Microbial Pathogenesis 124 (2018) 322–331

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Aliivibrio salmonicida requires O-antigen for virulence in Atlantic salmon (*Salmo salar* L.)

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ARTICLE INFO

Keywords:
Aliivibrio salmonicida
Atlantic salmon
Pathogenesis
Cold-water vibriosis
O-antigen
Lipopolysaccharide

ABSTRACT

Aliivibrio salmonicida is the causative agent of cold-water vibriosis, a hemorrhagic septicemia of salmonid fish. The bacterium has been shown to rapidly enter the fish bloodstream, and proliferation in blood is seen after a period of latency. Although the pathogenesis of the disease is largely unknown, shedding of high quantities of outer-membrane complex VS-P1, consisting of LPS and a protein moiety, has been suggested to act as decoy and contribute to immunomodulation. To investigate the role of LPS in the pathogenesis, we constructed O-antigen deficient mutants by knocking out the gene encoding O-antigen ligase *waaL*. As this gene exists in two copies in the *Al. salmonicida* genome, we constructed single and double in-frame deletion mutants to explore potential effects of copy number variation. Our results demonstrate that the LPS structure of *Al. salmonicida* is essential for virulence in Atlantic salmon. As the loss of O-antigen did not influence invasive properties of the bacterium, the role of LPS in virulence applies to later stages of the pathogenesis. One copy of *waaL* was sufficient for O-antigen ligation and virulence in experimental models. However, as a non-significant decrease in mortality was observed after immersion challenge with a *waaL* single mutant, it is tempting to suggest that multiple copies of the gene are beneficial to the bacterium at lower challenge doses. The loss of O-antigen was not found to affect serum survival *in vitro*, but quantification of bacteria in blood following immersion challenge suggested a role in *in vivo* survival. Furthermore, fish challenged with the *waaL* double mutant induced a more transient immune response than fish challenged with the wild type strain. Whether the reduction in virulence following the loss of *waaL* is caused by altered immunomodulative properties or impaired survival remains unclear. However, our data demonstrate that LPS is crucial for development of disease.

1. Introduction

Aliivibrio salmonicida is the etiological agent of cold-water vibriosis, a hemorrhagic septicemia of Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod (*Gadus morhua* L.). After experimental challenge of Atlantic salmon, bacteria have been found to enter the bloodstream within a few minutes of immersion exposure, and exponential proliferation in blood is observed after a period of latency [1–3]. In early stages of disease, bacteria are seen exclusively in the lumen of capillaries [4]. The first sites of cellular damage appears to be leukocytes and endothelial cells of the capillaries [4]. The bacterium seems to penetrate the cell membrane of endothelial cells and enter the cytoplasm, and complete endothelial disintegration is seen in later stages of disease [4].

The pathogenesis of cold-water vibriosis is poorly understood, and no classical virulence factors have been described that can explain the tissue damage observed in moribund fish. However, a highly immunogenic protein/lipopolysaccharide moiety (VS-P1) is released by *Al. salmonicida* in high quantities both *in vitro* and *in vivo* [5–7]. Extracellular VS-P1 has been postulated to bind effector components of the host immune system, functioning as a decoy and saving the bacterial cell from complement-mediated killing and phagocytosis [5,6]. Furthermore, the cell damage observed in fish suffering from cold-water vibriosis has been suggested to be related to the immune response raised against the invading pathogen [1].

Although the mechanism of VS-P1 release is unknown, membrane-bound blebs have been observed to bud off from the outer membrane of the bacteria in infected fish and adhere to fragmented cell membranes,

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https://doi.org/10.1016/j.micpath.2018.08.058
Received 16 June 2017; Received in revised form 12 April 2018; Accepted 25 August 2018
Available online 27 August 2018

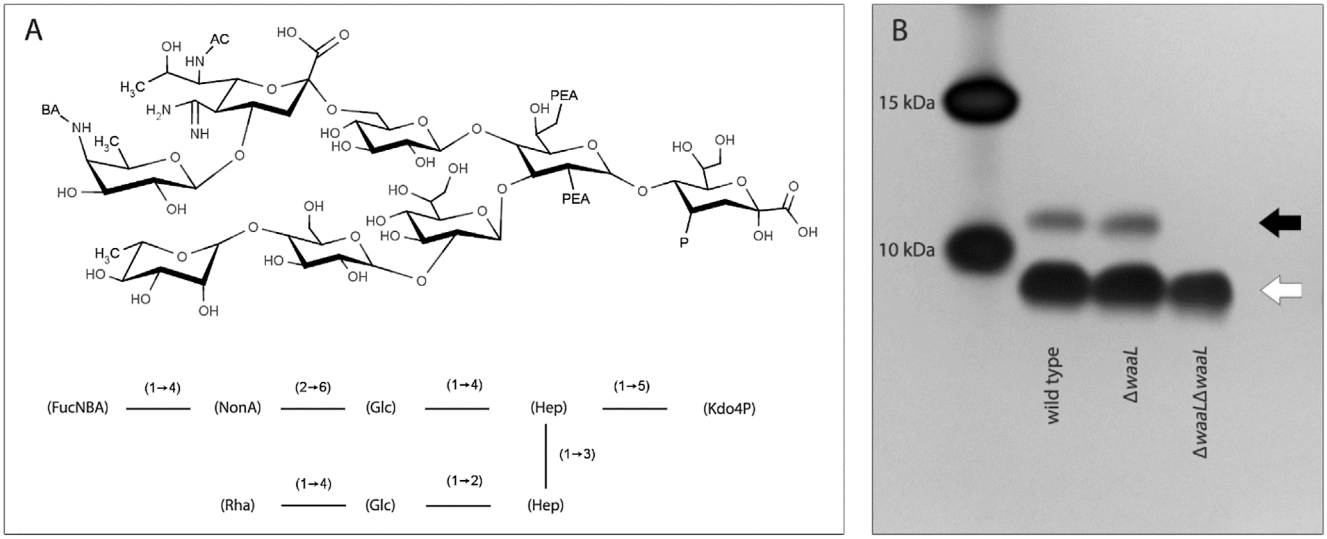

Fig. 1. A, Structure of the oligosaccharide part of the lipopolysaccharide of *Al. salmonicida* (strain NCMB 2262) as determined by Edebrink et al. [11]. FucN is 4-amino-4,6-dideoxy-α-D-galactopyranose, BA is (R)-3- hydroxybutanoyl, NonA is 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxy-L-glycero-α-D-galacto-nonulosonic acid, Glc is glucopyranose, Hep is glycero-manno-heptopyranose, Rha is α-L-rhamnopyranose, Kdo is 3-deoxy-α-D-manno-oct-2-ulosonic acid, and PEA is phosphoethanolamine. B, SDS-PAGE showing LPS structures of wild type, Δ*waaL* and Δ*waaL*Δ*waaL*, extracted by a phenol-water method. Arrows indicate a faster migrating high density band (open arrow) and slower migrating low density band (filled arrow), of which the latter is absent in the Δ*waaL*Δ*waaL* strain.

