<thead>
<tr>
<th>O-Serogroup</th>
<th>Strain</th>
<th>Agglutination titers of rabbit anti-<em>T. maritimum</em> sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Titer of Anti-<em>T. maritimum</em> NUF684 against</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FKC          HKC          FKC          HKC          FKC          HKC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unabsorbed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>NUF492</td>
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</tr>
<tr>
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<td>NUF493</td>
<td>8,192</td>
</tr>
<tr>
<td>A</td>
<td>NUF684</td>
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</tr>
<tr>
<td>B</td>
<td>NUF433</td>
<td>32,768</td>
</tr>
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<td>NUF434</td>
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<td>O-Serogroup</td>
<td>Strain</td>
<td>Titer of Anti-( T.\ maritimum ) NUF684 against</td>
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<td>--------</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td>FKC</td>
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<td></td>
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Table 4.4. Agglutinin titers of antisera absorbed with HKCs and FKCs

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Chapter 5

Investigation on the 19.6 kDa Protein of Fish Pathogenic

Tenacibaculum maritimum
5.1. Introduction

Although the phenotypic, antigenic, and molecular characteristics of *T. maritimum* have been examined by several authors (Toranzo *et al.*, 2005), the actual factors determining the virulence of this pathogen have not yet been elucidated. Some synergistic interactions of the toxins contained in extracellular products and a hemolysin might be involved in *T. maritimum* infections (Baxa *et al.*, 1988a). Pathological properties of the bacterium, such as a strong adherence to the skin mucus of different fish species and the capacity to resist its bactericidal activity (Magariños *et al.*, 1995) was also reported. Moreover, the role of *T. maritimum* outer membrane protein to take up iron during infection (an essential factor in the bacterial pathogenicity) (Avendaño-Herrera *et al.*, 2005a) and some cell-surface components related to gliding and adhesion ability (Rahman *et al.*, 2014) have been pointed out as possible virulence factors.

Gram-negative bacteria are surrounded by two membranes, an inner cytoplasmic membrane and an outer membrane that faces the environment. The outer membrane is highly asymmetric because it’s outer leaflet is composed of lipopolysaccharides (LPS), but the inner leaflet is composed of the same phospholipid classes as the inner membrane (Tamm *et al.*, 2004). The sites of interaction between bacteria and host reside in the bacterial outer membrane and, hence, the outer membrane components often play important roles in the interaction of pathogenic bacteria. During the course of studies on the protein profile of *T. maritimum* strains, a distinctive expression of the band at 19.6 kDa was revealed from the sonicated cell extracts of usual gliding strains (chapter 1). The non-gliding strain NUF1129 produced this band visibly less than usual strains. Since the ECP and the polytron homogenized aliquots of a gliding strain, NUF1128, also
show the similar band during SDS-PAGE analysis, the 19.6 kDa protein is thought to be an outer membrane protein of *T. maritimum*. The goal of the present study was to isolate the 19.6 kDa protein from the pathogenic strain NUF1128, identify its gene and determine its role in the pathogenesis of tenacibaculosis.

5.2. Materials and Methods

5.2.1. Bacterial strain

*T. maritimum* pathogenic strain, NUF1128, and the non-pathogenic strain, NUF1129, were used in the present study. The bacteria were grown in TYS broth medium consisted of 0.3% Bacto™ Tryptone (Difco) and 0.2% Bacto™ Yeast Extracts (Difco), pH 7.4-7.6, in filtered seawater for 24 h at 27°C in a shaking incubator. The stock cultures were kept at -80°C, and the main stocks were maintained in liquid nitrogen.

