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Host specificity and clade dependent distribution of putative virulence genes in *Moritella viscosa*

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ABSTRACT

Moritella viscosa is the aetiological agent of winter-ulcer disease in farmed salmonids in the North Atlantic. Previously, two major (typical and variant) genetic clades have been demonstrated within this bacterial species, one of which is almost solely related to disease in Atlantic salmon (*Salmo salar*). In the present study infection trials demonstrated that 'typical' *M. viscosa* isolated from Norwegian Atlantic salmon was highly virulent in this fish species but resulted in lower levels of mortality in rainbow trout. 'Variant' *M. viscosa* isolated from rainbow trout resulted in modest mortality levels in both Atlantic salmon and rainbow trout. To investigate the possible genetic background for inter-strain virulence differences, 38 *M. viscosa* isolates of diverse geographical origin and host species and a number of other *Moritella* spp. were investigated for the presence/absence of putative virulence related homologs. All isolates were positive for DNA sequences coding for; the Type VI secretion ATPase (*clpV*), hemolysin co-regulated protein (*hcp*), bacterioferritins (*bfrA* and *bfrB*), lectin (*hemG*), phospholipase D (*pld*), multi-functional autoprocessing repeats-in-toxin (*martxA*), aerolysin (*aer*), invasin (*inv*), and cytotoxic necrotizing factor (*cnf*), with the exception of one isolate in which *cnf* could not be confirmed. The product of an ABC transporter metal-binding lipoprotein (*mat*) was consistently detected although 11 isolates, all phylogenetically related, appear to produce a truncated version. A putative insecticidal toxin complex (*mitABC*) was detected almost exclusively in 'typical' Atlantic salmon isolates, and our data indicate that this complex of genes is expressed and co-transcribed. Transmission electron microscopy investigation revealed pili and flagella surface structures on nine *M. viscosa* representing both typical and variant isolates. Our results provide strong support for the existence of host specificity/high virulence in 'typical' *M. viscosa* related to Atlantic salmon. The gene distribution also provides further support for the genetic division within *M. viscosa*, and constitutes a basis for further study of the importance of the *mitABC* complex in winter-ulcer pathogenesis.

1. Introduction

The aetiological agent of winter-ulcer disease in salmonids, *Moritella viscosa*, induces chronic skin ulcers at low temperature that may be followed by terminal septicemia [1,2]. Despite widespread vaccination, the disease remains a welfare problem in Norway and results in significant economic losses [3]. Farmed salmonids in Iceland [1], the Faroe Islands [4], Scotland [5] and Canada [6] are also affected. Experimental challenges have further demonstrated that turbot *Scophthalmus maximus*, Atlantic cod

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Table 1
Characteristics of the *M. viscosa* isolates.

Moritella viscosa[a]	Isolation origin [year]	ECP lethality[b]	Atlantic salmon challenge		
			Method[c]	LD_{50}	Ref.
Hosted in *S. salar*					
NCIMB 13584^T	Norway [1988]	Yes	ip	6×10^5	[10]
NVI 5433	Norway [2006]	No	ip	$>2 \times 10^6$	[10]
NVI 4731	Norway [2003]	Yes			
LFI 5006[d]	Norway [2002]	Yes			
NVI 5443	Norway [2006]	n.d.			
NVI 4679	Norway [2003]	n.d.			
NVI 4397	Norway [2001]	n.d.			
NVI 4179	Norway [2000]	n.d.			
NVI 3999	Norway [1999]	n.d.			
NVI 1527	Norway [1990]	n.d.			
NVI 3632	Norway [1996]	n.d.			
06/09/139	Norway [2006]	n.d.	bath	$<1 \times 10^6$ ml^{-1}	[34]
K58	Iceland [2008]	Yes	im	$<1.5 \times 10^1$	[1]
			ip	2×10^5	[10]
K56	Iceland [2008]	Yes	im	$<1.1 \times 10^2$	[31]
F153	Iceland [2008]	n.d.			
K2	Iceland [2008]	n.d.			
MT 2528	Scotland [2001]	Yes	ip	2×10^4	[10]
MT 2858	Scotland [2004]	Yes			
MT 2555	Scotland [2002]	n.d.			
990129-1/3B	Faroe Island [1999]	Yes	ip	2×10^5	[10]
990217-1/1A	Faroe Island [1999]	Yes			
990217-1/2A	Faroe Island [1999]	n.d.			
Vvi-11	Canada [2005]	No	ip	Avirulent	[10]
Vvi-7	Canada [2005]	No	ip	Avirulent	[10]
Hosted in *O. mykiss*					
NVI 4917	Norway [2004]	Yes	ip	7×10^5	[10]
NVI 5450	Norway [2006]	Yes			
NVI 5168	Norway [2005]	Yes			
NVI 6185	Norway [2008]	n.d.			
NVI 6184	Norway [2008]	n.d.			
NVI 5683	Norway [1999]	n.d.			
NVI 4958	Norway [2004]	n.d.			
NVI 4869	Norway [2004]	n.d.			
NVI 3968	Norway [1999]	n.d.			
F162/01	Iceland [2008]	Yes			
Hosted in *G. morhua*					
NVI 5482	Norway [2006]	Yes	ip	2×10^5	[10]
NVI 5507	Norway [2006]	n.d.			
NVI 5471	Norway [2006]	n.d.			
Hosted in *C. lumpus*					
F57	Iceland [2008]	Yes	im	1.7×10^6	[31]
			ip	$>2 \times 10^6$	[10]
Moritella sp. from sea water[a]					
M. marina	North Pacific Ocean at 1200 m [1964]	No			
M. sp. PE36	North Pacific Ocean at 3600 m [1985]	n.d.			
M. dasanensis	Arctic Ocean at surface [2008]	n.d.			

[a] Isolates; *M. viscosa* [4], *M. marina* [35], *M.* sp. PE36 [36], *M. dasanensis* [37].
[b] Reference [10].
[c] ip, Intraperitoneal; im, Intramuscular; n.d., Not determined.
[d] LFI 5006 is an isolate from dead Atlantic salmon (2002) experimentally challenged with LFI 5000, which originally was isolated from Atlantic salmon suffering from a natural occurring outbreak in 1997.

Gadus morhua and Atlantic halibut *Hippoglossus hippoglossus* [7,8] are susceptible to *M. viscosa* infection.

Two major clades have been identified in *M. viscosa* and it has been speculated that one clade may represent a clone highly virulent for farmed Atlantic salmon *Salmo salar* [4]. Factors involved in bacterial infections may be complex and multi-factorial. Gene products expressed and secreted to the bacterial surface or to the environment may have specific properties related to toxicity, adhesion, colonization, motility, invasion, iron- and nutrition-acquisition, evasion or inhibition of host defenses, features that may be necessary in virulence. Little is known of *M. viscosa* virulence. Extracellular products (ECPs) destroy host cell membranes and the cytoskeleton [9]. However, there is no correlation between Atlantic salmon lethality and the cytotoxic and hemolytic activities observed from different extracts *in vitro* [10]. The extracellular MvP1 peptidase, although non-lethal causes hemorrhage and tissue necrosis in salmon [11]. Increased transcription of putative virulence genes has been associated with cell rounding [12].

In view of previous speculation regarding host specificity, we decided to investigate 1) the virulence of two strains of *M. viscosa*, representing the two main clades within the species [4], in both rainbow trout and Atlantic salmon and 2) the presence/absence of selected putative virulence genes in isolates of both major clades and related bacterial species. In this way we wanted to develop

basic knowledge which may eventually help in reducing the occurrence of winter-ulcer disease.

2. Materials and methods

2.1. Bacterial isolates

The 38 *M. viscosa* isolates studied span the known geographical area of occurring outbreaks and includes representatives from different fish species suffering from winter-ulcer disease (Table 1). The strain collection is previously characterized by standard biochemical and phenotypic methods and identified by sequence analysis as *M. viscosa* [4,10,13], except for strains NVI 3632, 990217-1/2A, and MT 2555, which phylogenetic relationship was determined in this study.

