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Research article

Infection Kinetics of *Tenacibaculum maritimum* on the Abraded Skin of Japanese Flounder *Paralichthys olivaceus*

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ABSTRACT—*Tenacibaculum maritimum* is the gliding bacterium that causes tenacibaculosis, an ulcerative disease in marine fish. In this study, we conducted a pathogenicity test to assess the effect of skin abrasion on the infectivity of gliding and non-gliding strains of *T. maritimum*, NUF1128 and NUF1129, respectively, and investigated the infection kinetics by enumeration and immunohistochemical observation of the adhered and proliferated *T. maritimum* on the abraded skin of Japanese flounder *Paralichthys olivaceus*. In the pathogenicity test, Japanese flounder whose dorsal skin was abraded with a cotton swab or blade or tip of dorsal fin was clipped with scissors were immersed for 30 min in seawater containing 10^6 CFU/mL of cultured NUF1128 or NUF1129 cells. As a result 100% mortality was achieved in the fish groups pretreated with blades or scissors followed by challenged with NUF1128. NUF1129 was unable to induce infection regardless of the treatments applied. The infection kinetic studies revealed that NUF1128 adhered more readily than NUF1129 to dermal connective tissues which were exposed by abrasion with blades and subsequently proliferated mainly in the dermal connective tissues and perimysium.

Key words: *Tenacibaculum maritimum*, tenacibaculosis, infection kinetics, abraded skin, immersion challenge, *Paralichthys olivaceus*, Japanese flounder

Tenacibaculum maritimum (formerly *Flexibacter maritimus*), a Gram-negative and filamentous bacterium, has been described as the etiological 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 Japanese 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*., 2006a). *T. maritimum* is a ubiquitous and opportunistic pathogen (Avendaño-Herrera *et al*., 2006a), 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*., 2006a). Thus, the primary sites of *T. maritimum* infection are considered as body surfaces (Bernardet, 1998), specially the skin (Faílde *et al*., 2013). 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* (Faílde *et al*., 2014) and turbot *Psetta maxima* (Faílde *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 (Avendaño-Herrera *et al*., 2006a). However, other authors have succeeded to reproduce the disease using prolonged immersion of fish (Avendaño-Herrera *et al*., 2006b) or high inoculum doses (van Gelderen *et al*., 2011). Recent studies demonstrated that immersion challenge

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(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.

Previously we reported that the gliding strain *T. maritimum* NUF1128 was virulent ($LD_{50} = 10^{6.0}$ colony forming unit [CFU]/mL), whereas the non-gliding strain NUF1129 was avirulent ($LD_{50} > 10^{8.9}$ CFU/mL) to Japanese flounder by immersion route (Rahman *et al*., 2014). The affected fish exhibited skin necrosis and fin fraying, the typical signs of tenacibaculosis. We considered that netting of fish upon challenge caused injuries on the skin and fins, to which *T. maritimum* infected. In the present study, we conducted a pathogenicity test using Japanese flounder to demonstrate the effect of skin abrasion on the infectivity of the gliding and non-gliding *T. maritimum* strains (NUF1128 and NUF1129). 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 challenge was carried out to investigate the adhesion and proliferation of *T. maritimum* NUF1128 and NUF1129 on the abraded skin of Japanese flounder by numeration and immunohistochemical detection of the bacteria.

Materials and Methods

Bacteria

T. maritimum strains NUF1128 and NUF1129 derived from a naturally infected puffer fish *Takifugu rubripes* were used in this study. The strains were stored at −80°C in TYS broth [0.3% Bacto™ Tryptone (Difco) and 0.2% Bacto™ Yeast Extracts (Difco) in filtered seawater, pH 7.4−7.6] supplemented with 10% glycerol. When using, the stocked cultures were grown on TYS agar plates (TYS broth supplemented with 1.5% agar) at 27°C.