5.2.2. Preparation of outer membrane protein

**Polytron homogenization:** *T. maritimum* strains were grown in TYS broth at 27°C for 24 h with shaking at 120 rpm. Bacterial cells were collected from 3 L of broth by centrifugation for 10 min at 9,000 × g. The pellet was washed three times followed by resuspended in sterile PBS at a concentration of 0.4 g wet weight/ mL and subjected to high speed polytron homogenization (Kinematica, GmbH LITTAU, Switzerland) at a rate of 3 min/ mL. The homogenates were centrifuged at 9,000 × g for 30 min.
**Protein extraction:** The resulting supernatants as ultracentrifuged at 30,000 $\times$ g for 2 h at 4°C to sediment the outer membrane components of *T. maritimum*. The sediments were washed three times with PBS, suspended at 1 g wet weight of preliminary bacterial cells/ mL in 50 mM Tris-HCl, pH 8.7, containing 0.8% n-Octyl-β-D-thiogluco side (Dojindo Laboratories) (NOTG buffer) and left to stand for 2 h at room temperature to extract protein. The supernatant was collected by ultracentrifugation (at 30,000 $\times$ g, 4°C for 2 h), concentrated by filtration with an ultrafilter unit (molecular cut off 10,000 Da) (Advantec) and dialyzed against distilled water (DW) overnight. Finally, the dialyzed sample was freeze dried, resuspended in DW and kept at –20°C until use.

### 5.2.3. SDS-PAGE study

The polytron homogenate, prepared earlier, was examined by SDS-PAGE to find out the existence of a 19.6 kDa polypeptide using 12.5% gels as described in chapter 1. Moreover, the freeze dried sample was also subjected to SDS-PAGE study for the confirmation of 19.6 kDa band.

### 5.2.4. Isolation of 19.6 kDa polypeptide

**Negative staining:** After SDS-PAGE, the polyacrylamide gel was soaked in 0.2 M imidazole, 0.1% SDS for 15 min and shaken gently followed by washed briefly (30 sec) with MilliQ. Negative stain was done with 0.2 M ZnSO$_4$ for 20-30 sec until protein band at the position of 19.6 kDa became clearly visible. Staining reaction was stopped with water (MilliQ).
Extraction of 19.6 kDa protein from gel: The 19.6 kDa band portion of the sample was cut off from polyacrylamide gel, and ground by adding electrophoresis buffer (running buffer, used for SDS-PAGE) with a pestle motor (Biomasher, Nippi inc). The resulting slurry was incubated for 1 h at room temperature, applied to the sample cup of centrifugal filtration device (ATTO) and centrifuged at 15,000 × g for 10 min to elute the filtered liquid containing the 19.6 kDa polypeptide.

5.2.5. Digestion of 19.6 kDa polypeptide with proteases

The 19.6 kDa polypeptide was digested with various proteases i.e., α-chymotrypsin (from bovine pancreas, Sigma), papain (from papaya, Sigma) and endoproteinase Glu-C (from Staphylococcus aureus V8, Sigma) to get the N-terminal-deblocked polypeptide(s). About 7 µL of buffer, containing the mixture of 0.625 mL of 0.5 M Tris-HCl buffer (pH 6.8), 0.1 g SDS and 0.25 mL of MilliQ, pH 7.2, was added separately to 33 µL of gel extracts and digested with 0.5 µL of α-chymotrypsin (2 mg/ mL) or papain (2 mg/ mL) or endoproteinase (100 µg/ mL) at 28°C for 3 h.

5.2.6. Tricine SDS-PAGE study

Followed by enzyme digestion, the resulting proteins were fractionated by Tricine SDS-PAGE (16% polyacrylamide gel) by the method of Schagger and Von Jagow (1987). The separating gel was 16.5% T, 3% C, the “spacer” gel was 10% T, 3% C, and the stacking gel was 4% T, 3 % C (T denotes the total concentration of both monomers i.e., acrylamide and bisacrylamide; C
denotes the concentration of bisacrylamide relative to the total concentration \( T \). All solutions were stored at 4°C. The electrophoresis was performed in a mini-slab electrophoresis apparatus AE-6530 (ATTO), and each gel was stained with 0.25% Coomassie Brilliant Blue R-250 (Wako).

5.2.7. **N-terminal amino acid sequencing**

The N-terminal amino acid sequence was determined using a PPSQ-33A amino acid sequencer system (Shimadzu). The enzyme digested 19.6 kDa polypeptide was electrophoresed on a tricine SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After the membranes were stained with Coomassie Brilliant Blue R-250 followed by distained, the protein band was excised from the membrane and analyzed using the sequencer.

