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Tenacibaculum sp. associated with winter ulcers in sea-reared Atlantic salmon *Salmo salar*

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ABSTRACT: Coldwater-associated ulcers, i.e. winter ulcers, in seawater-reared Atlantic salmon *Salmo salar* L. have been reported in Norway since the late 1980s, and *Moritella viscosa* has been established as an important factor in the pathogenesis of this condition. As routine histopathological examination of winter ulcer cases in our laboratory revealed frequent presence in ulcers of long, slender rods clearly different from *M. viscosa*, a closer study focusing on these bacteria was conducted. Field cases of winter ulcers during 2 sampling periods, 1996 and 2004–2005, were investigated and long, slender rods were observed by histopathological examination in 70 and 62.5% of the ulcers examined, respectively, whereas cultivation on marine agar resulted in the isolation of yellow-pigmented colonies with long rods from 3 and 13% of the ulcers only. The isolates could be separated into 2 groups, both identified as belonging to the genus *Tenacibaculum* based on phenotypic characterization and 16S rRNA sequencing. Bath challenge for 7 h confirmed the ability of Group 1 bacterium to produce skin and cornea ulcers. In fish already suffering from *M. viscosa*-induced ulcers, co-infection with the Group 1 bacterium was established within 1 h. Ulcers from field cases of winter ulcers and from the transmission experiments tested positive by immunohistochemistry with polyclonal antiserum against the Group 1 bacterium but not the Group 2 bacterium. Our results strongly indicate the importance of the Group 1 bacterium in the pathogenesis of winter ulcers in Norway. The bacterium is difficult to isolate and is therefore likely to be underdiagnosed based on cultivation only.

KEY WORDS: Winter ulcers · *Tenacibaculum* · Atlantic salmon · Transmission experiment · *Moritella viscosa* · Cod · Halibut

INTRODUCTION

The occurrence of winter ulcers, i.e. coldwater-associated skin lesions in Atlantic salmon *Salmo salar* L., usually seen at sea temperatures below 8°C, is currently a significant bacterial disease problem in salmon farming in Norway. Although cumulative mortalities are usually below 10%, a large proportion of the fish on a site may be affected and there are often considerable economical losses due to downgrading at slaughter. As fish may exhibit extensive ulceration over extended periods, winter ulcers are also creating an important fish welfare concern. While aetiology and pathogenesis are not fully elucidated, the bacterium *Moritella viscosa* (prev. *Vibrio viscosus*) is considered to be an important factor in the pathogenesis of winter ulcers in Norway, Iceland and Scotland (Lunder et al. 1995, Benediktsdóttir et al. 1998, Bruno et al. 1998). *M. viscosa* has been shown to cause skin ulcers, septicaemia and mortality in transmission experiments (Lunder et al. 1995, Løvoll et al. 2009). Despite the availability and widespread use of vaccines against *M. viscosa*, winter ulcers remains a significant threat. *Vibrio wodanis* is also frequently isolated from fish with

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winter ulcers (Lunder et al. 1995, Benediktsdóttir et al. 1998); however, demonstration of pathogenicity for this bacterium under experimental conditions has so far been unsuccessful (Lunder et al. 1995). Many culture-based investigations of winter ulcers have resulted in the identification of mixed cultures of mainly *Vibrio* spp. (Benediktsdóttir et al. 1998, H. Nilsen & A. B. Olsen unpubl.).

Worldwide, members of the *Cytophaga-Flavobacterium-Bacterioides* group are reported to cause mortality and economical losses because of skin ulcers and eroded fins of both freshwater and seawater fish species (Wakabayashi et al. 1986, Kent et al. 1988, Bernardet 1989, Bernardet & Kerouault 1989, Bernardet et al. 1990). The bacteria appear as yellow colonies with long, slender filamentous rods and are relatively difficult to culture. Four species are considered pathogenic to salmonids: *Flavobacterium branchiophilum* (bacterial gill disease in freshwater), *F. columnare* (freshwater above 14°C), *F. psychrophilum* (mainly rainbow trout in freshwater) and *Tenacibaculum maritimum* (prev. *Flexibacter maritimus*) in seawater (Borg 1960, Wakabayashi et al. 1986, 1989, Kent et al. 1988, Bernardet 1989, Bernardet & Grimont 1989, Bernardet & Kerouault 1989, Bernardet et al. 1990). *T. maritimum* has never been isolated in Norwegian ulcer cases.

Over the years, at the National Veterinary Institute Bergen (NVIB), routine histopathological examination of skin ulcers in sea-farmed Atlantic salmon during the winter season has revealed the presence of long, slender bacteria appearing either alone or as a mixed infection with *Moritella viscosa* and/or other bacteria. The aim of the present study was to identify — through microbiological, histopathological, immunohistochemical and transmission studies — the importance of these bacteria for the development of winter ulcers in Atlantic salmon.

MATERIALS AND METHODS

Sampling. Samples for pathological, histopathological and microbiological investigation of skin ulcers in sea-reared Atlantic salmon were collected from January to April 1996 and between December 2004 and April 2005 (Table 1). During the 1996 sampling, field veterinarians were asked to submit fish and formalin-fixed tissues for standardized laboratory examination. Samples representing 18 outbreaks of winter ulcers were submitted by 13 fish health practitioners. During 2004–2005, 2 outbreaks of winter ulcers were each sampled 3 times between December and April. Sampling was performed in these cases by staff of the NVIB.

