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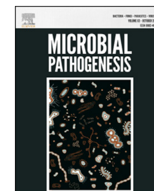

A unique role of flagellar function in *Aliivibrio salmonicida* pathogenicity not related to bacterial motility in aquatic environments

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Aliivibrio salmonicida is the causative agent of cold-water vibriosis, a septicemia of farmed salmonid fish. The mechanisms of disease are not well described, and few virulence factors have been identified. However, a requirement for motility in the pathogenesis has been reported. *Al. salmonicida* is motile by the means of lophotrichous polar flagella, consisting of multiple flagellin subunits that are expressed simultaneously. Here we show that flagellin subunit FlaA, but not FlaD, is of major importance for motility in *Al. salmonicida*. Deletion of *flaA* resulted in 62% reduction in motility, as well as a reduction in the fraction of flagellated cells and number of flagella per cell. Similarly, deletion of the gene encoding motor protein *motA* gave rise to an aflagellate phenotype and cessation of motility. Surprisingly, we found that *Al. salmonicida* does not require motility for invasion of Atlantic salmon. Nevertheless, in-frame deletion mutants defective of *motA* and *flaA* were less virulent in Atlantic salmon challenged by immersion, whereas an effect on virulence after i.p. challenge was only seen for the latter. Our results indicate a complex requirement for motility and/or flagellation in the pathogenesis of cold-water vibriosis, but the mechanisms involved remain unknown. We hypothesize that the differences in virulence observed after immersion and i.p. challenge are related to the immune response of the host.

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1. Introduction

Motility is well recognized as a virulence factor in bacteria. In *Vibrio anguillarum*, motility is required for virulence in fish challenged by immersion, but it is not needed for disease progression once the bacterium has invaded the fish [1]. Similarly, *Aliivibrio fischeri* depends on motility for invasion of the light organ of the Hawaiian bobtail squid *Euprymna scolopes* and symbiosis establishment [2]. A requirement for motility in virulence has also been observed for *Aliivibrio salmonicida*,[1] the causative agent of cold-water vibriosis in salmonids. An uncharacterized motility-deficient mutant was found to be less virulent by immersion challenge and cause a delay in disease development after intraperitoneal challenge [3].

Flagella are helical propellers protruding from the external surface of bacteria, providing a means of locomotion that enables swimming towards favorable environments. In addition to their role in motility, flagella may also function in adhesion, biofilm formation, secretion and immune system modulation [4]. Most *Vibrio* spp. are equipped with lophotrichous or monotrichous polar flagella covered by a sheath [5]. The sheath appears to be an extension of the outer membrane, although its function is not well understood.

The flagellar structure is often described in three parts: the basal body containing a rotary motor and embedding the flagellum in the cell envelope, the hook functioning as a joint, and the filament extending from the hook [6]. Flagellar assembly is a complex process involving more than 50 genes. As the production of flagella requires a major commitment of energy, regulation is kept under strict control [7]. The control system is coupled with assembly and involves several checkpoints as construction progresses from the inner structures to the outer ones [8].

The flagellar filament is the largest part of the flagellum and consists of self-assembling flagellin subunits arranged in a helix. While many flagellated bacterial species contain one or two flagellin genes, some organisms have genes encoding several

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[1] *Vibrio salmonicida* was reclassified as *Aliivibrio salmonicida* in 2007 [38]. However, as the abbreviation *A. salmonicida* is associated with the fish pathogen *Aeromonas salmonicida*, *Aliivibrio salmonicida* is abbreviated *Al. salmonicida* throughout this paper.

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flagellin subunits. *Vibrio parahaemolyticus, V. anguillarum* and *Vibrio cholerae* harbor multiple flagellin genes with a similar chromosomal organization [5]. For each flagellin gene, the sequence homology to the orthologous gene in the other organisms is higher than to the other flagellin genes in the same organism. In contrast, *Al. fischeri* and *Al. salmonicida* seem to constitute a different clade in terms of flagellin gene organization [9,10]. Flagellin genes *flaA* and *flaB* have orthologs in other *Vibrio* spp., while *flaCDEF* appear unique to *Aliivibrio*. Interestingly, the *flaA* orthologs of *V. parahaemolyticus*, *V. anguillarum*, *V. cholerae* and *Al. fischeri* have been shown to have distinct transcription and/or function compared to the other flagellin genes [5,9].

Why these organisms possess several flagellin genes is unknown. Flagellin is considered a microbe-associated molecular pattern (MAMP) and is readily recognized by Toll-like receptor 5 (TLR5) of the innate immune system of both mammals and teleosts [11,12]. In order to evade an immune response, several bacteria utilize antigenic variation. The multiple flagellins of vibrios may be involved in a similar role. However, the flagellins of *Al. salmonicida* are expressed simultaneously *in vitro* [10].

Flagellar motility is powered by the flagellar motor, which consists of multiple stator elements surrounding a rotor. Interactions between stators and rotor generate a torque which drives flagellar rotation [13]. The stator complex, consisting of proteins MotA and MotB, functions as an ion channel and provides energy from an electrochemical gradient of ions across the cytoplasmic membrane. Both H^+ and Na^+-driven motors have been described, and the polar flagella of *Vibrio* spp. are powered by Na^+-driven motors [13]. The torque generated by the motor is transmitted through the hook to the propelling filament [6].

The pathogenesis of cold-water vibriosis is poorly understood. However, *Al. salmonicida* has been described to be motile *in vivo* by several authors [3,14,15]. As the production of flagella provides a target for innate immunity in addition to being energetically costly, the flagellar structure and/or function is likely to be advantageous to the bacterium.

Our aim was to determine effects of motility and flagellation on host colonization and disease development of cold-water vibriosis. By constructing defined in-frame deletion mutants for *flaA* and *flaD*, encoding flagellin subunits, and *motA*, encoding a stator component, we set out to determine the role of these genes in virulence in experimental models challenging Atlantic salmon (*Salmo salar* L.) by immersion and intraperitoneal (i.p.) injection.

2. Methods

2.1. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Al. salmonicida* LFI1238 was grown on blood agar consisting of blood agar base No. 2 (Oxoid, Cambridge, UK) supplemented with 5% ox blood and 0.9% or 2.5% NaCl (BA0.9 or BA2.5), in Luria Bertani broth supplemented with 0.9%, 1%, 2.5% or 3% NaCl (LB0.9, LB1, LB2.5 or LB3), or on Luria Bertani agar (LA0.9, LA1, LA2.5, LA3) solidified by addition of 1.2% agar-agar (Merck, Darmstadt, Germany) to the different LB media. Unless otherwise stated, LFI1238 was cultivated at 12 °C. *Escherichia coli* strain S17-1 λpir was grown in LB1 or on LA1 agar at 37 °C.

For construction of in-frame deletion mutants, R6K origin suicide vector pDM4 kindly provided by Debra Milton [1] was used. Selection of S17-1 λpir transformants was carried out by adding 25 μg/ml chloramphenicol (Sigma-Aldrich, St. Louis, MS, USA) (25CAM) to LA1, and selection of LFI1238 transconjugants was carried out by adding 2 μg/ml chloramphenicol (2CAM) to LA2.5. Counter-selection of LFI1238::pDM4 was performed by adding 5% sucrose to LA2.5.

Growth curve experiments were carried out by cultivation of strains in LB0.9 or LB3 at 8 °C with agitation (125 rpm). Optical density at 600 nm (OD_{600}) was measured at three hour intervals using a Genesys 20 photospectrometer (Thermo Scientific, Waltham, MA, USA). All experiments were carried out in biological duplicates.

