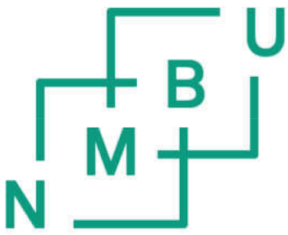
Microbe-associated molecular patterns of *Aliivibrio salmonicida*: roles in the pathogenesis of cold-water vibriosis

Simen Foyn Nørstebø

Thesis for the degree of *Philosophiae Doctor*



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“My reality needs imagination like a bulb needs a socket.

My imagination needs reality like a blind man needs a cane.”

Tom Waits

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Preface

I would like to acknowledge the people of Lindern for their contributions in creating a superb workplace. Especially, I am grateful to the tremendous Baktlab crew for the way they have made me feel included during the years I have spent in their presence, and for the (mostly) good vibes that make Baktlab “the lab with the cheerful atmosphere”.

To Ane and Henning, my supervisors: you have made me believe that I am able to contribute to the progress of science. Thank you for letting me experience the savory zing of academic freedom, and for allowing me to pursue my own ideas (or at least, tricking me into believing so). At the same time, I don’t know where this journey would have ended if you had not, when required, provided me with reality checks in various forms.

Also, I am grateful to Pat Fidopiastis and Michael Wade for their generous hospitality during my stay in San Luis Obispo. You truly made my experience a great one. Our collaboration on proteomics work has certainly been an adventure, and I look forward to its continuation.

I would also like to thank my friends and family for tolerating my intermittent hermit-like behavior. I know that I at times have been hard to reach.

Finally, I am in desperate need of advice on how to repay my beloved Anna and Elias for weeks, months and years of supportive demeanor. I am struggling to visualize the stocks of licorice, chocolate raisins and toy cars I probably owe you.

Abbreviations

C3	Complement component 3
CFU	Colony-forming units
FlaA	Flagellin subunit FlaA of the flagellar filament
FlaD	Flagellin subunit FlaD of the flagellar filament
Fur	Ferric uptake regulator
i.p.	Intraperitoneal
IL-1β	Interleukin 1 beta, cell signaling protein
in vitro	"within the glass"
in vivo	"within the living"
IROMPs	Iron-regulated outer-membrane proteins
IS	Insertion sequence
iTRAQ	Isobaric tags for relative and absolute quantitation
LB	Luria Bertani (Lysogeny) broth
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
Mab	Monoclonal antibody
MAMPs	Microbe-associated molecular pattern
MotA	Part of the stator element of the flagellar motor complex
OmpU	Protein found in the outer-membrane
OMVs	Outer membrane vesicles
ORF	Open reading frame
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptor
RT-qPCR	Quantitative reverse transcription PCR (polymerase chain reaction)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T1SS	Type I secretion system
T2SS	Type II secretion system
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha, cell signaling protein
TonB	Energy transduction system for outer-membrane transport
VS-P1	*Vibrio salmonicida* protein 1 (outer-membrane complex)
WaaL	O-antigen ligase

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Simen Foyn Nørstebø, Leif Lotherington, Marius Landsverk, Ane Mohn Bjelland and Henning Sørum
Submitted to *Microbial Pathogenesis*

Summary

Aliivibrio salmonicida is the cause of cold-water vibriosis, a hemorrhagic septicemia of farmed salmonids. Previous reports have shown that *Al. salmonicida* is able to circumvent defense systems of the fish host, and it has been suggested that the tissue damage observed in moribund fish is associated with the immune response raised towards the invading pathogen. This project was initiated to identify components of importance for virulence and immunogenicity, in order to increase the understanding of interactions between *Al. salmonicida* and its salmonid host.

For microbial detection and induction of defense systems, the host immune system relies on microbe-associated molecular patterns (MAMPs), structures specific to microbes that serve as signatures for microbial presence. In this thesis, two well-known MAMPs have been investigated: flagellin of the flagellar motility apparatus, and lipopolysaccharide (LPS) of the outer membrane. In addition to their roles as targets for the immune system, both the flagellum and LPS serve important functions that aid bacterial survival. The results presented here demonstrate that neither flagellar motility nor LPS are required for invasion of salmon. However, both structures were shown to be involved in later stages of disease development.

Results of challenge experiments demonstrated a function in virulence for the flagellar flagellins. Interestingly, bacteria cultured in implants within the peritoneal cavity of salmon showed an increase in production of flagellins, but not other components associated with motility. Combined, these results indicate a motility-independent requirement for flagellation in the cold-water vibriosis pathogenesis. However, the mechanisms involved remain unknown.

In addition, the O-antigen moiety of LPS was shown to be essential for virulence. The results of this thesis indicate that O-antigen contributes to survival within the host and modulates the magnitude of the immune response raised in experimentally infected salmon. These observations may relate to the presence of LPS in VS-P1, an outer membrane complex that is shed from the bacterial surface and is hypothesized to decoy the immune response away from bacterial cells.

While the findings of this thesis elucidate certain aspects of mechanisms involved in virulence in *Al. salmonicida*, new questions have also been raised. Knowledge about microbial pathogenesis is crucial for control of diseases, and this work may contribute to improvement of prophylactic strategies for cold-water vibriosis as well as other bacterial fish diseases.

Sammendrag (Summary in Norwegian)

Aliivibrio salmonicida forårsaker kaldtvannsvibriose, en hemorrhagisk septikemi hos laksefisk i oppdrett. Tidligere studier har vist at *Al. salmonicida* er i stand til å unngå fiskens forsvarssystemer, og det har blitt foreslått at vevsskadene i syk fisk har sammenheng med immunresponsen mot bakterien. Dette prosjektet ble satt i gang for å identifisere komponenter med betydning for virulens og immunogenitet, for på den måten å øke forståelsen av samspillet mellom *Al. salmonicida* og laksen som vert.