5.2.8. **Inverse PCR amplification**

**Inverse-PCR:** Two degenerated primers oppositely directed were designed from the N-terminal amino acid sequence of 16.8 kDa polypeptide band obtained from \( \alpha \)-chymotrypsin digestion. The genomic DNA from NUF1128 was prepared using Wizard Genomic DNA Purification Kit (Promega) and digested with one of several restriction enzymes, and each digested fragments were treated with phenol-chroloform-isooamylalchol (25:24:1) and precipitated with ethanol and self-ligated using DNA Ligation Kit, Mighty Mix (Takara) at 16°C for 30 min. Inverse-PCR was carried out with Prime Star GXL DNA Polymerase (Takara) using T. mari 19.6 kD-F4 and T.
mari 19.6 kD-R3 as primers (Table 5.1) and each self-ligated DNAs as template on C1000 Thermal Cycler (Bio-Rad).

**Sequencing of inverse-PCR products:** The templates derived from *Eco*RI, *Pst*I and *Bgl*II-digested DNAs yielded PCR products of approximately 8, 10 and 10 kbp, respectively. The PCR products from *Eco*RI and *Bgl*II-digested DNAs were purified from electrophoresis gels using QIAEX II Gel Extraction Kit (Qiagen) and used as the templates for sequencing. DNA sequencing was carried out with BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3130 and 3130x1 Genetic Analyzers (Applied Biosystems) in the Center for Frontier Life Sciences, Nagasaki University using primers T. mari 19.6 kD-F4, F6, F7, R3 and R4, (Table 5.1) sequentially. DNA sequence around the two degenerative primers was amplified using T. mari 19.6 kD-F5 and R5 and sequenced using these primers.

**Sequencing of the DNA region encoding 19.6 KDa protein in NUF1129:** PCR was performed using T. mari 19.6 kD-F8 and R7 as primers and the genomic DNA of NUF1129 as template and then the PCR products were sequenced using these primers.

**5.2.9. Sequence analysis**

Sequenced data were assembled and analyzed using DNASIS program (Hitachi Software). Nucleotide and protein similarity searches were performed using BLAST programs available through the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).
5.3. Results and Discussion

5.3.1. SDS-PAGE analysis and protein extraction

After polytron homogenization, the crude aliquot of the outer membrane components from the strain NUF1128 exhibited distinctive expression of a 19.6 kDa polypeptide band during SDS-PAGE study (Fig. 5.1) but no 19.6 kDa band was visible in the case of NUF1129 (data not shown). As mentioned earlier (chapter 1), mutation in the cell surface component of NUF1129 probably disrupted the protein secretion system (Rhodes et al., 2011) that ultimately resulted no expression of 19.6 kDa band from the polytron homogenate. A further SDS-PAGE study confirmed that extraction of outer membrane 19.6 kDa protein was achieved efficiently by using 0.8% NOTG buffer but failed with TSE buffer (200 mM Tris-HCl, 500 mM sucrose, 1 mM EDTA, pH 8.0) (data not shown). NOTG is an efficient non-ionic surfactant specifically designed for the solubilization of membrane proteins (Saito and Tsuchiya, 1984; Tsuchiya and Saito, 1984) and was successfully used for the extraction of a 19.3 kDa protein, identical to fimbrial subunit (FimA), of *E. tarda* (Sakai et al., 2003).

5.3.2. Tricine SDS-PAGE analysis

Coomassie Brilliant Blue R-250 stained protein bands in the tricine SDS-PAGE study indicated that the 19.6 kDa polypeptide was cleaved to two major peptides along with other smaller peptides by α-chymotrypsin (Fig. 5.2, lane 3). In the case papain digestion, the 19.6 kDa band broke down into several peptides of different molecular weights that were less sharp than
the α-chymotrypsin digested major peptides (Fig. 5.2, lane 4). The endoproteinase Glu-C was unable to digest properly with rarely visible existence of two minor fragments (Fig. 5.2, lane 5). From this experiment, one of the major peptides 16.8 kDa, obtained from the α-chymotrypsin digestion, was decided to cut off after blot-transfer to a PVDF membrane for N-terminal amino acid sequencing.