2.2. Construction of in-frame deletion mutants

In-frame deletion mutants LFI1238Δ*flaA*, LFI1238Δ*flaD*, LFI1238Δ*flaA*Δ*flaD* and LFI1238Δ*motA* were constructed by allelic exchange as described by others [1,18]. Primers used were ordered from Invitrogen (Carlsbad, CA, USA) and are listed in Table 2. Plasmid purification and gel extraction were performed using QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit respectively (both Qiagen, Hilden, Germany) as recommended by manufacturer. For construction of LFI1238Δ*flaA*, segment flaA-AB immediately upstream of *flaA* was amplified by PCR using primers flaA-A and flaA-B, and segment flaA-CD downstream of *flaA* was amplified using primers flaA-C and flaA-D. Fusion of flaA-AB and fla-CD, employing a complimentary sequence, was conducted by overlap PCR using the following program: 7 cycles with no added primers (94 °C for 30 s, 54 °C for 30 s, 72 °C for 2 min) and 30 cycles with primers added (same conditions). The resultant construct and suicide vector pDM4 were digested with restriction enzymes *XhoI* and *SpeI* (New England Biolabs, Ipswich, MA, USA), ligated (T4 DNA ligase, Invitrogen) into pDM4Δ*flaA*, and transformed in *E. coli* S17-1 λpir. Following this, pDM4Δ*flaA* was introduced into *Al. salmonicida* LFI1238 by conjugation and integrated in its chromosome by allelic exchange as previously described [18]. To complement the constructed LFI1238Δ*flaA*, the full-length gene *flaA* including flanking regions was inserted into pDM4 creating pDM4Δ*flaA*c, followed by chromosomal integration in LFI1238Δ*flaA* as described above. In-frame deletion mutants LFI1238Δ*flaD*,

Table 1
Bacterial strains and plasmids used. * This study.

Strain or plasmid	Description	Reference
Aliivibrio salmonicida LFI1238	Wild type strain	[16]
Escherichia coli S17-1 λpir	Donor strain for conjugation	[17]
LFI1238Δ*flaA*	LFI1238 with in-frame deletion of *flaA*	*
LFI1238Δ*flaAc*	LFI1238Δ*flaAc* strain complemented with full length LFI1238 *flaA* gene	*
LFI1238Δ*flaD*	LFI1238 with in-frame deletion of *flaD*	*
LFI1238Δ*flaA*Δ*flaD*	LFI1238 with in-frame deletions of *flaA* and *flaD*	*
LFI1238Δ*motA*	LFI1238 with in-frame deletion of *motA*	*
pDM4	R6K origin suicide vector; contains *cat* and *sacB*	[1]
pDM4Δ*flaA*	pDM4 containing Δ*flaA* allele	*
pDM4Δ*flaD*	pDM4 containing Δ*flaD* allele	*
pDM4Δ*flaAc*	pDM4 containing full length *flaA*	*
pDM4Δ*motA*	pDM4 containing Δ*motA* allele	*

Table 2
Primers used for construction of in-frame deletion mutants.

Description:	Primers:	Sequence (5' – 3'):	Comments	Construct size:
Primers for construction of LFI1238Δ*flaA*:	flaA-A	CGTCTCGAGCAGTTGCAAAGTAGAGTT	5′ end contains *XhoI* restriction site	244 bp
	flaA-B	CGCAGCTACGTTAGTATTTACATTTACAGCCATGG		
1110 bp deletion targeting *flaA* (VSAL_RS12190)	flaA-C	ACTAACGTAGCTGCGTAACGAACAACAGTC	5′ end contains a 15 bp sequence complementary to the 5′ end of flaA-B	242 bp
	flaA-D	GGACTAGTGTAAGTCATCGACCGTTGTGTG	5′ end contains a *SpeI* restriction site	
Verification primers for LFI1238Δ*flaA*	flaA-G	ACTATGACTGAGTAGTTCACAGTTTGC	Targets construct flanking introduced deletion	1707/597 bp (wild type/ mutant)
	flaA-H	GCGGTTGAACACTTAATGCAG		
Primers for construction of LFI1238Δ*flaD*:	flaD-A	TATGAGCTCTCAAGCTAAGCAAGCG	5′ end contains a *SacI* restriction site	514 bp
	flaD-B	AATCTTATATCTCCGCTTTGGTTTCG		
1143 bp deletion targeting *flaD* (VSAL_RS12140)	flaD-C	GCGGAGATATAAGATTCTTGTGAGAATGGG	5′ end contains a 16 bp sequence complementary to the 5′ end of flaD-B	476 bp
	flaD-D	GTACTAGTTAGAGATGCCGTCGTTTGC	5′ end contains a *SpeI* restriction site	
Verification primers for LFI1238Δ*flaD*	flaD-G	GCTCAAATTCTTCAACAAGCAAGTTCG		2242/1099 bp (wild type/ mutant)
	flaD-H	GTCAGCGTCAGTGTTTGAACC		
Primers for complementation of LFI1238Δ*flaA*	flaAc-A	ATCTCGAGAAGCAAGAGCAGAAGTAGG	5′ end contains *XhoI* restriction site	2818 bp
	flaAc-D	TAACTAGTACGAACGGCAACATCTAACC	5′ end contains a *SpeI* restriction site	
Primers for verification of complemented LFI1238Δ*flaAc*	flaAc-G	AGCAGAAGGGATTAAATACGAAGG		3121/2011 bp (complemented/mutant)
	flaAc-H	GACGATTGCAAAGCCAAATCG		
Primers for construction of LFI1238Δ*motA*:	motA-A	CGCTCGAGGCCACTTTCTAACTGATTAACG	5′ end contains *XhoI* restriction site	407 bp
	motA-B	AGTCTATTCTTCGCCTATTAACGTTGCTAAATCC	5′ end contains a 15 bp sequence complementary to the 5′ end of motA-C	
732 bp deletion targeting *motA* (VSAL_RS05175)	motA-C	GGCGAAGAATAGACTAGGAGCTCATGATGGAAG		432 bp
	motA-D	CCACTAGTGTCGATGTGGACGATGATTCTCC	5′ end contains a *SpeI* restriction site	
Verification primers for LFI1238ΔmotA	motA-G	CAGCTTGAAGGAGAATATCG		1714 bp
	motA-H	ACTCTTGCTGACTCTGG		

LFI1238Δ*flaA*Δ*flaD* and LFI1238Δ*motA* were created as described for LFI1238Δ*flaA*, using the primers listed in Table 2.

2.3. Soft agar motility assay

For motility studies, semi-solid LA0.9 and LA2.5 plates were made by addition of 0.3% agar-agar to LB0.9 and LB2.5 media, and 0.005% TTC (2,3,5-Triphenyl tetrazolium chloride, Sigma-Aldrich) for enhanced visualization of bacterial growth. Bacterial cultures grown overnight were spotted onto agar plates and incubated at 8 or 12 °C, followed by daily measurements of growth zones. The experiments were performed in pentaplicates and repeated twice. Growth rates for mutant strains are shown relative to wild type assayed under the same conditions. Comparisons between mutant strains and wild type were performed using Student's *t*-test, where a p value less than 0.05 was considered statistically significant.