2.2. Infectious challenge

Unvaccinated Atlantic salmon (ca. 110 g, $n = 200$) and rainbow trout *Oncorhynchus mykiss* (ca. 46 g, $n = 200$), routinely health monitored, with no history of infectious disease, and screened by ELISA [14] for the absence of specific antibodies against *M. viscosa*, were adapted to seawater for 13 and 26 days, respectively, prior to challenge. The fish were maintained in seawater (salinity 31–35‰) in separate tanks at 7.0–7.4 °C. Prior to exposure, experimental fish were split into 4 challenge tanks i.e. 2 tanks each containing 80 salmon and 2 tanks each containing 80 rainbow trout. Single control tanks each containing 40 salmon or trout were also maintained. Bacterial isolates were cultured in Brain Heart Infusion (BHI) medium containing 2% NaCl for 48 h, following resuscitation from −80 °C. NVI 3632 had previously been passaged through Atlantic salmon prior to the experiment and NVI 5450 was low passage after original isolation in 2006. The water volume in the challenge tanks was reduced to approximately 100 L and bacteria were added to a final concentration (retrospectively established by serial dilution and colony counting) of 1.2×10^6 CFU ml^{-1} for the Atlantic salmon isolate NVI 3632 (two tanks, one containing 80 salmon, the other containing 80 rainbow trout), and 5×10^5 CFU ml^{-1} for the rainbow trout isolate NVI 5450 (two tanks, one containing 80 salmon, the other containing 80 rainbow trout). During the bath challenge, the water flow was stopped, aeration was provided and the oxygen saturation monitored. After 60 min water flow of ca. 1.7 L per kg fish per min was resumed. Fish were fed to appetite and mortalities removed twice daily until termination of the trial 18 days post-infection. Head kidney tissues were streaked onto blood agar containing 2% NaCl from all dead fish to confirm *M. viscosa* infection. The presence and degree of ulceration was also registered for all surviving fish at termination, and significant difference between groups was estimated using contingency tables (Pearson Chi-square) in JMP v10 (SAS Institute Inc.). Differences were considered significant if $p < 0.05$. The experiment was approved by the National Animal Research Authority in Norway.

2.3. Prediction of putative virulence genes and phylogenetic analysis

The draft genome [4] of *M. viscosa* NCIMB 13584^T comprising 1206 contigs is predicted to contain 4810 open reading frames (ORFs). Putative virulence related genes within the genome sequence were identified utilizing predicted coding DNA sequences (CDA) and their translated nucleotide query in BLASTx homology searches. The distribution of putative virulence homologs within the genus *Moritella* was analyzed utilizing BLASTn and tBLASTx homology searches against the shotgun genomes of *Moritella* sp. PE36 (accession no.: ABCQ00000000), *Moritella dasanensis* ArB 0140 (accession no.: AKXQ00000000) and *Moritella marina* ATCC 15381 (accession no.: ALOE00000000). The phylogenetic relationships between strains used in this study was constructed utilizing *gyrB* sequences obtained in this study with accession no.; KJ746474 (strain NVI 3632), KJ746475 (strain 990217-1/2A), and KJ746476 (strain MT 2555) as described in Grove et al. (2010), *gyrB* sequences with accession no. GU124771–GU124811 [4], and *gyrB* sequences extracted from the shotgun genomes of the *Moritella* spp. Sequences were aligned in BioEdit using ClustalW [15]. The phylogenetic relationships between the 41 nucleic sequences were determined from 926 positions using Maximum Likelihood (ML) (GTR) and Neighbor-Joining (NJ) (Kimura 2-parameter) each with 1000 bootstrap trials in MEGA5 [16].

Relationships between studied flagellin protein sequences were obtained from predicted *M. viscosa* translated gene sequences in this study or retrieved from UniProt (*Moritella* sp. PE36: A6FHY1, A6FHY2, A6FHX8, A6FHX9, A6F9P1; *Vibrio parahaemolyticus*: Q03473, Q56702, Q56703, Q56704, Q56712, Q87081, Q9ZBA2; *Aeromonas salmonicida*: A4SP60, A4SP61, O30378). The amino acid sequences were aligned and analyzed (MEGA5) by ML with the WAG + G model applying NJ (JTT) for selection, aligned positions $n = 130$. The evolutionary distances were also computed using NJ with the Poisson correction in a 425 position dataset. Both methods were conducted with 1000 bootstrap trials.

Table 2
Oligonucleotide primers used for detection of virulence associated genes.

Primer designation	Forward primer, reverse primer (5′ → 3′)[a]	Product size
bfrA F	AACTATGAAAGGCAACAGTAA	1011
bfrB R	AAGCACAACAACAGCCACAG	
mat F	GGCTGGAAACAAGTGGTGTT	1304
mat R	TCAATATTCCCAGCGAAAGG	
pld F	GGCTGGGTTGATTGGAAAT	1968
pld R	CCGCAAACCTAAATGGAAAA	
inv F	AGGATATTTAAATGCCCGATT	1257
inv R	TTCGGACGTTGTTGTCACAT	
aer F	CCTCCAAGGTGATGGAAGAA	1362
aer R	AACAACTGCCTAAGGGATACCA	
hcp F	TTCTTGCCATGTGTTACCGA	616
*clpV*1 R	ATGCTGTTTTTGCGATAGGG	
hemG F	CATCCTGCCTTCGAATGTTT	484
hemG R	ATGGGTTCTAGGTGGTCGTG	
cnf F	AATGTTAGTTGCCGCCGTAG	1390
cnf R	CCGAAACCAACGTCAGAAAT	
rtxA F	GGCCGGTCAAAATGGTATTA	884
*rtxA*R	ATCAAGCTGAACGACCACACT	
mitA F	ACACATGAAATCAATGTCAA	1112
mitA R	TCCATTTGAAGGTGTTCATCA	
mitC F	CGGAGCAAACCAATACACCT	1667
mitC R	CAATTTACTGTTGTCCATGCTG	
Insecticidal toxin complex reverse-transcription primers		
qmitA F	TCCGCCACCTTTATCCAACC	144
qmitA R	AACAGTACGCTGCACCTCAA	
qmitB F1	ACAGCAGGGAGGAACCATTG	102
qmitB R1	CGGCCACTGCTGATAGGTAG	
qmitB F2	TTGAAGCGCACACAGAAACG	147
qmitB R2	CAGCATATCCTTGTGCACGC	
qmitC F1	TGGCATATAGCGCTTGCTGA	175
qmitC R1	CGCCATAGACAAAGCGTTCG	
qmitC F2	CTTCCCGTTCAATTCGGTGC	125
qmitC R2	CCCATACGTCCGCCTAAACA	

[a] Oligonucleotide sequences were constructed from the draft genome of *M. viscosa* NCIMB 13584^T used in this study.

2.4. DNA isolation and gene detection

DNA was prepared from *M. viscosa* plated onto Luria–Bertani (LB) agar plates containing 2.0% NaCl (LB2) incubated at 8 °C using

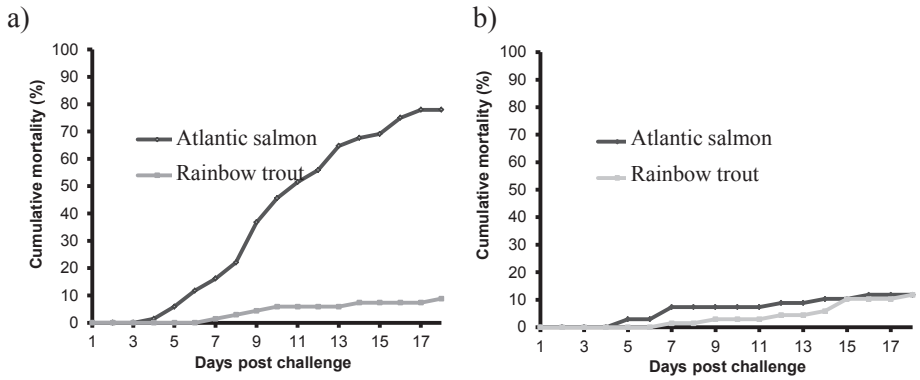


Fig. 1. Cumulative mortality for Atlantic salmon and rainbow trout, registered until 18 days post challenge. The mortality is given as percent. a) Atlantic salmon and rainbow trout challenged with *M. viscosa* originally isolated from Atlantic salmon. b) Atlantic salmon and rainbow trout challenged with *M. viscosa* originally isolated from rainbow trout.

Table 3
Homology to targeted putative virulence factors analyzed in this study.

