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Previously unrecognised division within *Moritella viscosa* isolated from fish farmed in the North Atlantic

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ABSTRACT: Previously undocumented phenotypical and genetic variation was identified amongst isolates of *Moritella viscosa* collected from various geographical locations and from different fish species. The studied isolates could be split into 2 major phenotypically and genetically different clusters, one of which was consistent with the species type strain (NCIMB 13548). Isolates consistent with the type strain originated exclusively from Atlantic salmon farmed in Norway, Scotland and the Faroe Isles, although a single isolate from farmed Norwegian cod clustered closely with this group. The 'variant' cluster comprised isolates originating from Norwegian farmed rainbow trout, Icelandic farmed rainbow trout and salmon, Canadian farmed (Atlantic) salmon, Icelandic lumpsucker and only exceptionally from Norwegian salmon. With the exception of the single aforementioned cod isolate, all isolates from Norwegian farmed cod belonged to the variant cluster. Phenotypically, the clusters could be absolutely separated only by elevated haemolytic activity in the variant strain, although approximately half of these isolates also produced acid from mannose, in contrast to the typical (type) strain. While 16S *rRNA* gene sequencing was unable to separate the 2 clusters, Western blot analyses, plasmid profile analysis, pulsed field gel electrophoresis and *gyrB* gene sequence analysis produced clusters consistent with the phenotypic data. Macroscopically and histologically the disease in rainbow trout caused by the variant strain was consistent with that previously described in Atlantic salmon. The results of the present study may indicate a degree of host specificity of the typical strain for Atlantic salmon.

KEY WORDS: Rainbow trout · Atlantic salmon · *Oncorhynchus mykiss* · *Salmo salar* · *Gadus morhua* · *Moritella viscosa* · *Cyclopterus lumpus* · Winter-ulcer

INTRODUCTION

Moritella viscosa is believed to be the dominating aetiological agent of 'winter-ulcer' in Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* farmed in the Northern Atlantic area. Despite the widespread availability of commercial *M. viscosa* vaccines providing high levels of protection against experimental challenge, and increasing levels of field protection, winter-ulcer continues to be the major bacteria-related loss in Norwegian salmonid farming.

Although first reported in Norway (Lunder 1992), the bacterium has also been isolated regularly from diseased farmed salmonids in Iceland (Benediktsdóttir et al. 1998) and less frequently in the Faroe Islands (Inger Dalsgaard pers. comm.), Scotland (Bruno et al. 1998) and Canada (Whitman et al. 2000). While Atlantic cod *Gadus morhua* have been shown to be experimentally

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susceptible to *Moritella viscosa* infection (Gudmundsdóttir et al. 2006) and the bacterium has also been isolated from farmed cod (Colquhoun et al. 2004), captive wild-caught plaice *Pleuronectes platessa* (Lunder et al. 2000) and lumpsucker *Cyclopterus lumpus* (Benediktsdóttir et al. 2000), it is not yet clear whether it can be considered a true disease agent in these species. In experimental challenges, turbot *Scophthalmus maximus* and, to a lesser degree, Atlantic halibut *Hippoglossus hippoglossus* have been shown to be susceptible to infection with *M. viscosa* (Björnsdóttir et al. 2004, Gudmundsdóttir et al. 2006).

A degree of geographically based amplified fragment length polymorphism (AFLP) clustering within *Moritella viscosa* isolates has been demonstrated (Benediktsdóttir et al. 2000) and the existence of 4 different serotypes was recently shown (Heidarsdóttir et al. 2008). However, until the present study the Norwegian *M. viscosa* population had been considered relatively homogeneous, an assumption probably based on lack of evidence to the contrary, rather than direct evidence of homogeneity.

The present study was initiated against a background of systemic *Moritella viscosa* infections in farmed rainbow trout in Norway which, in recent years, have occurred with irregular frequency but high virulence and associated mortalities. As *M. viscosa* isolated from these outbreaks appeared to differ slightly phenotypically from those normally isolated from Atlantic salmon in Norwegian waters, the present study was instigated to survey heterogeneity amongst Norwegian *M. viscosa*, with focus on both 'typical' isolates from salmon and the increasing yet still relatively small number of 'atypical' isolates archived from rainbow trout, cod and also exceptionally from Atlantic salmon. For comparative purposes, representative isolates from diverse geographical origins and host species were included.

MATERIALS AND METHODS

Bacterial isolates and maintenance. Norwegian isolates were collected as submissions to the clinical diagnostic bacteriology service provided by the National Veterinary Institute. Isolates from Canada, Iceland, Scotland and the Faroe Islands were kindly donated by various researchers (Table 1).

Phenotypical characterization. Cells from cultures grown for 48 h at 15°C on blood agar with 2% NaCl (BAS) were used in all tests. Unless otherwise noted, the final NaCl content of test media was 2%. The studied isolates were subjected to the standard (limited) range of phenotypical tests currently used in the identification of *Moritella viscosa* in our laboratory, comprising description of colony morphology and haemolysis on BAS; Gram stain; motility; oxidative/fermentative production of acid from glucose; production of gas from glucose; sensitivity to vibriostat (O/129); production of catalase; production of arginine dihydrolase, lysine- and ornithine decarboxylases (Møller 1955); production of gelatinase; pro-

Table 1. Bacterial strains in *Salmo salar, Oncorhynchus mykiss, Gadus morhua and Cyclopterus lumpus*. NVI: National Veterinary Institute, Norway; JCM: Japan Collection of Microorganisms; NCIMB: National Collection of Industrial Marine Bacteria