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

Strain or plasmid	Description	Reference
Aliivibrio salmonicida LFI1238	Wild type strain	[22]
Escherichia coli S17-1 λpir	Donor strain for conjugation	[23]
LFI1238Δ*waaL*	LFI1238 with in-frame deletion of one copy of the *waaL* gene	*
LFI1238Δ*waaL*Δ*waaL*	LFI1238 with in-frame deletion of two copies of the *waaL* gene	*
pDM4	R6K origin suicide vector; contains *cat* and *sacB*	[24]
pDM4Δ*waaLA*	pDM4 containing Δ*waaL* allele	*
pDM4Δ*waaLB*	pDM4 containing nested Δ*waaL* allele	*

cell organelles and intercellular material [4]. Bjelland et al. [1] hypothesized that these blebs were outer-membrane vesicles containing VS-P1. In experimentally challenged Atlantic salmon, immunohistochemistry has revealed diffuse intra- and extracellular staining specific for VS-P1 in tissue of heart, spleen and kidney [8,9].

Al. salmonicida harbors a short-chain LPS resembling that of rough-type bacteria (Fig. 1A) [10,11]. The organism is described as serologically homogenous [7,12,13], and two serotypes are recognized. Serotype C1 has predominantly been isolated from Atlantic salmon, while serotype C2 has only been reported in three isolates from diseased cod [14]. The LPS structures of the two serotypes are closely related, and C2 differs from C1 only by the absence of a 4,6-dideoxy-4-[(R)-3-hydroxybutaneamido]-D-galactose (Fuc*p*4NBA) residue [15]. Although the alteration in LPS structure affects the antigenicity of the bacterium, both serotypes are capable of causing disease [14].

In contrast to higher vertebrates, fish are resistant to endotoxic shock [16]. Nevertheless, LPS has been found to stimulate the production of cytokines and influence cellular and humoral immunity in several fish species [16]. Also, LPS of fish-pathogenic bacteria have been reported to participate in resistance to complement-mediated killing, phagocytosis, and in adhesion [17–20].

This work was initiated in order to investigate roles of LPS of *Al. salmonicida* in the pathogenesis of cold-water vibriosis. To achieve a phenotype with a truncated LPS structure, we constructed in-frame deletion mutants lacking *waaL*, a gene encoding a putative O-antigen ligase. In general, O-antigen ligases participate in LPS biosynthesis, binding O-antigen to the core oligosaccharide-lipid A complex [21]. The putative *waaL* gene of *Al. salmonicida* LFI1238 is found within a 29 kb perfect duplication region encoding 27 genes, of which the majority are predicted to encode products involved in biosynthesis of LPS [22]. Previously, Hjerde and co-workers have postulated a gene-dosage effect for the duplicated genes, leading to an increase in LPS production [22]. To investigate roles of this duplication in the pathogenesis of cold water vibriosis, deletion mutants lacking one or two copies of *waaL* (Δ*waaL* and Δ*waaL*Δ*waaL*) were constructed. Effects of the *waaL* deletions on LPS structure were assessed by SDS-PAGE and effects on virulence were investigated by experimental challenge of Atlantic salmon.

2. Methods

2.1. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used are listed in Table 1. Strains of *Al. salmonicida* were cultivated on blood agar base no. 2 (Oxoid, Cambridge, UK) with 5% ox blood and 0.9% or 2.5% NaCl added (BA0.9 or BA2.5), in Marine broth (Difco, Detroit, MI, USA) containing 2% NaCl, or in Luria Bertani broth (LB) containing 0.9%, 1%, 2.5% or 3% NaCl (LB0.9, LB1, LB2.5 or LB3). When appropriate, LB media were solidified by addition of 1.2% agar-agar (LA1 or LA2.5). Unless otherwise stated, broth cultures were incubated at 12 °C overnight and plates were incubated at 12 °C for 3–5 days. *Escherichia coli* S17-1 λpir was cultivated in LB1 or on LA1 at 37 °C overnight. Selection of *E. coli* transformants or *Al. salmonicida* conjugants containing R6K origin suicide plasmid pDM4 was performed by adding respectively 25 μg ml^{-1} or 2 μg ml^{-1} chloramphenicol (Sigma-Aldrich, St. Louis, MS, USA) (25CAM or 2CAM) to LB1, LA1 or LA2.5. Counter-selection of pDM4 was performed by adding 5% sucrose to the LA2.5.

Growth curves for strains of *Al. salmonicida* were obtained by cultivation in LB0.9 or LB3 at 8 °C (150 rpm), measuring optical density of the cultures at 600 nm (OD_{600}) every 2–6 h. Growth curve experiments were performed in duplicates.

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

Description:	Primers:	Sequence (5' – 3'):	Comments	Construct size:
Primers for construction of LFI1238Δ*waaL*: 1347 bp deletion targeting *waaL*	waaL-A1	ATACTAGTGTACTGGTCGTGCTGAACC	5′ end contains *Spe*I restriction site	247 bp
	waaL-A2	CGCTCAGTATGGCGAGCTTTACTTATTAACAATCGC	5′ end contains a 15 bp sequence complementary to the 5′ end of waaL-A3	
	waaL-A3	TCGCCATACTGAGCGCCTTAG		257 bp
	waaL-A4	TACTCGAGCGACCAAACAAATCAAAGG	5′ end contains a *Xho*I restriction site	
Primers for construction of LFI1238Δ*waaL* Δ*waaL:*	waaL-B1	ATCTCGAGGCGATTGTTAATAAGTAAAGCTC	5′ end contains a *Xho*I restriction site	284 bp
	waaL-B2	CCACGTAAGAGTCAGGATAAATAATAGG		
861 bp deletion targeting a region of *waaL* inside the deleted fragment of LFI1238Δ*waaL*	waaL-B3	CTGACTCTTACGTGGAAGATTTACAAACCAAAGGG	5′ end contains a 15 bp sequence complementary to the 5′ end of waaL-B2	263 bp
	waaL-B4	TAACTAGTGTATGGCGATGCCAACG	5′ end contains a *Spe*I restriction site	
Verification primers for LFI1238Δ*waaL* and LFI1238Δ*waaL* Δ*waaL*	waaL-G	GATGTGGCTGCGGTTAACTTGTGG	Targets regions immediately outside the introduced deletions; used in combination with other primers to verify introduced deletions	–
	waaL-H	CGAATTGGAATACCAGCAAACCAAGG		

2.2. Mutagenesis

Mutagenesis was performed as previously described [24,25]. Primers used were ordered from Invitrogen (Carlsbad, CA, USA) and are listed in Table 2. In short, in-frame deletion mutants of *Al. salmonicida* LFI1238 were constructed by conjugation of R6K origin suicide vector pDM4 containing a deletion allele, followed by allelic exchange integrating the deletion allele in the original locus of the gene. As *waaL* is present in two copies in the LFI1238 genome, a nested approach was utilized in order to target both copies of the gene in the constructed double mutant.