5.3.3. Protein sequencing

The portion of the PVDF membrane corresponding to the 16.8 kDa polypeptide was N-terminally sequenced, and a structure consisted of 16 amino acid residues, SQVSVGGQDADDTVFT, was revealed. A search by the protein BLAST program revealed high homology (identity, 81.0%; E value, 0.43) to the hypothetical protein (accession no. WP_024740854.1) of T. maritimum NBRC 15946T (Wakabayashi et al. 1986; Suzuki et al. 2001). Thirteen out of 16 amino acids of the NUF1128 16.8 kDa protein were identical to those of the T. maritimum NBRC 15946T protein. Based on this information, following two degenerated oligonucleotides were designed for inverse PCR: 5′-TTGNCCNCCWACWSWACTTG-3′ (forward primer, T. mari 19.6kD-R3) and 5′-GAYGCHGAYGAYGAYCHGTWTTYAC -3′ (reverse primer, T. mari 19.6kD-F4)

5.3.4. Analysis of the nucleotide sequence and the deduced structure of 19.6 kDa polypeptide

The complete nucleotide sequence of T. maritimum 19.6 kDa polypeptide was compiled from the overlapping sequences of the PCR and inverse PCR products that ultimately revealed an ORF
size of 588 bp. The deduced amino acid sequence revealed that 19.6 kDa polypeptide has 196 amino acid residues which contained a signal peptide consisting of 18 amino acids (Fig. 5.3). The molecular mass of the polypeptide without signal peptide was calculated as 19,000 and that of the N-terminally sequenced structure was 16,800. A search by protein BLAST for 19.6 kDa polypeptide showed a high sequence homology of 96% (E value, 8e-127) with the hypothetical protein of *T. maritimum* NBRC 15946\(^T\) (sequence ID: WP_024740854). The predicted amino acid sequence exhibited specific hits on the OMP_b-brl (outer membrane protein beta barrel domain; E value = 5.61e-17; accession no. pfam13505) and non-specific hits on the OmpW (outer membrane protein W; E value = 1.23e-03; accession no. COG3047) under the superfamily of OM_channels (E value = 5.61e-17) during conserved domain database search by NCBI.

In addition to the 19.6 kDa polypeptide, a total size of the inverse PCR product was found as 3,468 bp with several groups of the deduced amino acid sequences. Database search by the NCBI showed high sequence homology of 100% (215 amino acids residues; E value, 2e-148), 99% (167 amino acids residues; E value, 8e-101) and 99% (162 amino acids residues; E value, 9e-112) (Fig. 5.3) with three hypothetical proteins (accession nos. WP_024740853, WP_024740851 and WP_024740855) respectively of *T. maritimum* NBRC 15946\(^T\) that also possessed the putative conserved beta barrel domain under the superfamily of OM_channels. OM_channels superfamily is porin superfamily whose outer membrane channels share a beta-barrel structure that differ in strand and shear number.

Sequencing of the DNA region encoding 19.6 kDa protein in NUF1129 revealed that NUF1129 also possessed the same sequence as NUF1128 (Fig. 5.4) *i.e.*, the gene encoding 19.6 kDa protein is also present in NUF1129 but gene expression is absent.
The outer membranes of Gram-negative bacteria, mitochondria, and chloroplasts all contain transmembrane β-barrel proteins (self-closed β-sheets) commonly called outer membrane proteins (OMPs), which serve essential functions in cargo transport and signaling and are also vital for membrane biogenesis (Tokuda 2009; Bos et al., 2007). Despite the common β-barrel scaffold, OMPs have evolved to perform many different functions including acting as porins, transporters, enzymes, and receptors (Galdiero et al. 2007; Walther et al. 2009; Wimley 2003). Although the functions of T. maritimum beta barrel domain are not yet known, the hypothetical protein related to the 19.6 kDa with specific hits on the OMP_b-brl and non-specific hits on the OmpW is predicted to be involved in putative channel (Protein Data Bank ID: 2F1V, 2F1T) (Fairman et al. 2011) for the transport of small hydrophobic molecules across the bacterial outer membrane. Definitive determination of the function of T. maritimum outer membrane 19.6 kDa protein in the context of the pathogenesis of tenacibaculosis awaits further study.
Fig. 5.1. SDS-PAGE analysis of whole cell proteins and polytron homogemate of *T. maritimum* NUF1128 stained with Coomassie Brilliant Blue R-250. M, molecular weight markers (Bio-Rad).
Fig. 5.2. Tricine SDS-PAGE analysis of *T. maritimum* 19.6 kDa polypeptide stained with Coomassie Brilliant Blue R-250. **Note:** Lane 1, polypeptide SDS-PAGE molecular weight standards (Bio-Rad); lane 2, 19.6 kDa polypeptide as control; lane 3-5, digested polypeptide profile of 19.6 kDa using α-chymotrypsin, papain and *V₈*, respectively. Lane 6-8, polypeptide profile of α-chymotrypsin, papain and *V₈*, respectively.
Chapter 5