Vertens immunsystem bruker mikrobe-assosierte molekylære mønstre (MAMPs), strukturer som er spesifikke for mikrober, til å gjenkjenne mikrober og sette i gang forsvarssystemer. Denne avhandlingen har tatt for seg to velkjente MAMPs: flagellin, en del av bakteriens bevegelsesapparat, og lipopolysakkarid (LPS), som finnes i bakteriens yttermembran. I tillegg til å fungere som mål for immunsystemet har både flagellen og LPS viktige funksjoner som bidrar til bakteriell overlevelse. Resultatene som presenteres her viser at verken flagellen eller LPS behøves for invasjon av laks. Begge strukturene har imidlertid funksjoner i senere stadier av sykdomsutviklingen.

Resultater fra smitteforsøk viser at flagellens flagelliner har en funksjon i virulens. Bakterier som ble dyrket i implantater i bukhulen hos laks økte produksjonen av flagelliner, men ikke andre komponenter med betydning for bevegelighet. Satt i sammenheng viser disse resultatene at flagellen har funksjoner i patogenesen av kaldtvannsvibriose som ikke avhenger av bevegelighet. Disse funksjonene er enda ikke kjent.

Det ble også vist at O-antigen i LPS har betydning for virulens. Resultatene i denne avhandlingen tyder på at O-antigen bidrar til overlevelse av bakterier inne i fisken. I tillegg ser O-antigenet ut til å ha betydning for omfanget til fiskens immunrespons. Disse observasjonene kan ha sammenheng med at LPS finnes som en del av VS-P1, et yttermembrankompleks som slippes ut fra bakteriens overflate og er antatt å fungere som «lokkemat» for immunapparatet slik at bakteriene slipper unna.

Funnene i denne avhandlingen belyser visse aspekter ved virulensmekanismer hos *Al. salmonicida*, men leder også til nye spørsmål. Kunnskap om mikrobiell patogenese er viktig for sykdomskontroll, og dette arbeidet kan bidra til utvikling av forbedrede forebyggende strategier for kaldtvannsvibriose og andre bakterielle fiskesykdommer.

Introduction

History of fish farming

In China, aquaculture may have existed for as long as 4000 years[1]. The oldest known text describing farming of fish was written by Fan Li around 500 B.C[2]. In 2014, China was by far the largest producer of farmed fish and shellfish, accounting for more than half of the world aquaculture production (61.6% of world total production in tons)[3].

Although the European production of farmed fish and shellfish only accounts for 4.0% of world production in weight, it constitutes 8.5% in monetary value[3]. Norwegian aquaculture production is the largest in Europe, representing 4.4% of the world production value[3].

The commercial break-through of the Norwegian aquaculture industry took place in the 1970s[4,5]. However, the trial and error of early pioneers in the period between 1950 and 1970 was essential for development of sustainable technical solutions, enabling a move from rearing of trout in land-based fresh water ponds to Atlantic salmon kept in sea-based net-pens. In the years following 1970, new commercial players added to the interest of Atlantic salmon farmed at sea, and the annual production volume of farmed fish increased exponentially from less than 1 000 tons in 1970, to about 8 000 tons in 1980, and 150 000 tons in 1990[4]. However, the rapidly increasing production rate gave rise to problems concerning animal welfare, environmental contamination and infectious diseases. During the 1970s, 1980s and early 1990s, the bacterial diseases vibriosis (*Vibrio anguillarum*), cold-water vibriosis (*Aliivibrio salmonicida*) and furunculosis (*Aeromonas salmonicida*) represented a major problem to the growing Norwegian aquaculture industry[5,6]. With the introduction of vaccines and improved management and husbandry practices, the losses accompanying bacterial diseases diminished and the Norwegian aquaculture industry underwent an extensive expansion[7].

Over the last five years, the annual production of farmed Atlantic salmon and rainbow trout has ranged between 1 239 000 and 1 376 000 tons[8]. Today, salmon lice and viral diseases such as pancreas disease and infectious salmon anemia are regarded among the largest threats to health of farmed salmonids, while bacterial diseases are mostly stable at low levels[8]. Nevertheless, winter ulcer disease is seen along the entire coastline of Norway

and represent a welfare problem as well as a cause of financial loss. Furthermore, the number of outbreaks of yersiniosis has been increasing over the past ten years, also contributing to the loss of fish[8]. History has shown that the range of infectious diseases threatening fish health is under constant change, and knowledge about these diseases is fundamental for identification of the necessary preventive measures.

Cold-water vibriosis

History of cold-water vibriosis

Cold-water vibriosis is a bacterial septicemia of farmed Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhyncus mykiss*) and Atlantic cod (*Gadus morhua* L.)[9–12].

The disease was first seen in 1977 in a location close to Hammerfest in Finnmark, Norway[13,14]. In 1979 and 1980, it was the cause of great loss at locations in Hitra and Frøya[9,15]. As the etiology remained undetermined, the disease was named "Hitra disease" after the region in which it was first recorded[9]. In the following years, the numbers of outbreaks increased rapidly, and the disease soon became the largest cause of loss in Norwegian aquaculture[15]. In 1983, the disease spread as far south as Stavanger, affecting areas with high densities of fish farms[10]. The years of 84/85 and 86/87 encompassed peaks in outbreak numbers, the latter seing more than 300 reported outbreaks, geographically ranging from Finnmark to Rogaland[16].

In the early 1980s, Norwegian research institutions had limited resources available for research on fish diseases. As a response to the increasing disease problems seen in the growing aquaculture industry, the unions "Norske fiskeoppdretteres forening" and "Fiskeoppdretternes salgslag A/L" proposed a joint initiative in 1982 for economic support of research on disease control in farmed fish[4]. Funding was to be administered through the National research council "Norges Fiskeriforskningsråd" (NFFR), and the research program "Frisk fisk" (Healthy fish) was initiated. The "Frisk Fisk" program represented an increased commitment to fish health research in Norway, enabling better coordination between research institutions.

Although the first report on cold-water vibriosis describes an association with a Gram-negative, vibrio-like bacteria[9], the etiology of the disease was subject to dispute for some

time. Several authors claimed the symptoms to be a manifestation of a nutritional disorder, and the isolated bacteria to be secondary invaders[13,17–19]. Nevertheless, a *Vibrio* sp. was found to fulfil Koch's criteria in several challenge studies and was subsequently described as the causative agent of cold-water vibriosis[9,10,20,21]. In 1986, the organism was characterized and proposed as a new species, *Vibrio salmonicida*[10,11].