For comparative purposes, 8 historical cases were included (Table 2), comprising samples for histopatho-

Table 1. *Salmo salar*. Results from histopathological and immunohistopathological investigation of winter ulcers in Atlantic salmon with focus on the detection of long, slender rods during 2 sampling periods. IHC: immunohistochemistry; n: total number investigated

Year	Month	Fish weight (kg)	Winter ulcer outbreaks (n)	Skin ulcers with long, slender rods detected by histopathology (%)	IHC-positive (%) Group 1 antiserum	IHC-positive (%) Group 2 antiserum
1996	Jan–Apr	0.5–3.0	18	70[a] (n = 56)	100 (n = 18)	0 (n = 18)
2004–2005	Dec–Apr	0.5–3.7	2[b]	62.5 (n = 32)	100 (n = 13)	0 (n = 13)

[a]Representing all 18 outbreaks. [b]Each outbreak was sampled 3 times (Outbreak 1: Dec, Feb, Apr; Outbreak 2: Feb, Mar, Apr)

Table 2. *Salmo salar*, *Hippoglossus hippoglossus*, *Gadus morhua* and *Oncorhynchus mykiss*. Results of immunohistochemical investigation of historical samples with long, slender rods detected by histopathology. IHC: immunohistochemistry; +: positive result; –: negative result; nt: not tested

Year	Case	Species	Organ	Samples investigated	IHC Group 1 antiserum	IHC Group 2 antiserum	IHC *Moritella viscosa* antiserum
1988	F1409/88	Atlantic salmon	Skin ulcer	1	+	nt	nt
2000	F41/00	Atlantic salmon	Gill[a]	1	+	–	–
2001	F187/01	Atlantic salmon	Dorsal fin	1	+	–	–
2002	F48/02	Atlantic salmon	Eye (cornea)	1	+	–	–
2002	F101/02	Halibut	Skin ulcer	2	+	–	–
2005	F119/05	Atlantic cod	Skin ulcer	1	+	–	–
2005	F90/05	Rainbow trout	Skin ulcer	1	+	–	–

[a]The main infection in gill was another bacterium

logical examination of gill, eye and dorsal fin of Atlantic salmon and skin ulcers from Atlantic salmon diagnosed with winter ulcers in 1988, rainbow trout *Oncorhynchus mykiss* brood fish, Atlantic cod *Gadus morhua* and halibut *Hippoglossus hippoglossus*. Additional bacterial isolates were collected from historical winter ulcer cases submitted to the laboratory at NVIB by field veterinarians.

Bacteriology. For bacteriological examination, samples from kidney and the border of skin ulcers were inoculated onto blood agar (4% bovine/ovine blood) (BA), blood agar supplemented with 1.5% NaCl (BAS) and marine agar (Difco Marine Agar 2216) (MA). The plates were incubated for 7 d, BA at 22°C and MA and BAS at 15°C.

Yellow colonies on MA were examined by phase-contrast microscopy. Colonies consisting of long, slender rods were further characterized for the presence of pigment, colony and cell morphology and Gram-stain affinity. The bacteria were tested for growth on BA, BAS and Anacker and Ordal's medium (Anacker & Ordal 1959). Growth temperatures were tested by incubation on MA at 4, 15, 22 and 30°C. Anaerobic growth was tested on MA incubated for 5 d at 22°C using the GENbox test system (bioMérieux). Catalase production was tested using 10% H_2O_2, and the presence of cytochrome oxidase was demonstrated using the filter paper strip test (Merck). The presence of flexirubin-type pigments was demonstrated by covering the colonies with 10% KOH solution (Fautz & Reichenbach 1980). Isolates were examined for an extracellular galactosamine glycan by flooding colonies with a 1% Congo-red solution (Johnson & Chilton 1966). API ZYM strips (bioMérieux), incubated at 22°C for 18 to 20 h, were used to test 19 enzymatic activities. Identification of *Moritella viscosa* was performed according to standard National Veterinary Institute (NVI) procedures.

16S rRNA gene sequencing. Nearly complete 16S rRNA genes were amplified using PCR and primers described by Weisburg et al. (1991). The amplicon was then sequenced using the same primers and the internal sequencing primers V1 (5′-ACT GCT GCC TCC CGT-3′), V2 (5′-CTA CCA GGG TAT CTA ATC-3′), V3 (5′-GTA GTC CAC GCC GTA AAC G-3′), V4 (5′-GTT TAT CAC CGG CAG TCT C-3′), V5 (5′-GTC CAC ACT CCT ACG GGA GGC-3′) and V6 (5′-GGG GAY GAC GTC AAG TC-3′). A DYEnamic™ ET dye terminator cycle sequencing kit and a MEGABACE 1000 capillary sequencer (Amersham Biosciences) were used. Contiguous sequences were assembled, aligned and compared using Sequencher 4.5 (Gene Codes).

Phylogenetic analysis. The obtained sequences were compared with existing sequences in GenBank using BLAST search analysis (Altschul et al. 1990). The obtained sequences and sequences accessed from GenBank were then aligned in Clustal X (Thompson et al. 1997) and neighbour-joining (Kimura 2-parameter) analysis was performed in PAUP* 4.0 (Swofford 2000). Missing and ambiguous bases were excluded. Bootstrap confidence values were obtained with 1000 resamplings.