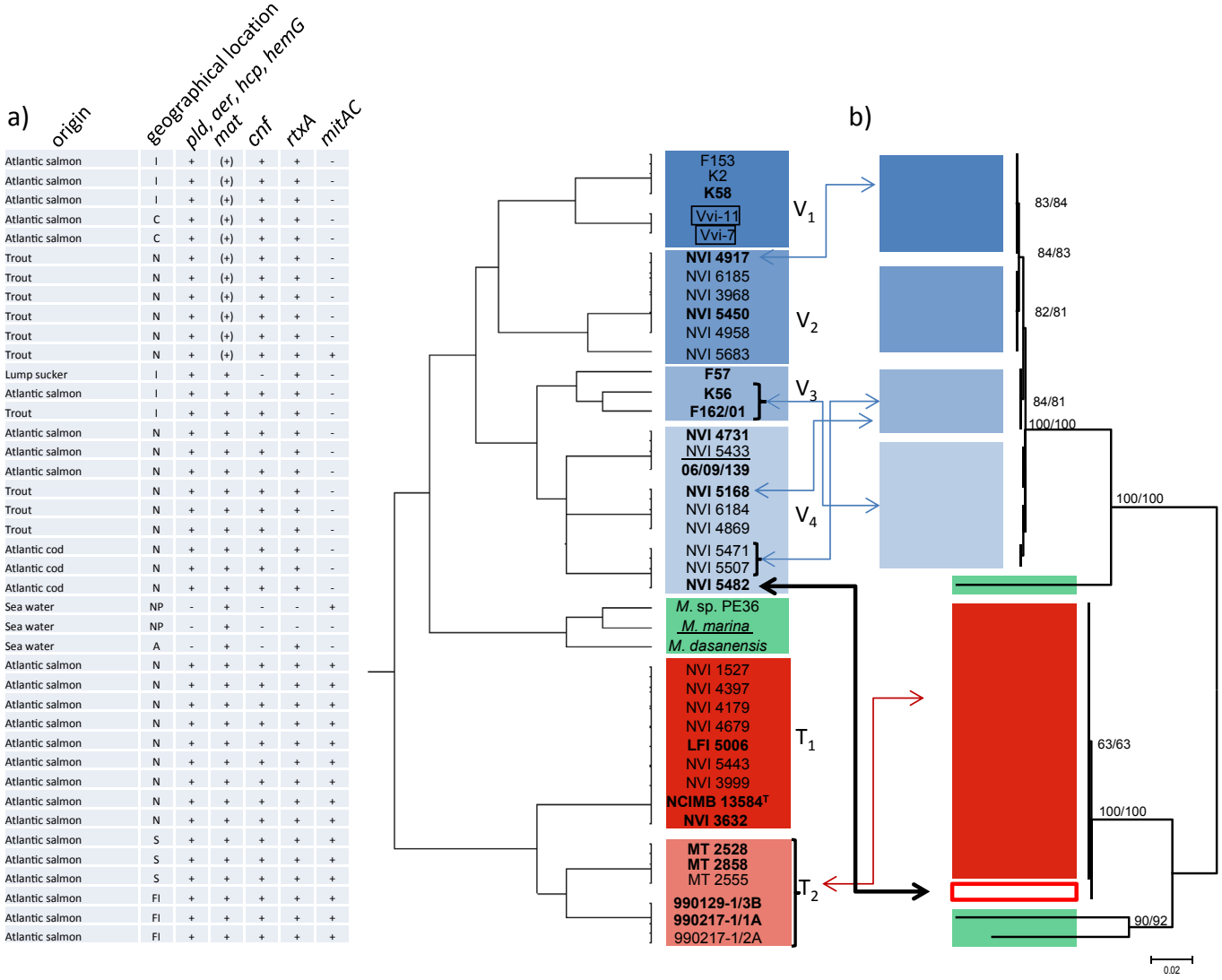
M. viscosa NCIMB 13584^T			Homology to characterized sequences of other species			
Putative gene	Predicted no. of aa	Accession no.	% aa identity/ similarity	Predicted no. of aa	Species and homolouge locus to characterized sequence (Ref.)	Reference to function
Bacterioferritin A, *bfrA*	157	KF822680	60/72	154	*Pseudomonas putida* (NP_742648) [38]	Maintain iron homeostasis during environment adaption and protect against oxidative stress [39]
Bacterioferritin B, *bfrB*	154	KF822681	56/74	157	*Pseudomonas putida* (NP_743243) [38]	
Cytotoxic necrotizing factor, *cnf*	1019	KF822678	61/78	1014	*Escherichia coli* (AF483829) [40]	Modulate actin fibers by activating regulatory GTPases in eukaryotic cells [20]
Aerolysin, *aer*	433	KF822677	30/48	493	*Aeromonas hydrophila* (AAA72103) [23]	Channel-forming toxin binding to host cell-surface structures [24]
Hemagglutinin, *hemG*	273	KF822683	50/65	267	*Myxococcus xanthus* (AAA25399) [41]	Lectin able to agglutinate erythrocytes [42] with anti-viral potency [43]
Hypotetical protein (invasin), *inv*	322	KF822679	34/51	303	*Escherichia coli* (EFX08374)	Homologus to larger invasin-like proteins involved in adherence and invasion of eukaryotic cells [44]
ABC transporter metal-binding lipoprotein, *mat*	342	KF822686	26/47	309	*Streptococcus pneumoniae* (NP_346089) [45]	Transport of Mn^{2+}, pneumococcal surface adhesion (*psaA*) and virulence [25]
Phospholipase D, *pld*	617	KF822682	52/68	587	*Yersinia pestis* (NP_857852) [46]	Required for *Y. pestis* survival and a transmissible infection from the flea vector [47]
Multifunctional autoprocessing repeats-in-toxin, *martxA*	3990	KF822687	71/83[a]	5206	*Vibrio vulnificus* (NP_937086) [21]	Multifunctional toxins likely to be involved in pathogenesis [21]
T6SS VasG chaperone, *clpV*	895	KF822684	100/100	895	*Moritella viscosa* (tMVIS0616) [17]	T6SS is important for virulence of several bacteria [48]
T6SS Hemolysin co-regulated protein, *hcp*	172	KF822685	100/100	172	*Moritella viscosa* (tMVIS0615) [17]	
Insecticidal toxin component A, *mitA*	2576	KF822688	48/64[b]	2378	*Photorhabdus luminescens* (NP_928299) [49]	Toxin complexes with insecticidal activity [50] and cytotoxic to mammalian cultured cells [27]
Insecticidal toxin component B, *mitB*	1410	KF822689	39/43	1476	*Photorhabdus luminescens* (NP_928295) [49]	
Insecticidal toxin component C, *mitC*	987	KF822690	46/62	938	*Photorhabdus luminescens* (NP_928298) [49]	
Type IV prepilin, *tapA*	193	KJ746482	33/69	142	*Aeromonas hydrophila* (P45791) [51]	Adherence, attachment and invasion [52]
Fimbrial protein, *pilA*	159	KJ746483	35/54	154	*Pseudomonas aeruginosa* (P17836) [53]	
Fimbrial protein, *fimA*	176	KJ746484	32/47	162	*Dichelobacter nodosus* (P11933) [54]	
Flagellin component A, *flaA*	274	KJ746477	45/63	284	*Vibrio parahaemolyticus* (Q03473) [55]	Motility and virulence [56]
Flagellin component B, *flaB*	275	KJ746478	45/63	284	*Vibrio parahaemolyticus* (Q03473) [55]	
Flagellin component C, *flaC*	273	KJ746479	44/65	284	*Vibrio parahaemolyticus* (Q03473) [55]	
Flagellin component G, *flaG*	137	KJ746480	24/55	144	*Vibrio parahaemolyticus* (Q56704) [57]	
Lateral flagellin, *lafA*	348	KJ746481	42/55	284	*Vibrio parahaemolyticus* (Q03473) [55]	

[a] N- and C-terminal parts.
[b] Amino acids 1476–2376.

the DNeasy blood and tissue kit (Qiagen) adapted to bacterial cultures according to the manufacturer's instructions. PCR amplification was performed in 50 μl volumes using 40–200 ng template and a final concentration of 1× Taq buffer, 3 U *Taq* DNA polymerase (Invitrogen), 0.2 μM of each primer (Table 2), 0.24 mM dNTP, 2.0 mM $MgCl_2$. The thermal profile was 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min and one cycle of 72 °C for 4 min. Aliquots (35 μl) of the final reaction mixture were visualized in 1.2% agarose gel with 1-kb O'GeneRuler DNA molecular weight ladder (Thermo Scientific) stained with SYBR safe (Invitrogen). PCR products for sequence determination were purified using QIAquick® Gel Extraction Kit (Qiagen) according to instructions and sequenced at the GATC Biotech, DNA sequencing services and bioinformatics (Germany). Detection in *M. viscosa* 06/09/139 was identified through BLAST homology searches. The *M. viscosa* 06/09/139 genome can be accessed at: https://stormbringer.cs.uit.no:60060/ [17].