In farms suffering severe cases of cold-water vibriosis, the mortality ranged between 50-90%[22]. To reduce losses, treatment with orally administered oxytetracycline, trimethoprim/sulfadiazine and nitrofurazolidone was attempted[13,22,23]. However, the first multiple resistant strain of *Al. salmonicida* was isolated in 1985, and more than half of a collection of strains isolated between July 1986 and July 1987 showed tetracycline resistance[22,24]. In response to the development of resistance in *Al. salmonicida*, increasing efforts were put into the development of a functional vaccine.

In 1987, vaccination with a formalin-inactivated bacterin was shown to provide protection against cold-water vibriosis[25]. Following this, a controlled vaccine trial was conducted in a commercial fish farm setting, using the vaccine formulation APOVAX-HS[26]. The vaccine proved to be an efficient prophylactic measurement against cold-water vibriosis. Since 1988, the majority of Atlantic salmon and rainbow trout produced in Norway have been vaccinated[27]. Vaccines were initially administered by immersion, and later by intraperitoneal injection due to the superior efficacy and lower vaccine consumption[28,29]. In the late eighties, outbreaks of furunculosis were frequently seen in fish farms from the southern parts of Norway to the county of Troms[30]. As non-adjuvanted vaccines were found to give insufficient protection against furunculosis, oil adjuvants were introduced in fish vaccines in the early 1990s in order to increase efficacy towards *Aeromonas salmonicida*[27]. Due to the multivalent nature of the vaccines used, oil adjuvants also became part of vaccines against *Al. salmonicida.* As the vaccination coverage increased throughout the 1990s, the number of annual outbreaks of cold-water vibriosis dropped considerably[7]. Consequently, the consumption of antibacterial drugs in Norwegian fish farming decreased from a peak of 49 tonnes of active substance in 1987 to 983 kg of active substance in 1996, even though the rate of production increased over the same time span[31]. In 2016, the production rate of farmed salmonids was almost four times that of

1996, while the consumption of antibacterial drugs was reduced nearly five-fold at 212 kg active substance[8].

However, in the period between 2011 and 2013, a moderate increase in numbers of cold-water vibriosis outbreaks was registered[32]. The increase has been postulated to be related to vaccination regimens[32]. Since 2014, no outbreaks of cold-water vibriosis have been reported in Norway[8,33,34].

Epidemiology

Cold-water vibriosis is considered to be endemic along the entire Norwegian coast, especially in the northernmost counties of Finnmark and Troms. In addition to Norway, the disease has been registered in Scotland, Iceland, Faroe Isles, Nova Scotia/New Brunswick, Canada and Maine, US[20,35,36] .

The route of transmission has been suggested to be between salmonids in the same farm or in neighboring locations. Transmission between salmon and cod has also been described[37]. The concentration of *Al. salmonicida* in the water of fish farm surroundings fluctuates throughout the year, and is highest during winter[38]. The organism is capable of long-term survival in sea water[39,40], and has been isolated from sediments below fish farms both with and without foregoing cold-water vibriosis outbreaks, but not from areas without fish farming activity[39,41]. Furthermore, *Al. salmonicida* has been identified in the feces of survivors of infection studies[42]. For these reasons, an asymptomatic carrier state has been proposed[38,42].

Outbreaks of cold-water vibriosis are seen in salt and brackish water. The disease occurs mainly when the water temperature is below 10°C, mostly corresponding to the period between late fall and spring[10]. Atlantic salmon is more susceptible to disease than rainbow trout and Atlantic cod[10,43]. Disease is often seen in relation to stress, and operations such as moving, marking, sorting, slaughter or feed changes may precede an outbreak[13]. Often, the disease targets the biggest and fattest fish, contributing to high economic costs of an outbreak[13].

The port of entry has been suggested to be through the gills, skin or gastrointestinal tract[44]. Recently, Kashulin and Sørum challenged Atlantic salmon by immersion of different

parts of the fish and found that all skin areas are important uptake sites for *Al. salmonicida*, while immersion of the gills did not contribute to uptake of bacteria[45].

Under experimental conditions, *Al. salmonicida* has been isolated from fish blood few minutes after immersion challenge[45]. Similarly, Bjelland and co-workers found bacteria in blood in addition to scant amounts in gills, mouth and intestine two hours after immersion challenge[44]. A latency period was observed before bacteria could be isolated from other organs. The authors hypothesized that a period of bacterial propagation and triggering of the host immune system was required to elicit disease[44].

Symptoms and pathology

The progression of cold-water vibriosis is usually acute, although peracute mortality is occasionally seen. Early in the course of disease, fish display swimming disturbances, stay close to the surface, appear lethargic and stop feeding. The skin can appear dark in color, and exophthalmos may be seen.

The disease is characterized by generalized hemorrhagic septicemia, resulting in anemia and extended internal and external hemorrhages (Figure 1). Externally, fish develop hemorrhages of the skin, fin basis and anal region.