Strain designation	Species isolated from	Geographical origin
Moritella viscosa		
NCIMB 13584	*S. salar*	Norway
NVI 2989	*S. salar*	Norway
NVI 6216	*S. salar*	Norway
NVI 1527	*S. salar*	Norway
NVI 4397	*S. salar*	Norway
NVI 5443	*S. salar*	Norway
NVI 6562	*S. salar*	Norway
NVI 4679	*S. salar*	Norway
NVI 3999	*S. salar*	Norway
NVI 4179	*S. salar*	Norway
NVI 4731	*S. salar*	Norway
NVI 5433	*S. salar*	Norway
LFI 5006[a]	*S. salar*	Norway
990129-1/3B[b]	*S. salar*	Faroe Islands
990217-1/1A[b]	*S. salar*	Faroe Islands
MT 2858[c]	*S. salar*	Scotland
MT 2528[c]	*S. salar*	Scotland
K2[d]	*S. salar*	Iceland
K58[d]	*S. salar*	Iceland
F153[d]	*S. salar*	Iceland
K56[d]	*S. salar*	Iceland
Vvi-7	*S. salar*	Canada
Vvi-11	*S. salar*	Canada
NVI 5168	*O. mykiss*	Norway
NVI 4917	*O. mykiss*	Norway
NVI 4692	*O. mykiss*	Norway
NVI 4958	*O. mykiss*	Norway
NVI 5450	*O. mykiss*	Norway
NVI 5683	*O. mykiss*	Norway
NVI 6185	*O. mykiss*	Norway
NVI 3968	*O. mykiss*	Norway
NVI 6184	*O. mykiss*	Norway
NVI 4869	*O. mykiss*	Norway
F162/01[d]	*O. mykiss*	Iceland
NVI 5471	*G. morhua*	Norway
NVI 5507	*G. morhua*	Norway
NVI 5204	*G. morhua*	Norway
NVI 4547	*G. morhua*	Norway
NVI 5482	*G. morhua*	Norway
F57[d]	*C. lumpus*	Iceland
M. japonica JCM 10249	Seawater	Japan
M. marina NCIMB 1144	Seawater	?

Strains kindly donated by:
[a]Nofima marine Tromsø; [b]Dr. Inger Dalsgaard, Technical University of Denmark; [c]Dr. David Bruno, FRS Marine Laboratory, Aberdeen, Scotland; [d]Dr. Eva Benediktsdóttir, University of Reykjavik, Iceland

duction of acid from cellobiose, mannose and trehalose; and growth at 4°C and 30°C. All test media with the exception of those used to determine temperature range were incubated at 15°C. Otherwise, biochemical characterisation was carried out using standard methodology. Arginine dihydrolase and lysine- and ornithine decarboxylase tests were observed for 7 d.

Pulsed field gel electrophoresis (PFGE). Bacterial isolates were grown on blood agar supplemented with 2% NaCl for 2 d at 15°C. The gel plugs were made as described by Martinez-Urtaza et al. (2004), with the following modification: the cell concentration was decided using a spectrophotometer, and the optical density was adjusted to 1.16 to 1.39 (at 610 nm). A small (approximately 4 × 5 mm) portion of the plug was first incubated in *Not*I buffer (New England Biolabs) for 1 h at 37°C. The buffer was then replaced by fresh buffer containing 20 U *Not*I and incubated for 3 h at 37°C. Restriction fragments were subjected to electrophoresis in 1% agarose (SeaKem Gold Agarose, Cambrex) gels in 0.5× Tris-Borate-EDTA (TBE) buffer using a CHEF-DR III system (Bio-Rad). *Salmonella* Braenderup H9812 digested with 20 U *Xba*I (New England Biolabs) at 37°C for 3 h was used as a marker. Running conditions were 6 V cm^{-1} at a field angle of 120°, at 12°C for 22 h. Pulse times were increased step-wise as follows: 5 s for 4 h, 9 s for 4 h, 12 s for 4.5 h, 20 s for 4.5 h, 25 s for 2.5 h and 30 s for 2.5 h. Following electrophoresis the gels were stained in ethidium bromide (2 µg ml^{-1}) for 20 min, destained in water for 15 min and photographed under UV-transillumination (GeneGenius, Syngene). PFGE results were analyzed using the Bionumerics Software Package (Bio-Maths) and dendrograms constructed using the Dice Coefficient of similarity and cluster analysis with the unweighted pair-group method with arithmetic averages (UPGMA).

Plasmid profiling. Cultures were grown (with shaking) at 15°C in Lysogeny Broth (LB) (Bertani 1951) supplemented with 2% NaCl to mid-logarithmic phase, and cells collected by centrifugation at 4°C (10 min, 4000 × *g*). Plasmid isolation was performed using the Qiaprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. Plasmids were separated by gel electrophoresis using 1% agarose gels at 90 V for 1 h at room temperature. Supercoiled DNA ladder (Invitrogen) was used as a molecular marker.

Sequencing of 16S *rRNA* and *gyrB* genes. The genes studied were amplified and sequenced using the primers described in Table 2. Although the 16S analysis was limited to *Moritella viscosa* isolated in Norway, isolates originating from salmon, rainbow trout and cod were included. The obtained 16S *rRNA* sequences were submitted to Genbank under accession numbers GU138619–GU138626.

Primers for amplification of *gyrB* sequences were designed using a draft genome sequence of the type strain (NCIMB 13548) as reference (access to this sequence is available via the corresponding author). A fragment of the *gyrB* gene, of approximately 1670 to 2500 bp, was amplified and sequenced in isolates 2033, 3999, 4179, 5450, 5507 and 5683, while a fragment of approximately 1000 bp was sequenced in the remaining isolates. Internal primers for sequencing were designed using either the draft sequence or by primer walking in isolates with significant sequence variation. The obtained *gyrB* sequences were submitted to Genbank under accession numbers GU124771–GU124812.