2.5. Reverse transcription PCR

M. viscosa NCIMB 13584^T was grown in LB broth containing 3.5% NaCl at 9 °C to late exponential phase (OD_{600} = 1.2) before 500 μl culture was added to 1 ml RNAprotect™ (Qiagen). The suspension was centrifuged at 5000× *g* for 5 min. The resulting cell pellet was subjected to total RNA extraction using the RNeasy® Mini Kit (Qiagen) including the DNase treatment utilizing the RNase-Free



origin	geographical location	pld, aer, hcp, hemG	mat	cnf	rtxA	mitAC
Atlantic salmon	I	+	(+)	+	+	-
Atlantic salmon	I	+	(+)	+	+	-
Atlantic salmon	I	+	(+)	+	+	-
Atlantic salmon	C	+	(+)	+	+	-
Atlantic salmon	C	+	(+)	+	+	-
Trout	N	+	(+)	+	+	-
Trout	N	+	(+)	+	+	-
Trout	N	+	(+)	+	+	-
Trout	N	+	(+)	+	+	-
Trout	N	+	(+)	+	+	-
Trout	N	+	(+)	+	+	+
Lump sucker	I	+	+	-	+	-
Atlantic salmon	I	+	+	+	+	-
Trout	I	+	+	+	+	-
Atlantic salmon	N	+	+	+	+	-
Atlantic salmon	N	+	+	+	+	-
Atlantic salmon	N	+	+	+	+	-
Trout	N	+	+	+	+	-
Trout	N	+	+	+	+	-
Trout	N	+	+	+	+	-
Atlantic cod	N	+	+	+	+	-
Atlantic cod	N	+	+	+	+	-
Atlantic cod	N	+	+	+	+	-
Sea water	NP	-	+	-	-	+
Sea water	NP	-	+	-	-	-
Sea water	A	-	+	-	+	-
Atlantic salmon	N	+	+	+	+	+
Atlantic salmon	N	+	+	+	+	+
Atlantic salmon	N	+	+	+	+	+
Atlantic salmon	N	+	+	+	+	+
Atlantic salmon	N	+	+	+	+	+
Atlantic salmon	N	+	+	+	+	+
Atlantic salmon	N	+	+	+	+	+
Atlantic salmon	N	+	+	+	+	+
Atlantic salmon	N	+	+	+	+	+
Atlantic salmon	S	+	+	+	+	+
Atlantic salmon	S	+	+	+	+	+
Atlantic salmon	S	+	+	+	+	+
Atlantic salmon	FI	+	+	+	+	+
Atlantic salmon	FI	+	+	+	+	+
Atlantic salmon	FI	+	+	+	+	+

Fig. 2. The dendrogram in a) from the hierarchical cluster analysis is based on the origin of isolation, geographical location and the prevalence of putative virulence genes in 38 *M. viscosa* isolates including *Moritella* sp. PE36, *M. marina* and *M. dasanensis*. Column 1 shows the origin of isolation species or site. Column 2 shows the geographical origin: N, Norway; I, Iceland; FI, Faroe Island; C, Canada; S, Scotland; NP, North Pacific; A, Arctic. Column 3–7 shows putative virulence genes where PCR products are denoted as: +, detected; −, unobserved; or (+), truncated product. Genes *bfrA*, *bfrB*, *inv*, *clpV* were positive in all bacteria and left out of the analysis. Results from *M. viscosa* 06/09/139, *Moritella* sp. PE36, *M. marina* and *M. dasanensis* were obtained from genome BLAST hits. The dendrogram in a) is compared with phylogeny (b) by Maximum Likelihood analysis of the *gyrB* genes from *Moritella* sp. PE36 (accession no.: ABCQ00000000), *M. dasanensis* ArB 0140 (accession no.: AKXQ00000000), *M. marina* ATCC 15381 (accession no.: ALOE00000000) and *M. viscosa* isolates [4] and this study. Bootstrap values above 60% based on 1000 replicas from Maximum Likelihood and Neighbor-Joining analysis are shown. The scale bar represents the number of substitutions per site. Color-coding of the two main phylogenetic clades is in accordance with the clustering in the dendrogram shown on the right. Split between the variant isolates (sub-grouped into V_1, V_2, V_3, and V_4) colored blue, and the typical salmon isolate (sub-grouped into T_1 and T_2) colored red. Other *Moritella* species are in green. Isolates known to produce lethal ECP or cause mortality are denoted in bold, non-lethal producing ECP isolates are underlined. Avirulent strains are boxed. Strains with altered distribution between sub-clades within the same main clade are shown with thin arrows. Thick arrow represents strain that swapped main clade in the two methods.

DNase Set (Qiagen) according to the manufacturer's instructions. Purified RNA extract was confirmed inactive for DNase activity by digestive analysis. RNA concentration was measured using a NanoDrop ND-1000 with resulting $A_{260/280}$ ratio of 2.15. Total RNA (2 μg) was used as template for reverse transcription (RT) PCR reaction using the QuantiTect® Reverse Transcription Kit utilizing the RT primer mix (Qiagen). The resulting cDNA was utilized as template using the gene-specific primers (Table 2) amplifying the targeted DNA product by a regular PCR method. Genomic DNA isolated from *M. viscosa* NCIMB 13584^T served as positive control. Reverse transcription mixture without addition of reverse transcriptase served as negative control. PCR amplification was performed in 50 μl volumes using 100 ng template, 1× Taq buffer, 2 U *Taq* DNA polymerase (Invitrogen), 0.2 μM of each primer, 0.24 mM dNTP mix, 2.0 mM $MgCl_2$. The thermal profile was 94 °C for 10 min, 35 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 2 min and one cycle of 72 °C for 5 min. Aliquots (35 μl) of the final reaction mixture was visualized in a 1.0% agarose gel with a 100-bp O'GeneRuler DNA molecular weight ladder (Thermo Scientific) stained with SYBR safe (Invitrogen).

2.6. Transmission electron microscopy

M. viscosa strains were maintained on blood agar containing 2% NaCl at 8 °C. Sample grids were made by placing Formvar-coated copper grids for 10 min at room temperature on droplets of *M. viscosa* resuspended in LB2. The grids were washed quickly on PBS and fixed with 0.5% glutaraldehyde in 0.1 M NaCacodylate buffer for 4 min. Grids were washed three times in drops of 0.1 M NaCacodylate buffer followed by four washes in drops of dH_2O and subsequently negative stained 1 min with 2% uranyl acetate before a short rinse in dH_2O. After drying, the samples were viewed in a Phillips 208 S transmission electron microscope (TEM).

2.7. Hierarchical clustering

In a further attempt to discern patterns among the isolates, a multivariate analysis by hierarchical clustering using the ward linkage method in JMP v10 was done. The analysis of the isolates was based on the detection of PCR products of putative virulence factors, their host or environment of isolation, and their geographical origin.

3. Results

3.1. Strain/host specificity

Total mortality (Fig. 1) associated with infections of 'typical' *M. viscosa* (strain NVI 3632) were 78% and 9% in Atlantic salmon and rainbow trout, respectively. Infection by 'variant' *M. viscosa* (strain NVI 5450) resulted in mortality levels of 12% in both Atlantic salmon and rainbow trout. Ulceration in fish surviving the three week observation period showed significant ($p = 0.0325$) difference between groups; with ulceration on 15/17 (88%) Atlantic salmon and 55/73 (75%) rainbow trout infected with the 'typical' *M. viscosa*, and in 50/70 (71%) Atlantic salmon and 41/71 (58%) rainbow trout infected with the 'variant' strain. No mortality or ulcer development was observed in the control groups.