Antisera. Antisera were raised in gray chinchilla rabbit against representatives of subsequently described Groups 1 (isolate F140/96) and 2 (isolate F95C/98). The isolates were grown in marine broth (Difco Marine Broth 2216) (MB) and incubated at 15°C for 2 d. The harvested cells were washed 3 times in phosphate-buffered saline (PBS), pH 7.3, resuspended in PBS and formaldehyde (40%) was added for a final concentration of 0.5%. For the F140/96 isolate, the suspension was incubated at 15°C for 1 h, washed 3 times and then standardized to McFarland standard 3. A volume of 1 ml of the formalin-killed cells emulsified with Freunds Complete adjuvant was injected intravenously on Days 1, 4, and 7 and a volume of 2 ml was injected on Days 12, 15 and 20. Serum was collected on Day 28. For the F95C/98 isolate, the procedure described by Sørensen & Larsen (1986) was followed.

Specificity of antisera. Specificity of the antisera was tested using the slide-agglutination test. The bacteria were mixed with undiluted antiserum on a microscopic slide using a careful rocking motion. A distinct agglutination within 1 min was recorded as positive. Additional bacteria tested with this method were *Tenacibaculum maritimum* (NCIMB 2154), *T. ovolyticum* (NCIMB 13127), *T. gallaicum* (CECT7123), *T. soleae* (CECT7292), *T. discolor* (NCIMB14278), *T. lutimaris* (DSM 16505), *T. skagerrakense* (DSM 14836), *T. mesophilum* (IFO 16307), *T. amylolyticum* (IFO 16310), *Flavobacterium psychrophilum* 386/94, *Moritella viscosa* (88/478), *Vibrio wodanis* (88/441) and *V. anguillarum* (ATCC 14181).

Histopathology. Samples from skin lesions were fixed in 10% buffered formalin and embedded in paraffin, according to standard procedures. Sections (3 to 5 µm) were stained with haematoxylin and eosin (H&E) and using the May-Grünwald-Giemsa (MGG) method.

Immunohistochemistry. Sections of skin lesions revealing long, slender rods were treated with the antisera described, using avidin-biotin alkaline phosphatase procedures. All samples from 1996, the first sampling period, were tested with an avidin-biotin enzyme complex (ABC) method using new fuchsin as the chromogen. All samples collected later and a selection of the 1996 samples were tested with the enzyme-labeled streptavidin procedure (LAB) with fast red as the chromogen. Specifically, after deparaffinization (and, for the 1996 samples, pre-treatment with metha-

nolic H_2O_2), the sections were overlaid with 5% bovine serum albumin (BSA) for 20 min. The sections were then blotted without washing and incubated with the antiserum, diluted 1:1000, for 30 min at room temperature. The secondary antibody, biotinylated goat anti-rabbit (DakoCytomation E 0432) diluted 1:300, was added after washing in wash buffer. Following 30 min incubation and additional washing, ABC-reagent or streptavidin alkaline phosphatase was added, respectively. Substrate-chromogen solution was applied and, after the development of desired colour, sections were gently rinsed and counterstained with haematoxylin.

Preparations of bacterial cultures for challenge. For challenge, the presumptive *Tenacibaculum* sp. Group 1 isolate F95B/98 and Group 2 isolate F95C/98 were chosen. The *Moritella viscosa* isolate (LF1 5006) was obtained from Atlantic salmon suffering from winter ulcers in sea cages at the Tromsø Aquaculture Research Station.

The bacteria were grown from MB/glycerol culture at –80 °C, *Moritella viscosa* on BAS plates and F95B/98 and F95C/98 on MA. Several pure colonies were inoculated in MB and pre-cultured at 9°C (Expts 1 and 2) or 12°C (Expt 3) for 48 h. Before use in bath challenge experiments, the bacteria were further sub-cultured for 24 h in larger volumes of MB until final absorbance at 600 nm reached 0.6 to 1 (U 1100 spectrophotometer, Hitachi). The cell concentration was determined by counting colony forming units (CFU) using a Hawksley counting chamber (Expts 1 and 2) or manually with a colony counter pen (Expt 3).

Fish. Three batches of smoltified Atlantic salmon of average weight 250 to 300 g were used. The smolts were held in tanks (Expts 1 and 2: 400 l; Expt 3: 250 l) with UV-treated filtered seawater with a salinity of 34.5‰ taken from a depth of 120 m. The fish were exposed to 12 h of artificial light and were fed continuously during the daylight period with commercial feed until they were used in 3 separate challenge experiments. The water temperature was 9.5°C throughout the challenge periods in Expts 1 and 2, and was 12°C in Expt 3. The different groups were tagged by injection of fluorescent dyes (elastomer tagging) above the eye in Expts 1 and 2, or by clipping of the left or right abdominal fin in Expt 3. Prior to tagging and exposure, the fish were anaesthetized using benzocaine in seawater (50 mg l^{-1}). During the period of exposure, the water level was reduced to 100 l, oxygenated and the bacterial cultures were added directly to the water.

Expt 1: Challenge with *Tenacibaculum* spp. Fish with and without the induction of mechanical skin damage were kept in the same tank and were bath-challenged simultaneously with the presumptive *Tenacibaculum* sp. isolates F95B/98 and F95C/98. Prior to bath challenge, 15 of 20 fish were deeply scarified by removing scales covering an area of 1 × 0.5 cm by extensive scraping with a scalpel leaf, approximately 1 cm below dorsal fin. For 7 of the scarified fish, 2 to 3 drops of each bacterial culture were applied on the scarified area for 12 s. The fish were then bath-challenged simultaneously for 1 h with both isolates, F95B/98 (1.2×10^6 CFU ml^{-1}) and F95C/98 (1.8×10^6 CFU ml^{-1}).