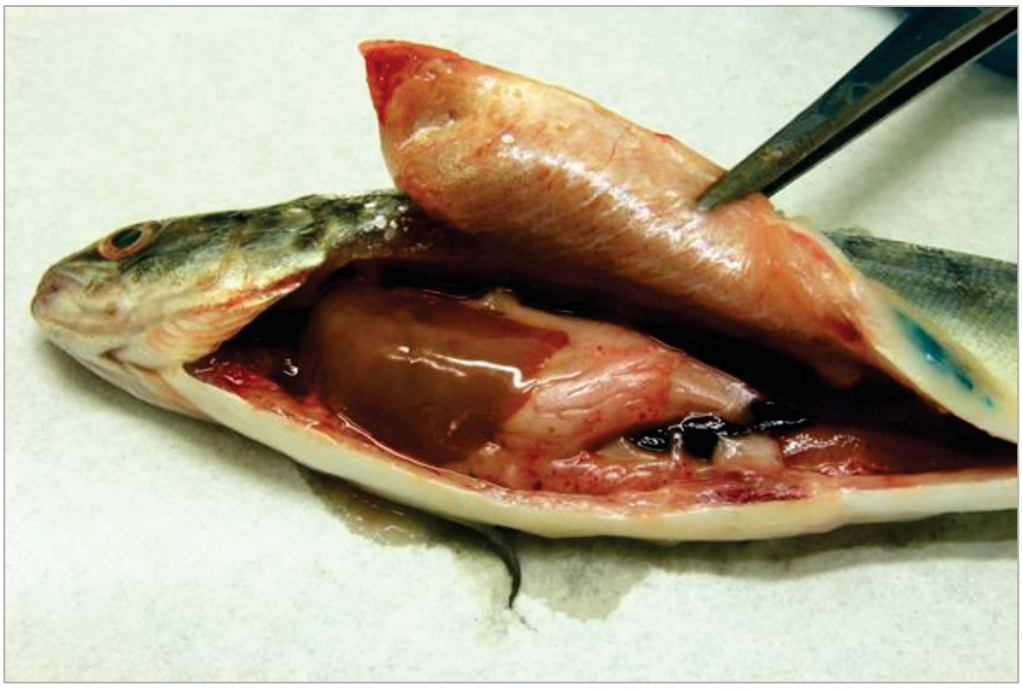

Figure 1: Diseased fish exhibit generalized hemorrhagic septicemia characterized by internal and external petechial hemorrhages.

The scale pockets of the skin may appear edematous, giving the skin surface an uneven appearance. Internally, hemorrhages are seen on the surfaces of all organs, and especially on the integument surrounding the internal organs. Ascites is commonly seen, being serous to serohaemorrhagic in character. The swimming bladder may also be filled with fluid of similar characteristics. The liver often appears enlarged, pale and yellow to brown in color. The spleen is enlarged and pale grey to red. Intestinal content is often loose and watery, yellow and mixed with blood.

In cod, the hemorrhagic tendency is less pronounced and largely located to the head region. Exophtalmia, keratitis and swollen liver and spleen are common findings[46].

In a study conducted by Totland and co-workers, structural changes in Atlantic salmon undergoing cold-water vibriosis were described following experimental immersion challenge[47]. In the initial stage of infection, bacteria were only detected in the lumen of capillaries. The first cells targeted were the endothelial cells of capillaries and leukocytes. Structural changes were seen in the form of indentations on the cell surface, as bacteria penetrated the membrane of endothelial cells and entered cytoplasm. As the disease progressed, the damage to the epithelial cells appeared to become irreversible and was followed by complete cellular disintegration. Subsequently, bacteria were detected extravascularly and in the surrounding tissue. The tissues with the greatest blood flow, such as the heart, red muscle fibers and the *lamina propria* of the intestine were most severely damaged, and the degree of damage was closely related to the number of bacteria found.

In another challenge experiment, active uptake of bacteria in phagocytes of the sinusoids in head kidney tissue was described few hours after experimental intravenous challenge[48]. Although recruitment of mononuclear cells and evidence of phagocytosis and cellular degradation were observed at later time points in the same experiment, disintegration and necrosis of sinusoidal endothelial cells were also seen. Furthermore, bacteria were observed extra- and intracellularly in the sinusoids and in the parenchyma, and immunochemistry revealed diffuse intra- and extracellular staining specific for bacterial products in tissue of heart, spleen and kidney[48] .

Aliivibrio salmonicida

The genus *Vibrio* constitutes a large and diverse group of heterotrophic bacteria. Vibrios are found in abundant numbers in marine environments such as marine coastal waters and sediments, estuaries and aquaculture facilities.

The majority of *Vibrio* spp. are non-pathogenic, and some species have been found to contribute to the carbon cycle, benefit squid in production of counter-illumination and fix nitrogen in plants[49]. However, the organisms producing disease are the most well-studied.

Vibrio spp. are found as etiological agents of diseases in both vertebrates and invertebrates, but may be best known for their role as human pathogens. Among them are *Vibrio cholerae*, the causative agent for cholera, and *Vibrio parahaemolyticus* and *Vibrio vulnificus,* both associated with seafood-borne disease[50–52]. Several *Vibrio* spp. also act as fish pathogens, e.g. *V. (Listonella) anguillarum, Al. salmonicida*, *Vibrio ordalii*, *Vibrio harveyi, Vibrio damsela* and and *Vibrio vulnificus*[53,54].