2.4. Transmission electron microscopy

Bacterial cultures for transmission electron microscopy (TEM) were grown in LB3 at 8 °C (150 rpm) overnight and were negatively stained with 2% (w/v) uranyl acetate on carbon coated copper grids (FF400-Cu; Electron Microscopy Sciences, Fort Washington, PA, USA). Specimens were examined using a FEI Morgagni 268 transmission electron microscope (FEI Company, Eindhoven, The Netherlands) equipped with a Veleta TEM CCD camera (Olympus Soft Imaging System, Münster, Germany), operating at an accelerating voltage of 80 kV. Number of flagella and maximum flagellar length (μm) were noted for a minimum of 50 cells for each strain. Differences between mutant and wild type cells were tested using Student's *t*-test. The null hypotheses were rejected at a 5% significance level.

2.5. Challenge trials

2.5.1. Trial 1: intraperitoneal challenge experiment

Atlantic salmon parr ($n = 330$) at a mean weight of 47 g were kept in a common tank supplied with flow-through of carbon filtered fresh water at 11 °C. Before challenge, strains were recently passaged in Atlantic salmon to ensure optimal virulence [19]. Wild type *Al. salmonicida* LFI1238 (WT) and mutant strains were grown overnight in LB0.9 at 10 °C (150 rpm) and diluted to OD_{600}: 0.3. In all three trials, challenge doses were based on experience from earlier experiments [3,18,20,21]. Challenge doses were determined by serial dilution and are listed in Table 3. Fish were anesthetized in water baths with 0.0025% benzocaine (Benzoak VET; ACD Pharmaceuticals, Leknes, Norway), split into seven groups and challenged by intraperitoneal (i.p.) injection of 0.1 ml bacterial culture or phosphate-buffered saline (PBS). To distinguish between groups, fish were subsequently marked by a combination of fin clipping and fin marking with 1.5% Alcian blue using a Dermojet high-pressure injection pen (Akra Dermojet, Pau, France). Challenged fish were mixed, transferred to 200 L holding tanks and monitored for a period of 25 days. During the course of the experiments, fish were fed *ad lib*. Tanks were monitored twice daily and moribund fish were removed.

2.5.2. Trial 2: immersion challenge experiment

Atlantic salmon smolt ($n = 350$) at a mean weight of 80 g were divided into six experimental groups and one control group. Each group of 50 smolts were kept in separate tanks (150 L) supplied with flow-through of sea water at a temperature of 8 °C and salinity of 35 ppm. Fish were challenged by immersion for 45 min in sea water with added bacteria cultured in LB3. The control group was mock challenged by adding sterile LB3. Shortly after challenge initiation, tank water was sampled and challenge doses were determined by serial dilution (Table 3). By the end of the challenge period, tanks were flushed with sea water. After the challenge, fish were fed *ad lib* and monitored for mortality over a period of 35 days.

2.5.3. Trial 3: invasion experiment

Atlantic salmon smolt ($n = 28$) at a mean weight of 172 g were divided into five experimental groups and one control group. Fish were challenged by immersion for ten minutes in suspensions of LB3-cultured bacteria added to sea water holding 8 °C. For the

Table 3
Challenge doses for i.p. and immersion challenge experiments.

Strain	Trial 1: Challenge dose i.p. (CFU/fish)	Trial 2: Challenge dose immersion (CFU/ml sea water)	Trial 3: Challenge dose immersion (CFU/ml sea water)
LFI1238 Wild type	2.85×10^7	4.97×10^6	1.33×10^7
LFI1238Δ*flaA*	3.17×10^7	1.32×10^7	2.00×10^7
LFI1238Δ*flaAc*	3.39×10^7	1.12×10^7	Not determined
LFI1238Δ*flaD*	3.13×10^7	7.37×10^6	Not included
LFI1238Δ*flaA*Δ*flaD*	3.02×10^7	6.50×10^6	1.97×10^7
LFI1238Δ*motA*	3.61×10^7	1.54×10^6	1.27×10^7

control group, sterile LB3 was added in place of bacterial culture. Challenge doses were determined by serial dilution of tank water and are listed in Table 3. After challenge, fish were transferred to additional tanks filled with sea water holding the same temperature and kept for fifteen minutes. Finally, fish were euthanized and subjected to blood sampling.

2.5.4. Sampling

Diseased fish in trial 1 and 2 were autopsied, and head kidney was sampled and plated on BA2.5 to verify the presence of *Al. salmonicida*. In trial 1, additional sampling of five fish from each experimental group were performed at time points 12, 24 and 72 h post challenge (hpc). Following euthanization in water baths containing 0.0125% benzocaine, the spleen was dissected, immediately transferred to RNAlater (Qiagen) and stored at −20 °C until analysis. In trial 3, blood was sampled from the caudal vein of each fish using a vacutainer and blood collection tubes with EDTA anticoagulants. For each fish, volumes of 100 µl were plated on BA2.5 in duplicates for determination of colony forming units per ml (CFU ml^{-1}) blood. Log-transformed CFU ml^{-1} blood of the challenge groups were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test for comparison of wild type and mutant groups. P values < 0.05 were considered statistically significant.

2.6. RNA extraction

For the flagellin gene expression analysis, strains were grown in LB3 at 10 °C (125 rpm) to OD_{600}: 0.7–0.8 in triplicates, and volumes of 200 µl of bacterial suspension were transferred to RNAlater (Qiagen) and stored at −20 °C until RNA extraction. Bacterial cells or spleen tissue were homogenized and lysed using Qiazol with a Tissuelyser II (both Qiagen) according to the manufacturer's protocol. After phase separation, the liquid fraction was transferred to a new tube and subjected to RNA extraction by the use of an RNAeasy Mini Kit (Qiagen) as described by the manufacturer. RNA concentration and purity was evaluated by measurements of A260/280 and A260/230 using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA), and gel electrophoresis was conducted for visualizing degree of degradation.

2.7. Two-step reverse transcription qPCR

Complementary DNA (cDNA) was synthesized by the use of QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions, including a genomic DNA (gDNA) wipeout treatment. For each reaction, 1 µg RNA was used as template. After synthesis, cDNA was diluted to 5 ng/µl and kept at −20 °C until qPCR. qPCR was carried out using SYBR GreenER qPCR Supermix Universal Kit (Invitrogen) and primers listed in Table 4 in 20 µl reactions run in triplicates. Each reaction contained 10 µl master mix, 200 nM of each primer, 50 nM ROX dye and 15 ng template cDNA. The program was run as following in a Mx3005P thermal cycler (Agilent Technologies, Santa Clara, CA, USA): (I) 50 °C for 2 min, 95 °C for 10 min and (II) 40 cycles of 95 °C for 15 s, 60 °C for 1 min (with ROX- and SYBR data collection) and (III) 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s (for melting curve analysis). For each qPCR run, a no template control and No RT control was included.

2.8. Gene expression analysis

Gene expression profiles were derived through a comparative Cq approach (ΔΔCq) [25]. Gene expression normalization factors for each sample were calculated based on the geometric mean of reference genes and used to correct for different amounts of starting material. For analysis of flagellin gene expression, the reference genes used were: acetyl-CoA carboxylase subunit β (*accD*), glyceraldehydes-3-phosphate dehydrogenase (*gapA*) and 16S ribosomal RNA [21]. For analysis of immune gene expression, reference genes used were *Elongation factor 1Aa* ($EF1A_A$), Elongation factor 1Ab ($EF1A_B$) and β-actin [23]. For each gene analyzed, primer efficiency was calculated in LinRegPCR (version: September 2014) [26] and used for transformation of Cq values to gene quantities. Gene quantities were then normalized against sample normalization factors and are shown as fold changes (±standard error of the mean) relative to control samples. For each gene and time point analyzed, differential expression between strains or experimental fish groups were tested by Student's t-test, rejecting the null hypothesis at a 5% significance level.