Expt 2: Challenge with *Moritella viscosa* and *Tenacibaculum* spp. The purpose of this experiment was to determine whether skin ulcers caused by *M. viscosa* infection had any impact on infection with presumptive *Tenacibaculum* sp. isolates F95B/98 and F95C/98. Atlantic salmon were distributed in 2 tanks of 400 l, 20 fish in each tank, and bath challenged for 1 h with 2 doses (1.3×10^8 and 6.4×10^8 CFU ml^{-1}) of *M. viscosa* LF1 5006. At Day 5 post-challenge, mortality was low in both groups (1 and 5 dead fish in low and high dose, respectively) and the 2 groups were mixed. Fifteen and 11 fish from low and high dose groups, respectively, were transferred to one tank (Tank A) whereas 4 fish from each group were kept in Tank B (mono-infection with *M. viscosa*). At Day 6, the fish in Tank A were bath-challenged for 1 h simultaneously with both F95B/98 (6.4×10^5 CFU ml^{-1}) and F95C/98 (1.2×10^6 CFU ml^{-1}). Twenty non-treated fish were kept in a third tank (Tank C).

Expt 1 lasted for 28 d and Expt 2 lasted for 24 d. Mortality was recorded daily and dead and moribound fish were removed and examined. In Expt 1, the first samples were taken after 4 d (2 fish) and in Expt 2 after 1 d (1 fish). Post-mortem examination was performed and samples were taken for histopathology (normal skin, skin lesions and internal organs) and bacteriology (normal skin, skin lesions and kidney). A selection of skin lesions were tested for bacteria using immunohistochemistry.

Expt 3: Challenge with Group 1 strain of *Tenacibaculum* sp. The purpose of this experiment was to determine whether infection with only the Group 1 F95B/98 isolate of presumptive *Tenacibaculum* sp. and prolonged exposure time produced skin lesions. Prior to bath challenge, 10, 6 and 8 fish were subjected to either deep, superficial or no scarification, respectively. The fish were equally distributed in 2 tanks and fish in Tank 1 were bath challenged for 7 h with the isolate F95B/98 (4.2×10^7 CFU ml^{-1}) whereas fish in Tank 2 constituted non-infected controls. The fish were observed every day for 14 d and abnormal behaviour and external lesions were recorded. From Day 3, one fish in the exposed group was sampled every second day. Post-mortem examination was performed and samples were taken for histopathology (skin lesions and internal organs) and bacteriology (normal skin, skin lesions and kidney,

MA, BA, BAS). At the end of the experiment, all surviving fish in the exposed group were killed and samples were collected. A selection of skin lesions were tested for F95B/98 using immunohistochemistry.

To investigate whether the MB itself affected the fish, remaining individuals from the control group of Expt 3 were divided into 2 groups. Four deeply skin-scarified fish and one non-scarified fish were exposed to sterile MB (3 l/100 l water) for 5.5 h. Three deeply skin-scarified fish and one non-scarified fish served as controls in a separate tank. The fish were kept and observed for 8 d.

RESULTS

Gross pathology

Gross pathological examination of fish collected from field cases during the 2 study periods revealed a prevalence of one ulcer per fish of 71% (53/75 fish from 18 outbreaks) and 74% (28/38), respectively. Otherwise, the fish suffered from multiple ulcers. For a minority of fish (4%), the ulcer included the dorsal fin. In some individuals (13%), erosion of the mouth and head regions was seen.

Isolation of long, slender bacteria

In 1996, investigation of skin lesions by cultivation on MA from a total of 75 fish (18 outbreaks) resulted in isolation of long, slender bacteria from 2 ulcers (3%), one from each of 2 outbreaks, whereas in 2004–2005 such bacteria were isolated from both outbreaks investigated and from 9 of the 25 (36%) ulcers examined by cultivation. The bacteria were not isolated from the kidneys.

Phenotypic characterization of the bacteria

Isolates consisting of long, slender rods on MA could be separated into 2 groups according to colony colour. The first group (Group 1) was moderately yellow in colour whereas the second group (Group 2) displayed bright yellow colonies. In contrast to Group 1 isolates, Group 2 isolates did grow on BAS. No growth on Anacker and Ordal's medium was observed. The bacteria formed thread-like, flexible cells; Group 1 cells measured between 10 and 60 µm whereas Group 2 cells were shorter, 6 to 20 µm. Both presented spherical, degenerative inflations and coccoid bodies with age. The Group 1 and 2 isolates were Gram-negative, strictly aerobic, cytochrome oxydase- and catalase-positive (weak reaction), degraded gelatin and did not have flexirubin-type pigments, but were Congo red positive. They grew well on MA and growth was seen at 4, 15 and 22°C, but not at 30°C. When tested by API ZYM, they were positive for alkaline phosphatase, esterase, esterase lipase, lipase (very weak), leucine arylamidase, valine arylamidase, cystine arylamidase, phosphatase acid, naphtolphospo-hydrolase and negative to trypsine, chymotrypsine, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, β-glucosidase, *n*-acetyl β-glucosaminidase, α-mannosidase and α-fucosidase.

Molecular analysis

The results from the 16S rDNA analysis are summarized in Fig. 1. The 16S rDNA sequences from the NCBI databases most similar to the 2 presumptive *Tenacibaculum* spp. F95B/98 and F95C/98 in the present study all belong to the genus *Tenacibaculum*.

By NCBI BLAST, the 16S rDNA sequence from *Tenacibaculum* sp. F95B/98 had 97% identity with the 16S rDNA sequence from *T. solea* LL04 (AM746476.1) and 96% with *T. ovolyticum* IFO15947 (AB078058) and IFO 15993 (AB032508) as its closest relatives. Similarly, the 16S rDNA sequence of *Tenacibaculum* sp. F95C/98 had 96% identity with the same 3 sequences.