3.2. Detection of putative virulence genes in M. viscosa

Analysis of the putative protein-encoding sequences from the draft genome of *M. viscosa* NCIMB 13584^T revealed ORFs with significant similarity to virulence genes found in other Gram-negative bacteria (Table 3). Amongst others, ORFs predicting a putative aerolysin (*aer*), ABC transporter (*mat*) protein, two bacterioferritins (*bfrA* and *bfrB*), a lectin (*hemG*), a phospholipase D (*pld*), an invasin gene (*inv*) and an ORF encoding a cytotoxic necrotizing factor-like (*cnf*) gene were identified. Genes homologous to the T6SS were also predicted, i.e. *clpV* and *hcp*, along with a multifunctional autoprocessing repeats-in-toxin (*martxA*) gene. An insecticidal toxin complex homolog was predicted within a ~15 kb region (described in detail later). Also fimbrial and pilin systems (*fimA*, *tapA*, and *pilA*) were revealed together with homologs of structural components of both polar and lateral flagella (*fla* and *laf*).

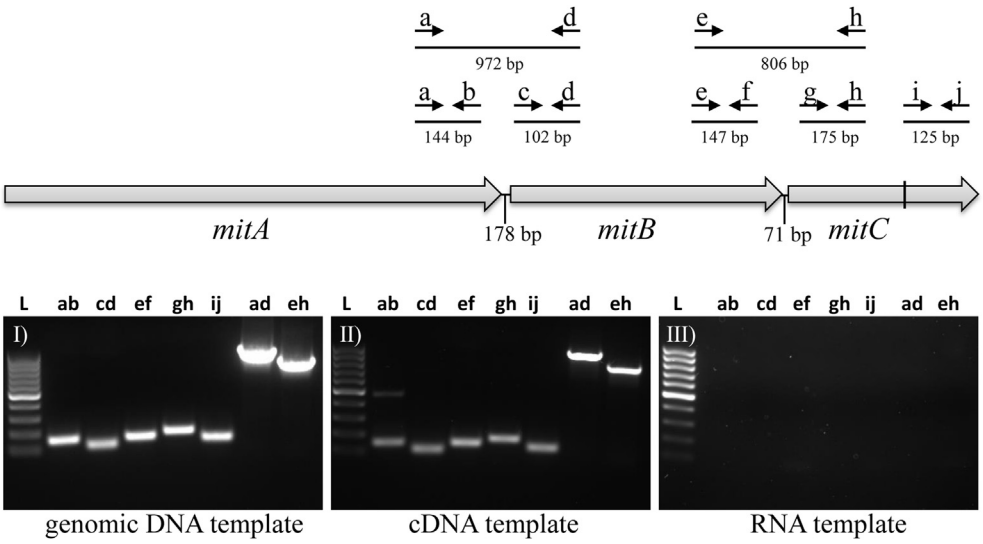


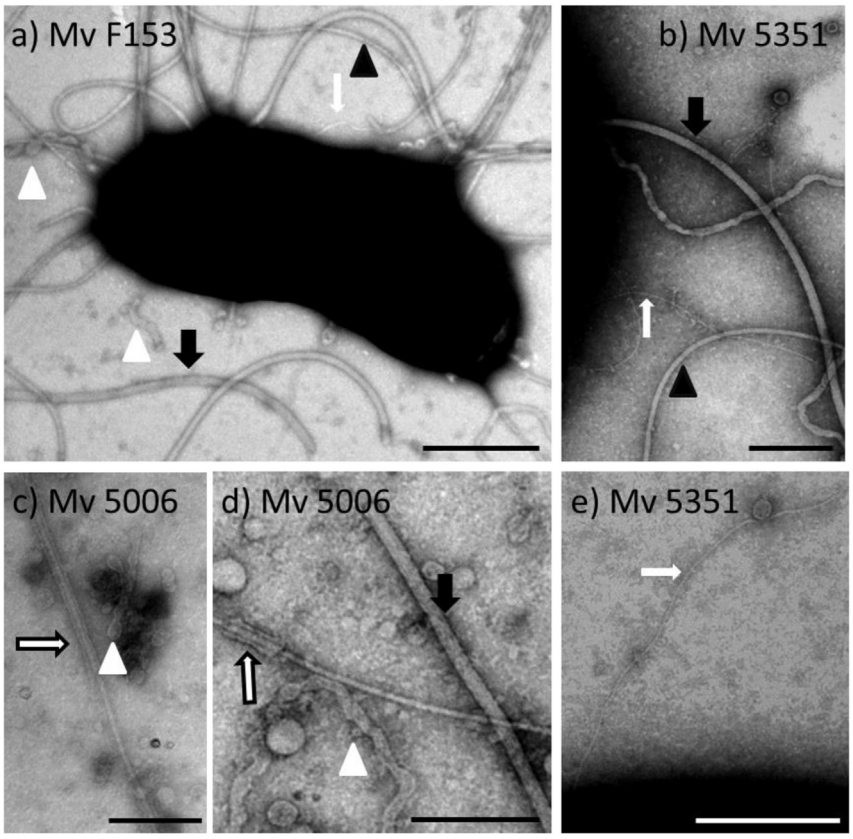
Fig. 3. RT-PCR analysis of transcription from the *M. viscosa* insecticidal toxin-like operon complex. The arrows represent the transcriptional directions and coding regions of the genes, *mitA*, *mitB*, and *mitC* representing class A, B, and C-like proteins. Primers used in the RT-PCR are labeled as a (qmitA F), b (qmitA R), c (qmitB F1), d (qmitB R1), e (qmitB F2), f (qmitB R2), g (qmitC F1), h (qmitC R1), i (qmitC F2), and j (qmitC R2). Binding sites to primers are shown on the operon and length of the products obtained. Gel I: positive controls, PCR products from genomic DNA template. Gel II: PCR products from the reverse transcripted RNA template. Gel III: negative controls, PCR products from the RNA template without reverse transcriptase. Primer pairs used for generating amplicon products are symbolized above each lane. L = 100-bp molecular marker.

3.3. Distribution of putative virulence genes within M. viscosa

The putative virulence genes were broadly distributed (Fig. 2). Evidence of the existence of *bfrAB*, *pld*, *inv*, *aer*, *clpV*, *hcp*, *hemG*, and *rtxA*, was identified by PCR in all *M. viscosa* isolates studied from different fish species and geographic origins. The putative *mat* gene was also detected in all strains, although 11 isolates, all phylogenetically related within variant sub-clade V_1 and V_2 (Fig. 2) produced a truncated version of the product. Sequenced loci coding for complete and truncated products were identical with the exception of a 357-bp deletion within the C-terminal part of the protein (Appendix A). The *cnf* product was detected in all isolates, except for isolate F57 (isolated from a healthy lump sucker), which is a low-virulent strain to Atlantic salmon [10]. The *mitA* and *mitC* genes of the insecticidal toxin complex were identified almost exclusively and in all members of the 'typical' Atlantic salmon *gyrB* clade.

3.4. Analysis of the M. viscosa insecticidal toxin-like operon

BLASTx of the predicted genes within the *M. viscosa* insecticidal toxin-like (*mit*) region revealed a cluster of three genes displaying considerable identity with insecticidal toxin operons in other bacterial taxa, including *Photorhabdus luminescens* (Table 3), *Xenorhabdus nematophilus* and *Yersinia* sp. (results not shown). The predicted genes in *M. viscosa* are all in the same orientation (Fig. 3). The 5′-3′ direction of the genes initiates with a 7728-bp ORF termed *mitA* encoding a type A-like protein, which is believed to form a transmembrane pore in complex with B-type protein that



M. viscosa strains	Origin	Location	Thick flagella	Thin flagella	Pili
NCIMB 13584^T	*S. salar*	Norway	yes	yes	yes
LFI 5006	*S. salar*	Norway	yes	yes	yes
NVI 3999	*S. salar*	Norway	Nv.	yes	yes
F153	*S. salar*	Iceland	yes	yes	yes
Vvi-7	*S. salar*	Canada	yes	yes	yes
NVI 4917	*O. mykiss*	Norway	Nv.	yes	yes
NVI 5450	*O. mykiss*	Norway	yes	yes	yes
NVI 5168	*O. mykiss*	Norway	yes	yes	yes
NVI 5507	*G. morhua*	Norway	yes	yes	yes

Fig. 4. Expression of surface organelles by *M. viscosa* viewed by transmission electron microscopy. Imaging analysis verifies the expression of pili in a), b) and e) (white arrow), thin flagella in a) and b) (black arrowhead) and thick flagella in a), b), c) and d) (black arrow) by most *M. viscosa* strains as summarized in the lower table. Nv = not verified. The thick flagella with disrupted sheeting are shown as black arrows with white filling in c) and d). White arrowheads = membrane blebs in a), c), and d). Space bar = 500 nm in a) and 200 nm in b–e.