3. Results

To determine roles of motility in colonization and virulence in a salmonid host, we constructed four in-frame deletion mutants of *Al. salmonicida* LFI1238. Genes encoding flagellins FlaA and FlaD and flagellar motor protein MotA were targeted, resulting in LFI1238 mutants Δ*flaA*, Δ*flaD*, Δ*flaA*Δ*flaD* and Δ*motA*. One mutant (Δ*flaA*) was complemented by insertion of the full-length gene in its original locus, resulting in Δ*flaAc*.

3.1. Growth assay

All mutant strains appeared macroscopically indistinguishable from wild type when grown on blood agar plates or LA plates at 0.9–3% salinity. To examine whether the introduced mutations affected bacterial growth, growth assays were conducted in LB0.9 or LB3 at 10 °C comparing mutants to the parental strain. For Δ*motA*, stationary phase of growth was observed at a higher cell density than wild type under both conditions, reaching OD_{600}: 2.04 at a salinity of 3% (wild type: 1.65) and OD_{600}: 1.01 at a salinity of 0.9% (wild type: 0.7). Growth of Δ*flaA*, Δ*flaA*Δ*flaD* and Δ*flaD* did not differ from wild type (data not shown).

Table 4
Primers used for gene expression analyses by RT-qPCR.

Description:	Primers:	Sequence (5' − 3'):	Construct size:	Ref.
Acetyl-CoA carboxylase subunit β (VSAL_RS05900)	accD-F	TTGCTGGTCGTCGTGTTATT	149 bp	[18]
	accD-R	TTTAGCCATCAAACCACCAA		
16S ribosomal RNA (VSAL_RS00545)	16S-F	CTTGACGTTAGCGACAGAAGAA	100 bp	[18]
	16S-R	CGCTTTACGCCCAGTAATTC		
Glyceraldehydes-3-phosphate dehydrogenase (VSAL_RS09725)	gapA-F	TTTGTTTTCCGTGCATCTGT	120 bp	[21]
	gapA-R	GTTGAAACGACCGTGAGTTG		
Flagellin subunit A (VSAL_RS12190)	flaA-F	CCATCTGTACGTTCTGACGACGAC	140 bp	[10]
	flaA-R	GAAACCGCTTCGCCTTTCTTCGTA		
Flagellin subunit B (VSAL_RS12180)	flaB-F	TCAGATACATTAGCGATGGGCGGT	135 bp	[10]
	flaB-R	CGTGATCTCTTGTGCTTTGCCTTC		
Flagellin subunit C (VSAL_RS12145)	flaC-F	AAGCAGGCGAAGAGAAAGAG	127 bp	[10]
	flaC-R	TGAAGCTCACCCTTTTCTGA		
Flagellin subunit D (VSAL_RS12140)	flaD-F	AGGCGCTGAGAACTCAGAAT	134 bp	[10]
	flaD-R	TCGCGTTACTTTGACCATTG		
Flagellin subunit E (VSAL_RS12135)	flaE-F	ACCTACGTGCAGACGAAGCTAACA	130 bp	[10]
	flaE-R	TGATCTTCGCCAGATTTGTCTGTG		
Flagellin subunit F (VSAL_RS13155)	flaF-F	GAGAATTCGCAATTTAATGTTCA	139 bp	[10]
	flaF-R	CAGAAGCGCTTAACTCATTTGT		
Elongation factor 1Aa (AF321836.1)	EF1Aa-F	CCCCTCCAGGACGTTTACAAA	57 bp	[23]
	EF1Aa-R	CACACGGCCCACAGGTACA		
Elongation factor 1Ab (BG933853.1)	EF1Ab-F	TGCCCCTCCAGGATGTCTAC	57 bp	[23]
	EF1Ab-R	CACGGCCCACAGGTACTG		
β-actin (BG933897.1)	B-actin-F	CCAAAGCCAACAGGGAGAAG	91 bp	[23]
	B-actin-R	AGGGACAACACTGCCTGGAT		
Interleukin 1 beta (AY617117.1)	IL-1b-F	GCTGGAGAGTGCTGTGGAAGA	73 bp	[24]
	IL-1b-R	TGCTTCCCTCCTGCTCGTAG		
Tumor necrosis factor alpha (NM_001123589.1)	TNFa-F	AGGTTGGCTATGGAGGCTGT	173 bp	[24]
	TNFa-R	TCTGCTTCAATGTATGGTGGG		

3.2. Soft agar motility assay

Motility of the strains studied were determined by inoculation of semi-solid agar plates containing either 0.9% or 2.5% NaCl incubated at 8 and 12 °C, followed by daily measurements of growth zones to calculate the rate of zone extension. Motility of wild type LFI1238 was found to be dependent on NaCl concentration and temperature (data not shown), where the highest rate of motility was observed at 12 °C and 2.5% NaCl. In accordance with earlier observations [10], NaCl concentration had a more pronounced effect than temperature on motility.

Compared to wild type, Δ*flaA* displayed a 62% (CI: 60–64) reduction in motility under the conditions assayed (Fig. 1). The relative reduction in motility was found to be lower at 0.9 than at 2.5% NaCl, but this finding was not statistically significant (*p*: 0.0572). The complemented Δ*flaAc* displayed wild type motility. Similar to Δ*flaA*, Δ*flaA*Δ*flaD* displayed 65% reduction in motility (CI: 62–67). For Δ*flaD*, a 14% (CI: 8–19) reduction in motility was observed at 0.9% salinity (*p*: 0.0004), whereas motility did not differ from wild type at 2.5% salinity. Δ*motA* was found to be non-motile under all conditions tested (Fig. 1).

3.3. Transmission electron microscopy

Wild type LFI1238 appeared as flagellated, curved rods, each cell displaying between 1 and 9 flagella (Fig. 2A). Ninety-one percent of the cells observed were flagellated and the mean number of flagella per flagellated cell was 2.8 ± 0.17. The mean flagellar length of wild type cells was 4.04 ± 0.15 μm. For Δ*flaA*, a reduced fraction of flagellated cells (45%) as well as number (1.4 ± 0.1; $p < 0.0001$) and length of flagella (2.49 ± 0.25 μm; $p < 0.0001$) were observed compared to the wild type. No changes in flagellation were observed in Δ*flaAc* (96% flagellated cells, mean number of flagella: 2.6 ± 0.2, mean flagellar length: 3.84 ± 0.20 μm) and Δ*flaD* (90%

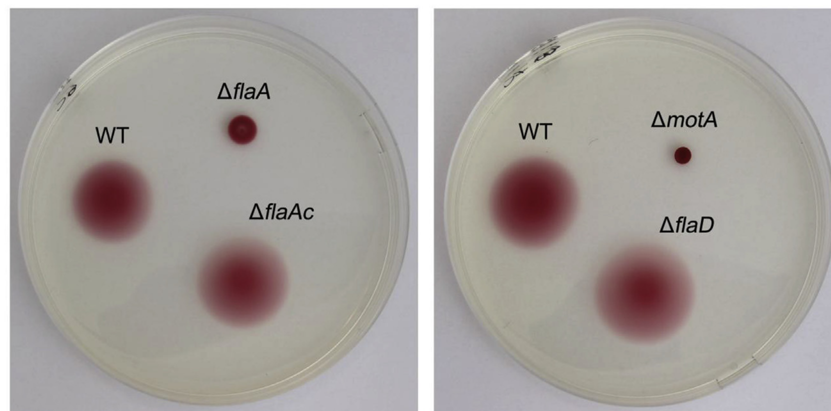

Fig. 1. Motility assay showing zonal expansion of strains grown in 2.5% NaCl at 8 °C for five days. Compared to wild type, Δ*flaA* displayed reduced motility, while Δ*motA* appeared non-motile. Δ*flaD* and Δ*flaAc* did not differ from wild type.