The deletion allele was constructed by overlap PCR. For LFI1238Δ*waaL*, segment waaL-A1A2 (247 bp) immediately upstream of *waaL* was amplified using primers waaL-A1 and waaL-A2. Segment waaL-A3A4 (259 bp), consisting of the last 47 bp of *waaL* and the downstream sequence, was amplified using primers waaL-A3 and waaL-A4. Restriction sites were included in the 5′ end of waaL-A1 (*Spe*I) and waaL-A4 (*Xho*I). The 5′ end of waaL-A2 included a 15 bp sequence complementary to waaL-A3. Fusion PCR creating segment waaL-A1A4 was performed in a two-step manner. First, a PCR reaction was conducted with no added primers using waaLA1A2 and waaLA3A4 as template and the following temperature settings: Denaturation at 95 °C for 3 min, followed by 7 cycles of 95 °C for 45 s, 40 °C for 30 s and 72 °C for 1 min. Immediately after, primers waaL-A1 and waaL-A4 were added, and an additional program was run: 30 cycles of 95 °C for 45 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. The resultant construct *waaLA* and vector pDM4 were digested using restriction enzymes *Xho*I and *Spe*I (New England Biolabs, Ipswich, MA, USA), and cut products were ligated (T4 DNA ligase; Invitrogen) creating pDM4Δ*waaLA*. Purification of plasmids and gel extraction of DNA segments were performed using QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit respectively (both Qiagen, Hilden, Germany), according to the manufacturer's instructions. Following ligation, pDM4Δ*waaLA* was introduced in *E. coli* S17-1 λpir by transformation. Potential transformants were plated on LA1 (25CAM) and CAM-resistant colonies were verified by PCR using primers waaL-A1 and waaL-A4.

For conjugation, donor strain S17-1 containing pDM4Δ*waaLA* was cultivated in LB1 (25CAM) at 37 °C to OD_{600}: 0.9 and recipient strain *Al. salmonicida* LFI1238 was grown in LB2.5 at 12 °C to OD_{600}: 2.6. Recipient cells (750 µl) and donor cells (1500 µl) were washed in LB1 and suspended together in a small volume. For mating, cells (5–10 µl) were spotted on BA0.9 and incubated at room temperature for 4.5 h and 12 °C overnight. Next, spotted cells were resuspended in 2 ml LB2.5 (no antibiotics) and incubated at 12 °C for 24 h. For selection of transconjugants, volumes of 30–100 µl were plated on LA2.5 (2CAM) and incubated at 12 °C for 5 days. Potential transconjugants were transferred to an additional LA2.5 (2CAM) plate and incubated at 12 °C for 5 days. To verify chromosomal integration of pDM4Δ*waaLA,* PCR was conducted using two pairs of primers targeting the deletion construct and the flanking region on both sides (waaL-A1/waaL-H and waaL-G/waaL-A4).

Resolution of the integrated pDM4 was performed by sucrose counter-selection, inducing a second allelic exchange event and leaving only the deletion allele Δ*waaL* in the original locus. LFI1238::pDM4Δ*waaL* was cultivated in LB2.5 (no antibiotics) at 12 °C for 24 h. Volumes of 10 and 100 µl were plated on LA2.5 (containing 5% sucrose) and incubated at 12 °C for 5 days. Colonies growing in the presence of sucrose were plated in parallel on LA2.5 (2CAM) and LA2.5 (5% sucrose), and sucrose-resistant and CAM-sensitive clones were subjected to PCR using primers (waaL-G/waaL-H) spanning the boundaries of the introduced deletion. In addition, primers targeting an amplicon inside the deleted fragment were used to control the intactness of the second copy of the gene. Finally, the constructed LFI1238Δ*waaL* was verified by Sanger sequencing (GATC, Konstanz, Germany).

For deletion of the remaining copy of the gene, gene segments waaL-B1B2 (237 bp) and waaL-B3B4 (263 bp) were fused together by overlap PCR, constructing *waaLB*. Being located inside the deleted waaL-A1A4 segment of Δ*waaL*, exclusive homology with the remaining gene copy was ensured. Digestion, ligation, transformation in S17-1 and conjugation was done as described above. For conjugation, LFI1238Δ*waaL* was used as recipient. To verify the successful construction of LFI1238Δ*waaL*Δ*waaL*, a combination of primers targeting both genes and adjacent regions was employed.

2.3. LPS profiling

LPS was isolated from the wild type, Δ*waaL* and Δ*waaL*Δ*waaL* strains using a modified phenol-water extraction procedure [26]. Cells were grown in Marine broth at 10 °C, collected by centrifugation and washed once in PBS (pH: 7.4) and once in distilled water. The resultant pellet was dissolved in solubilization buffer (4% β-mercapto-ethanol [Sigma-Aldrich], 2% sodium dodecyl sulfate [SDS; Sigma-Aldrich], 2 mM $MgCl_2$, 10 mM Tris-Cl [pH: 8.0]) and incubated at 65 °C for 60 min, before proteinase K (Invitrogen) was added to a final concentration of 10 µg ml^{-1}. Samples were digested overnight at 37 °C, and LPS was precipitated from the solution twice by addition of 0.3 M sodium acetate (final concentration; Sigma-Aldrich) and two volumes of 100% ethanol followed by overnight incubation at −20 °C. Following this, LPS was dissolved in 10 mM Tris-Cl (pH: 7.4) and incubated with DNase I and RNase A (both Invitrogen) overnight at 37 °C for digestion of contaminating nucleic acids. Next, the solution was mixed with an equal volume of phenol (Sigma-Aldrich) (65 °C) and incubated at 65 °C for 20 min while vortexing frequently. After cooling on ice, the solution was centrifuged at 6000 g for 15 min (4 °C). The aqueous phase was transferred to a new tube, and the phenol phase was re-extracted as described. The aqueous phases from both rounds of extraction were

pooled together, before LPS was precipitated twice as described above. For SDS-PAGE, the LPS samples were mixed with sample buffer (30 mM Tris-HCl [pH: 6.8], 0.45 mM EDTA [Sigma-Aldrich], 1% SDS, 20% glycerol, 4% β-mercapto-ethanol and bromophenol blue), boiled for 5 min and resolved in a 12% Criterion XT Bis-Tris gel with the XT MES buffer system (Bio-Rad). Bands were visualized by silver staining using a Pierce Silver Stain Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.4. Serum assay

Strains were exposed to serum of Atlantic salmon (*Salmo salar* L.) to investigate serum resistance. Serum was collected from 17 Atlantic salmon smolt previously not exposed to *Al. salmonicida* and pooled together. As a control, serum was heat-inactivated at 44 °C for 20 min to inactivate complement activity as previously described [27]. Strains were grown overnight in LB0.9 at 8 °C (200 rpm), washed once in cold PBS and resuspended in the same buffer to OD_{600}: 0.2. For each strain, 25 µl of bacterial suspension and 75 µl untreated serum, heat-inactivated serum or LB0.9 were mixed and incubated at 8 °C (50 rpm). After 0, 2, 24, 48 and 72 h, colony-forming units (CFU) were determined by serial dilution followed by plating on BA2.5. The experiment was performed in triplicates and the results are presented relative to the starting amount as means ± standard error of the mean (SEM).

2.5. Challenge experiments

Virulence of the Δ*waaL*, Δ*waaL*Δ*waaL* and wild type strains were determined in challenge experiments by challenging Atlantic salmon (*Salmo salar* L.) through immersion or intraperitoneal injection (i.p.) of bacterial suspension. Prior to challenge, the strains were passaged in Atlantic salmon to avoid loss of pathogenicity due to passage on artificial substrates [13]. Challenge doses were based on experience from earlier experiments [28]. Experimental fish were kindly provided by Sørsmolt (Sørsmolt AS, Sannidal, Norway). Ahead of the immersion challenge, fish were smoltified by manipulation of the light regime. Optimal state of smoltification was estimated by skin coloring and verified by transfer of a few individuals to sea water for a period of eight days. After observation of negative symptoms, the remaining fish were moved.