DNA sequencing was performed using DYEnamic™ET dye terminator chemistry (Amersham Biosciences) and a MEGABACE 1000™ capillary sequencer (Amersham). Sequences were compiled using either the Sequencher program (Gene Codes Corp, Genetics Computer Group [GCG], Oxford Molecular) or Vector NTi (Invitrogen), and their identity confirmed using BLAST search analysis (Altschul et al. 1997). Obtained sequences and sequences accessed from Genbank were aligned in ClustalX (Thompson et al. 1997) prior to Neighbour-joining (Kimura 2-parameter)

Table 2. Primer oligonucleotides used for PCR amplification and sequencing

Name	Gene	Sequence	Reference
FD1	16S *rRNA*	5′-AGA GTT TGA TCC TGG CTC AG-3′	Weisburg et al. (1991)
RP2	16S *rRNA*	5′-ACG GCT ACC TTG TTA CGA CTT-3′	Weisburg et al. (1991)
MvgyrBF	*gyrB*	5′-TGG CGC AGT ATC TGA TTG AG-3′	This study
MvgyrBR	*gyrB*	5′-GCA ACC GCT TAA TCA TAG CC-3′	This study
MvgyrB 251F	*gyrB*	5′-ATG AAG AAG AGG GTG TTT-3′	This study
751F	*gyrB*	5′-GGA ATT ACT GTT GAA GTT-3′	This study
2001F	*gyrB*	5′-ATC TAT CCG CCA ACA TGG-3′	This study
1251F	*gyrB*	5′-TCC TGC ACT TTC TGA ACT-3′	This study
1759F	*gyrB*	5′-GTA AAT GGT TAT CGT AAG-3′	This study
2155R	*gyrB*	5′-AAT TAG TTA CTT CAC GAC-3′	This study
1665R	*gyrB*	5′-TGT TAA GTC TGC ATC ATC-3′	This study
1149R	*gyrB*	5′-CGC ACG TGA TGC ATC GAT-3′	This study
665R	*gyrB*	5′-ATA CCA CCT TCA TAA ACG-3′	This study
165R	*gyrB*	5′-ATG ACC CGC TAA TGC TTC-3′	This study

analysis in PAUP* 4.0 (Swofford 2002). Ambiguous bases were excluded from the analysis. Bootstrap confidence values were obtained with 1000 re-samplings.

Western blotting. Samples were prepared for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Lunder et al. (2000), except that samples in the present study were not treated with proteinase K and were only heated at 37°C for 10 min. Antigens were separated by SDS-PAGE using the Amersham Pharmacia Multiphor II Flatbed Electrophoresis System and 8 to 18% precast gradient gels (ExcelGel SDS Gradient, 80-1255-53, Amersham Pharmacia) and subsequently transferred to nitrocellulose membranes (162-0234, Bio-Rad) using the method of Olsen & Wiker (1998). Precision Protein Standards (10 to 250 kDa) (161-0362, Bio-Rad) were used as molecular weight (MW) markers. Immunostaining of transferred *Moritella viscosa* antigens was performed by the following procedure: (1) blocking with 5% skimmed milk in phosphate-buffered saline (PBS) with 0.1% Tween-20 (PBS/Tween); (2) incubation with primary rabbit anti-*M. viscosa* antiserum in PBS/Tween; (3) incubation with peroxidase conjugated donkey anti-rabbit (NA934, Amersham) in PBS/Tween; (4) colour development using 3,3-diaminobenzidine tetra-hydrochloride (D5637, Sigma) in 0.1 M $NaCH_3CO_2$, 0.005% H_2O_2 (pH 4.0). All steps were carried out at room temperature and were separated by 3 washes in PBS/Tween. Three different rabbit anti-*M. viscosa* antisera were employed as primary antiserum: α-Vvi-1 (1:5000) (kindly provided by M. Pallapothu, Novartis Animal Health Canada Inc.; raised against *M. viscosa* isolated from Norwegian Atlantic salmon), α-5450 (1:1000) (raised against *M. viscosa* 5450 isolated from Norwegian rainbow trout, National Veterinary Institute) and α-NCIMB 13548 (1:500) (raised against *M. viscosa* NCIMB 13548, the species type strain isolated from Norwegian Atlantic salmon, National Veterinary Institute).

Histopathological studies. Histological samples were taken during 2 cases of ulcer development in rainbow trout during January and March of 2007. Gills, heart, liver, kidney, spleen, pancreatic tissue and skeletal musculature were sampled. The samples were fixed in 4% neutral buffered formalin, embedded in paraffin wax and routinely processed. The sections were stained by haematoxylin and eosin (H&E).

RESULTS

Histopathology

The observed pathological changes were in accordance with previous descriptions of infection with *Moritella viscosa* (Lunder et al. 1995, Bruno et al. 1998). The histopathology of skin ulcers revealed subepidermal oedema, dermal inflammation, haemorrhage and inflammation in subcutis and underlying red muscle and some necrosis of white muscle fibers. Liver tissues demonstrated moderate to marked focal congestion with dilated, blood-filled sinusoids.

An increased number of circulating neutrophils was observed. No striking differences in pathological changes were observed on comparison of disease caused by the typical or variant cluster in Atlantic salmon and rainbow trout respectively.

Phenotypical characterisation

With the exception of non-*Moritella viscosa* species, all tested isolates produced viscous haemolytic colonies on BAS. Slight differences in colony pigmentation were perceived, with isolates belonging to the subsequently denoted typical strain appearing to be greyer in colour than the variant isolates which were considered to have a slight yellowish/orange pigmentation on BAS. All isolates were motile, short, Gram-negative pleomorphic rods which produced acid from glucose under anaerobic conditions, did not produce gas from glucose, were slightly sensitive to vibriostat (O/129), produced catalase and gelatinase, but did not produce acid from cellobiose or trehalose. All isolates grew well at 4°C but not at 30°C. Degree and type of haemolysis and production of acid from mannose were identified as variable in the *M. viscosa* isolates studied. The most apparent difference was that relating to the degree of haemolysis observed on plating on BAS (Fig. 1). The isolates originating from salmon farmed in Norway, Scotland and the Faroe Islands produced a relatively weak beta-haemolysis, while isolates from rainbow trout and salmon farmed in Iceland produced a visibly stronger haemolytic zone over a larger area around the colony. More extensive haemolysis, i.e. larger diffusion zones, were also observed in 2 Canadian isolates, 2 Icelandic isolates and 1 isolate from cod. Acid production from mannose (Fig. 1) appeared to be a somewhat quantitative trait rather than absolute. With the exception of 3 isolates which displayed very weak acidification of the media, all Norwegian, Scottish and Faroe Atlantic salmon isolates were clearly negative, while Canadian salmon isolates, 4 of 5 Icelandic salmon isolates and 64% of rainbow trout isolates were clearly positive. While slight acidification was apparent in one isolate from cod, the remaining 4 isolates tested were clearly negative. Very weak production of lysine decarboxylase (Fig. 1) was identified in a larger proportion (62.5%) of the Norwegian, Scottish and Faroe Atlantic salmon isolates compared to the remaining isolates (20.8%).