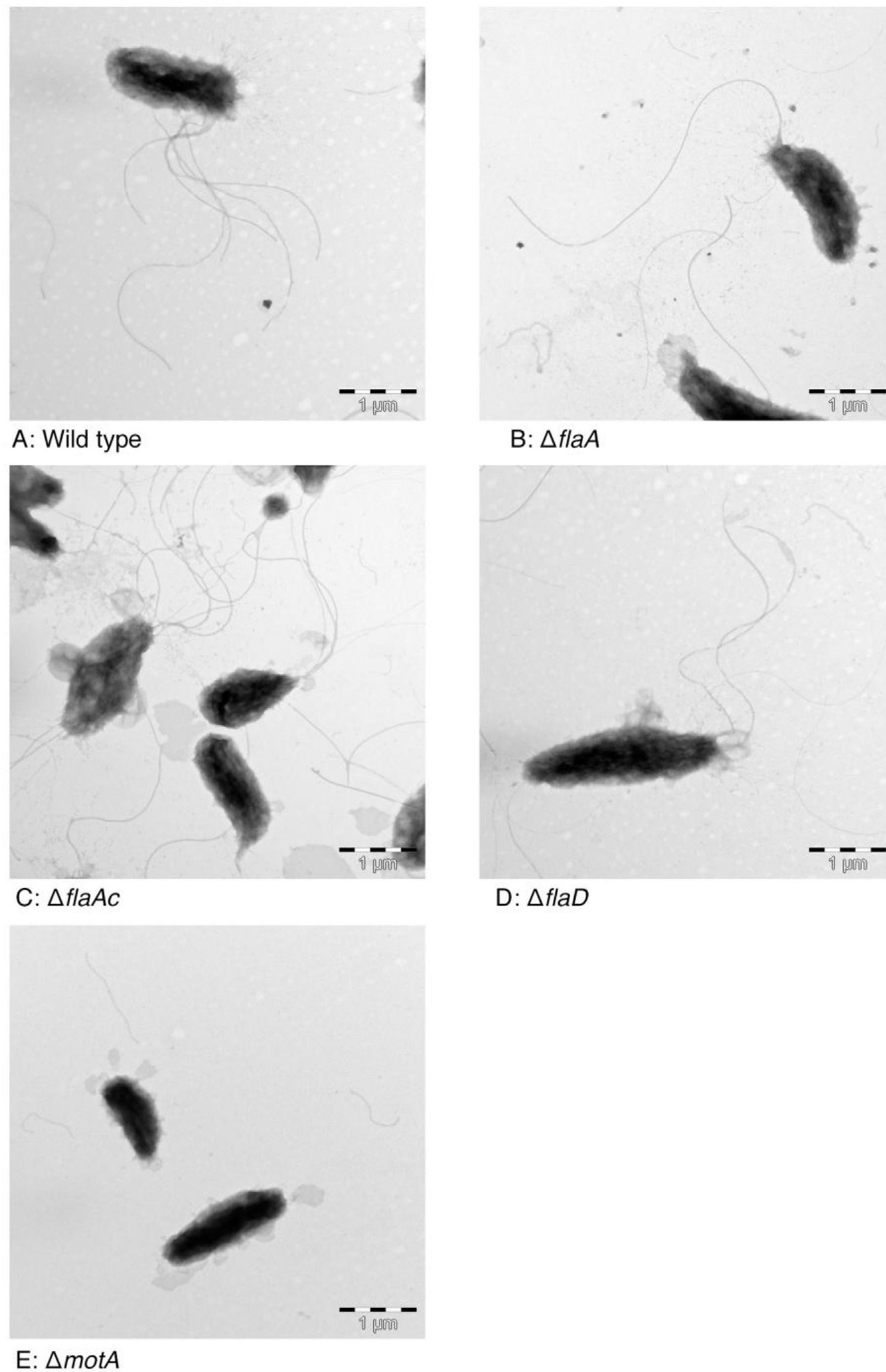

Fig. 2. Transmission electron micrographs showing flagellar structures of *Al. salmonicida* LFI1238 wild type (A), Δ*flaA* (B), Δ*flaAc* (C), Δ*flaD* (D) and Δ*motA* (E) strains. All cells were grown in LB3 at 8 °C (150 rpm) overnight before imaging.

flagellated cells, mean number of flagella: 2.5 ± 0.1, mean flagellar length: 4.42 ± 0.16 μm). In the Δ*motA* strain, all cells investigated appeared aflagellate (2E).

3.4. Relative expression of flagellin genes

To investigate whether the loss of one flagellin gene influenced the regulation of other flagellin genes, the relative expression of flagellin genes *flaA*, *flaB*, *flaC*, *flaD*, *flaE* and *flaF* in the wild type strain, mutants Δ*flaA*, Δ*flaD* and the complemented Δ*flaA*c were investigated by RT-qPCR. In Δ*flaA*, a significant increase in transcription of flagellin genes *flaB* (relative fold change: 2.09 ± 0.30, *p*: 0.0273) and *flaF* (1.47 ± 0.037, *p*: 0.0033) was observed relative to wild type (Fig. 3A). In Δ*flaD*, a tendency towards increased transcription of *flaA*, *flaB* and *flaF* was observed relative to wild type (Fig. 3B), although the increase was not significant. No transcripts of the deleted genes were detected in either Δ*flaA* or Δ*flaD*, while the transcription of *flaA* in Δ*flaA*c was restored to wild type levels.

Similarly, flagellin gene transcription in Δ*motA* was analyzed to investigate potential regulatory coupling. *ΔmotA was* shown to

