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Research article

Biological and Serological Characterization of a Non-gliding Strain of *Tenacibaculum maritimum* Isolated from a Diseased Puffer Fish *Takifugu rubripes*

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ABSTRACT—*Tenacibaculum maritimum* is a Gram-negative, gliding marine bacterium that causes tenacibaculosis, an ulcerative disease of marine fish. 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 from a puffer fish *Takifugu rubripes* suffering from tenacibaculosis, and studied the biological and serological characteristics of a representative isolate of the compact colony phenotype, designated strain NUF1129. The strain was non-gliding and avirulent in Japanese flounder *Paralichthys olivaceus* in immersion challenge test and showed lower adhesion ability to glass wall in shaking broth culture and to the body surface of flounder. It lacked a cell-surface antigen commonly detected in gliding strains of the bacterium in gel immunodiffusion tests. SDS-PAGE analysis 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*.

Key words: *Tenacibaculum maritimum*, tenacibaculosis, non-gliding, virulence, adherence, cell-surface antigen

The genus *Tenacibaculum* 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, gliding bacterium and causes tenacibaculosis, an ulcerative disease of marine fish. The disease was first described by Masumura and Wakabayashi (1977) and, later, Wakabayashi *et al.* (1986) reported *Flexibacter maritimus* (= *T. maritimum*) as the pathogen after isolating from diseased red sea bream *Pagrus major* and black sea bream *Acanthopagrus schlegeli* in Japan. Other susceptible species include Japanese flounder *Paralichthys olivaceus* (Baxa *et al.*, 1986) and yellowtail *Seriola quinqueradiata* (Baxa *et al.*, 1988b) in Japan, 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. 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.*, 2006) and iron-uptake mechanisms (Avendaño-Herrera *et al.*, 2005), 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

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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*.

Materials and Methods

Bacterial strains

Fourteen *T. maritimum* strains including NUF1129 derived from diseased fish were used in this study (Table 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 = ATCC43398^T (NR_113825) and ATCC43398^T (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^{TM}Tryptone (Difco), 0.2% Bacto^{TM} 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.

Observation of motility

Gliding motility was assessed by the plate technique (Perry, 1973), in which a few glass beads (φ0.1 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.

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

Table 1. Sources of *T. maritimum* strains with their biological characteristics

Strain	Host fish	Location and year of isolation	Colony type	Gliding motility	Adhesion to glass wall*	Cell length (Mean ± SD) (μm)	Enzyme activity	
							Chodroitinase	Gelatinase
NUF433	Japanese flounder	Nagasaki/1989	Rhizoid	+	+	5.7 ± 1.3	+	+
NUF434	Japanese flounder	Nagasaki/1989	Rhizoid	+	+	5.4 ± 1.9	+	+
NUF492	Japanese flounder	Kumamoto /1991	Rhizoid	+	+	5.3 ± 1.0	+	+
NUF493	Japanese flounder	Kumamoto/1991	Rhizoid	+	+	4.6 ± 0.8	+	+
NUF684	Japanese flounder	Nagasaki/1993	Rhizoid	+	+	4.9 ± 0.8	+	+
NUF685	Japanese flounder	Nagasaki/1993	Rhizoid	+	+	4.9 ± 1.1	+	+
NUF686	Japanese flounder	Nagasaki/1993	Rhizoid	+	+	4.9 ± 1.2	+	+
NUF1035	Japanese flounder	Nagasaki/2006	Rhizoid	+	+	4.4 ± 0.9	+	+
NUF1081**	Puffer fish	Nagasaki/2009	Rhizoid	+	+	5.5 ± 1.4	+	+
NUF1126	Japanese flounder	Nagasaki/2009	Rhizoid	+	±	4.6 ± 1.1	+	+
NUF1127	Japanese flounder	Nagasaki/2009	Rhizoid	+	+	4.7 ± 0.8	+	+
NUF1128	Puffer fish	Nagasaki/2010	Rhizoid	+	+	5.2 ± 1.0	+	+
NUF1129	Puffer fish	Nagasaki/2010	Compact	–	–	2.4 ± 0.5	+	+
NS110	Puffer fish	Nagasaki/2010	Rhizoid	+	+	4.5 ± 1.4	+	+

NUF = Nagasaki University Fisheries, NS = Nagasaki-ken Suisan-shikenjo (Nagasaki Prefectural Institute of Fisheries).
* +, adherent; ±, weakly adherent; –, non-adherent; **NUF1081 was used for preparing the rabbit antiserum against formalin-killed cells used in this study.

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 7.9×10^4 to 7.9×10^7 CFU/mL and 2.8×10^5 to 2.8×10^8 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 4.0×10^3 to 4.0×10^6 CFU/fish and 1.4×10^4 to 1.4×10^7 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_{50} value was calculated using the method described by Reed and Muench (1938).