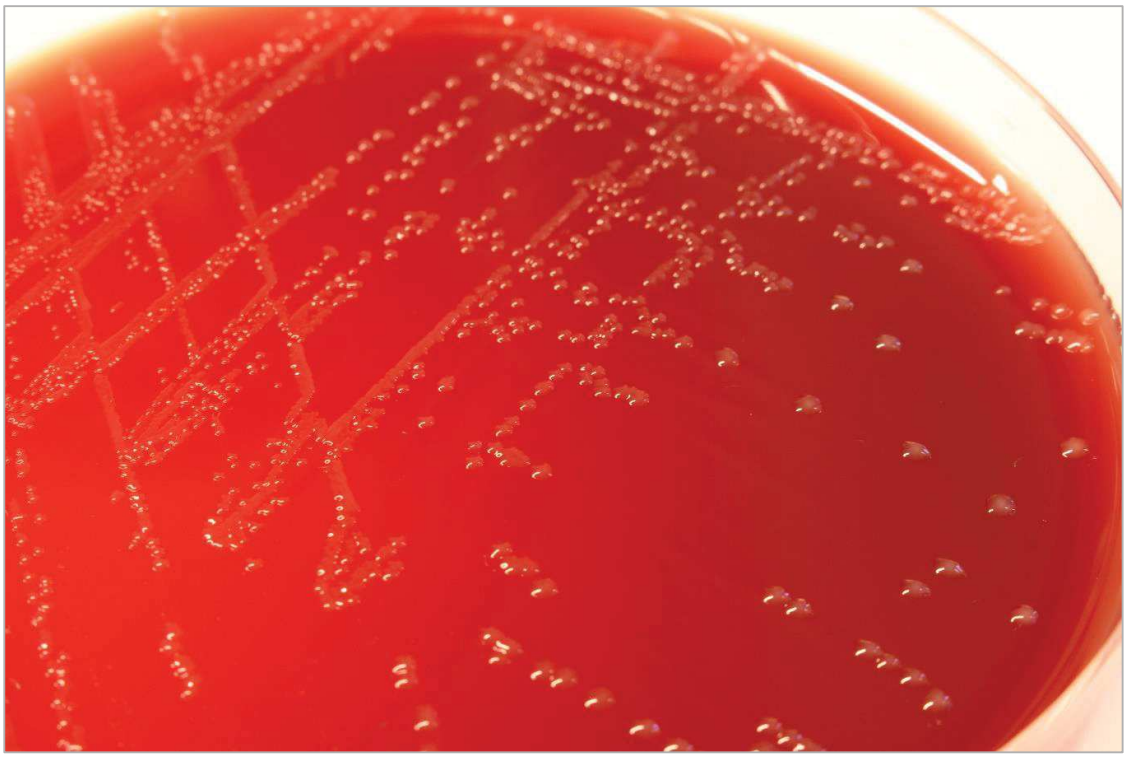

Figure 2: Al. salmonicida grown on ox blood agar supplemented with 2.5% NaCl for five days at 8°C.

Al. salmonicida was originally classified in genus *Vibrio*, but later reclassified as *Aliivibrio* together with *Aliivibrio fischeri*, *Aliivibrio logei* and *Aliivibrio wodanis*[55]. Belonging to the familiy Vibrionaceae*, Al. salmonicida* is a gram-negative, facultative anaerobic, curved rod bacterium. It is moderately halophilic, growing at salinities ranging between 0.5 and 4.0%[10]. Optimal growth is seen at 1.5 - 2% NaCl[11]. Being psychrophilic, growth is seen in the temperature range 1 – 22°C[10]. Optimal growth is seen at 15°C when grown on solid surfaces, and 10°C when cultured in liquid media[56].

After cultivation on ox blood agar plates for three to five days, colonies measure 1-3 mm in size, and are round, convex and greyish opaque in appearance (Figure 2). Hemolysis is not seen. Microscopically, the cells of a 24 h culture are 0.5 um by 2-3 um. The bacterium is highly motile and carries up to ten polar flagella (Figure 3)[15].

Al. salmonicida is a geneticially, biochemically and serologically homogenous species[10,15,24,57,58]. Several authors have found the plasmid contents of isolates to be stable over time, and a 21-24 MDa plasmid to be present in almost all isolates investigated[37,58]. However, none of the plasmids seem to be related to virulence[59].

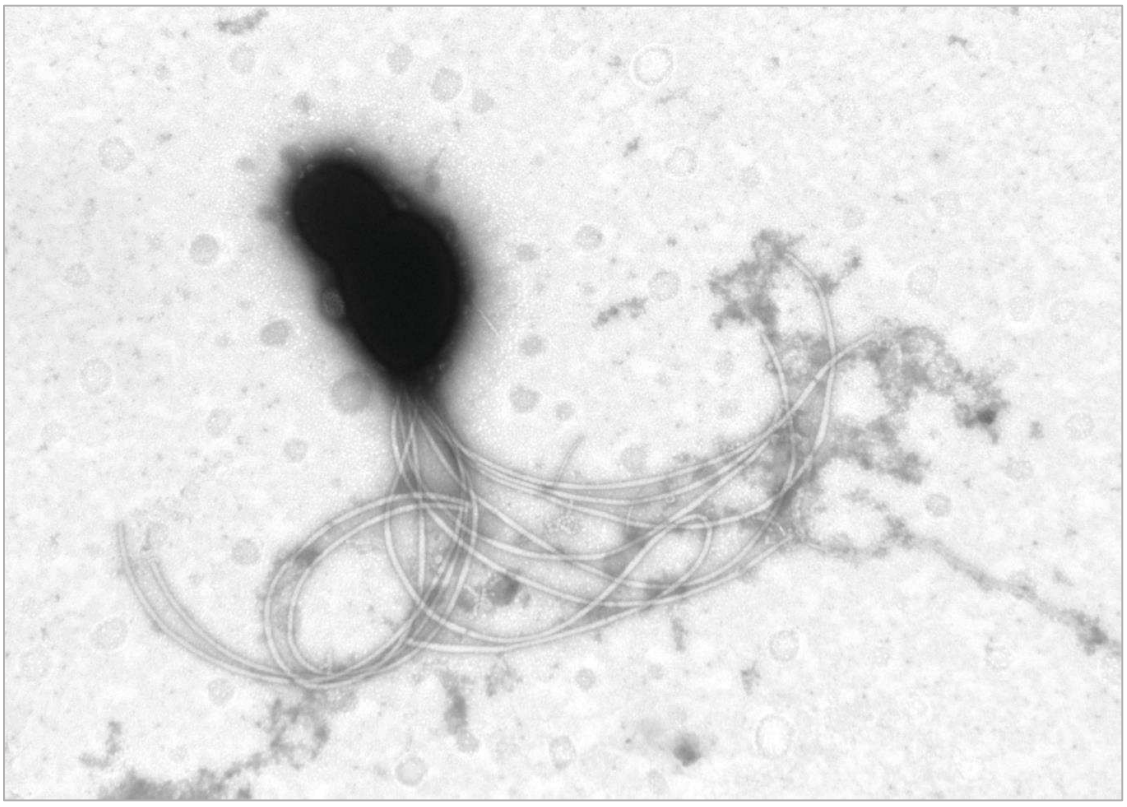

Figure 3: Transmission electron microscopy (TEM) image of Al. salmonicida LFI1238.

The genome of *Al. salmonicida* strain LFI1238 was sequenced and annotated in 2008[60]. The LFI1238 genome comprises 4.6 Mb DNA, and consists of 4286 open reading frames (ORFs) distributed in two chromosomes (chr I: 3.3 Mb and chr II: 1.2 Mb) and four plasmids. Like for other vibrios, the largest chromosome mainly contains essential genes, while the additional chromosome predominantly contains accessory genes. The genome of *Al. salmonicida* includes a high number of inactivated pseudogenes, indicative of a genome reduction as commonly seen for specialized pathogens. The high occurrence of insertion sequence (IS) elements in the genome have been linked to the inactivation of at least 156 ORFs[61].