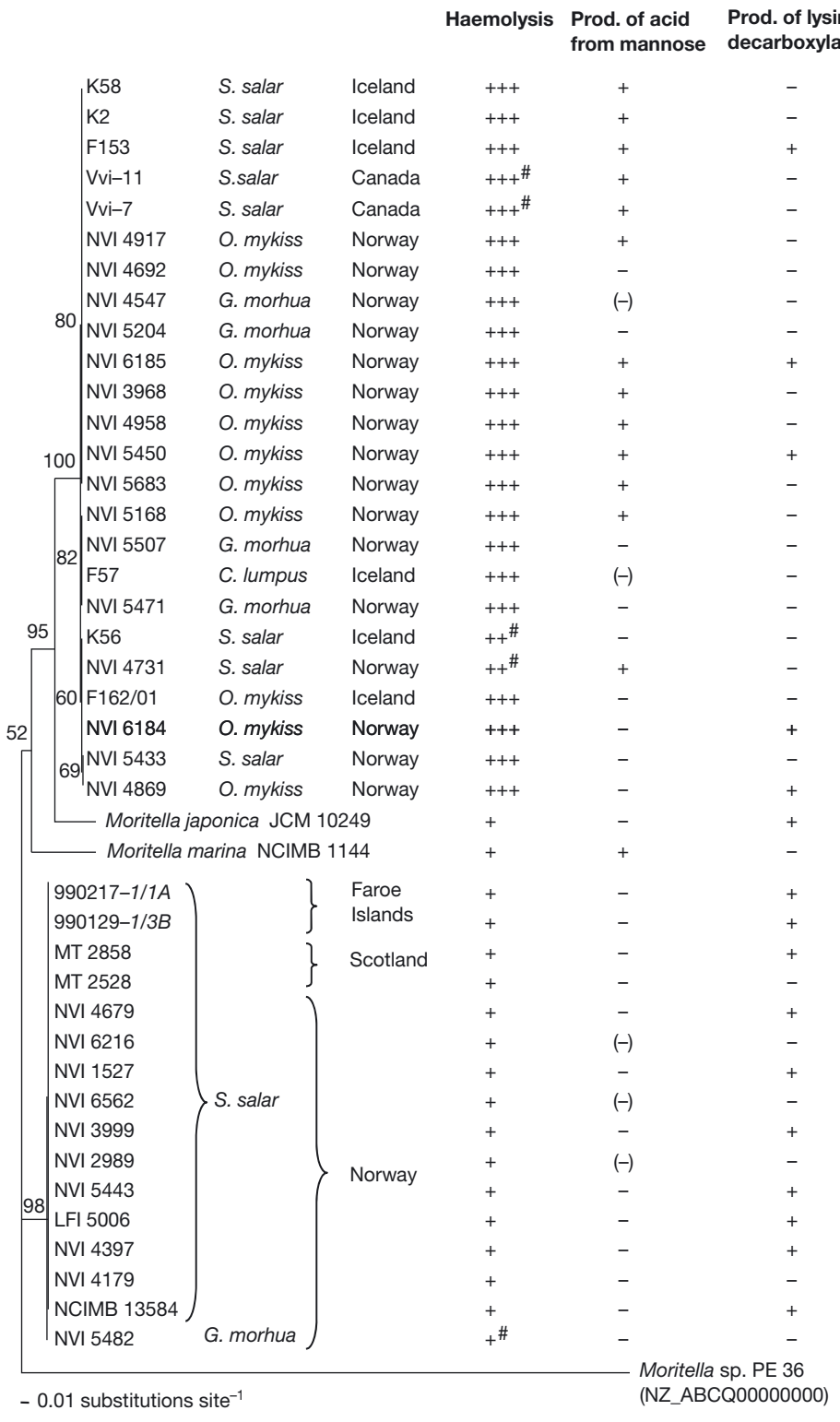

Fig. 1. Neighbour-joining analysis of the *gyrB* gene (926 bp) of *Moritella marina*, *M. japonica*, *Moritella* PE36 and *M. viscosa* isolated from various fish species from different geographical areas. Bootstrap values are percent of 1000 replicates. For aesthetic reasons some bootstrap values between closely related branches have been omitted. Haemolytic properties and the ability of each strain to produce acid from mannose and lysine decarboxylase are also included. Parentheses indicate slight acidification of the media. Haemolysis was evaluated as weak (+); moderate (++); or strong (+++). # indicates a relatively larger zone of haemolysin diffusion. Fish species are *Gadus morhua*, *Salmo salar*, *Oncorhynchus mykiss*, *Cyclopterus lumpus*

Plasmid profiling

The isolates examined fell into 2 clearly separated groups (Fig. 2). One group comprised isolates from salmon in Norway, Scotland and the Faroe Islands in which each isolate produced a clear plasmid profile of 4 to 7 bands, many of which were common to several or all isolates. The second group comprised isolates from various fish species in Canada, Iceland and Norway which produced plasmid profiles consisting of between 0 and 4 bands, with the majority of isolates displaying no obvious plasmid bands.

PFGE analysis

Moritella viscosa isolates formed 2 distinct clades (Fig. 3), consistent with the division indicated by phenotypical differences and plasmid profiling. One clade comprises closely related isolates, originating exclusively from Atlantic salmon in Norway, Scotland and the Faroe Islands, along with a single less related isolate from Norwegian cod. The second distinct cluster consists of rather less related isolates from salmon, rainbow trout and lumpsucker from Iceland, Norwegian rainbow trout and Atlantic salmon from Canada. *M. japonica* and *M. marina* did not cluster with either of the *M. viscosa* groups.