For the first immersion challenge experiment, Atlantic salmon smolts ($n = 140$) with a mean weight of 80 g were split in three experimental groups and one control group. Challenge was conducted by immersion in 75 L oxygenated sea water (8 °C) with added LB3-cultured bacteria for 45 min. The control group was mock challenged with sterile LB3 in an identical manner. Shortly after challenge initiation, tank water was sampled and challenge doses were found to be: Δ*waaL*: 1.19×10^7 CFU ml^{-1}, Δ*waaL*Δ*waaL*: 4.87×10^6 CFU ml^{-1} and wild type: 4.97×10^6 CFU ml^{-1} sea water. After challenge, the tank volumes were reduced to 30 L, before sea water was added to 150 L. For the remaining course of the experiment, tanks were supplied with flow-through of sea water (8 °C, 35 ppm salinity). The experiment was terminated after 35 days.

For the i.p. challenge experiment, Atlantic salmon parr ($n = 166$) were kept in a holding tank supplied with aerated fresh water until initiation of the experiment. Strains were grown overnight in LB0.9 at 10 °C (150 rpm) and diluted to OD_{600}: 0.3. Fish were anesthetized by immersion in 0.0025% benzocaine (Benzoak VET; ACD Pharmaceuticals, Leknes, Norway) and challenged by intraperitoneal injection of 0.1 ml of bacterial suspension or PBS. Challenge doses were: Δ*waaL*: 3.41×10^7 CFU, Δ*waaL*Δ*waaL*: 2.59×10^7 CFU and wild type: 2.85×10^7 CFU. To differentiate between groups, challenged fish were 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). Following challenge, the fish were mixed and moved to multiple 150 L holding tanks supplied with flow-through of carbon filtered fresh water holding 11 °C and monitored for 25 days. At time points 12, 24 and 72 h post challenge, five fish from each experimental group were euthanized in a water bath containing 0.0125% benzocaine, and the spleen of each fish was dissected and transferred to 1 ml RNAlater (Qiagen). Spleen samples were incubated at 4 °C overnight and kept at −20 °C until analysis.

During the course of both experiments, fish were fed *ad lib* and tanks were monitored for mortality twice daily. Samples from head kidney of all diseased fish were plated on BA2.5 to verify the presence of *Al. salmonicida*. Differences in survival between experimental groups were evaluated by Wilcoxon and log-rank tests.

A second immersion challenge was conducted for quantification of bacteria present in blood of fish after challenge. Atlantic salmon smolt ($n = 54$) with a mean weight of 172 g were split in three experimental groups consisting of 15 fish each and one control group consisting of 9 fish. Challenge was performed by immersion in 20 L of sea water holding 8 °C with bacterial cultures added. Shortly after initiation of challenge, water was sampled for determination of challenge doses by serial dilution and found to be: 1.33×10^7 CFU ml^{-1} (wild type), 9.07×10^6 CFU ml^{-1} (Δ*waaL*) and 2.06×10^7 CFU ml^{-1} sea water (Δ*waaL*Δ*waaL*). After 10 min, fish were moved to 150 L holding tanks supplied with flow-through of sea water (8 °C). At time points 15 min, 24 h and 48 h after challenge, five fish were removed from each group and euthanized in a water bath containing 0.0125% benzocaine. Blood samples were collected from the caudal vein using a vacutainer, and volumes of 100 µl were plated on BA2.5 in duplicates for CFU determination.

The challenge experiments were approved by Norwegian Research Animal Authorities (FOTS ID: 7808, 7810 and 11808).

2.6. RNA extraction

To extract salmon RNA from spleen tissue, 10–20 mg of RNAlater-preserved tissue was transferred to a tube containing a 5 mm steel bead (Qiagen) and 1 ml Qiazol (Qiagen). Samples were kept at room temperature for approximately 10 min and homogenized in a Tissuelyser II (Qiagen) at 25 Hz for 5 min. After homogenization, samples were briefly incubated at room temperature, mixed with 200 µl chloroform and separated by centrifugation at 11 400 rpm for 20 min at 4 °C in a Himac CT15RE tabletop centrifuge (Hitachi Koki Co., Ltd., Tokyo, Japan). The aqueous phase (containing RNA) was transferred to a new tube, mixed with an equal volume of 70% ethanol and applied to a RNAeasy Mini spin column (Qiagen). Total RNA was extracted using the RNAeasy Mini Kit according to the protocol of the manufacturer. Concentration and purity of the RNA samples were evaluated by measuring the A260/280 ratio on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmingtion, DE, USA), and gel electrophoresis was conducted for visualization of RNA degradation.

2.7. Two-step RT-qPCR

Complementary DNA (cDNA) was synthesized from RNA using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. For each reaction, 1 µg RNA was used as template, and the protocol included a gDNA wipeout treatment. To control for remaining gDNA in the samples, reverse transcriptase was omitted from a randomly selected sample per round of cDNA synthesis. Before use in qPCR, cDNA samples were diluted to 5 ng µl^{-1}, aliquoted in small volumes and stored at −20 °C. qPCR was conducted using SYBR GreenER qPCR Supermix Universal Kit (Invitrogen) in 20 µl reactions, using primers listed in Table 3. Each reaction containing 10 µl master mix, 200 nM of each primer, 50 nM ROX dye and 15 ng template cDNA. All reactions were run in triplicates in a MX3005P thermal cycler (Agilent Technologies, Santa Clara, CA, USA) with the following temperature settings: (1) 50 °C for 2 min, 95 °C for 10 min, (2) 40 cycles: 95 °C for 15 s, 60 °C for 1 min (ROX- and SYBR data collection), (3) melting curve

Table 3
Primers used for gene expression analyses by RT-qPCR.

Description:	Primers:	Sequence (5' – 3'):	Construct size:	Ref.
Elongation factor 1Aa (AF321836.1)	EF1Aa-F	CCCCTCCAGGACGTTTACAAA	57 bp	[29]
	EF1Aa-R	CACACGGCCCACAGGTACA		
Elongation factor 1Ab (BG933853.1)	EF1Ab-F	TGCCCCTCCAGGATGTCTAC	57 bp	[29]
	EF1Ab-R	CACGGCCCACAGGTACTG		
β-actin (BG933897.1)	B-actin-F	CCAAAGCCAACAGGGAGAAG	91 bp	[29]
	B-actin-R	AGGGACAACACTGCCTGGAT		
Interleukin 1-β (AY617117.1)	IL-1b-F	GCTGGAGAGTGCTGTGGAAGA	73 bp	[30]
	IL-1b-R	TGCTTCCCTCCTGCTCGTAG		
Tumor necrosis factor α (NM_001123589.1)	TNFa-F	AGGTTGGCTATGGAGGCTGT	173 bp	[30]
	TNFa-R	TCTGCTTCAATGTATGGTGGG		
Interleukin 6 (XM_014143031.1)	IL-6-F	ACCAACAGTTTGTGGAGGAGTT	105 bp	[30]
	IL-6-R	AGCAAAGAGTCTTGGAGAGGTG		
Interleukin 8 (CXCL8) (XM_014187025.1)	IL-8-F	ATTGAGACGGAAAGCAGACG	136 bp	[30]
	IL-8-R	CGCTGACATCCAGACAAATCT		
Complement component 3 (XM_014186867.1)	C3-F	TCCCTGGTGGTCACCAGTACAC	157 bp	[31]
	C3-R	ATGATGGTGGACTGTGTGGATC		

analysis: 95 °C for 1 min, 55 °C for 30 s, 95 °C for 30 s. For each run, a no template control and no reverse transcriptase control were included.