The virulence factors of *Al. salmonicida* are largely unknown, but some components with putative roles in virulence have been identified. *Vibrio salmonicida* protein 1 (VS-P1) is a surface layer product that is hypothesized to mediate resistance towards the host immune system[62–64]. Temperature-dependent iron sequestration has been proposed to function in virulence, and genes for siderophore production, three TonB systems, and one heme uptake system are annotated in the genome[60,65]. Also, quorum sensing, motility and the production of hydrogen peroxide has been suggested as possible virulence factors[44,66,67].

Although several authors have postulated that the observed damage in moribund fish is caused by bacterial toxins, no toxin or cytolytic activity have been observed[11,60,63,66,68,69]. Bjelland *et al*. measured the transcription of a number of putative virulence genes of the bacterium in the early phase of an experimental infection[69]. Surprisingly, all genes measured were reduced in expression as compared to *in vitro*, bringing the authors to hypothesize a general muting of the gene expression in order to avoid immune system detection[69].

A relatively high challenge dose is required in order to cause disease in Atlantic salmon, and an even higher dose is required in rainbow trout and cod[43,58]. The LD_{50} for *Al. salmonicida* has, after i.p. injection, been reported to range between 10^6 and 10^8 colony-forming units (CFU) per fish[58], as compared to 10^5 CFU for *Aeromonas salmonicida*[70] and 10^2 - 10^5 for *V. anguillarum*[71]. As the challenge doses required for onset of cold-water vibriosis greatly exceed the bacterial levels expected in a fish farm setting, prerequisite factors such as stressful environmental or nutritional conditions seem to be required. In

accordance with this, cortisol-treatment of Atlantic salmon increases the susceptibility to cold-water vibriosis[72].

Host – microbe interactions

Prokaryotes are superior to eukaryotes in their metabolic diversity and are able to colonize a wide range of habitats, some in which the extreme conditions do not permit eukaryotic survival. Following this, it is not surprising that bacteria are found associated with other organisms, such as other bacteria, protozoa, fungi, plants and animals. Animal tissue represents a rich source of nutrients for bacterial growth, as it contains sugars, amino acids and simple nitrogen-containing compounds[73].

Although the exploitation of resources always constitutes a burden on the host organism, the majority of these relationships do not affect homeostasis of the host. Furthermore, some bacteria are of benefit to the organism which they colonize, returning the favor by providing services to their host. For instance, many microbes produce essential nutrients in exchange of resources, assist in metabolic activities such as nitrogen recycling, or provide protection against parasites or predators[74]. However, in some cases a relationship inflicts harm and causes disease in the host organism. Generally, interactions between a host and microbe have been categorized as either commensalism, mutualism or parasitism, depending on the outcome for both sides involved. Commensalism implies that the colonized host suffers no damage, while a mutualistic relationship confers benefits for both host and microbe. Parasitic microbes are capable of causing disease and are therefore classified as pathogens, possessing specific virulence factors that contribute to host damage. However, in recent years one has come to realize that many of the same mechanisms are involved in both pathogens and symbionts[74,75]. Thus, the spectrum between mutualism and parasitism is continuous and may depend on the circumstances of the interaction.

One example of a beneficial host – microbe interaction is that of *Al. fischeri*, an organism closely related to *Al. salmonicida*, and the nocturnal squid *Euprymna scolopes*[55,76]. *Al. fischeri* colonizes the light organ of its squid host and produces bioluminescence, which camouflages the shadow the squid would cast in moonlight and provides protection against predators[76]. Intriguingly, *Al. fischeri* constitutes as little as 0.01% of the total

bacterial population in the surrounding sea water, and is specifically harvested from the surrounding waters within few hours after the squid is hatched[77,78]. Several specific mechanisms of both host and symbiont facilitate the establishment of this symbiotic relationship and will be discussed in more detail later.

Motility of Vibrio *spp. in host-microbe interactions*

In order to reach beneficial habitats such as the surface of potential host organisms, being able to move is of immense benefit. Motility enables bacteria to migrate towards favorable environments and escape from unfavorable ones. Although a wide range of different strategies for bacterial movement have been identified, flagellum-mediated motility is the most thoroughly studied[79]. Flagellar motility often assists in initiation of host – microbe interactions[80]. In addition, flagella may be involved in pathogenesis through other mechanisms, including adhesion, biofilm formation, secretion and immune system modulation[81]. For several *Vibrio* spp., motility is required for invasion and colonization of their hosts[82–85].

The flagellar apparatus of Vibrios

Many *Vibrio* spp. have polar flagella that enable swimming in liquid environments at speeds up to 60 µm/s, and some are also equipped with lateral flagella which are used for swarming on solid surfaces[86,87]. Flagella are filamentous organelles that extend from the cytoplasm to the exterior of the cell. The flagellar structure consists of a basal body, a hook and a flagellar filament (Figure 4)[88,89]. Polar flagella of *Vibrio* spp. are often covered by a sheath that appears to be an extension of the cell membrane[86,90]. However, its function is not well understood.

The flagellar apparatus of Vibrios is a complex system of more than 50 gene products, and its gene regulation is kept under strict control[91]. The control system is coupled with assembly and involves several checkpoints as construction progress from the inner structures to the outer ones[88,92].

The basal body consists of integral membrane components including a rotary motor. The motor is composed of multiple stator elements surrounding a rotor. Interactions between stators and rotor generate a torque which drives flagellar rotation[93]. 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.