16S *rRNA* analysis

Exclusion of missing and/or ambiguous characters resulted in analysis of 1332 bases. While 16S rRNA analysis (Fig. 4) clearly distinguished the various *Moritella* species, identical sequences were obtained from all isolates of Norwegian *M. viscosa* studied, irrespective of host species.

gyrB analysis

Removal of gaps and ambiguous bases resulted in analysis of a 926 bp sequence common to all sequenced isolates. A total of 40 *Moritella viscosa* isolates from different fish and various geographic origins as well as *M. marina* NCIMB 1144, *M. japonica* JCM 10249 and PE 36 (Acc. Nr. NZ ABCQ01000076) were analysed. Neighbour-joining analysis of *gyrB* (Fig. 1) clearly separated the various species. *M. viscosa* isolates were also split into 2 relatively deeply divided clusters, consistent with the division identified by phenotypical testing, plasmid profiling and PFGE. One cluster consisted, with the exception of a highly similar but not identical isolate from cod in Norway, of identical isolates (including the type strain), originating from Atlantic salmon farmed in Norway, Scotland and the Faroe Islands. The second cluster comprised a closely related but slightly

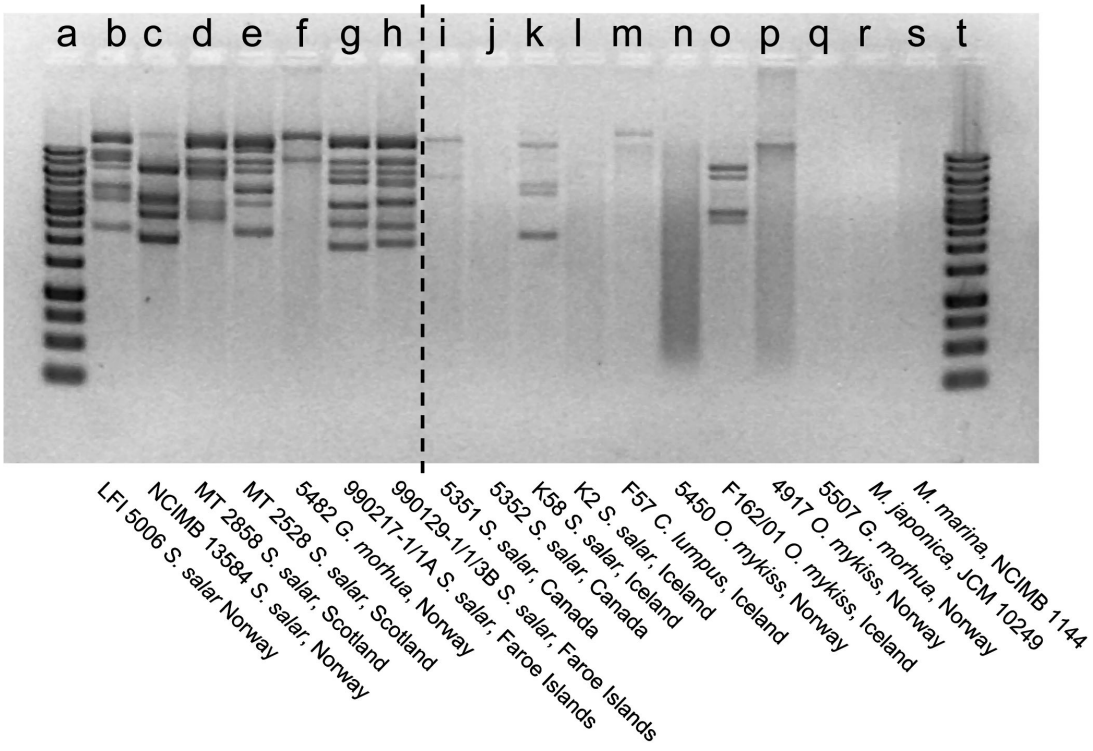

Fig. 2. Plasmid profiles of *Moritella viscosa* isolated from various species of fish from different geographical areas. Lanes (a) and (t): molecular weight markers. The dashed line separates the 2 *gyrB* clusters

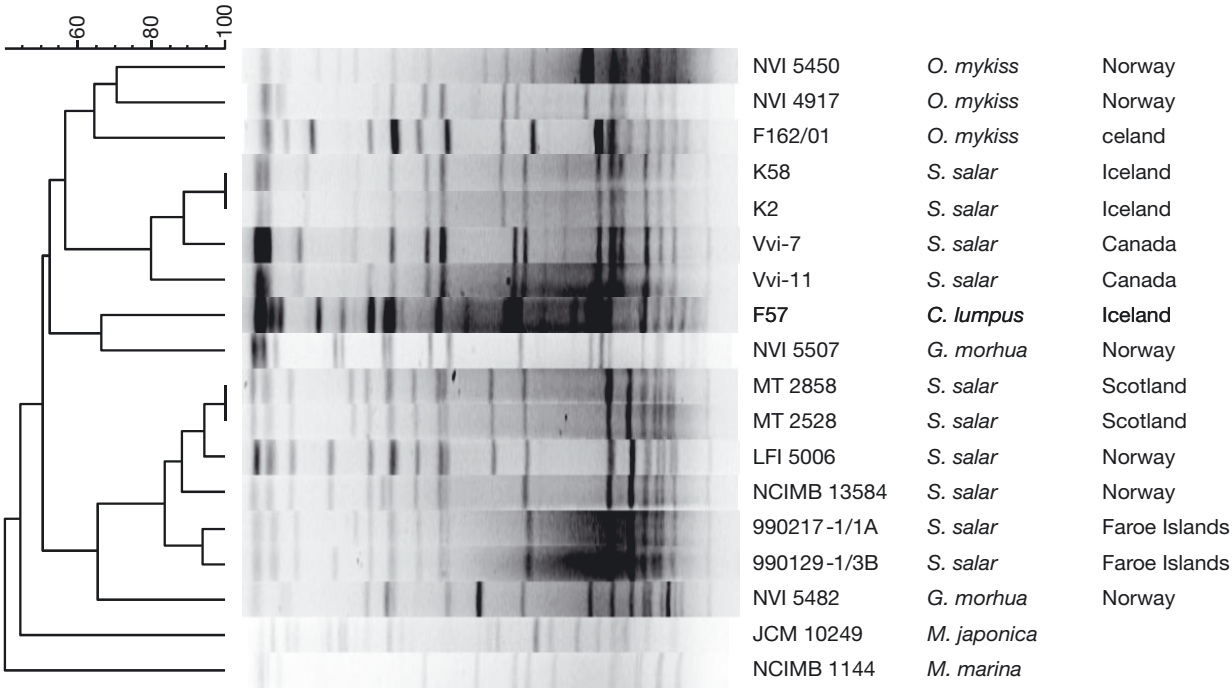

Fig. 3. Pulsed field gel electrophoresis analysis of *Moritella viscosa* isolated from various species of fish from different geographical areas. Isolate designation, fish species and country of isolation are shown

more heterogeneous group of isolates from various fish species in Norway, Iceland and Canada. *M. japonica* and *M. marina* were placed between the 2 *M. viscosa* clades. No isolates with the type strain *gyrB* sequence were identified in species other than salmon, although the single cod isolate which was identified as closely related to the type strain clade by PFGE is also very closely related by *gyrB* analysis.