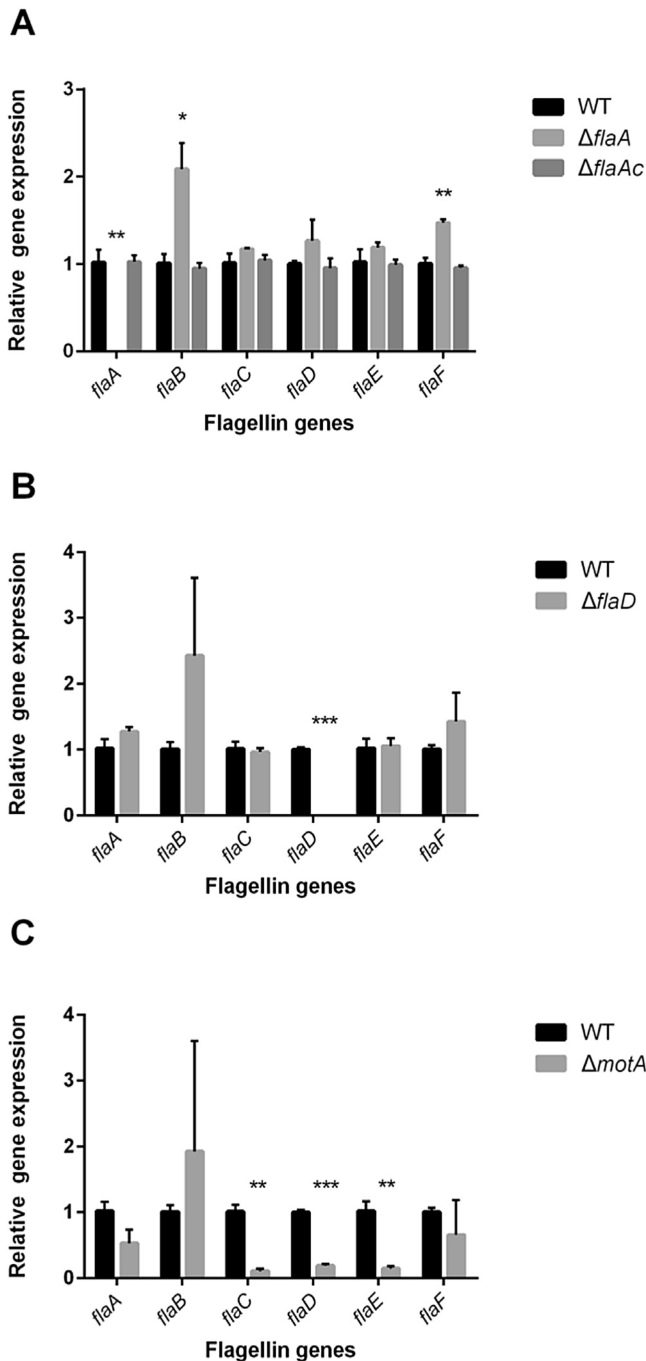

Fig. 3. Transcription of flagellin genes *flaABCDEF* in strains Δ*flaA*, Δ*flaAc* (A), Δ*flaD* (B) and Δ*motA* (C) relative to wild type. Statistical analysis was performed using Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), comparing transcription levels of each flagellin gene in the mutants against the wild type strain.

significantly downregulate *flaC, flaD* and *flaE* relative to wild type (Fig. 3C). For *flaA*, *flaB* and *flaF*, down-regulation was seen in some, but not all of the replicates assayed.

3.5. Challenge studies

To explore effects of flagellation and motility in virulence, groups of fish were challenged intraperitoneally and by immersion with similar challenge doses of wild type and the constructed mutants. In the first trial, a total mortality of 91.7% was observed between day four and nine in the fish challenged i.p. with wild type LFI1238, with a mean onset of death of 6.1 days. In comparison, fish challenged with Δ*flaA* showed a delayed onset of death (8.6 days; Log-rank: $p < 0.0384$; Wilcoxon: $p < 0.0022$), although the total mortality was similar at 94.3% (Fig. 4A). Total mortality for the group challenged with the complemented Δ*flaAc* was similar to that of the wild type, although mean onset of death was expedited by one day. The group of fish challenged with Δ*flaA*Δ*flaD* underwent a similar mortality curve compared to Δ*flaA*, exhibiting a total mortality of 91.1%, a delayed onset of death (8.7 days; Log-rank: $p < 0.0476$; Wilcoxon: $p < 0.0241$). For Δ*flaD* and Δ*motA*, total mortality and mean onset of death did not differ significantly from wild type (Fig. 4B and C).

In the second trial, 74.3% of the fish challenged by immersion with wild type LFI1238 and none in the group challenged with Δ*flaA* died (Log-rank and Wilcoxon: $p < 0.0001$) (Fig. 4D). Fish challenged with the complemented Δ*flaAc* displayed a similar mortality to wild type (total mortality: 69.7%). In the Δ*flaA*Δ*flaD* group, total mortality was 11.8% (Log-rank and Wilcoxon: $p < 0.0001$). A minor increase in survival rate relative to wild type was seen for fish challenged with Δ*flaD* (total mortality of 60.6%; Log-rank: $p < 0.0530$; Wilcoxon: $p < 0.0159$) (Fig. 4E). In the Δ*motA* group, a total mortality of 5.3% was observed (Log-rank and Wilcoxon: $p < 0.0001$) (Fig. 4F).

Diseased fish developed pathological signs typical for cold-water vibriosis, including external hemorrhages and reddening of skin at the fin basis, petechial hemorrhages and hyperemia of the serosa, and an enlarged, pale liver. In the Δ*flaA* and Δ*flaA*Δ*flaD* groups, a slight increase in serosal petechiae, a decrease of serosal hyperemia and an increase in liver pathology were observed compared to wild type.

In the i.p. challenge experiment, *Al. salmonicida* was isolated from head kidney of all diseased fish. No growth could be detected in head kidney from survivors. In the immersion challenge experiment, *Al. salmonicida* was recovered from head kidney of the majority of diseased fish, whereas bacteria could not be detected in blood or head kidney of survivors. However, *Al. salmonicida* was absent in five diseased fish challenged with *ΔflaA*, three challenged with Δ*flaA*Δ*flaD*, one challenged with Δ*flaD*, four challenged with Δ*motA* and one challenged with Δ*flaAc*. In its place, *Aliivibrio wodanis* and/or *Moritella viscosa* were isolated, and skin ulcerations typical for winter ulcer disease were observed. Following this, it is likely that the observed mortality in these fish was caused by winter ulcer disease rather than cold-water vibriosis. For that reason, we excluded these individuals from the survival analysis presented above.

By including winter ulcer diseased fish in the immersion experiment, total mortalities in the challenge groups were as following: wild type: 74.3%, Δ*flaA*: 13.9%, Δ*flaA*Δ*flaD*: 20.6%, Δ*flaD*: 63.6%, Δ*motA*: 15.8% and Δ*flaAc*: 70.6%.

The reduction in mortality observed for fish challenged by immersion with the motility-deficient mutants suggests that motility is involved in host invasion. To investigate whether the strains were able to pass the fish integument, a third challenge experiment was conducted. Groups of fish were challenged by immersion in bacterial suspension for ten minutes, and blood was sampled fifteen minutes after challenge and plated for determination of CFU. Similar levels of bacteria were isolated from the groups challenged with wild type, Δ*flaA* and Δ*flaA*Δ*flaD* (Fig. 5). In the Δ*motA* group, a tendency towards increased bacterial retrieval levels was observed, although this was not found to be statistically significant.