Pathogenicity test

T. maritimum NUF1128 and NUF1129 grown on TYS agar were inoculated and 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) 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 days 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. 1A) (Miwa and Nakayasu, 2005) and blades (a portion of dorsal skin was scratched with a blade, Fig. 1B), fin clipping (a tip of the dorsal fin was clipped with dissection scissors) and no treatment were employed on forty anesthetized fish (five fish/treatment/strain) before immersion challenge. Upon pretreatment 2-phenoxyethanol diluted with water was directly added to the rearing tank, and anesthetized fish was picked up with a hand to prevent injuries caused by netting. 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 2.0×10^6 CFU/mL and 1.3×10^6 CFU/mL, respectively, which were around the LD_{50} value of NUF1128 (Rahman *et al*., 2014). Non-abraded five fish were immersed in seawater without the bacterial suspension for control. After immersion for 30 min, fish were transferred with hands to rectangular 30-L aquaria (five fish of each experimental group/aquarium) containing approximately 25 L of seawater equipped with continuous water flow and aeration. The fish received no feed for 7 days of the experimental period. The water temperature ranged from 22.0 to 23.5°C during the experimental period. Gross pathological changes and moribundness were checked daily.

Infection kinetics of T. maritimum *in Japanese flounder*

Immersion challenge: Juvenile Japanese flounder (average body weight, 42.6 ± 8.0 g) were stocked into two 200-L tanks 2 days before challenge. Fish were anaesthetized with 2-phenoxyethanol as above, and a portion of the dorsal skin was abraded by scratching with blades prior to immersion challenge. Seventy fish were kept into two 100-L tanks (35 fish/tank) with aerated water to which the bacterial suspension of NUF1128 or NUF1129 prepared as above was added. The challenge doses were 1.8×10^6 CFU/mL and 1.7×10^6 CFU/mL, respectively. After 30 min, the fish were transferred into two 200-L tanks supplied with constant aeration and continuous water flow and reared for 48 h with no feed. For the control, ten fish were immersed in seawater without the bacterial suspension and kept in a 30-L aquarium. Water temperature varied from 23.0 to 24.5°C during the experimental period.

Enumeration of *T. maritimum* in tissue samples: Five challenged fish from each 200-L tank were sampled randomly at 30 min, 2 h, 6 h, 24 h and 48 h post challenge for counting viable bacteria in the skin tissues of challenged fish. Approximately 1 cm^2 of skin from the abraded site was 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 days. Bacterial counts of the skin were expressed as CFU/cm^2. Some confusing colonies were subcultured on TYS agar and subjected to agglutination tests with rabbit anti-*T.*

maritimum NUF1081 serum (Rahman *et al*., 2014) for confirmation. Due to severe infection and mortality in NUF1128-challenged group, the number of sampled fish at the last sampling (48 h) was insufficient.

Histopathology and immunohistochemistry: Two challenged fish from each 200-L tank were sampled randomly at 30 min, 2 h, 6 h, 24 h and 48 h post challenge and were subjected to histological and immunohistochemical examination. The skin with underlying muscle, spleen, kidney and liver of sampled fish were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (3 μm in thickness) were stained with hematoxylin and eosin (H-E) for light microscopic observation. For immunohistochemical detection of *T. maritimum*, sections were incubated with 0.3% hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidase activity in tissues followed by blocked using 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 the 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. The sections were counter-stained with hematoxylin. *T. maritimum* was detected as brown color in sections. For negative control a representative skin section from NUF1128-challenged fish at 24 h post challenge was incubated with rabbit anti-*L. garvieae* NUF1019 serum instead of anti-*T. maritimum* NUF1081 serum.

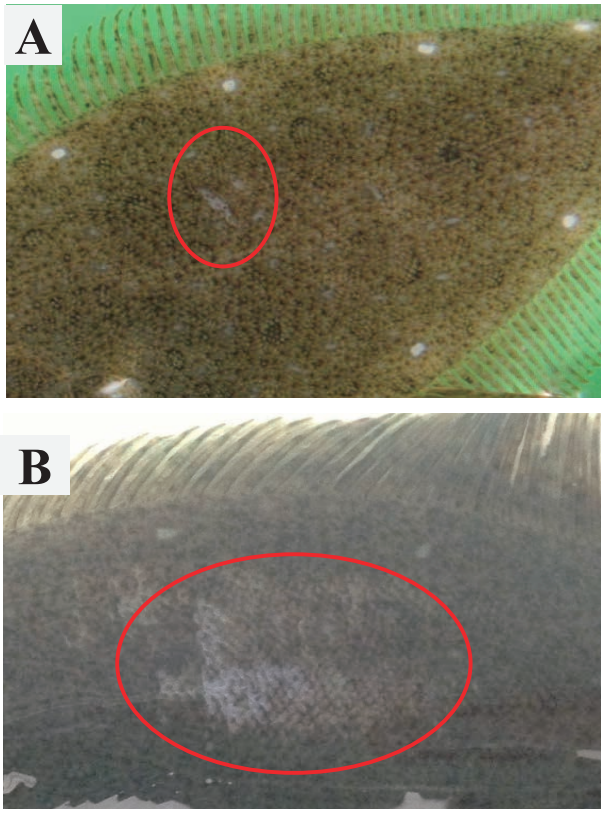

Fig. 1. The appearances (red circles) of the abraded skin of Japanese flounder. (A) Abrasion by a cotton swab and (B) abrasion by a blade.