Table 4
Homology analysis of the putative virulence factors identified in other *Moritella* species.

Homology to ORFs of other *Moritella* species										
M. viscosa^T		*Moritella* sp. PE36			*M. dasanensis*			*M. marinum*		
Putative ORF	Predicted no. of aa	Predicted no. of aa	Homologous locus	% identity/ similarity	Predicted no. of aa	Homologous locus	% identity/ similarity	Predicted no. of aa	Homologous locus	% identity/ similarity
bfrA	157	97	1099400000761	95/95	155	AKXQ01000032	85/93	156	ALOE01000031	86/95
bfrB	154	153	1099400000761	94/98	156	AKXQ01000032	82/90	153	ALOE01000031	84/90
inv	322	312	1099400000766	83/92	312	AKXQ01000033	76/86	304	ALOE01000020	79/88
mat	342	326	1099400000766	91/97	309	AKXQ01000033	93/96	314	ALOE01000020	94/97
clpV[a]	895	865	1099400000714	43/62	860	AKXQ01000019	41/61	896	ALOE01000017	38/56
		886	1099400000711	43/61	769	AKXQ01000015	41/62	771	ALOE01000006	41/63
		898	1099400000738	37/56		nd			nd	
rtxA	3990		nd		4911	AKXQ01000028	(76/86: 85/ 91)[a]	nd		
mitA	2576	2627	1099400000641	41/56[b]		nd			nd	
mitB	1410	1527	1099400000641	35/52		nd			nd	
mitC	987	967	1099400000641	51/74		nd			nd	

[a] N- and C-terminal regions of the protein.
[b] aa1618-2626, nd = not detected.

translocate the functioning/toxic C-protein into the cell [18]. Downstream of *mitA* are two coding regions, *mitB* (4230-bp) and *mitC* (2963-bp) encoding type B-like and C-like homologs, respectively. The sequence of MitC encodes a stop codon at aa position 695, but this region has also an apparent frameshift mutation from frame +1 to frame +3.

The transcriptional pattern of the *M. viscosa* insecticidal toxin-like genes was analyzed by RT-PCR. Primer pairs were designed such that polycistronic mRNA could be detected if produced between the 3′ end of one gene and the 5′ end of the adjacent downstream gene. RT-PCR data showed a transcript of all predicted *mitABC* genes indicating a functional transcription of these genes in *M. viscosa*. Also the amplified regions using primers that crossed the intergenic space between *mitA* and *mitB*, and *mitB* and *mitC* produced amplicons correlating with the predicted 972-bp and 806-bp product size, respectively. This result indicates that the *mitA*, *mitB* and *mitC* are co-transcribed as a polycistronic mRNA in *M. viscosa*.

3.5. Expression of surface organelles in M. viscosa and flagellin phylogeny

Several loci devoted to the expression of surface organelles were identified in the genome of *M. viscosa* NCIMB 13584^T. Here nine *M. viscosa* isolates from both the typical and variant sub-clades were selected for TEM analysis. Thin peritrichous flagella and at least one polar or sub-polar thick flagellum were identified, except in two isolates (Fig. 4). Lack of visual detection could be caused by assay sensitivity (cell handling/treatment) or reflect that these systems are not constitutively expressed [12]. Observed flagella were sheathed as viewed in Fig. 4(c) and (d). Single pili of 6–8 nm in diameter were detected in all isolates (Fig. 4(a), (b) and (e)). No bundle forming pili could be viewed. Also membrane blebs and vesicles were observed in all isolates. Although no attempts were made in identifying the nature of the structural subunits of the flagella, five putative ORFs in the *M. viscosa* genome were annotated as putative flagellin genes (Table 3). Four were located in succession of each other (results not shown). Phylogenetically, FlaA (KJ746477) and FlaB (KJ746478) are highly identical and together with FlaC (KJ746479) cluster to homolog sequences constituting the structural subunits of the polar flagella in *V. parahaemolyticus* and *A. salmonicida* (Appendix B). The downstream and shorter KJ746480 sequence cluster with FlaG homologs of the same system. The putative flagellin of KJ746481 is part of a different operon and clusters together with a homolog to the lateral LafA component.

3.6. Homology within the genus Moritella

The distribution of the targeted genes was further investigated in three related *Moritella* species with no known disease causing effect, utilizing the draft genomes of *Moritella* sp. PE36, *M. marina* and *M. dasanensis*. BLASTn and tBLASTx searches revealed sequences homologous with *bfrA*, *bfrB*, *inv*, *mat*, and *clpV* in all genomes (Table 4). An *rtxA* homolog was detected in *M. dasanensis*, and genes homologous (although with low identity) to the insecticidal toxin-like complex (*mitABC*) were detected in *Moritella*. sp. PE36. The analysis did not identify significant homologies with *M. viscosa pld*, *cnf*, *aer*, *hemG* or *hcp* sequences.

3.7. Comparison of hierarchical clustering to phylogeny

Hierarchical clustering based on host species, geographical origin and genetic polymorphism, grouped the 38 *M. viscosa* isolates into two main clusters (Fig. 2(a)). The topology of the hierarchical dendrogram is similar to that of the phylogenetic analysis of *gyrB* gene sequences (Fig. 2(b)). This demonstrates that the phylogenetic evolutionary distance corresponds to host, geographical location and genetic polymorphism. The trees are split between the ‘variant’ isolates colored blue and the ‘typical’ salmon isolates colored red, with sub-clade classification (variant: V_1, V_2, V_3 and V_4; and typical: T_1 and T_2). Only isolate 5482 changes clade with a few isolates swapping within sub-clusters.

4. Discussion

The present study provides for the first time experimental support for host specificity in ‘typical’ *M. viscosa* and Atlantic salmon. That a ‘typical’ *M. viscosa* strain resulted in high acute mortality in Atlantic salmon and a more chronic ulcerative infection in rainbow trout (similar to that caused by the ‘variant’ *M. viscosa* in both species of fish), suggests that some factor highly toxic for Atlantic salmon exists in this clade. The genome of *M. viscosa* NCIMB 13584^T was utilized to identify possible virulence-related genetic differences and host specific variation between the ‘typical’ and the ‘variant’ group of *M. viscosa*.

M. viscosa pld, *cnf*, *aer*, and *hemG* sequence homologs were not predicted in the genomes of other *Moritella* species not known to

cause infection. These may constitute virulence or specific niche related factors associated with the general fish pathogenic abilities of *M. viscosa*. The temperature dependent fish cell adhesion [9], could be mediated by the lectin (*hemG*) as homologs bind carbohydrates on glycoproteins exposed on host cell surfaces [19]. Homologs of *cnf* and *rtxA* (*rtxA* is also present in *M. dasanensis*, Table 4) are actin degrading toxins [20,21]. Cnf is responsible for tissue damage and is associated with urinary tract, skin and soft tissue *Escherichia coli* infections [20]. Interestingly, up-regulation of *cnf* in *M. viscosa* is associated with tissue degradation during ulcer development [12]. The *cnf* gene could not be detected by PCR screening in the low-virulent F57 strain (lump sucker) in this study, but injection of F57 ECP results in Atlantic salmon mortality [10]. This could indicate Cnf as a virulence factor for *M. viscosa* but not as a central factor for Atlantic salmon mortality. Also *M. viscosa rtxA*, whose homolog is a major virulence factor in *Vibrio anguillarum* [22], is up-regulated in parallel with cell rounding and actin rearrangement of fish cells [12]. The putative *M. viscosa* aerolysin (*aer*), homologous with the *Aeromonas hydrophila* Aer [an exported channel-forming toxin that induce membrane destruction and terminal lysis [23,24]], could be part of the cytolytic pore forming products proposed secreted by *M. viscosa* [9].