Western blot analysis

By Western blotting, all 3 antisera were able to clearly distinguish the various *Moritella* species (Fig. 5). Although *M. viscosa* showed band patterns that were predominantly similar, some noteworthy differences were also identified (Table 3). Using the α-5450 sera raised against *M. viscosa* from Norwegian rainbow trout, isolates from Norwegian rainbow trout showed a double band at ~200 to 250 kDa, of which the ~200 band was the dominant. This contrasted with all salmon isolates from Iceland/Canada and most salmon isolates from Norway/Scotland/Faroes in which the 250 kDa band dominated. Notably, the Icelandic rainbow trout isolates had no detectable ~200 kDa band. In cod isolates from Norway, lumpsucker from Iceland and one salmon isolate from Scotland, both protein bands were almost equal in strength. Western blotting using α-NCIMB 13548 (type strain from Norwegian Atlantic salmon) and α-Vvi-1 antisera also suggested antigenic differences between analysed isolates in this MW region (Table 3). Further, the α-Vvi-1 was able to distinguish between isolates by showing differential band patterns at both ~110 kDa and 130 to 135 kDa (Table 3). For all employed antisera, the lumpsucker isolate was distinguished by a band at ~80 kDa which in all other bands appeared to be 5 to 10 kDa heavier. In summary, despite relative small differences, the Western blot analysis was able to separate the salmonid *M. viscosa* isolates into 4 antigenically distinct groups: Norwegian rainbow trout, Icelandic rainbow trout, Icelandic/Canadian salmon and finally Norwegian/Scottish/Faroese salmon.

DISCUSSION

The genus *Moritella* currently consists of 7 validly described species (www.bacterio.cict.fr), most of which are associated with deep marine environments, and contains *M. viscosa*, an established cause of winter-ulcer in fish farmed in countries bordering the northern Atlantic Ocean.

Although antigenic heterogeneity has been previously identified within *Moritella viscosa* (Benediktsdóttir et al. 2000, Heidarsdóttir et al. 2008), little has been performed in the way of phenotypic and genetic comparison of this bacterium since the original species descriptions (Benediktsdóttir et al. 2000, Lunder et al. 2000). Until the present study, only 2 *M. viscosa* 16S *rRNA* sequences (Lunder et al. 2000, Colquhoun et al.

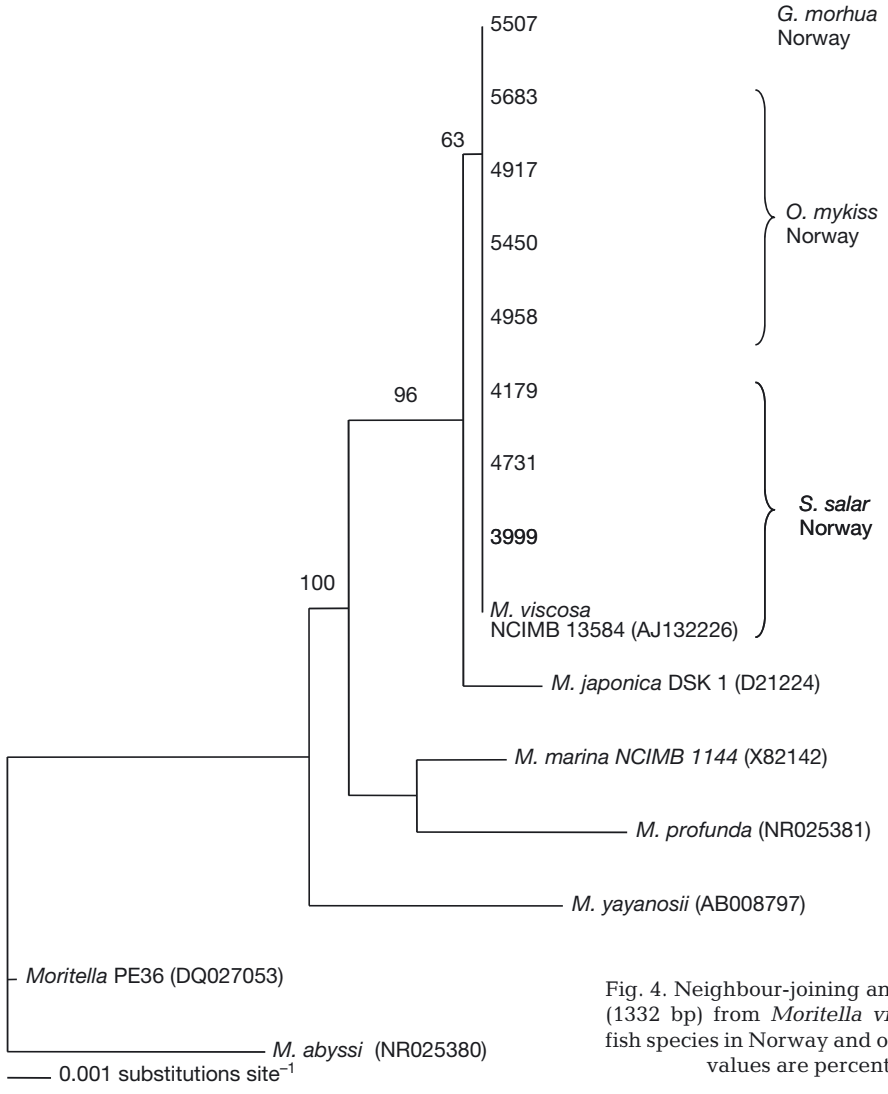

Fig. 4. Neighbour-joining analysis of the 16S *rRNA* gene (1332 bp) from *Moritella viscosa* isolated from various fish species in Norway and other *Moritella* spp. Bootstrap values are percent of 1000 replicates

2004), both from Norwegian farmed fish, were available in Genbank (www.ncbi.nlm.nih.gov/), and intra-species phenotypical differences were undocumented.