On phylogenetic comparison of the obtained 16S rDNA sequences (Fig. 1), the 7 isolates studied clustered into 2 closely related yet separate clusters with *Tenacibaculum ovolyticum* as their closest known relative, with one cluster containing the 4 isolates belonging to phenotypical Group 1 exclusively, and the second containing Group 2 isolates. Intra-cluster sequence identities in both cases were 99% whereas the 2 novel clusters exhibited sequence identity values of 98% with each other.

The 16S rRNA gene sequences were submitted to GenBank under the following accession numbers: F95B/98, GU124769; F140/96, GU124770; F78-6R/98, GU124767; F112C/98, GU124761; F95C/98, GU124769; F16_98/F98_98, GU124761; and F78-6-ulcer/98, GU 123761.

Histopathology

Histopathological examination revealed the presence of long, slender rods in skin ulcers in all 18 outbreaks investigated in 1996 and at all sample points at both farms sampled during 2004–2005. The prevalence of the bacteria in the ulcers as observed by histopathology was 70 and 62.5% for the 2 sampling periods, respectively (Table 1).

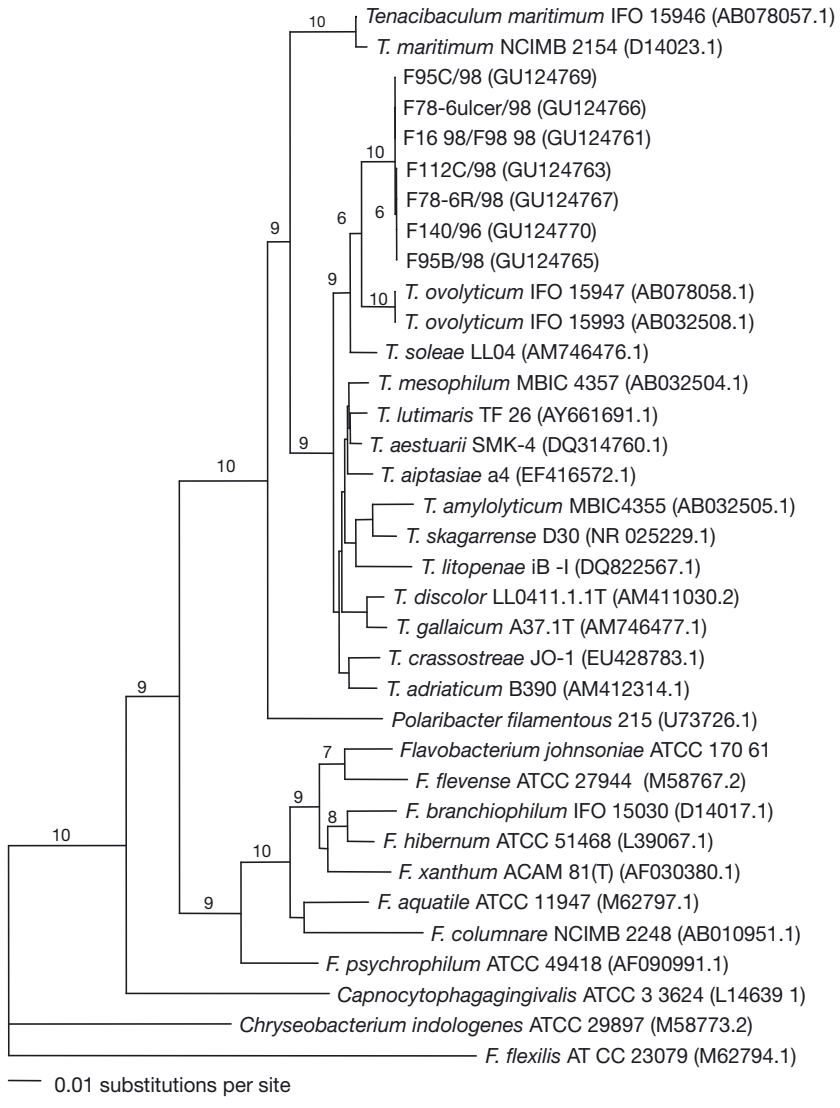


Fig. 1. Phylogenetic tree showing relationships between four Group 1 isolates (F95B/98, F140/96, F78-6R/98 and F112C/98), three Group 2 isolates (F95C/98, F78-6ulcer/98 and F16_98/F98_98) and related organisms based on nearly complete 16S rDNA sequences. Scale bar represents the number of base substitutions per site. Accession numbers for sequences derived from reference strains are in parentheses

The bacteria were only observed in open lesions in which the epidermis was disrupted. The long, slender rods were most often located in the superficial and exposed parts of the lesions. The bacteria appeared to be aligned along the collagen fibres of the dermis and could be present in large numbers. Long, slender rods were also, on occasion, observed extending deeply into intermuscular connective tissue and muscle fibres. Histological examination of skin lesions also revealed small, curved bacteria (possibly *Moritella viscosa*) as a prominent but not consistent finding.

In the areas where the long rods were the dominating bacterium, no inflammatory responses, with the exception of occasional findings consistent with oedema, were seen. In one historical case including damaged eyes (Table 2), long, slender rods were seen to colonise the cornea and were the only bacteria observed.

Specificity of antisera

By slide-agglutination test, there was no cross-reaction between the Group 1- and Group 2-type bacteria or with the panel of bacteria tested.