3.6. Immune response by RT-qPCR

To investigate potential differences in innate immunity between

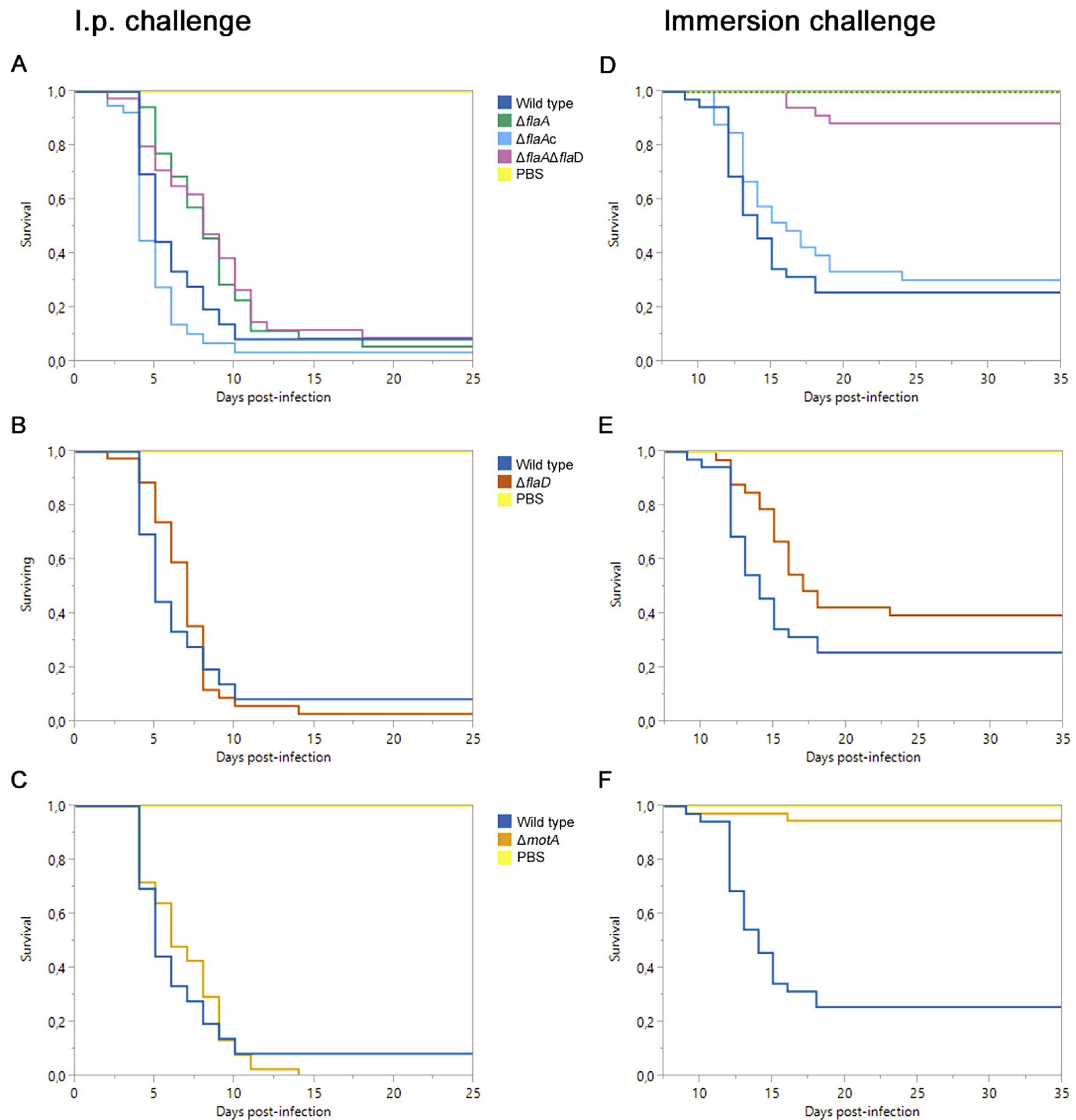

Fig. 4. Survival plots after challenge of Atlantic salmon by i.p. injection (A, B, C) and immersion (D, E, F) with *Al. salmonicida* LFI1238 wild type (blue), Δ*flaA* (green), Δ*flaAc* (light blue), Δ*flaA*Δ*flaD* (purple), Δ*flaD* (red) and Δ*motA* (orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

groups of fish challenged with wild type LFI1238 and isogenic motility mutants, relative mRNA expression of genes encoding pro-inflammatory cytokines IL-1β and TNFα was evaluated by RT-qPCR. Overall, all groups exhibited high levels of variation in expression of the genes measured. For IL-1β, an initial high expression was observed in fish challenged with wild type bacteria, showing similar levels 12 and 24 h post challenge (hpc) (348.2 ± 11.2, 394.5 ± 116.0). At 72 hpc, expression levels dropped moderately (204.3 ± 54.3). IL-1β expression in fish challenged with the motility mutants exhibited a similar pattern with initially high levels of expression, followed by a drop after 72 hpc (Fig. 6A). For TNFα, the highest expression in fish challenged with wild type was seen 12 hpc (255.6 ± 22.0), followed by a drop to 24 hpc (161.9 ± 28.7). At 72 hpc, a modest increase from 24 hpc was observed (220.2 ± 74.0). Similarly, a drop at 24 hpc relative to 12 and 72 hpc were seen in the other groups (Fig. 6B), although the overall levels of variation were high.

4. Discussion

A requirement for motility in host invasion and virulence has been reported for several *Vibrio* spp. including *Al. salmonicida* [1–3,9,27–30]. However, the reported impact of *Al. salmonicida* motility on virulence is based on observed mortality of motility-deficient strains in i.p. and immersion challenge trials, and it remains unclear whether the effects are related to host invasion or other functions of the flagella. To further clarify how motility and flagellation in *Al. salmonicida* are involved in the pathogenesis of

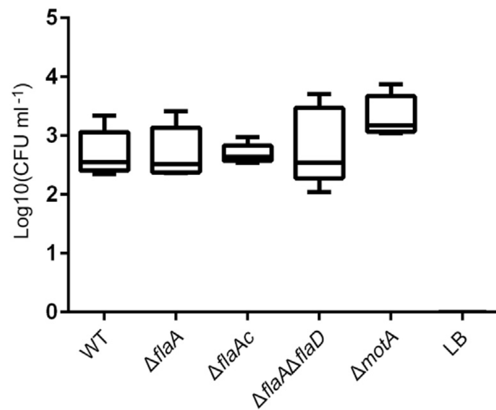

Fig. 5. Bacteria isolated from blood of fish challenged by immersion for ten minutes in bacterial suspension of wild type (WT), Δ*flaA*, Δ*flaAc*, Δ*flaA*Δ*flaD* or Δ*motA*. Blood was sampled fifteen minutes after challenge. Data are shown as box-and-whiskers plots representing log-transformed colony-forming units per ml (CFU ml^{-1}) blood.

cold-water vibriosis, we constructed in-frame deletion mutants for *flaA*, *flaD* and *motA*.

The genes *flaA* and *flaD* encode two of the six flagellin subunits found in the genome of *Al. salmonicida* LFI1238. A substantial contribution to motility was found for *flaA*, but not *flaD*. While the Δ*flaA* strain was markedly less motile than the wild type strain under all conditions tested, the Δ*flaD* strain only displayed a minor reduction in motility when grown in media containing 0.9% NaCl. In both strains, the introduced deletions affected the pattern of transcription of flagellar genes. The loss of *flaA* resulted in a compensatory increase in transcription of flagellin genes *flaB* and *flaF*. In Δ*flaD*, a minor increase in transcription of *flaA*, *flaB* and *flaF* was noted. Hence, sensing of expression levels of individual flagellin genes seems to influence flagellin gene regulation. TEM micrographs revealed a reduction in the number and length of flagella in Δ*flaA* relative to wild type. Previously, a link between flagellin composition and flagellar stability has been seen in flagellin mutants of *Vibrio vulnificus* [29]. Likewise, the loss of FlaA may have rendered the flagella more susceptible to breakage, resulting in the reduced length. The Δ*motA* strain, defective for a motor component, was found to be completely non-motile under the conditions tested. Furthermore, the strain demonstrated a transcriptional muting of *flaCDE* and appeared completely aflagellate. This is in contradiction to what has been reported for *V. parahaemolyticus*, *V. cholerae* and *Al. fischeri*, where mutagenesis of the *motAB* operon resulted in paralyzed, but flagellated cells [31–33]. However, as we have not complemented Δ*motA*, the observed aflagellate phenotype and down-regulation of flagellin genes may be results of polar effects on downstream genes. Alternatively, the observed flagellar loss could be an artefact caused by the preparatory process for TEM.

To elucidate whether motility is required for passage over the fish integument, we challenged Atlantic salmon by immersion in suspensions of wild type bacteria or motility-deficient mutants. By cultivation of bacteria from blood of fish sampled 15 min after challenge, we found that all motility-deficient mutants were able to enter the fish blood stream at rates similar to the wild type strain. The invasion rates registered are in agreement with previous observations [34]. Although we cannot infer the mechanisms of invasion from our data, motility does not seem to be involved.