The current study, which identified a previously unrecognised degree of phenotypical and genetic differences within the species *Moritella viscosa*, was instigated following a series of outbreaks of winter-ulcer, characterised by high levels of mortality, in farmed rainbow trout in Norway during 2005 and 2006. Bacterial isolates from these outbreaks, whilst phenotypically consistent with *M. viscosa*, differed in some respects to those normally isolated from Atlantic salmon in this country.

Clinically, the disease caused by the variant strain in rainbow trout in the studied case did not appear to differ markedly from that observed in winter-ulcer in Atlantic salmon caused by the typical strain. Although the material examined histologically in the present study was relatively modest, no evidence for significant differences in transmission or pathology exists either from the present study or previous studies related to Koch's postulates (Lunder et al. 1995, Bruno et al. 1998).

The study, which included isolates from all the major North Atlantic salmonid producing countries, found *Moritella viscosa*, *M. marina* and *M. japonica* to be phenotypically very similar, using standard diagnostic methodology. Whilst clearly genetically separated by both 16S *rRNA* and *gyrB* sequence analysis, *M. japonica* and *M. marina* could be distinguished phenotypically from *M. viscosa* only by their low colony viscosity. *M. viscosa* isolates were found to form a uniform cluster following analysis of 16S *rRNA* sequences, but formed 2 clearly separate genotypes following *gyrB* sequence analysis. Phenotypically the 2 genotypes of *M. viscosa* are highly similar, only clearly differing in

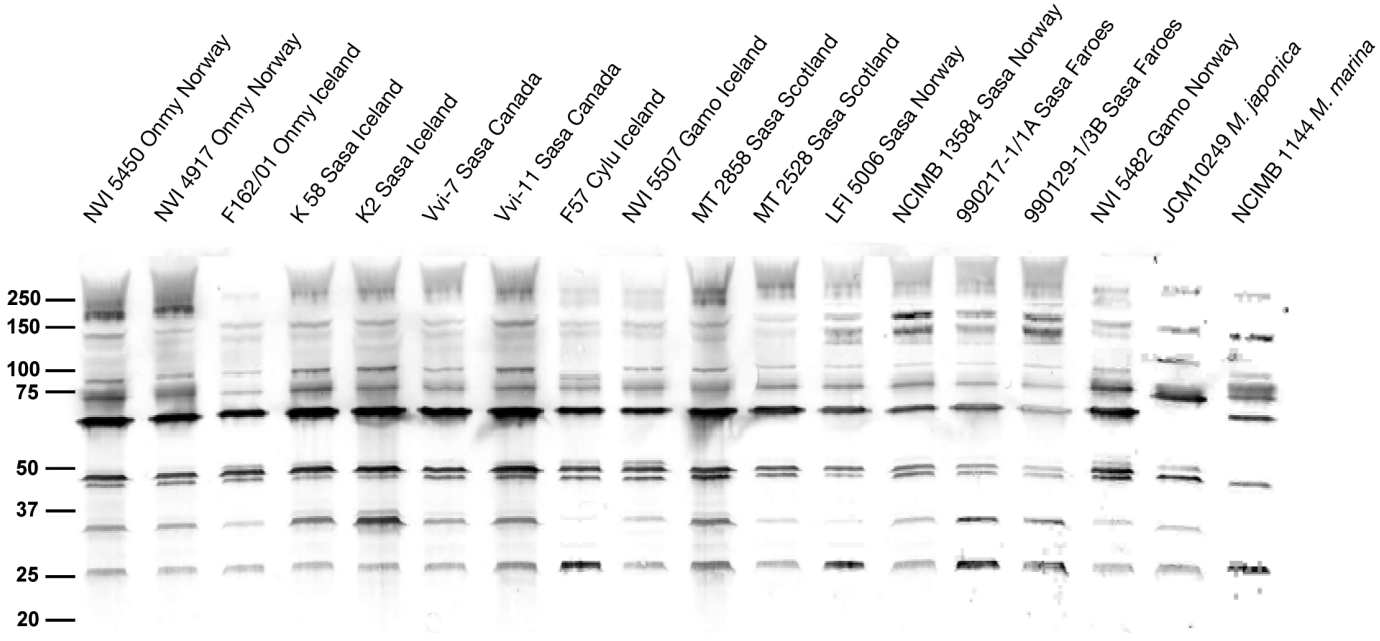

Fig. 5. Western Blot analysis of *Moritella* species and *M. viscosa* isolates using the α-5450 antiserum as primary antibody. Onmy, *Oncorhynchus mykiss*; Sasa, *Salmo salar*; Gamo, *Gadus morhua*; Cylu, *Cyclopterus lumpus*. Lane 1: molecular weight standard in kDa

Table 3. Summary of selected Western blotting data that distinguish *Moritella viscosa* strains isolated from rainbow trout and Atlantic salmon. Onmy: rainbow trout; Sasa: salmon; N, Norway; I, Iceland; C, Canada; S, Scotland; F, Faroese. Numbers indicate approximate molecular weight of bands, boldface type indicates strong/dominant band, while dash (–) indicates absence of detectable band. α-NCIMB 13548: rabbit antiserum raised against *Moritella viscosa* NCIMB 13548 (species type strain) isolated from Norwegian Atlantic salmon. α-5450: rabbit antiserum raised against *M. viscosa* strain 5450 isolated from Norwegian rainbow trout. α-Vvi-1: rabbit antiserum raised against *M. viscosa* strain Vvi-1 isolated from Norwegian Atlantic salmon

Antiserum	Onmy N	Onmy I	Sasa I/C	Sasa N/S/F
α-NCIMB	**250**	250	250	250
13548	**200**	–	–	200
α-5450	250	250	**250**	**250**[a]
	200	–	200	200
α-Vvi-1	**250**	**250**	**250**	**250**
	200	200	200	200
	135	135	135	135
	130	130	130	**130**
	110	–	110	–

[a]In one Scottish salmon strain (MT 2858), the bands were equally strong

haemolytic activity. Otherwise, variable test results including acid production from mannose and production of lysine decarboxylase were not absolutely related to genotype although these abilities or lack thereof appeared to be associated with certain genotypes, i.e. the Norwegian salmon cluster consistently failed to produce acid from mannose, while a higher proportion of these isolates produced lysine decarboxylase in our tests.