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 5.5×10^6 CFU/mL of NUF1128 or 2.7×10^7 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^2, 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^2 were calculated.

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.

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% NaN_3 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).

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).

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% $HgCl_2$ 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.

Results

Biological and morphological characteristics

Single cells of the strain NUF1128 were slender rods (Fig. 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. 1b) but the shortest (2.4 ± 0.5 μm) in length among the strains studied (Table 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. 1c), but colonies of the non-gliding strain NUF1129 appeared as slightly yellowish, round, convex ones on TYS agar plates (Fig. 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. 2a), while NUF1129 was found as non-adherent with homogenous bacterial suspension (Fig. 2b).

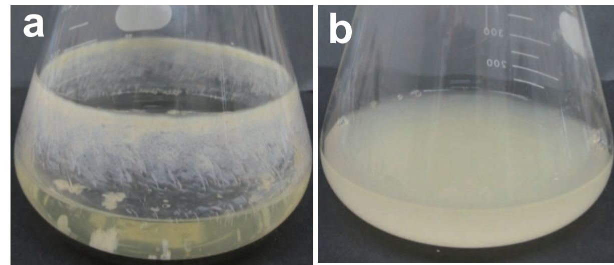

Fig. 2. Adhesiveness of *T. maritimum* NUF1128 (a) and NUF1129 (b) to glass wall of conical flasks grown at 27°C for 24 h with shaking in TYS broth. Note; NUF1128 was adhesive and showed aggregated growth, whereas NUF1129 was non-adhesive and produced homogenous suspension.

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 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 2).

Adherence to body surface

The results of the *in vivo* adhesion assay are shown in Table 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.

Serological characteristics

Using anti-*T. maritimum* NUF1081 serum, the microtiter agglutination test resulted in low titer (2^4) with

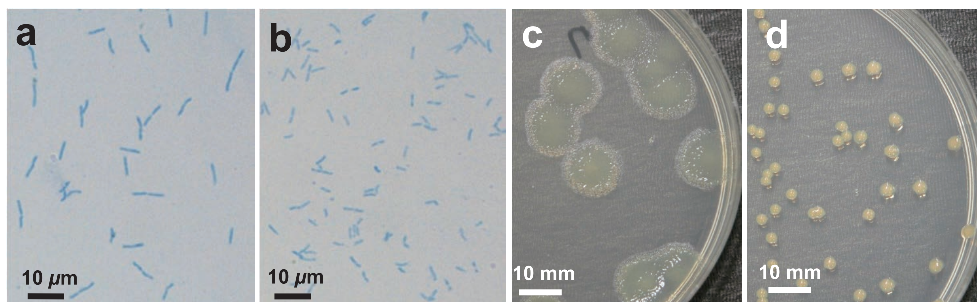

Fig. 1. Photomicrographs of *T. maritimum* NUF1128 (a) and NUF1129 (b) cells grown at 27°C for 24 h in TYS broth and colonies of NUF1128 (c) and NUF1129 (d) grown at 27°C for 36 h on TYS agar plates. Note; NUF1129 cells were shorter than NUF1128 cells, and NUF1128 exhibited rhizoid colonies while NUF1129 produced round compact ones.

Table 2. Virulence of *T. maritimum* NUF1128 and NUF1129 studied in Japanese flounder

Strain	Challenge method	Challenge dose (CFU/mL or fish)	No. of dead fish during the periods of (n = 5)				Mortality (%)	LD_{50} (CFU/mL or fish)
			0–1 d	2–3 d	4–6 d	7–15 d		
NUF1128 (Gliding strain)	Immersion	7.9×10^7	3	2	–	–	100	$10^{6.0}$
		7.9×10^6	0	3	2	–	100	
		7.9×10^5	0	0	1	1	40	
		7.9×10^4	0	0	0	0	0	
	Subcutaneous injection	4.0×10^6	0	0	0	0	0	$> 10^{7.1}$
		4.0×10^5	0	0	0	0	0	
		4.0×10^4	0	0	0	0	0	
		4.0×10^3	0	0	0	0	0	
NUF1129 (Non-gliding strain)	Immersion	2.8×10^8	0	0	0	0	0	$> 10^{8.9}$
		2.8×10^7	0	0	0	0	0	
		2.8×10^6	0	0	0	0	0	
		2.8×10^5	0	0	0	0	0	
	Subcutaneous injection	1.4×10^7	0	0	0	0	0	$> 10^{7.6}$
		1.4×10^6	0	0	0	0	0	
		1.4×10^5	0	0	0	0	0	
		1.4×10^4	0	0	0	0	0	

Table 3. Adhesion of *T. maritimum* NUF1128 and NUF1129 to the body surface of Japanese flounder*

Fish no.	Viable count of NUF1128 (CFU/cm^2)		Viable count of NUF1129 (CFU/cm^2)	
	Non-abraded region	Abraded region	Non-abraded region	Abraded region
1	2.6×10^3	3.4×10^3	$< 1.0 \times 10$	1.4×10^2
2	6.2×10^2	1.3×10^3	$< 1.0 \times 10$	5.2×10^3
3	3.4×10^2	1.8×10^3	$< 1.0 \times 10$	2.0×10^3
4	4.4×10^2	6.8×10^2	$< 1.0 \times 10$	1.2×10^3
5	3.8×10^2	1.8×10^2	4.0×10	4.0×10

* Fish were partially abraded and immersed in seawater containing 5.5×10^6 CFU/mL of NUF1128 or 2.7×10^7 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.