Immunohistochemistry

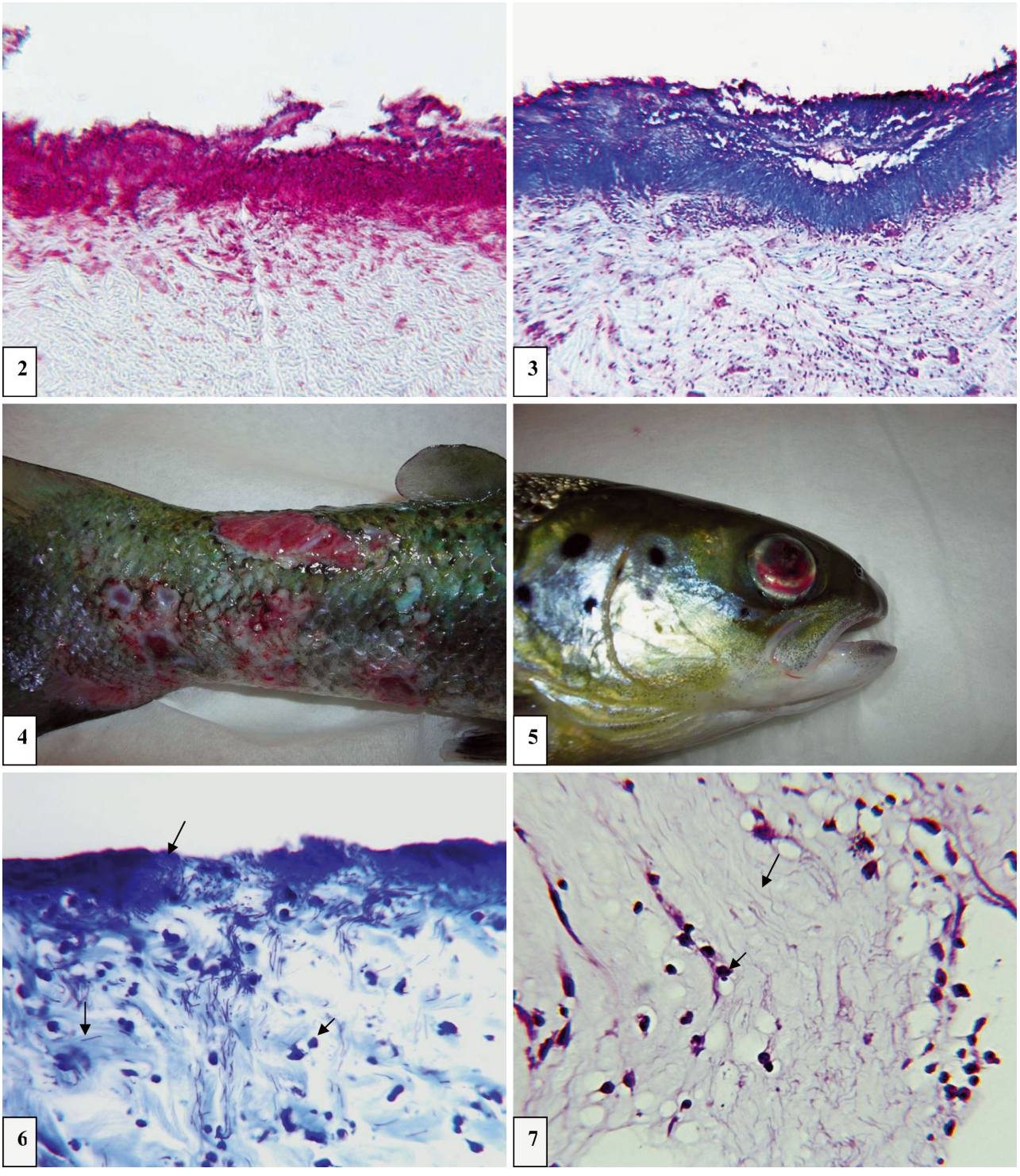
All tissue sections from field cases involving Atlantic salmon, halibut, Atlantic cod and rainbow trout showing long, slender rods stained positive with rabbit antisera against Group 1 (F140/96) but not with antisera against Group 2 (F95C/98), as shown by immunohistochemistry (Tables 1 & 2). Occasionally a small proportion of long rods in an ulcer did not stain with either antisera.

Challenge experiments

In Expt 1, where fish were bath challenged with both F95B/98 and F95C/98, all 15 scarified fish developed an ulcer in the scarified area of 2 to 3 cm in diameter. There was no difference between fish that had their scarified area challenged by drops of bacterial culture before bath challenge and scarified fish that were only bath challenged. None of the fish died. An inflammatory response, but no bacteria, was detected histologically and no antigen was detected with immunohistochemistry against either bacteria. No skin lesions were observed in non-scarified fish.

In Expt 2, 8 of the 26 fish challenged with F95B/98 and F95C/98 6 d following development of *Moritella viscosa*-induced ulcers died. Ulcers from 16 fish were examined.