Results

Pathogenicity test

The pathogenicity test using the strain NUF1128 showed 100% mortality in immersion challenged fish abraded with blades and fin-clipped with scissors

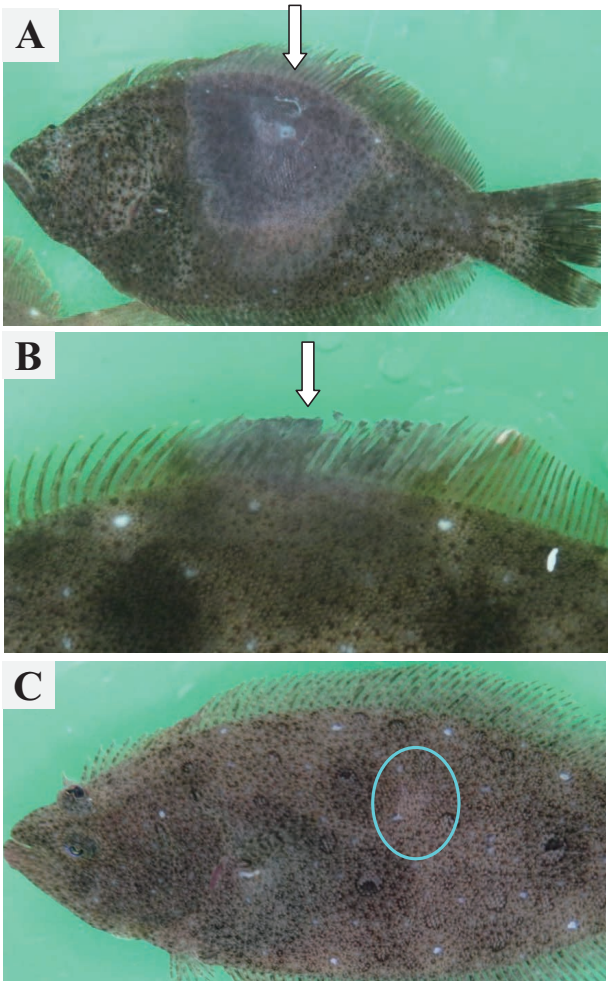

Fig. 2. Gross lesions on the body surface of Japanese flounder 2 days after challenged with *T. maritimum* NUF1128. **Note:** (A) A large necrotic lesion (arrow) started from the site abraded by a blade; (B) a lesion started from the fin (arrow) clipped by scissors; (C) abrasion by a cotton swab did not cause a lesion on the skin (circled area).

accompanied with visible lesions on the body surface and fin necrosis (Fig. 2A and B) and 20% mortality in non-pretreated and challenged fish (Table 1). The lesions started from the abraded or fin-clipped sites, spread towards the adjacent area and gradually turned into hemorrhagic. 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 other hand,

Table 1. Pathogenicity of *T. maritimum* NUF1128 and NUF1129 studied in Japanese flounder

Strain	Challenge dose (CFU/mL)	Pretreatment	No. of dead fish during the period of (n = 5)				Mortality (%)
			0–1 d	2 d	3 d	4–7 d	
NUF1128 (Gliding strain)	2.0×10^6	Swab abrasion	0	0	0	0	0
		Blade abrasion	1	3	1	–	100
		Fin clipping	0	2	3	–	100
		Not treated	0	1	0	0	20
NUF1129 (Non-gliding strain)	1.3×10^6	Swab abrasion	0	0	0	0	0
		Blade abrasion	0	0	0	0	0
		Fin clipping	0	0	0	0	0
		Not treated	0	0	0	0	0
Control	Not challenged	Not treated	0	0	0	0	0

Weight of challenged fish (Ave. ± S.D), 26.8 ± 5.1 g
Water temperature (Ave. ± S.D), 22.5 ± 0.5°C