Identification of homologs to *bfrA*, *bfrB*, *inv*, *mat*, and *clpV* (Table 4) in other *Moritella* species may indicate a function for these genes different or additional to fish pathogenicity. The *mat* gene encodes an ABC-type transport protein important for attachment and virulence in pneumococcal bacteria [25]. The truncated *mat* identified in 11 isolates studied (Fig. 2) appears to have occurred ancestrally in strains isolated from Canadian and Icelandic Atlantic salmon and Norwegian rainbow trout isolates. Although this study includes only a limited number of isolates, this mutation may reflect a reduced virulence potential, particularly for Atlantic salmon. However, isolates belonging to this group retain their ability to produce lethal ECP and infect Atlantic salmon experimentally [10]. Table 4 indicates that *clpV* and T6SS is a general trait in *Moritella* species. However, *M. viscosa* contains two putative T6SS (loci *mts1* and *mts2*), which most likely have been acquired in separate events due to low sequence and structure conservation [17]. Only *mts1* is similar to the two T6SS in *Moritella* sp. PE36 [17]. The *clpV* identified in this study is located within the *mts2* loci, which may explain the lack of *hcp* detection and the low *clpV* sequence homology to other *Moritella* species. The second *mts2* T6SS could be exclusive to *M. viscosa* and indicate an adaption of additional or separate functions i.e. towards infection. Functionality of *mts2* is confirmed in both virulent and non-virulent isolates [17] which encompass both 'typical' and 'variant' *M. viscosa*.

Blast searches (Table 3) together with phylogenetic analysis (Appendix B) predict that the *M. viscosa* genome could give rise to the surface expression of polar and lateral flagella and pili. TEM investigation of *M. viscosa* isolates representing different geographical regions and host species confirmed expression of both flagella types and pili surface organelles (Fig. 4). Although, motility, adherence and attachment factors are likely to constitute virulence factors of *M. viscosa*, these surface organelles are likely not the accountable factor for the induced higher mortality in "typical" *M. viscosa* towards Atlantic salmon as they are expressed in both "typical" and "variant" strains. This could indicate that it is the ability to produce and secrete an additional effector molecule such as e.g. a toxin that is the responsible factor for the increased Atlantic salmon mortality in "typical" *M. viscosa*. However, a variety of gene products could possibly act cooperatively making it difficult to pinpoint a single determinant responsible for the lethal outcome. Differential regulation or expression of the genome may also result in differences in disease pathogenesis between isolates.

Similarities between the cluster dendrogram and the *gyrB* phylogeny indicate that the evolutionary histories appear to be congruent with the geographical location, host species and genetic prevalence. The distribution of the insecticidal toxin-like complex (*mitABC*) splits *M. viscosa* and supports the phylogenetic division of *M. viscosa* as presented by Grove et al. (2010). The presence of clade-specific genes suggests differences in the selective pressure acting on the specific subpopulations. *M. viscosa* NCIMB 13584^T expressed and co-transcribed the *mitABC* genes under the growth conditions used in this study. Homologs to this toxin complex exert insecticidal activity [26], while others of the family are non-toxic until translocated from insects into mammalian host cells where they are postulated to modulate immune responses including actin modification [27–30]. *M. viscosa* is known to exert actin modulating activity in fish cells [9] and is postulated to suppress immune responses in salmon [14]. Whether the insecticidal toxin-like complex *mitABC* has a role in the ability of *M. viscosa* to infect fish cells remains to be elucidated, but the presence and expression of this complex suggests a functional role in 'typical' *M. viscosa* isolates.

Differences in cytotoxin production may reflect host adaptation. 'Variant' *M. viscosa* exhibit an elevated cytotoxic and hemolytic activity compared to 'typical' *M. viscosa* [4,10]. Correlation to our 'typical' and 'variant' *M. viscosa* classification is also apparent in an amplified length polymorphism (AFLP) study [31]. Furthermore, *M. viscosa* is a serological diverse group [32]. The study of Heidarsdottir et al. (2008) presents a lower sized antigen and a different serotype for Norwegian and Scottish Atlantic salmon isolates, which correlate to 'typical' and 'variant' *M. viscosa*. Thus antigenic diversity may represent a driving force for avoidance of immune responses in different hosts.

It is intriguing to speculate if the life cycle of *M. viscosa* could encompass hitherto unknown secondary or intermediate hosts in the marine environment. Could the putative insecticidal toxin complex (*mitABC*) be connected to interaction with e.g. marine arthropods? A large armory of virulence genes could be advantageous if infecting multiple hosts or in competition for resources in low nutritional marine environments; such as exploitation of e.g. different dead or dying planktonic organisms. Metagenomic data of marine bacteria indicate a high abundance of virulence-associated gene homologs, which could suggest that some bacteria infect or consume eukaryotes for nutrients [33]. The reservoir of putative virulence genes homologous to known animal, plant and insect pathogens could allow an opportunistic pathogenic lifestyle in higher eukaryotic organisms such as fish. The range of fish hosts susceptible to *M. viscosa* infection is wide. The number of putative virulence factors present in *M. viscosa* could give *M. viscosa* the ability to infect multiple fish species. However, pathogens cannot cause disease indiscriminately, and some fish species appear more resistant to *M. viscosa* than others [8]. This may infer that *M. viscosa* as a species has evolved compatibility factors that enable pathogenesis in a host-specific manner. None of the genes targeted in this study are necessarily directly linked to virulence, but only 'typical' *M. viscosa* carries the *mitABC* toxin complex. It could be speculated that the very presence and density of large scale Atlantic salmon farms in the North East Atlantic may have led to the expansion of what appears to be a highly virulent clone causing winter-ulcer in this fish species.