Histopathological examination revealed the occurrence of long, slender rods in 11 of the 16 ulcers (69%). All of these 11 ulcers were co-infected with small, curved rods, consistent with *Moritella viscosa*. In 3



Figs. 2 to 7. *Salmo salar*. Experimental infection of Atlantic salmon with *Tenacibaculum* sp. Fig. 2. Skin ulcer showing positive immunostaining with antiserum against the Group 1 bacterium (red colour) from fish simultaneously exposed to Group 1 and Group 2 bacterium by bath challenge for 1 h following development of *Moritella viscosa*-induced ulcers (Expt 2) (×400). Fig. 3. Same ulcer immunostained with antiserum against *M. viscosa* (red colour) (×400). Fig. 4. Skin ulcers in tail root area of fish bath-challenged for 7 h with the Group 1 bacterium (Expt 3). Fig. 5. Cornea ulcer and haemorrhage in the eye of fish from Expt 3. Fig. 6. Photomicrograph of skin ulcer from fish in Expt 3, showing infection in dermis with long, slender rods (long arrows) and inflammatory cells (short arrow) (May-Grünwald-Gemsa, MGG, ×400). Fig. 7. Photomicrograph of cornea of the eye from fish in Expt 3 infected with long, slender rods (long arrow) and inflammatory cells (short arrow) (H&E, ×400)

ulcers only, *M. viscosa*-like bacteria were found and, in 2 ulcers, which appeared to be in the reparative stage, no bacteria were detected. By immunohistochemical examination, the long, slender rods stained positively with antisera against the Group 1 bacterium (F95B/98) (Fig. 2), but not with antisera against the Group 2 bacterium (F95C/98). Figs. 2 & 3 illustrate concurrent infection in an ulcer with *M. viscosa* and the Group 1 bacterium. None of the non-exposed fish in Tank C developed ulcers.

In Expt 3, where fish were exposed to bacterium F95B/98 by bath challenge for 7 h, all 5 deeply scarified fish developed an ulcer in the scarified area. The 3 fish with superficial scarification also developed smaller ulcers in the damaged area. Multiple skin ulcers, especially in the caudal area, developed in 1 deeply and 2 superficially scarified fish (Fig. 4). Few to several small haemorrhagic, slightly protruding skin lesions were identified on the lateral surfaces of all but one fish. All deeply scarred fish also developed eye damage with corneal inflammation with part-perforation, bilaterally in 2 fish and unilaterally in 3 fish (Fig. 5). In the first fish examined, 3 d post-challenge, inflammation and long, slender rods positively stained by immunohistochemistry against the Group 1 bacterium and negatively stained against the Group 2 bacterium were seen in cornea and eye lumen, skin lesions on the abdomen and tail root and in the ulcer that developed in the scarified area (Figs. 6 & 7). Severe inflammation, oedema and muscle necrosis were detected in the lesions of all remaining fish, but no bacteria were identified. No fish in the control group or those exposed to MB developed ulcers or corneal pathology.

DISCUSSION

The prominent finding of long, slender bacteria in skin lesions from cases of winter ulcers in Atlantic salmon, as seen by histopathology in the present study, has not previously been reported. Studies based on cultivation alone report *Moritella viscosa* and to some extent *Vibrio wodanis*, as the most important specific bacteriological results related to this condition (Lunder et al. 1995, Benediktsdóttir et al. 1998, Bruno et al. 1998). In those studies, *M. viscosa* was the dominating finding in 30, 40 and 40% of the outbreaks investigated, respectively. This is in accordance with our findings based on cultivation alone, as *M. viscosa* was the most common bacterium isolated in the present study: it was detected in 42 and 48% of the lesions during our 2 sampling periods, respectively (data not shown).

The high prevalence of long, slender rods observed by histopathology compared with the low prevalence of such bacteria isolated, especially in the 1996 study, shows that these bacteria, if present, are likely to be highly underdiagnosed when skin lesions in field cases of winter ulcers are examined by cultivation alone, even with the use of MA.

Our inclusion of MA for isolation, in addition to the media used in previous studies (BA and BAS, Lunder et al. 1995; TSA with 2% NaCl, TSA-NaCl with 10% blood and modified Anacker and Ordal's medium, Bruno et al. 1998; and BAS, Benediktsdóttir et al. 1998), did, to some extent, increase the sensitivity of cultivation as a detection method. During the 2004–2005 study, we improved our isolation rate utilising firm scraping of sample material from the ulcer surface. There is, however, an obvious need for further improvement of both the growth conditions and the method of sampling from fish tissues.

The 2 strains, representing 2 distinct groups of long, slender rods isolated during our study of outbreaks of winter ulcers are consistent phenotypically and genetically with the *Cytophaga-Flavobacterium-Bacterioides* group. In common with many species within the *Flavobacteriaceae*, these bacteria were isolated from an aquatic environment. Both strains are Gram-negative, strictly aerobic, long rods and grow as yellow-pigmented colonies on MA. They form threadlike flexible cells and present spherical degenerative inflations and coccoid bodies with age. More specifically, the available phenotypic evidence is supported by the genetic testing and indicates that, although the studied isolates belong to the genus *Tenacibaculum* (Suzuki et al. 2001), they differ sufficiently from previously described *Tenacibaculum* spp. (Hansen et al. 1992, Suzuki et al. 2001, Frette et al. 2004, Yoon et al. 2005, Jung et al. 2006, Sheu et al. 2007, Heindl et al. 2008, Piñeiro-Vidal et al. 2008a,b, Wang et al. 2008, Lee et al. 2009) and may constitute 2 new species or subspecies within this genus.

When field ulcers with long, slender rods were tested by immunohistochemistry with antisera raised against Group 1 and Group 2 bacteria, positive staining against the Group 1 bacterium was identified in all samples investigated, whereas positive staining against the Group 2 bacterium was never observed. This indicated that Group 1 isolates may be of importance in the development of winter ulcers whereas Group 2 appear to be of no or minor significance. The lack of Group 2 bacterium could be explained by poor invasive properties. This hypothesis was subsequently confirmed in our challenge experiments. Following challenge with a suspension containing both bacteria, only the Group 1 bacterium was detected in ulcers. Our experiment with the Group 1 bacterium alone also confirmed this bacterium as a causative agent of skin and corneal ulcers.