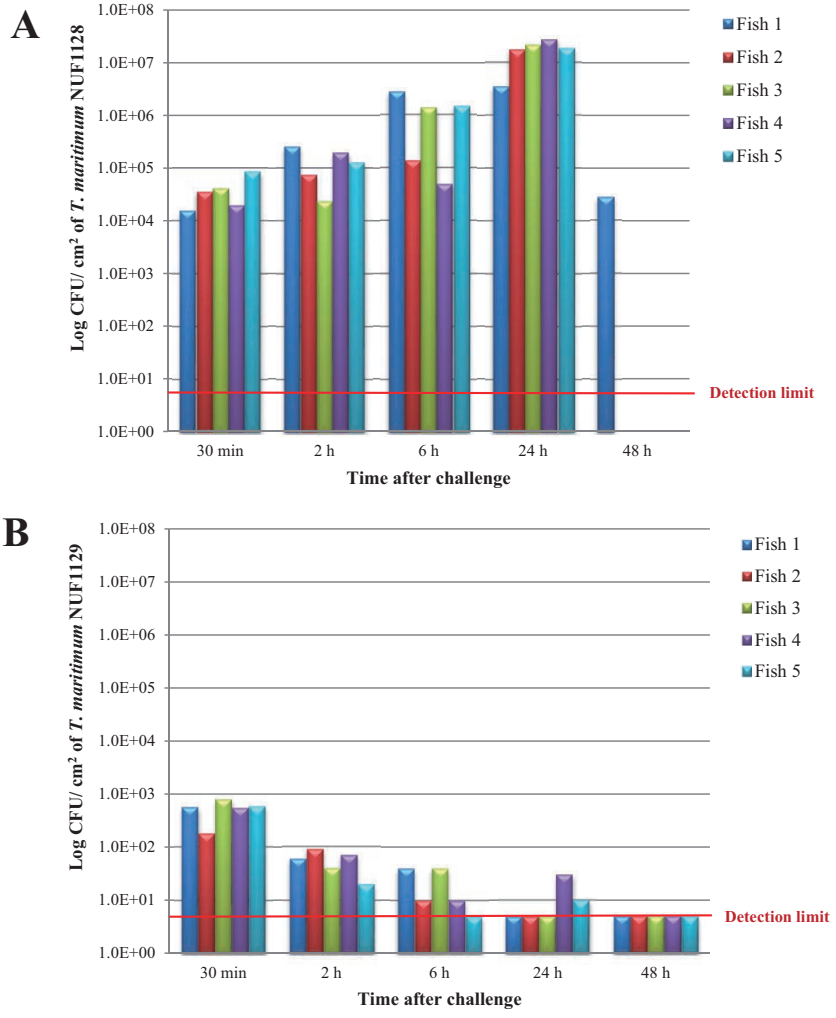

Fig. 3. Viable counts of *T. maritimum* in the skin of flounder abraded by blades followed by immersion-challenged with (A) NUF1128 and (B) NUF1129 at doses of 1.8×10^6 CFU/mL and 1.7×10^6 CFU/mL, respectively. Five fish were sampled at each time point.

the strain NUF1129 failed to induce any sign of infection or disease to experimental fish regardless of pretreatments applied. In the case of cotton swab abrasion, no fish either from NUF1128-challenged group or NUF1129-challenged group became infected or died (Table 1). Cotton swab abraded sites were found to be gradually recovered (Fig. 2C). No fish died in the control group (Table 1).

Infection kinetics in the tissues of abraded Japanese flounder

Viable count of *T. maritimum* in the abraded skin: The viable count of NUF1128 at 30 min post challenge (10^4 CFU/cm^2) was more than those of NUF1129 (10^2 CFU/cm^2). Thereafter, in the NUF1128-challenged group exponential proliferation of the bacteria occurred,