2.8. Gene expression analysis

To investigate potential differences in innate immunity between fish challenged with mutant and wild type strains, expression profiles for selected immune genes were derived from RT-qPCR data by performing a ΔΔCq analysis [32]. To normalize for variation in mRNA abundance between the analyzed samples, normalization factors for each sample were determined by calculating the geometric mean of reference genes $EF1A_A$, $EF1A_B$ and *β-actin*, previously described to be stably expressed in Atlantic salmon tissue [29]. For each gene assayed, amplification efficiency of the qPCR reactions was calculated using LinRegPCR (version: September 2014) [33]. For each sample and gene, Cq values were transformed to quantities and normalized against the sample normalization factor. Gene expression data are shown as fold changes (± standard error of the mean) relative to the control group sampled 12 h after mock challenge with PBS. For each sampling time point, differential gene expression in groups challenged with mutant and wild type strains were tested by Mann Whitney's *U* test. The null hypothesis was rejected at a 5% confidence level.

3. Results

To investigate roles of LPS in virulence, we constructed in-frame deletion mutants for a putative *waaL* O-antigen ligase (VSAL_I0160/VSAL_I0263). The gene shows low sequence similarity (< 30%) to known *waaL* orthologs, but the predicted secondary structure of the encoded protein contains twelve membrane-spanning domains and a large periplasmic loop, indicating that it is an integral membrane protein (Supplementary figure S1). As the genome of *Al. salmonicida* harbors two copies of the *waaL* gene, mutants defective of one or both copies of the gene were constructed.

Growth in LB broth containing 0.9 or 3% NaCl was measured to examine whether the constructed mutants affected *in vitro* growth. No differences in bacterial growth were observed for either the Δ*waaL* or the Δ*waaL*Δ*waaL* strain (data not shown).

3.1. SDS-PAGE of LPS

To establish whether the introduced deletions did indeed affect LPS biosynthesis, LPS was isolated from wild type bacteria and the mutant strains Δ*waaL* and Δ*waaL*Δ*waaL* for analysis by SDS-PAGE. Wild type LPS migrated as one dominant band of low molecular weight (8 kDa; Fig. 1B, open arrow) and a second band of 10 kDa (Fig. 1B, filled arrow). No differences were seen between the migration patterns of wild type and Δ*waaL*. For LPS of Δ*waaL*Δ*waaL*, the 10 kDa band of wild type LPS was absent, indicating a truncated structure.

3.2. Serum resistance experiment

As *Al. salmonicida* replicates in the bloodstream of experimentally infected fish and eventually causes septicemic disease, the organism is likely to possess strategies for survival in the presence of the host immune system. We wanted to investigate whether *Al. salmonicida* was resistant to killing by salmon serum, and if modifications made to the LPS structure by deletion of one or two copies of *waaL* was of importance for serum survival. A similar survival pattern was seen for all strains (Fig. 2). For the wild type strain, a minor increase in CFU was seen after 2 h of serum exposure, followed by a reduction of approximately one log per 24 h of incubation in serum. Cells incubated in heat-

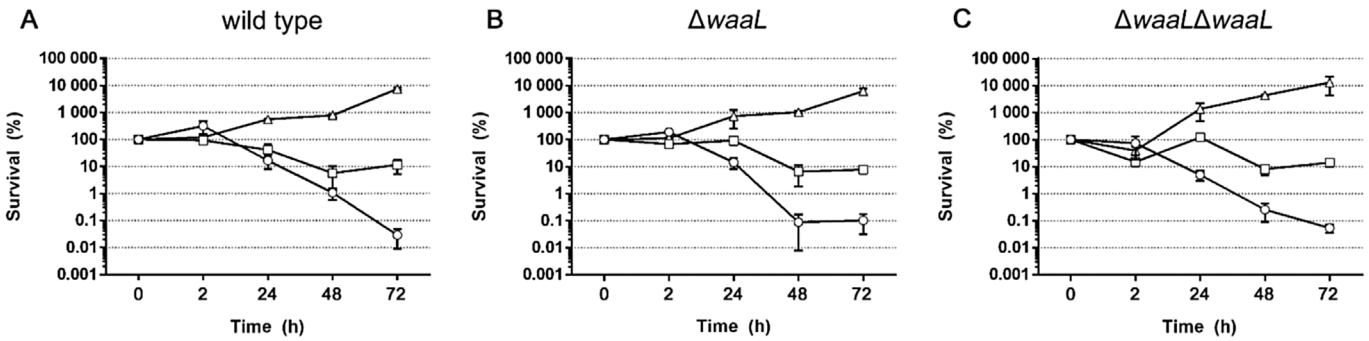

Fig. 2. Survival of wild type (A), Δ*waaL* (B) and Δ*waaL*Δ*waaL* (C) after incubation in nontreated serum (circles), serum heat-inactivated at 44 °C for 20 min (squares) or LB0.9 (triangles). Values are shown as mean ± SEM relative to the starting amount.

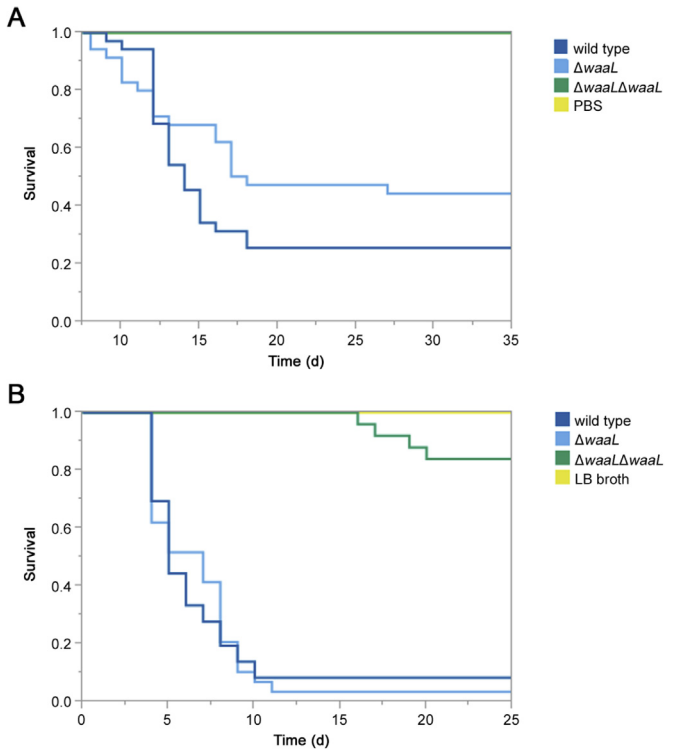

Fig. 3. Survival plots for immersion (A) and intraperitoneal (B) challenge of Atlantic salmon with the wild type (dark blue), Δ*waaL* (light blue) and Δ*waaL*Δ*waaL* (green) strains. As a negative control, fish were mock challenged with PBS or LB broth (yellow). Diseased fish from which the challenge strains could not be isolated are excluded in the plots. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inactivated serum showed a less marked reduction over the course of the experiment. In contrast to the wild type strain, Δ*waaL*Δ*waaL* displayed a reduction in viable numbers after 2 h of incubation in both serum and heat-inactivated serum. At later time points, the pattern of survival of Δ*waaL*Δ*waaL* was similar to the two other strains.