The division within the species *Moritella viscosa* illuminated in the current study is supported by all techniques used. The evidence indicates that at the phenotypic and 16S *rRNA* level, the 2 clusters are closely related. However, the combined evidence from *gyrB* analysis, plasmid profiling, PFGE and Western blotting would suggest that a relatively longstanding division exists between these 2 sub-populations. Although *gyrB* has been found to show little or no resolution in some closely related *Vibrio* species (Thompson et al. 2007), it has been used extensively in phylogenetic studies for this genus (e.g. González-Escalona et al. 2008, Thompson et al. 2009) which is relatively closely related to *Moritella*. The functional conservation in *gyrB* in *M. viscosa* is illustrated by the relatively low level of nucleic acid identity (92.5%) yet high level of amino acid identity (97.7%) and similarity (98.3%) maintained between the type strain (NCIMB 13584) and isolate 5450 from rainbow trout. Thus, it can be concluded that most if not all of the nucleic acid substitutions have occurred through random, non-selective mutation rather than horizontal gene transfer, and that a considerable period of time must have elapsed to allow such an accumulation.

Though the differences revealed by Western blotting were relatively small, they appear to follow the division between strains found by the phenotypical and genetic methods. Isolates from Atlantic salmon thus cluster into a Norwegian/Scottish/Faroese ('Eastern Atlantic') group and an Icelandic/Canadian ('Western Atlantic') group. Isolates from Norwegian rainbow trout are distinct from the 2 Atlantic salmon groups and from the single isolate analysed from Icelandic rainbow trout. In a Western blotting analysis, Heidarsdóttir et al. (2008) identified a strain difference in the MW of a 17/19 kDa outer membrane protein that was compatible with the presently observed division. Hence, while a 17/19 kDa protein was shown to be of lower MW in isolates from Norwegian/Scottish Atlantic salmon compared to isolates from Iceland/Canada, it was absent in an isolate from Icelandic rainbow trout.

Although the results of the present study clearly identify a deep division within the species *Moritella viscosa* represented by 2 distinct genetic clusters of isolates, it is not immediately clear whether the 2 identified groups of bacteria comprise ecologically distinct populations. The typical strain does not appear to cause disease in rainbow trout in Norway, while the variant strain causes disease only exceptionally in salmon in this country. If the studied strains are representative, the typical strain does not appear to have been isolated from diseased fish in Iceland or Canada, although the low numbers of isolates studied means that its absence from these waters cannot be assumed. It is, however, clear that in Icelandic and Canadian waters the variant strain is capable of causing disease in both salmon and rainbow trout.

Although overshadowed by salmon farming in terms of production (741 000 t compared to 86 000 t; Kjønhaug 2009) in Norway, rainbow trout are farmed in similar or identical areas of the Norwegian coastline as salmon. Retrospective analysis of isolates obtained from rainbow trout in the course of diagnostic work performed by the Norwegian National Veterinary Institute has failed to identify the presence of the typical strain in these cases, while the new variant strain has only exceptionally been identified in farmed Atlantic salmon. The reasons for this are not immediately clear, as isolates of this type appear to be relatively commonly isolated from salmon farmed in Iceland.

It is interesting to note that sea-farming of rainbow trout in Iceland was seriously affected by *Moritella viscosa* infection prior to the introduction of vaccination (Gudmundsdóttir & Bjornsdóttir 2007), while the incidence of such disease in this fish species in Norway is historically very low.

In Canada and Iceland the typical strain appears to be absent at least in the studied material, while the 'variant' strain was identified in all fish species studied. It could be argued that the apparent host specificity of the typical strain for Atlantic salmon is in fact the case and that it has not been identified in Iceland and Canada is due to its absence from these waters. That the variant strain is found in Atlantic salmon farmed in Iceland and Canada may correspond to the 'occasional' isolation of this strain in Atlantic salmon in Norway. As winter-ulcer is not a notifiable disease, absolute statistics are not available. However, from experience it appears that Norway has a much higher incidence of winter-ulcer in farmed salmon than Iceland or Canada. This could conceivably be explained by the presence of a strain particularly virulent for Atlantic salmon (i.e. the type strain) in Norwegian waters. The lower incidence of winter-ulcer in Scottish salmon may be related to higher winter water temperatures. It may therefore be speculated that a degree of host specificity may exist at least in the Norwegian/Scottish/Faroe Islands salmon strain of *Moritella viscosa*.

In the absence of evidence to the contrary, *Moritella viscosa* has until now, and for the purposes of vaccine production, been considered to be a largely homogeneous species. This presumption, despite the evidence presented by the present study, may be relatively safe as far as the overall susceptible population of fish is concerned. Winter-ulcer is a far more significant disease in Norwegian aquaculture compared to the other salmon farming countries, and Atlantic salmon account for a dominating proportion of the fish raised in this country. Isolates from Atlantic salmon in Norway must therefore account for the overwhelming majority of clinical *M. viscosa* isolates on a worldwide basis. Despite the antigenic differences identified during the present study and by Heidarsdóttir et al. (2008), cross-protection has been reported between Icelandic (presumed variant) and Norwegian (typical) strains (Greger & Goodrich 1999). The previous assumption of homogeneity may therefore not have been as significant in relation to protection of vaccinated stocks as it otherwise might have been.

In conclusion, it has been established that the species *Moritella viscosa* can be phenotypically and genetically split into 2 more or less homogeneous strains. It is not yet clear whether these strains are ecologically limited in relation to geographical distribution and host fish species specificity. Further characterisation regarding precise taxonomic placement of the newly identified variant strain is necessary and is currently being performed in our laboratories.

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