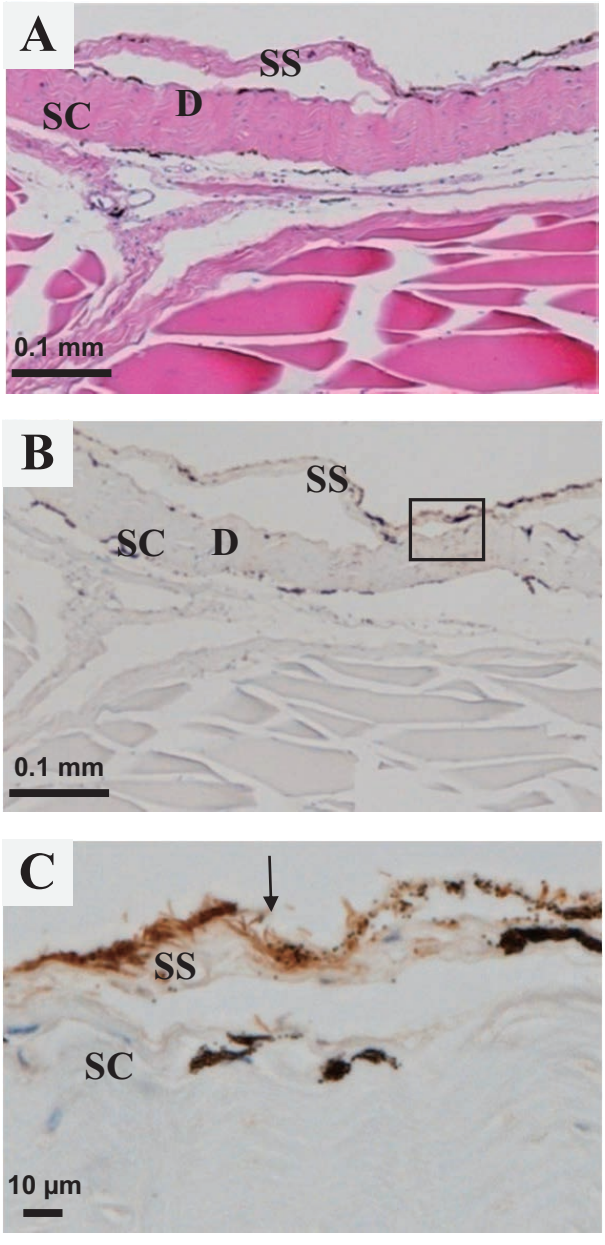

Fig. 4. Histopathological and immunohistochemical observation of the abraded skin after 30 min of immersion challenge with *T. maritimum* NUF1128. **Note:** (A) Stratum spongiosum of dermis is exposed due to complete missing of epidermis (H-E stain); (B and C) adhesion of *T. maritimum* (arrow) to the stratum spongiosum; (C) a magnified image of black border in B. D = dermis; SS = stratum spongiosum; SC = stratum compactum.