ORF1

**ORF1 (19.6 kDa protein)**

**ORF2 stop**

**ORF3**
Fig. 5.3. The nucleotide and amino acid sequences of the gene regions from *T. maritimum* NUF1128. Signal peptides are underlined; *=stop codon; blue alphabets indicate the starting of gene/protein sequence. **Note:** ORF1=486 bp (sequence ID WP_024740855); deduced sequence of the hypothetical protein contains 162 amino acid residues. Size of the predicted ORF2 (19.6 kDa protein)=588 bp (sequence ID: WP_024740854); deduced sequence of the 19.6 kDa polypeptide contains 196 amino acid residues. N-terminal amino acid sequence of 16.8 kDa polypeptide obtained by α-chymotrypsin digestion. ORF3=645 bp (sequence ID WP_024740853); deduced sequence of the hypothetical protein contains 215 amino acid residues. ORF4=501 bp (sequence ID: WP_024740851); deduced sequence of the hypothetical protein contains 167 amino acid residues.
Fig. 5.4. Alignment of the nucleotide sequences of the 588bp region encoding 19.6 kDa hypothetical protein from *T. maritimum* NUF1128 and NUF1129 generated using the ClustalW software. Note: The asterisk (*) shows sequences that are the same. The strain no. given on the left of each sequence lane and sequence no. is given on the right of each sequence lane.
**Table 5.1.** Primers used in this study

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H = A.C.T; W = A.T; Y = C.T.
General Discussion

and

Conclusions
Since 1976, tenacibaculosis has become a common problem among red and black bream fry cultured at various locations in Japan (Wakabayashi et al., 1986) and now-a-days, this disease is considered as one of the major threats for sea breams, Japanese flounder and puffer fish farms. The major aim of this thesis is to study the pathogenicity and serological properties of the Japanese strains of *T. maritimum* based on the comparison between gliding and non-gliding strains. Additionally, a challenge model was successfully conducted and progression of tenacibaculosis was investigated using experimentally infected Japanese flounder.

A compact colony phenotype of *T. maritimum* was isolated for the first time from the skin lesion of a naturally infected puffer fish *Takifugu rubripes*. The NUF1129 strain was designed from a compact colony and examined for motility, cell size and adhesion observation. To find out the antigenic differences, a gel immunodiffusion test was conducted using the sonicated extracts of *T. maritimum* strains which proved the lack of a cell surface antigen in NUF1129, probably protein in nature. The rabbit antiserum raised against the FKC of NUF1081, successfully recognized this antigenic variation.

Due to lack of surface antigen X, NUF1129 was suspected to lose the gliding motility, adhesion ability and ultimately turned into avirulent. Mutation in the cell surface component is thought to interrupt the protein secretion system common in *Bacteroidetes* group (Nelson et al., 2008; Sato et al., 2010; Rhodes et al., 2011) and resulted to accumulate enzyme(s) inside bacterium (chapter 1). That is why, antigen X was decided to isolate for further studies. Several attempts were undertaken to isolate the antigen X from the precipitation line. First attempt was digestion of low melting agarose (in which the precipitation line was formed) by agarase. As antigen X is heat-labile and disappears with the heat treatment of more than 55ºC for 15 min (chapter 1), heating with 65ºC for 15 min during agarase digestion probably denatured this
antigen. Moreover, chromatography with DEAE-sepharose and CM-sepharose failed to purify antigen X as it is slightly hydrophobic in nature. Hence, phenyl-Sepharose chromatography partially purified the high molecular weight surface antigen X. This is the first study to isolate a cell surface component from a Japanese *T. maritimum* strain which paves the opportunity to develop vaccines against tenacibaculosis for the aquaculture industry.