Nevertheless, all mutants exhibited decreased virulence after immersion challenge. The reduction in virulence correlated well with the observed motility defects. No fish died after challenge with Δ*flaA*, and a major reduction in virulence was observed in both the Δ*flaA*Δ*flaD* and Δ*motA* groups. In the group challenged with Δ*flaD*, only a modest reduction was seen compared to the wild type group. During the course of the immersion challenge trial, *Aliivibrio wodanis* and *Moritella viscosa* were isolated from head kidney of several diseased fish, and the same fish exhibited skin ulcerations consistent with winter ulcer disease. Outbreaks of winter ulcer disease have previously been registered in the research facility where the experiment was conducted, and the source of infection is presumably the intake of sea water. Although the impact of this unintentional co-infection is difficult to determine, it may have masked an even stronger difference in mortality between the wild type and mutant groups.

To further investigate the role of active motility and/or flagellar structures in disease development, we also challenged fish by i.p. injection of bacterial cultures. In contrast to the results from the immersion challenge, no difference was seen between the mortality curves for fish challenged i.p. with wild type bacteria and Δ*motA* or Δ*flaD*. In the groups challenged with Δ*flaA* and Δ*flaA*Δ*flaD*, a delay in disease development relative to the wild type group was noted, although the total mortality was similar 25 days after challenge. Previously, a similar delay in mortality has been

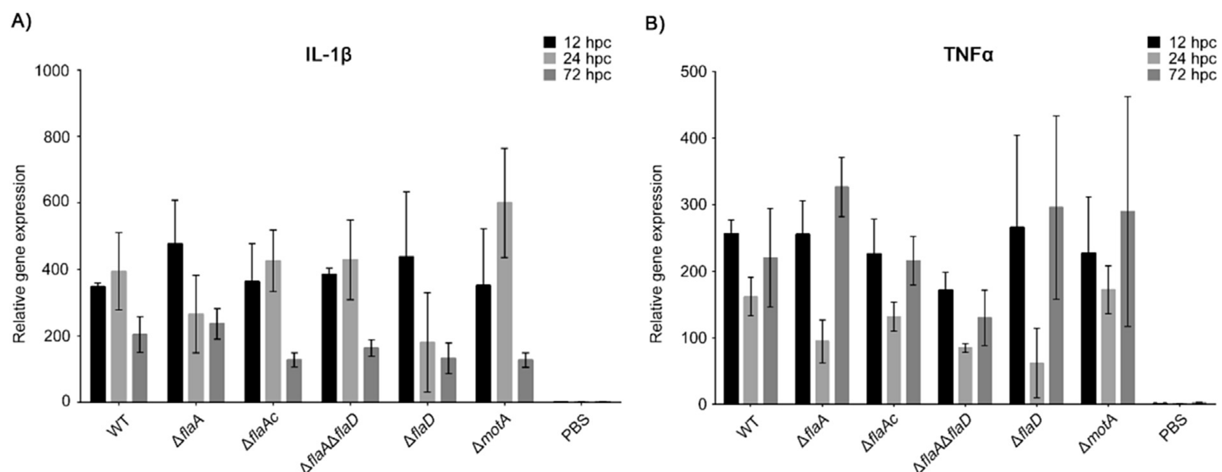

Fig. 6. Relative expression of pro-inflammatory cytokines IL-1β (A) and TNFα (B) in fish challenged with *Al. salmonicida* LFI1238 wild type (WT), Δ*flaA*, Δ*flaAc*, Δ*flaA*Δ*flaD*, Δ*flaD*, Δ*motA* and PBS. Fish were sampled 12, 24 and 72 h post challenge (hpc), and expression data are shown as mRNA fold increases (mean ± SEM) relative to control fish sampled 12 hpc.

observed after i.p. challenge of Atlantic salmon with a motility deficient mutant of *Al. salmonicida* [3].

Considering that we found host invasion to be independent of motility, the different outcomes of the i.p. and immersion challenge trials suggest a complex requirement for motility and/or flagellation in the pathogenesis of cold-water vibriosis. Challenge by immersion is thought to resemble the natural route of infection more closely than challenge by i.p. injection, and it is likely that the strategies of both host and microbe are adapted to this route of infection. From the perspective of the host, uptake of bacteria from surrounding waters may involve interactions between host and microbe that are beneficial for disease resistance and that are bypassed in i.p. injection.

Innate humoral defense factors found in blood, including complement, lysozyme, lectins, pentraxins and transferrin, provide an immediate defense towards the introduced pathogen [35]. In the peritoneal cavity, the resting population of leucocytes will readily phagocytose bacteria, but an influx of additional phagocytes may take 24–48 h [35]. Nordmo and co-workers found that, when challenging Atlantic salmon with different doses of *Al. salmonicida* by i.p. injection, a dose-effect relationship was seen between the dose and onset of disease [36]. A similar experiment involving challenge by immersion revealed a dose-effect relationship between dose and total mortality. Thus, the different mortality patterns observed in fish challenged by immersion and i.p. injection with Δ*flaA*, Δ*flaD* and Δ*flaA*Δ*flaD* may reflect the host immune system, and not properties of the mutants. The apparent wild type virulence observed in Δ*motA* after i.p. challenge may be related to the aflagellate state being energetically cost-effective, providing more energy for metabolic activities as reflected by the *in vitro* growth curves. Howbeit, such speculations should be made with care due to the lack of complementation of the Δ*motA* strain.

Flagellin is known as a potent MAMP contributing to inflammation. Differences in flagellar composition and integrity could influence the nature of flagellins available and influence immunomodulation. In both *V. cholerae* and *Al. fischeri*, flagellin monomers are found as part of the secretome [33,37]. A *V. cholerae* Δ*flaA* mutant lacked its flagellum, but was still able to activate cytokine production, although at lower levels than its isogenic parent strain [37]. Similarly, differences in abundance of monomeric flagellin between the strains in this study could impact the immune response of the infected host.

In order to elucidate potential differences in innate immunity raised in response to the constructed mutants, a panel of innate immunity parameters was evaluated. A similar response was observed for all groups in the study. However, large internal variation was seen in expression of the genes assayed, possibly masking minor differences between the groups. In addition, we have only measured immune gene transcription in i.p.-challenged fish. Evaluation of immune parameters of fish challenged by immersion may further elucidate roles of the flagella in immunomodulation and development of disease.

Our experimental evidence suggest that the flagella and/or motility of *Al. salmonicida* are involved in the pathogenesis of cold-water vibriosis through other means than invasion. However, the mechanisms involved cannot be determined from our data. Future studies should address alternative functions for flagellation.

5. Conclusion

We found motility of *Al. salmonicida* to be dispensable for invasion of Atlantic salmon. However, a major reduction in mortality was seen after immersion challenge with motility-deficient mutants. Flagellin subunit FlaA appeared to contribute more to motility and virulence than FlaD, although a non-significant effect on virulence was also seen for the latter. Challenge by i.p. injection of Δ*flaA* and Δ*flaA*Δ*flaD* resulted in delayed mortality relative to wild type. The non-motile Δ*motA* exhibited decreased virulence after immersion challenge, but did not differ from wild type after i.p. challenge. These results imply a complex requirement for motility and/or flagellation in the pathogenesis of cold-water vibriosis. However, the mechanisms involved remain unknown.

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