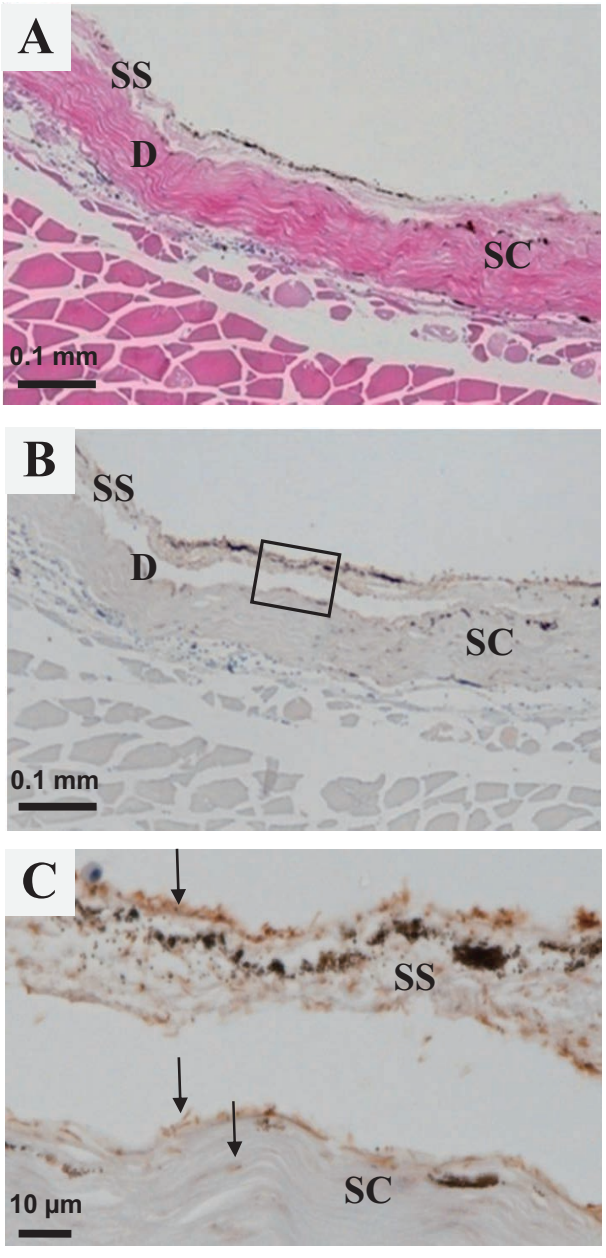

Fig. 5. Histopathological and immunohistochemical observation of the abraded skin after 6 h of immersion challenge with *T. maritimum* NUF1128. **Note:** (A) Exposed stratum spongiosum of dermis due to skin abrasion; (B and C) proliferation of *T. maritimum* (arrows) along the dermal connective tissues (stratum spongiosum and stratum compactum), (C) a magnified image of black border in B. D = dermis; SS = stratum spongiosum; SC = stratum compactum.

and, at 24 h post challenge, the viable counts increased to 10^7 CFU/cm^2 (Fig. 3A) associated with mortalities. On the other hand, in the NUF1129-challenged group, the bacterial population declined gradually (Fig. 3B), and no mortalities were observed.

Histopathology and immunohistochemistry: In immunohistochemistry pretreatment of sections with hydrogen peroxide and gelatin prior to the 1st antiserum step resulted in inhibition of non-specific coloring. And the section of negative control exhibited no antibody reaction.

The epidermis was found to be missed completely by blade abrasion, but both parts of the dermis, *i.e.,* stratum spongiosum and stratum compactum were