Acknowledgments

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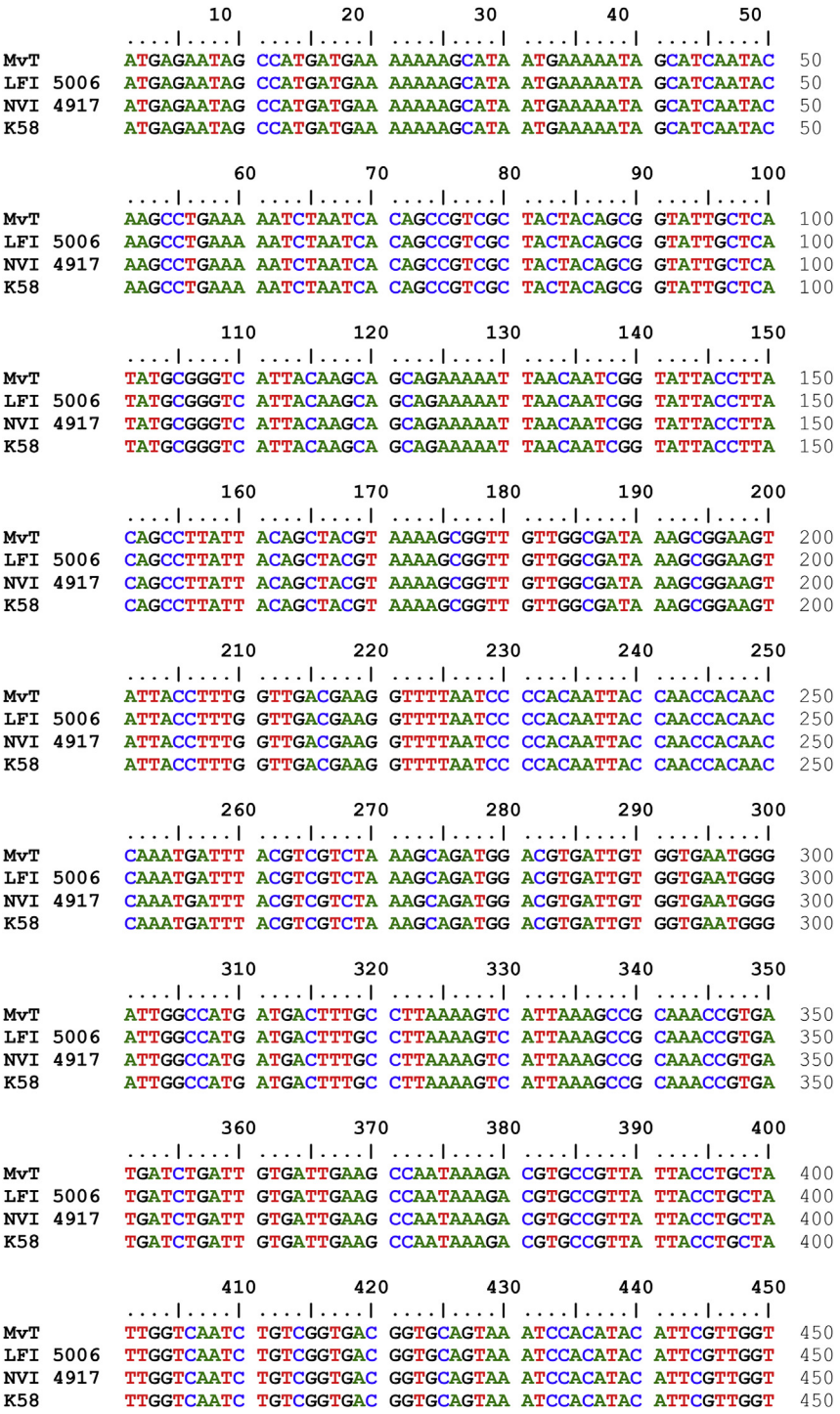
Appendix A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LFI 5006 ATGAGAATAG CCATGATGAA AAAAAGCATA ATGAAAAATA GCATCAATAC 50
NVI 4917 ATGAGAATAG CCATGATGAA AAAAAGCATA ATGAAAAATA GCATCAATAC 50
K58 ATGAGAATAG CCATGATGAA AAAAAGCATA ATGAAAAATA GCATCAATAC 50
60 70 80 90 100
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LFI 5006 AAGCCTGAAA AATCTAATCA CAGCCGTCGC TACTACAGCG GTATTGCTCA 100
NVI 4917 AAGCCTGAAA AATCTAATCA CAGCCGTCGC TACTACAGCG GTATTGCTCA 100
K58 AAGCCTGAAA AATCTAATCA CAGCCGTCGC TACTACAGCG GTATTGCTCA 100
110 120 130 140 150
MvT TATGCGGGTC ATTACAAGCA GCAGAAAAAT TAACAATCGG TATTACCTTA 150
LFI 5006 TATGCGGGTC ATTACAAGCA GCAGAAAAAT TAACAATCGG TATTACCTTA 150
NVI 4917 TATGCGGGTC ATTACAAGCA GCAGAAAAAT TAACAATCGG TATTACCTTA 150
K58 TATGCGGGTC ATTACAAGCA GCAGAAAAAT TAACAATCGG TATTACCTTA 150
160 170 180 190 200
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LFI 5006 CAGCCTTATT ACAGCTACGT AAAAGCGGTT GTTGGCGATA AAGCGGAAGT 200
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K58 CAGCCTTATT ACAGCTACGT AAAAGCGGTT GTTGGCGATA AAGCGGAAGT 200
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K58 CAAATGATTT ACGTCGTCTA AAGCAGATGG ACGTGATTGT GGTGAATGGG 300
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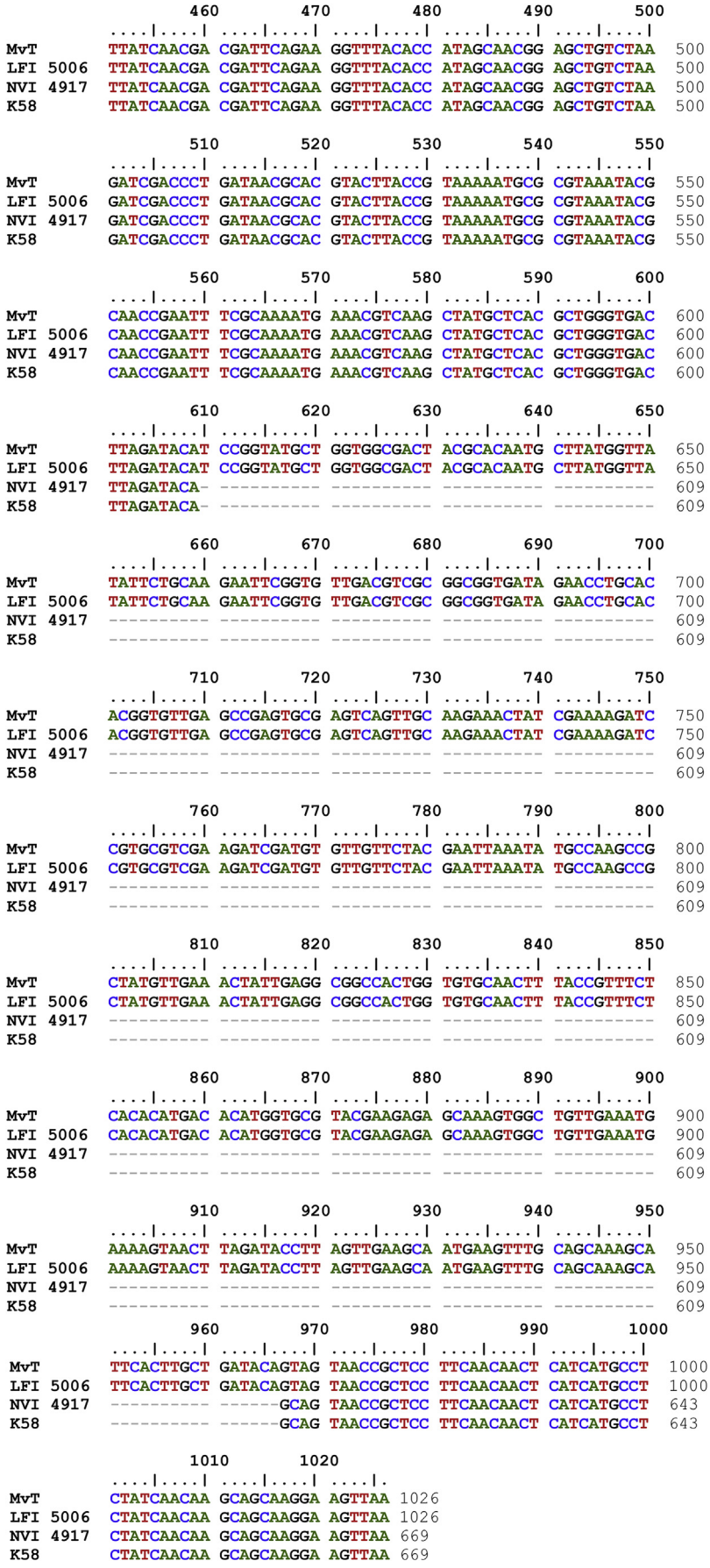

Fig. A. Aligned *mat* gene sequences for strains with full length gene (MvT and LFI 5006) and truncated gene (LFI 4917 and K58).

Appendix B

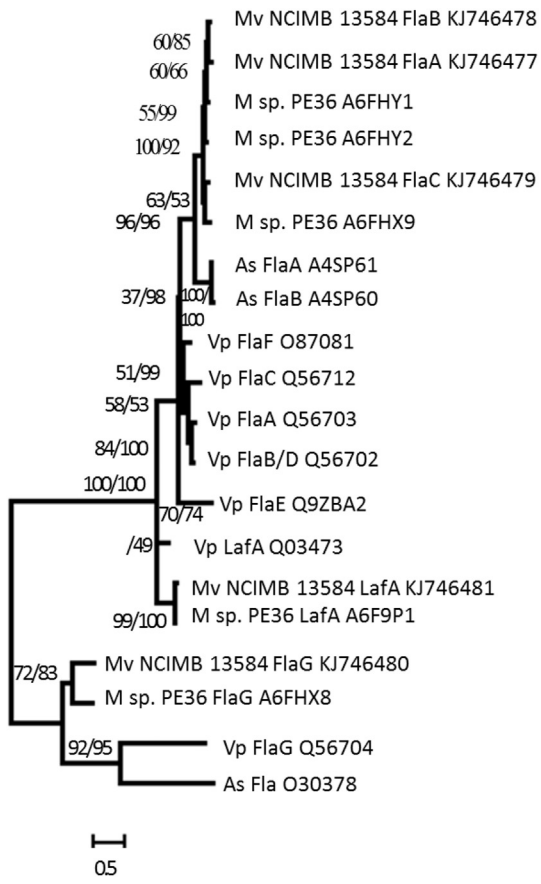

Fig. B. Phylogenetic analysis of the putative flagellins in the *M. viscosa* genome. The Neighbor-Joining tree illustrates the maximum likelihood based on amino acid sequences of *M. viscosa* type strain, *Moritella* sp. PE36, *Vibrio parahaemolyticus* and *Aeromonas salmonicida*. Bootstrap values for >50% of 1000 repetitions (maximum likelihood/neighbour joining) are shown adjacent to each branch. The scale bar represents the number of substitutions per site. There were a total of 130 and 425 positions in the final ML and NJ dataset, respectively.

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