Various experimental infection studies have been carried out with varying rates of mortality depending on the different methods used to infect each marine fish species (Wakabayashi *et al.*, 1984, Baxa *et al.*, 1987, Alsina and Blanch 1993, Bernardet *et al.*, 1994, Soltani *et al.*, 1996 and Powell *et al.*, 2004) which made difficult to fully understand of the mode of transmission and route of infection of this pathogen. The present study provides the first description of the kinetics of infection of gliding and non-gliding strains of *T. maritimum* by enumeration and immunohistochemical detection in the experimentally infected Japanese flounder using moderate inoculum doses to clarify the infection process. Wide variation in the pathogenicity was noted when *T. maritimum* was administered by immersion rout using blade-abraded or fin clipped fish. *T. maritimum* attaches itself strongly to the external skin and mucus of fish which do not contain compounds that inhibits the growth of this bacterium (Magariños *et al.*, 1995) and invades into the fish body though injured or abrasive sites. Abrasion with cotton swabs partially removed the epidermis but scratching with blades fully exposed the dermis by complete removal of epidermis. *T. maritimum* adhered to the dermal connective tissues that are composed of collagen compound, proliferated along the connective tissues and causes tenacibaculosis by secreting tissue degradation proteases (chapter 1). Although the collagenase activity is not yet reported from *T. maritimum*, a positive relationship between collagenous connective tissues and bacterial proliferation is suspected.
Serological and molecular typing methods have demonstrated the existence of distinct serological subgroups within *T. maritimum* (Ostland *et al.*, 1999, Santos *et al.*, 1999). To determine the serological relationships among Japanese *T. maritimum* strains, serological studies was conducted using (FKC) and (HKC) of *T. maritimum* strains. The absorbed anti-*T. maritimum* sera, after reacting with *T. maritimum* HKCs, demonstrated the existence of different O-serogroups (chapter 4). Findings were supported by the previous study (Avendaño-Herrera *et al.*, 2004) where antigenical difference were reported mainly due to differences in the ‘O’ chains of LPS. However, the presence of common heat-labile surface antigens from the cells of the strains isolated from diseased Japanese flounder and puffer fish are suggesting for a common FKC vaccine for tenacibaculosis.

A distinctive expression of a 19.6 kDa protein was reported previously to distinguish *T. maritimum* gliding strains from the non-gliding avirulent strain, NUF1129. Successful isolation, extraction, protein sequencing followed by inverse PCR analysis reported that 19.6 kDa protein is a hypothetical outer membrane protein of *T. maritimum* (outer membrane protein beta barrel domain) under the superfamily of OM_channels. The strain NUF1129 also possesses the nucleotide sequences encoding this protein but do not express. It is thinkable that mutation in the cell surface component of NUF1129 may influence related proteins or other surface proteins, their functions and expressions.

Lastly, it can be concluded that,

1. Cell surface antigen(s) that is not produced by NUF1129 is thought to be related to the gliding motility, rhizoid colony morphology and adherence, and influence the virulence in fish.
2. Partial purification of *T. maritimum* surface exposed antigen X was succeeded by hydrophobic interaction chromatography. Antigen X is a high molecular weight protein, consists of more than one polypeptide chains.

3. Pathogenic *T. maritimum* enters through the abraded body surfaces, adheres to the dermal connective tissues followed by proliferates along the dermal and perimysial connective tissues, secretes tissue-degradation enzymes including protease and chondroitinase that damage subjacent tissues and causes tenacibaculosis.

4. Different O-serogroups were found among *T. maritimum* strains. Presence of common heat-labile surface antigens of *T. maritimum* is suggesting for the future research based on the usage of a common FKC vaccine for tenacibaculosis among Japanese fish.

5. The 19.6 kDa protein is a hypothetical protein of *T. maritimum* cell surface that is predicted to be involved in putative channel. Further studies are required for elucidating its function.
Literature Cited


Literature Cited