3.3. Challenge experiment

To determine whether the modified LPS structure affected virulence, Atlantic salmon were challenged by immersion with the wild type, Δ*waaL* or Δ*waaL*Δ*waaL* strains. In fish challenged with wild type bacteria, a cumulative mortality of 74.3% was observed between day nine and eighteen (Fig. 3A). In the group challenged with the Δ*waaL* strain, 55.6% of the fish died between day 8 and 27 (Log-rank: $p < 0.1239$; Wilcoxon: $p < 0.2307$). No specific mortality was seen in the fish challenged with the Δ*waaL*Δ*waaL* strain (Log-rank: $p < 0.0001$; Wilcoxon: $p < 0.0001$). *Al. salmonicida* was isolated from head kidney of all diseased fish challenged with the wild type or Δ*waaL* strains, and no bacteria were isolated from head kidney of surviving fish. However, in the group challenged with the Δ*waaL*Δ*waaL* strain, 62.9% of the fish developed skin ulcerations during the course of the experiment, and 42.9% of the fish died between day 16 and 35 post challenge. Similarly, 5.7% of the fish in the negative control group developed skin ulcerations and died during the experiment. In these fish, *Moritella viscosa* and/or *Aliivibrio wodanis*, both associated with winter ulcer disease, were isolated from head kidney. *Al. salmonicida* could not be detected in any of the diseased or surviving fish of the Δ*waaL*Δ*waaL* group. The observed mortality was interpreted as a manifestation of winter ulcer disease. Consequently, these individuals were excluded from the presented survival analysis.

To further investigate the virulence of the Δ*waaL*Δ*waaL* strain,

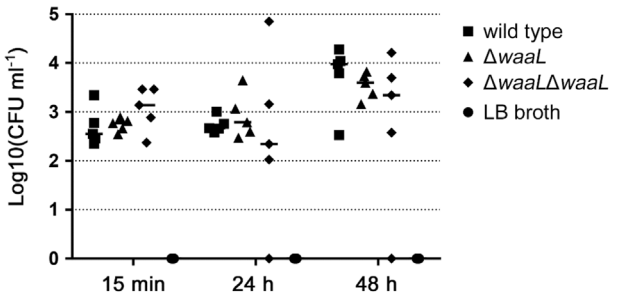

Fig. 4. Log-transformed values for CFU ml^{-1} blood of fish challenged with the wild type, Δ*waaL* and Δ*waaL*Δ*waaL* strains. Blood was sampled 15 min, 24 h and 48 h post challenge. Lines represent median values.

another challenge trial was run, in which groups of fish were challenged by intraperitoneal injection of the wild type, Δ*waaL* and Δ*waaL*Δ*waaL* strains. In this experiment, 91.7% of fish challenged with the wild type strain died between day four and ten (Fig. 3B). Similarly, 96.6% of the fish challenged with the Δ*waaL* strain died between day four and eleven. In the group challenged with the Δ*waaL*Δ*waaL* strain, mortality was first seen 16 days post challenge, and the cumulative mortality observed over the course of the experiment was 16% (Log-rank: $p < 0.0001$; Wilcoxon: $p < 0.0001$).

In all diseased fish in the i.p. challenge experiment, *Al. salmonicida* was isolated from head kidney. At the end of the experiment (day 25), no bacteria could be detected in surviving fish challenged with wild type or Δ*waaL* strains. Of the 21 fish that survived challenge with the Δ*waaL*Δ*waaL* strain, one was found positive for *Al. salmonicida.*

To investigate the capacity for survival in the fish host, a second immersion challenge experiment was conducted, and bacterial quantities in blood were determined in fish sampled 15 min, 24 h and 48 h after challenge. In fish challenged with the wild type strain, > 200 CFU ml^{-1} blood were detected in all fish sampled at the three time points (Fig. 4). An increase was observed from 24 to 48 h, possibly representing the initiation of logarithmic growth. Similar bacterial loads were retrieved from fish challenged with Δ*waaL*. In fish challenged with Δ*waaL*Δ*waaL*, a small drop in bacterial retrieval rates was seen between fish challenged 15 min and 24 h after challenge, followed by an increase at 48 h. However, in one fish sampled 24 h post challenge and one fish sampled 48 h after challenge with Δ*waaL*Δ*waaL*, bacteria could not be found (< 10 CFU ml^{-1}). Likewise, *Al. salmonicida* was not detected in fish mock challenged with sterile LB3.

3.4. Gene expression analysis

As the virulence of the Δ*waaL*Δ*waaL* strain was severely impaired compared with the Δ*waaL* and wild type strains after both i.p. and immersion challenge, we wanted to determine whether the immune response raised towards the invading pathogen also differed between the groups. Genes encoding pro-inflammatory cytokines IL-1β, TNFα, IL-6 and IL-8 and complement component C3 were selected for analysis, and fish were sampled 12, 24 and 72 h post challenge (hpc). Gene transcription was analyzed through a ΔΔCq approach, where *EFN1Aa*, *EFN1Ab* and *β-actin* were chosen as reference genes based on a previous paper showing stability of expression in Atlantic salmon [29]. M-values for the reference genes were as following: EFN1Aa: 0.728, EFN1Ab: 0.785 and β-actin: 0.888.

In fish challenged i.p. with the wild type strain, high initial expression was seen for the genes encoding pro-inflammatory cytokines IL-1β, TNFα and IL-6 (Fig. 5A–C). For genes encoding IL-8 and complement component C3, a gradual increase in expression was seen from 12 to 72 hpc (Fig. 5D and E). Overall, fish challenged i.p. with the Δ*waaL* strain exhibited an expression pattern similar to the wild type group. However, the expression of IL-6 at 12 hpc was lower than in fish challenged with wild type due to high IL-6 expression in one individual