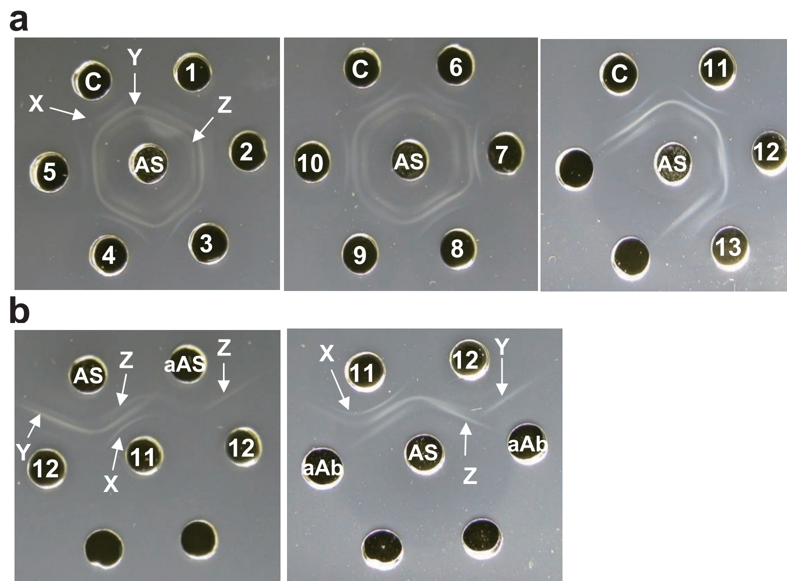

Fig. 3. Gel immunodiffusion analysis of cellular antigens of *T. maritimum* strains with rabbit antiserum (a) and with absorbed antiserum and adsorbed antibodies (b). AS, anti-NUF1081 FKC serum; aAS, anti-NUF1081 FKC serum absorbed with NUF1081 FKC; aAb, antibodies adsorbed to NUF1081 FKC; 1~13 and C, 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.

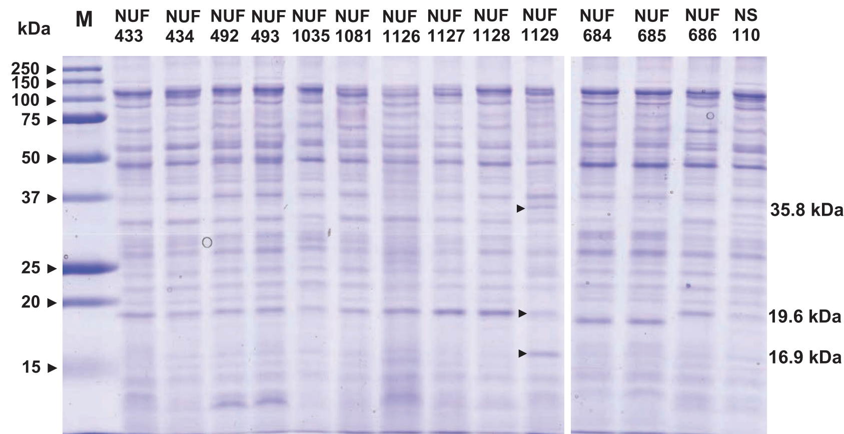

Fig. 4. SDS-PAGE analysis of whole cell proteins of *T. maritimum* strains stained with Coomassie Brilliant Blue R-250. M, molecular weight markers (Bio-Rad).

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-*T. maritimum* NUF1081 serum and sonicated cell extracts of the *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. 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. 3b).

Polypeptide profile

SDS-PAGE separation of the sonicated cell extracts revealed that all the *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. 4).

Chondroitinase and gelatinase activities

All the *T. maritimum* strains including NUF1129 showed positive for chodroitinase and gelatinase activities (Table 1). No clear zone around the colony was observed in the case of negative control.

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.* (2006), 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 (Faílde *et al*., 2013) 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 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. 3a). Interestingly, NUF1126 also lacked this antigen (Fig. 3a). NUF1126 exhibited gliding motility and formed rhizoid colonies, but was less adhesive to glass wall as NUF1129 (Table 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 a candidate for the antigen X, could 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). 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 and adherence and ultimately influence the virulence in fish. To confirm this, further studies are necessary to characterize the interesting cell-surface components.

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