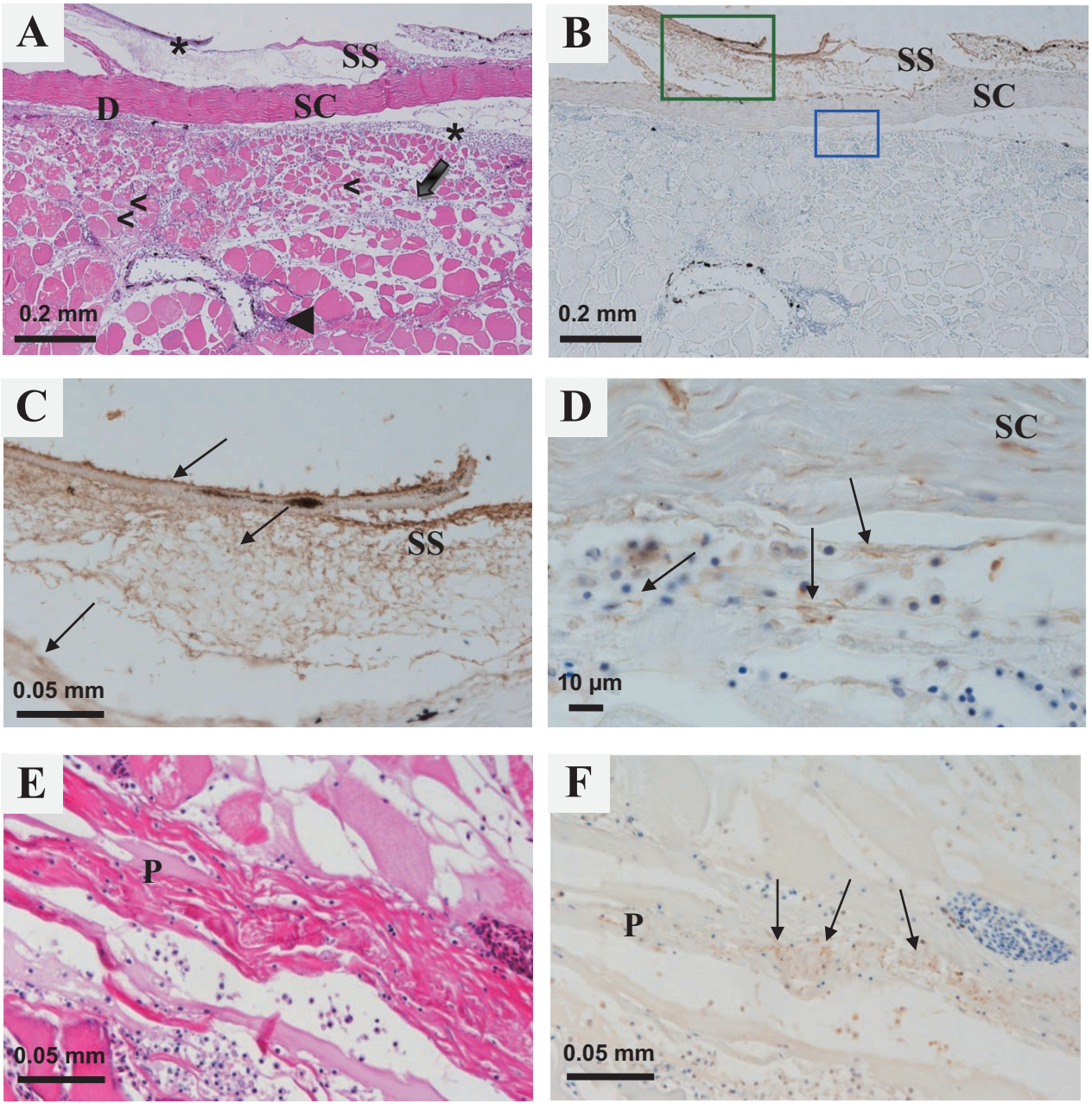

Fig. 6. Histopathological and immunohistochemical observation of the abraded skin after 24 h of immersion challenge with *T. maritimum* NUF1128. **Note:** (A) Necrosis (*) in the epidermis (adjacent to abrasion) and hypodermis, hemorrhage (◀) and degeneration of muscle tissues (<) due to the infection 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.

observed at abraded sites. After 30 min of immersion challenge, no obvious pathological changes or inflammatory responses were observed (Fig. 4A) although *T. maritimum* was found to adhere to the connective tissues of the exposed stratum spongiosum (Fig. 4B and C) of the skin samples of NUF1128-challenged group. The adhesion of *T. maritimum* was not observed in the NUF1129-challenged group.

At 6 h post challenge, no degenerative change was observed but an early inflammatory response was seen spreading adjacent to the abraded area (Fig. 5A). The invasion and proliferation of *T. maritimum* were clearly observed in the samples of NUF1128-challenged group along the dermal connective tissues (up to stratum compactum) (Fig. 5B and C) but not in the NUF1129-challenged group.

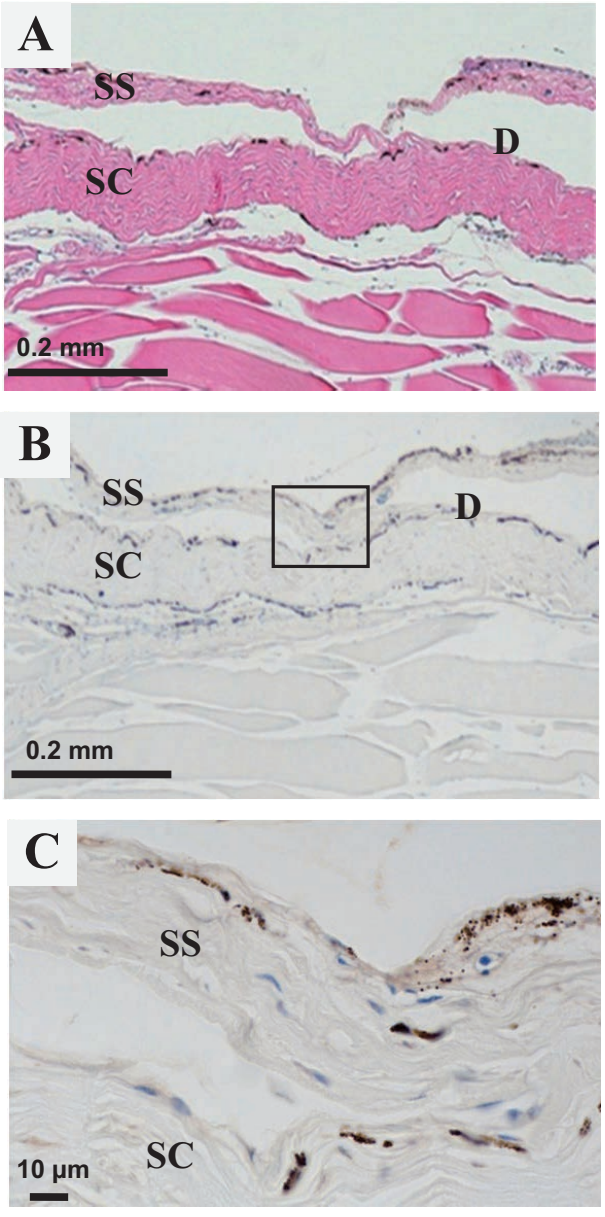

Fig. 7. Histopathological and immunohistochemical observation of the abraded skin after 30 min of immersion challenge with *T. maritimum* NUF1129. **Note:** (A) Dermis was found intact (H-E stain); (B) *T. maritimum* was not observed at the abraded site due to less adhesion of NUF1129; (C) a magnified image of black border in B. D = Dermis; SS = stratum spongiosum; SC = stratum compactum.