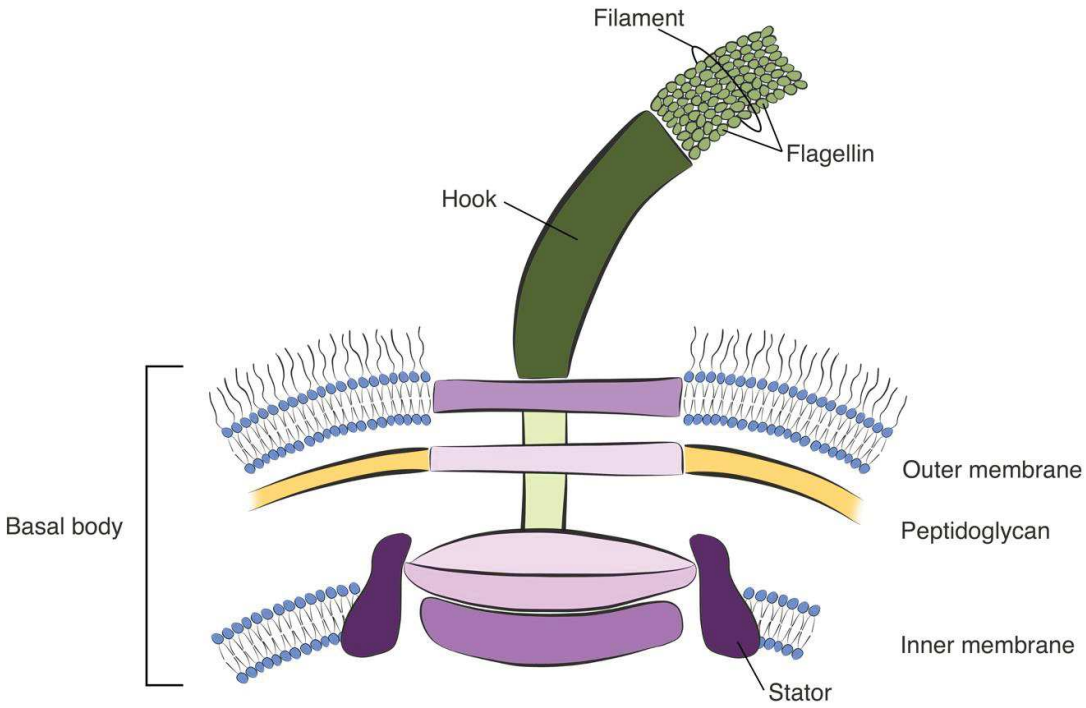

Figure 4: Schematic illustration of the bacterial flagellum.

Both H^+ and Na^+-driven motors have been described in bacterial flagellar systems, and the polar flagella of *Vibrio* spp. are powered by a Na^+-driven motor[93]. In *Vibrio alginolyticus,* the proteins orthologous to MotA and MotB are named PomA and PomB[94]. In addition to these two components, the *Vibrio* stator complex consists of two additional proteins, MotX and MotY, that are essential for motor function[95]. In order to power rotation of the flagellum, the torque generated by the motor is transmitted through the hook to the propelling filament[88].

The flagellar filament has a helical form, allowing it to function as a propeller. The helix is formed by eleven protofilaments, each consisting of self-assembling flagellin subunits stacked together[88]. While many flagellated bacterial species contain one or two flagellin genes, some organisms have genes encoding several flagellin subunits. *Vibrio parahaemolyticus, V. anguillarum* and *Vibrio cholerae* harbor multiple flagellin genes with a similar chromosomal organization[86]. 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* also possess multiple flagellin genes, but seem to constitute a different clade in terms of flagellin gene organization[96,97]. 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[86,96].

Motility in *Aliivibrio fischeri*

Al. fischeri has a requirement for motility in the establishment of symbiosis with its squid host. After first having aggregated in the ciliated epithelia on the light organ surface, the organism uses motility in entry of the light organ, and both increased and decreased motility result in colonization defects[85,96,98–100].

Al. fischeri isolated from the light organ 24 hours after colonization has been reported to be aflagellate, although the organism soon regains its flagellar state after expulsion[101]. The mechanism for down-regulation of motility is unknown, although a transcriptomic analysis of host and symbiont suggests that the expression of motility-related genes varies as the symbiont cycles through a daily rhytm of distinct metabolic states in response to different nutrient sources provided by the host[102].

Motility in *Vibrio cholerae*

In *V. cholerae*, a link between motility and virulence has been observed, but the connection is not well understood. Mutants with altered motility phenotypes have been shown to exhibit reduced attachment to intestinal brush borders and be less virulent than the wild type strain in some, but not all animal models[103–105]. The colonization defect has been shown to be independent of the flagellar structure[106]. A reciprocal regulation of genes related to virulence and motility has been suggested, and increased viscosity of the growth medium has been found to alter this expression[105,107]. The polar flagella of some *Vibrio* spp. have been implicated to function as mechanosensors, but such a role does not seem to apply to the flagellum of *V. cholerae*[108].

Intriguingly, non-chemotactic mutants of *V. cholerae* have been shown to outcompete their isogenic parent strain during infection of the small intestine[109,110]. This advantage

seems to relate to several factors. While wild type *V. cholerae* has a predilection for the lower half of the small intestine, the non-chemotactic mutants showed an expanded range and colonized the full length of the small intestine[109,111]. Also, the non-chemotactic mutants accumulated in the mucus covering intestinal villi, in contrast to wild type cells which traverse the mucosal barrier and migrate down the intestinal crypts where they are exposed to antimicrobial peptides detrimental to bacterial viability[110,112,113].

Motility in *Vibrio anguillarum*

V. anguillarum also uses motility to gain access to its fish host with a rather unpleasant consequence. Several authors have shown that motility-deficient mutants are reduced in virulence after immersion challenge, while virulence is not affected after i.p. challenge[83,114,115]. Thus, motility seems to be required for penetration of fish skin, but not for further development of vibriosis. However, the removal of the conserved C terminus of *flaA*, *flaD* and *flaE* resulted in decreased virulence also after i.p. challenge, although the mechanisms involved are not understood[83,116]. Furthermore, it was shown that chemotactic motility was required for invasion, as shown by the reduced virulence of a mutant defective of chemotaxis gene *cheR*[117]. The same gene was found to be involved in sensing of specific amino acids and carbohydrates, chemoattractants found in fish intestinal mucus[118].

Motility in *Aliivibrio salmonicida*

Like in *V. anguillarum, Al. salmonicida* has been shown to depend on motility for virulence. In experimental challenge studies, a motility-deficient *Al. salmonicida* mutant was attenuated in virulence after immersion and resulted in delayed mortality after i.p. challenge[44]. The motility of *Al. salmonicida* has been shown to be dependent on salinity, where motility is increased at higher salinities[97]. Nonetheless, motility is also seen at physiologic salinity, and *Al. salmonicida* has been observed to be flagellated *in vivo* by several authors [44,47,119]. In addition to salinity, quorum sensing is also involved in regulation of motility[66].