The specificity of our polyclonal antiserum may be questioned, and possible cross-reactions with related bacteria must be taken into account, but in the present case the infection was reproduced experimentally and was therefore unlikely to have been affected by the presence of a cross-reacting contaminating bacterium. In addition, no cross-reaction was observed when a panel including different members of the genus *Tenacibaculum* was tested by slide-agglutination.

Although skin ulcers were produced following the 1 h bath challenge, they were only observed in the scarified area. Prolonged exposure, i.e. 7 h, to the Group 1 bacterium did, however, result in the development of multiple ulcers and keratitis. A higher dose (4.2×10^7 CFU ml^{-1} compared with 1.2×10^6 CFU ml^{-1}) and/or higher water temperature (12°C compared with 9.5°C) could also have contributed to the result, but increased immersion time is in accordance with the recommendation by Avendaño-Herrera et al. (2006) for successful challenge with *Tenacibaculum maritimum* (increasing from 1–2 h to 18 h in their experiments with turbot). It may be speculated that the accumulation of large numbers of bacteria and the subsequent development of a biofilm on the skin surface is necessary before tissue damage and infection can occur (Avendaño-Herrera et al. 2006).

An important finding in the present study was that *Moritella viscosa*-related skin lesions appear to represent a pre-disposing factor for infection with Group 1 bacterium, as immersion for 1 h was sufficient to produce relatively heavy infections with this bacterium in such lesions. It was also in this experiment (Expt 2) that the redetection of the Group 1 bacterium with time was most consistent. This could be due to the facilitated access to favourable tissues for infection and proliferation of the Group 1 bacterium, but there is also a possibility of a synergistic relationship between these 2 bacteria, which should be looked into further.

Two findings could indicate a more effective inflammatory antigen-eliminating reaction in the cases when *M. viscosa* was absent. (1) No antigen was redetected after 4 d in ulcers that were produced by the 1 h challenge with both Group 1 and Group 2 bacteria (Expt. 1). (2) There was a decrease in bacteria over time in those ulcers produced with the Group 1 bacterium only (Expt. 3). This may also point towards the involvement and importance of exotoxins in the primary development of lesions.

The histopathological finding identified in the present study, in natural as well as experimental cases, resembled those described by Handlinger et al. (1997) in relation to natural infection with *Tenacibaculum maritimum*, a bacterium which has never been reported in Norway. As for *T. maritimum*, the long rods in the Norwegian cases may occur as a mat on the eroded surface of the ulcer and appear to have an affinity for collagenous tissue, e.g. dermis and intermuscular connective tissue. Orientation parallel to the collagen bundles is also described for the saddles produced by *Flavobacterium columnare* (prev. *Flexibacter columnaris*) (Morrison et al. 1981). In the present study, the affinity for collagen was also seen as infection in the collagenous cornea of the eye.

With regards to possible virulence factors of the Group 1 bacterium, the results so far are limited. Like most bacteria in the *Cytophaga-Flavobacterium-Bacteroides* complex, they have proteolytic properties, and identification of large numbers of cells in collagenous tissue also indicates collagenophilia. A collagen-binding adhesin was shown to be a virulence factor for some strains of *Staphylococcus aureus* causing keratitis in de-epithelialized corneas in a rabbit model (Rhem et al. 2000). Whether a similar mechanism is important for the virulence of our bacterium is not known. The observed degeneration and necrosis in white muscle may be related to bacterial toxic components. The histopathological results as described revealed some very interesting features of the bacterium that should be further investigated to elucidate the true nature of the infection.

Although confirmed as primary pathogens, natural infection by *Tenacibaculum maritimum* and *Flavobacterium columnare* seems to be seen only after abrasion of the skin surface. It should be noted that *Moritella viscosa* is also generally absent in early skin lesions with intact epidermis (data not shown) (Salte et al. 1994). Likewise, in our field cases the long, slender rods were only seen in open lesions with lost epidermis and were never detected on or in the epidermis in the periphery of the ulcer.

The exact pathogenic role of our bacterium for the development of winter ulcers has yet to be determined. The bacterium may mainly contribute to the production of ulcers in already mechanically abrated skin, due to handling, etc., or as a secondary infection in e.g. *Moritella viscosa*-induced skin lesions and thereby contribute to the aggravation of the ulcer development and probably also prevent healing. The Group 1 bacterium should, however, also be taken into account as a primary pathogen under field conditions, as we have shown that the virulence of the bacterium may overwhelm the defence of the host and produce ulcers of skin and eye.

The inclusion of a Group 1-positive skin ulcer from an outbreak in 1988 supports the finding that this bacterium is widely spread geographically within the area investigated over a considerable period of time. That Group 1 bacteria were also found in skin ulcers of cod, halibut and rainbow trout shows that a range of fish species may be affected and that these bacte-

ria may also be of importance in the development of ulcers in these species. This should be investigated further.

In conclusion, the high prevalence of large numbers of Group 1 bacteria within skin lesions in Atlantic salmon suffering from winter ulcers and the experimental reproduction of infection with this bacterium strongly indicate the importance of this group in the pathogenesis of winter ulcers in Norway. The available data indicate that the 2 groups of bacteria identified in the present study are phenotypically and genetically different and may constitute previously undescribed species or sub-species within the genus *Tenacibaculum*. The work of precise taxonomic placement of these 2 groups of bacteria is now under way.

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