At 24 h, the hypodermis and muscle showed degeneration at various degrees of severity in the skin of NUF1128-challenged group. Separation of muscular fibers and hemorrhages were also observed, and the area of necrosis was extended through the connective tissue of hypodermis reaching and affecting other muscular packets (Fig. 6A). Inflammatory cells often accumulated around the infection sites, although in most cases the bacterial antigens were not observed either in those cells or in the muscle fibers (Fig. 6B). *T. maritimum* was found to spread in the fish body by proliferation along the dermal connective tissues and perimysium (Fig. 6C, D, E and F). Pathological changes and bacterial multiplication were absent in the samples of NUF1129-challenged group (Fig. 7A, B and C).

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

Discussion

The pathogenicity test revealed that the strain NUF1128 was highly pathogenic resulting in 100% mortality of fin-clipped and blade-abraded fish within 3 d. Fin-clipping and blade-abrasion were thought to create entry points for *T. maritimum* and cause infection leading to the death of flounder. On the other hand, no fish became infected or died in the case of cotton swab abrasion. Histological observation of the cotton swab abraded site revealed the partial loss of epidermis (data not shown). Miwa and Nakayasu (2005) conducted an experimental infection with *Flavobacterium psychrophilum* and succeeded in producing ulcers on the body surface of ayu *Plecoglossus altivelis* which had been abraded with cotton swabs before challenge. Although our experiment followed their method, the abrasion with cotton swabs may be insufficient to create enough portal of entry for *T. maritimum* or the relative low dose employed (10^6 CFU/mL).

Our previous study showed mortalities (40–100%) of the non-pretreated flounder by immersion challenges with NUF1128 at doses of 10^5–10^7 CFU/mL (Rahman *et al.*, 2014). These mortalities were relative high in comparison with the present results of non-pretreated fish (20% at the dose of 10^6 CFU/mL). We presume that transferring the fish with nets in the previous study may create injuries on the body surface.

The bacterial entry into the body through undamaged skin seemed improbable since no *T. maritimum* antigens were observed in the intact skin. 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 were observed in the muscle below dermis. It is probable that once the bacteria reach 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 and cause pathological changes in flounder.

The results of quantification of the adhered bacteria as well as immunohistochemical observation indicated that NUF1129 was unable to adhere to the dermal connective tissues of Japanese flounder. Presumably this is why the avirulent strain NUF1129 did not produce any infection or disease in flounder regardless of blade abrasion or fin clipping.

T. maritimum was detected in the skin, gills, liver and spleen by culturing method, when red sea bream and black sea bream were experimentally infected with the bacterium using smear or immersion method (Kimura and Kusuda, 1983; Baxa *et al*., 1987). In addition, *T. maritimum* antigens were detected by immunohistochemistry in the internal organs of turbot and Senegalese sole inoculated by subcutaneous route with a relative high dose, 10^8 CFU/fish (Faílde *et al*., 2013, 2014). Conversely, in the present study, *T. maritimum* was not detected from the internal organs *i.e.,* spleen, kidney or liver. Considering that the primary sites of *T. maritimum* infection are body surfaces such as the head, mouth, fins and flanks (Bernardet, 1998) and that lesions occurred first on the skin surface followed by progressing into deeper layers (so called 'outside-in' skin lesion) (Vilar *et al.*, 2012), the conditions within the internal organs of flounder probably did not support the growth and proliferation of the bacteria.

As mentioned above the gliding strain NUF1128 was found highly pathogenic for Japanese flounder when the body surface was injured. Thus the condition of the body surface seems to affect the outcomes of challenge tests. To achieve reproducible results handling of experimental fish has to be carefully managed, and the present methods (blade abrasion or fin clipping prior to challenge) may be successful means for experimental *T. maritimum* infection in Japanese flounder.

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