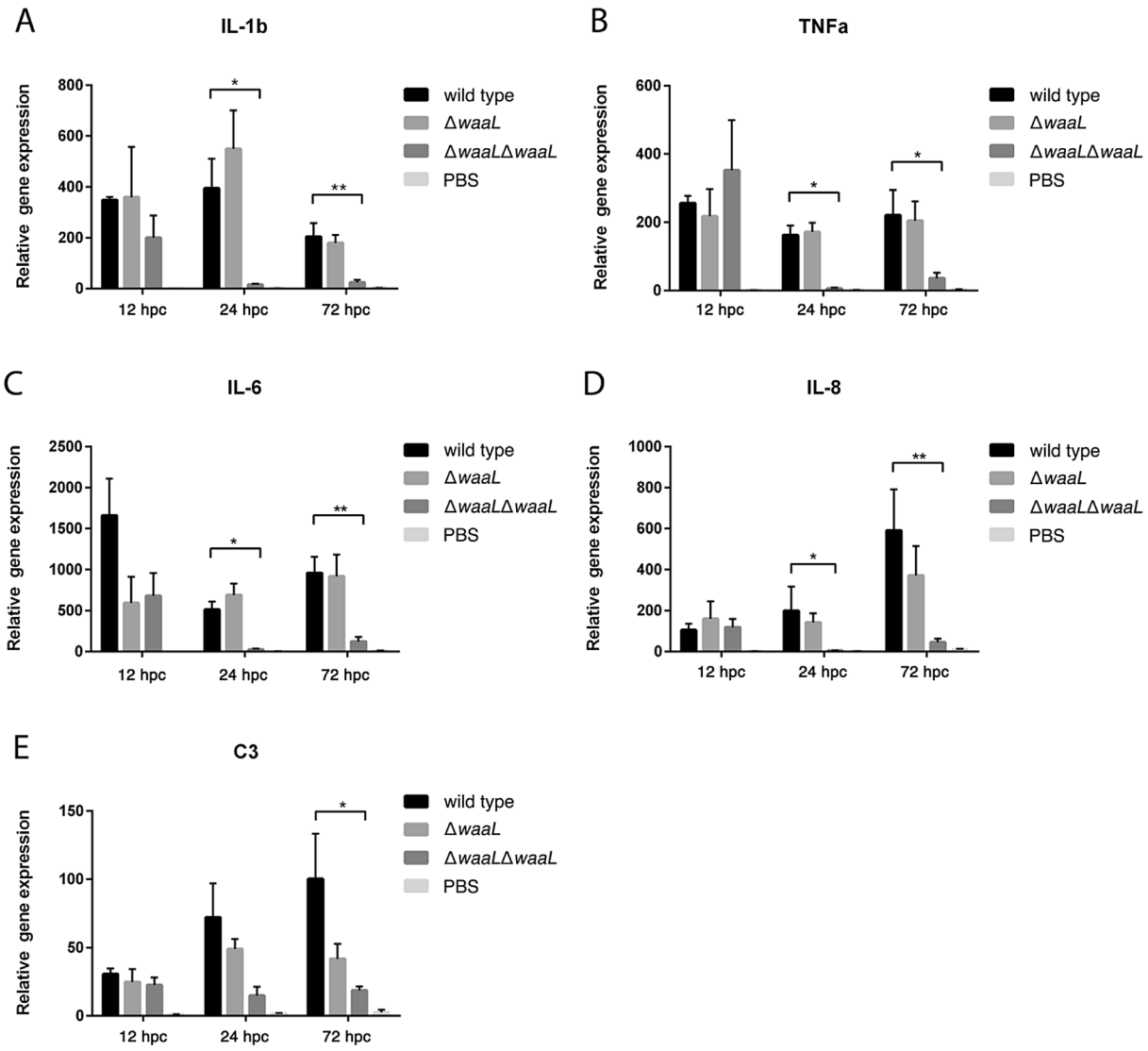

Fig. 5. Relative transcription of IL-1β (A), TNFα (B), IL-6 (C), IL-8 (D) and complement component C3 (E) of fish challenged i.p. with the wild type, Δ*waaL* and Δ*waaL*Δ*waaL* strains 12, 24 and 72 h post challenge (hpc). Transcription is shown relative to fish mock challenged with PBS sampled 12 hpc. Differential gene expression between the experimental groups for each time point and gene was tested by Mann Whitney's *U* test. *$p < 0.05$, **$p < 0.01$.

of the wild type group. Also, the increases in C3 expression observed 24 and 72 hpc were less marked than in the wild type group, but this was not found be statistically significant.

In fish challenged i.p. with strain Δ*waaL*Δ*waaL*, the initial gene expression pattern observed 12 hpc did not differ from the wild type group (except for IL-6). However, at 24 and 72 hpc, a significant reduction in relative transcription was observed for IL-1β (24 hpc – *p*: 0.0286; 72 hpc – *p*: 0.0079), TNFα (24 hpc – *p*: 0.0286; 72 hpc – *p*: 0.0317), IL-6 (24 hpc – *p*: 0.0286; 72 hpc – *p*: 0.0079) and IL-8 (24 hpc – *p*: 0.0286; 72 hpc – *p*: 0.079). Similarly, transcription of C3 was significantly reduced in Δ*waaL*Δ*waaL* relative to the wild type strain at 72 hpc (*p*: 0.0159).

4. Discussion

The release of high quantities of VS-P1 from *Al. salmonicida* during an infection has been suggested to function as a virulence factor, masking the presence of invading bacteria and modulating the immune response raised [5,7]. As LPS is found as part of the VS-P1 complex, we constructed in-frame deletion mutants lacking one or two copies of a putative O-antigen ligase *waaL* in order to obtain a phenotype with a truncated LPS structure and increase the understanding of how VS-P1 is involved in virulence. Generally, WaaL proteins are known to exhibit low similarity in their primary sequence, while their predicted secondary structures typically contain multiple transmembrane segments and a large periplasmic loop close to the C-terminus [34–37]. While the amino acid sequence of the protein encoded by the putative *waaL* gene of *Al. salmonicida* showed poor similarity to known WaaL orthologs, the *in silico* predicted structure of the same protein suggests that it shares structural features with known WaaL proteins (Supplementary figure S1).

The deletion of both *waaL* copies was found to affect the LPS structure. Analysis of isolated LPS from the wild type strain by SDS-PAGE revealed one major band of 8 kDa and one band of 10 kDa. A similar pattern has been observed in other Vibrionaceae spp. and is described to represent the core oligosaccharide and the core oligosaccharide plus O-antigen [38–41]. In LPS isolated from the Δ*waaL*Δ*waaL* strain, the 10 kDa band was absent, indicative of a truncated structure.

Through experimental challenge of Atlantic salmon by immersion and i.p. injection, we found the Δ*waaL*Δ*waaL* strain to be almost avirulent, clearly demonstrating that LPS is a virulence factor in *Al.*

salmonicida. For the Δ*waaL* strain, a non-significant reduction in cumulative mortality was seen after immersion challenge, whereas no difference was seen between fish challenged i.p. with the wild type and Δ*waaL* strains. However, the cumulative mortality observed in the wild type group of the i.p. trial was above 90%, probably reflecting the high challenge doses used in the experiment. These doses are likely to exceed those associated with outbreaks of disease in a fish farm setting, and a gene-dosage effect of the *waaL* duplication may have a greater impact on the virulence of *Al. salmonicida* under real life conditions.

In the immersion trial, a high prevalence of ulcerations was noted in fish challenged with the Δ*waaL*Δ*waaL* strain. The late onset of pathological signs, as well as the presence of *M. viscosa* and *Al. wodanis* in the head kidney of these fish, suggest that the ulcerations and the related mortality were manifestations of winter ulcer disease. Also, *Al. salmonicida* could not be identified in either morbid fish or survivors. Presumably, *M. viscosa* and *Al. wodanis* were introduced to the experimental facility through the intake of sea water. The prevalence of winter ulcer disease was far greater in the Δ*waaL*Δ*waaL* group (62.9%) than in the negative control group (5.7%), but the causality between the preceding bacterial challenge and the development of winter ulcer disease cannot be determined from our data. However, it is tempting to speculate that the infection with the Δ*waaL*Δ*waaL* mutant occupied some of the capacity of the host immune system and increased the impact of the ulcer condition.

The reduced virulence of Δ*waaL*Δ*waaL* observed in both challenge trials may be explained in at least three ways: (1) Reduced *in vivo* survival, (2) differences in host immunomodulation in response to the invading pathogen, or (3) loss of other functions required for virulence.

In order to successfully proliferate and cause disease in a teleost host, bacteria must overcome the repertoire of host defense mechanisms combating infections, including the complement system found in serum. In several known fish pathogens, LPS has been shown to provide serum resistance [42–44]. We found all strains to be semi-sensitive towards Atlantic salmon serum. While complement-mediated killing is generally seen to cause a major reduction in viable numbers (< 1% survival) within a few hours [42,43,45], we found approximately 12% of the initial inoculum to still be viable after 24 h of incubation in serum. Nevertheless, a clear trend of reduction was observed over the course of the experiment. The reduction in cell numbers was less pronounced after incubation in heat-treated serum, indicating that parts of the bactericidal components of the serum were heat-labile. Bacterial capsules and long O-antigen chains are known to protect the cell against complement-mediated killing by sterically hindering complement factors in accessing the cell surface [46]. Thus, the lack of capsule and the rough type LPS found in *Al. salmonicida* may explain the inability of growth in serum [10,47]. However, the slow rate of reduction is suggestive of some means of protection.

To investigate whether a similar drop in bacterial numbers was seen after host invasion, we quantified viable bacteria in blood of Atlantic salmon sampled 15 min, 24 and 48 h post immersion challenge. For wild type and Δ*waaL*, retrieval rates were comparable at all three time points. A relative increase in bacterial numbers found in blood between 24 and 48 h post challenge suggests that these strains are able to overcome defense mechanisms and proliferate within the host. The discrepancy between *in vitro* serum sensitivity and retrieval rates from blood of challenged fish suggests that the *in vivo* phenotype may differ from that *in vitro*, or that other factors are involved permitting *in vivo* growth. Possibly, adaption to the *in vivo* environment increases the potential for survival. Alternatively, interactions with host cells, such as macrophages and endothelial cells, may be of importance for the ability to survive. However, *Al. salmonicida* has previously been shown to be rapidly engulfed and degraded by macrophages *in vitro* [48].

The Δ*waaL*Δ*waaL* strain was retrieved from the majority of fish at all three time points, but large variation was seen between replicates 24 and 48 h post challenge. This may indicate a reduced survival potential for Δ*waaL*Δ*waaL*, denoting a role in survival for the LPS. Furthermore, the similar invasion rates observed in the wild type and Δ*waaL*Δ*waaL* groups immediately following challenge shows that the LPS structure is of little importance for invasion of Atlantic salmon.

The challenge dose required for onset of disease is relatively high for *Al. salmonicida* compared to that of other fish pathogens, such as *Aeromonas salmonicida* and *Vibrio anguillarum* [49]. Also, Kashulin has reported a drop in CFU of fish blood over the first few hours after challenge, followed by a rise in numbers at later time points [50]. The requirement for a high dose to overcome the defense mechanisms of the host may reflect the organism's semi-sensitivity to serum killing. The infected host manages to keep the infection at bay for some time, but given that the infectious pressure is sufficiently high, the host is overwhelmed and rapid bacterial proliferation is initiated.

As mentioned previously, VS-P1 has been postulated to serve as decoy and function in immunomodulation. As LPS is found as part of the VS-P1 complex, we were wondering whether alterations of the LPS structure influenced the immunomodulative properties of VS-P1. To evaluate the host immune response raised towards the invading bacteria, a panel of immune parameters was analyzed 12, 24 and 72 h post i.p. challenge. In fish challenged with Δ*waaL, t*he expression pattern of the analyzed genes was found to be similar to that of the group challenged with the wild type strain. In fish challenged with Δ*waaL*Δ*waaL*, a similar expression pattern was noted 12 h post challenge, but at later time points, the expression of all genes analyzed was significantly lower than in fish challenged with wild type. While the transcription of complement factor C3 was shown to increase over time in fish challenged with the wild type strain, C3 transcription in Δ*waaL*Δ*waaL*-challenged fish was stable at low levels. Possibly, the altered LPS structure of Δ*waaL*Δ*waaL* interferes with the postulated decoy function of VS-P1, resulting in a more directed and efficient immune response. The results from the *in vivo* growth experiment suggest that the bacterial loads in fish challenged with Δ*waaL*Δ*waaL* were reduced compared to the wild type group over the first two days following challenge. Thus, the relative reduction in immune gene transcription observed in fish challenged with Δ*waaL*Δ*waaL* could be related to either the loss of immunogenic properties, or a reduction in cell numbers at the time of sampling.

In addition to its role in serum resistance and immunomodulation, a function of LPS in adhesion has been postulated for several bacterial species [51–53]. In the enteric pathogens *V. mimicus* and *V. cholerae*, the polysaccharide moiety of LPS is involved in hemagglutination [54]. As hemagglutination activity has been found to correlate with intestinal adhesion, LPS was implicated as an adhesin. *Al. salmonicida* has been observed in intimate contact with endothelial cells under the progression of disease [4], but no adhesins have been described facilitating this contact. A role for LPS in adhesion could explain the reduction in virulence observed for Δ*waaL*Δ*waaL*. However, such a role cannot be determined from the data presented here.

In conclusion, we have shown that the LPS structure of *Al. salmonicida* is of importance for virulence in Atlantic salmon. Of the two genomic copies of O-antigen ligase *waaL*, one copy was found to be sufficient for onset of disease. Nevertheless, a non-significant decrease in mortality was observed after immersion challenge with a single copy *waaL* mutant, and it is tempting to suggest that multiple copies of the gene are beneficial to the bacterium at lower challenge doses. As the LPS structure did not influence invasive properties of the bacterium, the role of LPS in virulence applies to later stages of the pathogenesis. The loss of O-antigen was not found to affect serum survival *in vitro*, but quantification of bacteria in blood following challenge suggested a role in *in vivo* survival. Furthermore, fish challenged with the *waaL* double mutant induced a more transient immune response than fish challenged with the wild type strain. Whether the reduction in virulence following the loss of *waaL* is caused by altered immunomodulative properties or impaired survival remains unclear. Future studies should address the structure and immunogenicity of LPS isolated from the described mutants, and evaluate the protective properties obtained by immunization

with the Δ*waaL*Δ*waaL* strain.

Acknowledgements

The authors would like to acknowledge Stein Helge Skjelde (SørSmolt AS) for providing Atlantic salmon parr free of charge for the challenge experiments, as well as Even Thoen (Fellesakvariet, Norwegian Veterinary Institute, Oslo, Norway) and Oddbjørn Pettersen and colleagues (NIVA, Solbergstrand, Norway) for management of experimental facilities and technical assistance running challenge experiments.

This work was supported by the Norwegian University of Life Sciences.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.micpath.2018.08.058.

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