How motility contributes in the pathogenesis of cold-water vibriosis is not clear. However, the bacterium has been found to mount a stress response and increase the expression of

flagellins FlaC, FlaD and FlaE in response to fish mucus, suggesting that motility is utilized in passage of the skin mucus barrier of the fish[120].

Recognition of microbes

In order to defend itself against infectious disease, an organism must be able to recognize a wide range of invasive agents. The innate immune system of vertebrate animals relies on systems for recognition of ”self”, ”microbial non-self”, ”missing self” and ”altered self”[121]. Detection of ”microbial non-self” is based on recognition of conserved microbial products that are not present in host tissue, while detection of ”missing self” functions through identification of (the lack of) metabolic products that are unique to the host. Both strategies work to ensure a targeted immune response, neutralizing invaders and minimizing collateral damage. Detection of “altered self” recognizes markers of infection, enabling the removal or repair of own cells that are infected or transformed.

The conserved microbial products denoting the presence of microbes are commonly referred to as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs)[122–124].

These MAMPs are recognized by pattern recognition receptors (PRR), which elicit the appropriate downstream response through induction of regulatory cascades[123]. PRRs seem to be evolutionary conserved in all multicellular, eukaryotic organisms[125]. The best known example of PRRs are the Toll-like receptors (TLRs), which are found in both invertebrates and vertebrates alike[126]. Most vertebrate TLRs belong to six major families, and each family recognizes a general class of MAMPs and activate specific signal transduction pathways that are appropriate for the associated microbe[127,128]. In teleost fish, the number of TLR families are similar to that of other vertebrates, although a considerable diversity in TLR receptor repertoire, including several duplicated TLR genes, is seen between different fish species[129–131]. TLRs and the downstream signaling cascade of fish show many similarities to their mammalian counterpart, but several distinct differences in ligand specificities and functional roles of fish TLRs have been discovered, including several fish-specific TLRs that are not found in mammals[130–132].

TLRs are transmembrane proteins and may be expressed either on the surface of cells, or on endosomal membranes within cells[133]. Surface-bound TLRs recognize mainly microbial

membrane components, while intracellular TLRs recognize microbial nucleic acids. In addition to TLRs, PRRs also include C-type lectin receptors and cytoplasmic proteins such as nucleotide-binding oligomerization domain (NOD)-like receptors and retinoic acid inducible gene (RIG)-like receptors[133,134].

As a vertebrate host may interact with countless commensal microorganisms at any given time, it is important that the immune system is not continuously activated. Although the exact mechanisms for tolerance of non-pathogenic microorganisms are largely unknown, compartmentalization and anti-inflammatory cytokines seem to be important in this process[127].

The recognition of MAMPs by PRRs also contributes to beneficial interactions. The function of the immune system seems to be linked to the composition of gut microbiota[135]. Germ-free animals show deficits in the development of gut-associated lymphoid tissues, Peyer's patches and mesenteric lymph nodes, and are more susceptible to infection by a range of viral, parasitic and bacterial pathogens[135]. Furthermore, interactions between intestinal bacteria and the immune system have been suggested to direct the differentiation of pro- and anti-inflammatory T-cell populations in mammals[135]. Such interactions may be of importance for the development of inflammatory bowel disease, an intestinal disorder involving excessive inflammation of the gut, which is associated with a shift in normal microbiota[136,137].

The composition of the intestinal microbiota also seems to be shaped by PRR signaling[138]. Mice that are deficient of certain TLRs and components of the downstream signaling system display alterations in intestinal microbiota and are more prone to disease[139,140].

MAMPs

MAMPs are microbial components that are essential for survival of the microorganism in question and difficult to alter without the loss of function[127]. These structures are common to whole classes of microbes, regardless of pathogenicity. Several cell-wall components that are unique to bacteria, as well as bacterial genomic DNA, are known to function as MAMPs.

Bacterial nucleic acids

Bacterial DNA differs from eukaryotic DNA as it contains a large proportion of unmethylated cytosine-guanosine (CpG) dinucleotides, and is known as an immunostimulant[141]. CpG-DNA is recognized by TLR9, which resides in endosomal membranes and binds to degraded DNA that has been delivered to these intracellular compartments[141]. Up-regulation of TLR9 in response to bacterial challenge has been observed in several fish species[131].

Flagellin

The flagellin proteins of the flagellar filament are also potent activators of innate immunity. Mammalian TLR5 has been found to recognize a specific site on the conserved D1 domain of flagellin which is only available for TLR5 stimulation in its monomeric form[142]. While flagellin domains D2 and D3 are surface-exposed and show great variation, domains D0 and D1 are buried in the filament core and are highly conserved between species[143]. As these domains are required for assembly of the flagellar protofilaments and for functional motility, mutations of the TLR5 recognition site are generally not tolerated. Nevertheless, some α and ε Proteobacteria, such as *Helicobacter pylori* and *Campylobacter jejuni*, possess alterations in the TLR5 recognition site that allow TLR5 evasion while motility is maintained[144]. TLR5 activity has also been identified in fish[131]. In several fish species including rainbow trout and Atlantic salmon, TLR5 is found in both a soluble and membrane-bound form[129]. Ligand specificity for flagellin has been experimentally verified for both forms, although they seem to exhibit different expression profiles and have been suggested to be interconnected[129,131].

Surface-bound MAMPs

Dependent on the phylogenetic affiliation, bacteria contain various cell-wall components that are not found in eukaryotic cells. One such component is peptidoglycan, which is composed of long linear sugar chains of alternating *N*-acetyl glucosamine and *N*-acetyl muraminic acid that are cross-linked to form a mesh-like layer. Peptidoglycan is found in both Gram-positive and Gram-negative bacteria. Others include bacterial lipoproteins, lipoteichoic acids of Gram-positives and lipoarabinomannan of mycobacteria. These structures are recognized as MAMPs by TLR2, which forms heterodimers with certain other TLRs and also involves co-receptors for assistance in MAMP recognition[133]. Genomic

screening of several fish species has revealed the presence of putative *tlr2* genes, and carp challenged with both Gram-positive and Gram-negative bacteria have been shown to induce *tlr2* gene expression[129]. A putative *tlr2* gene has also been identified in rainbow trout, but little is known about its function[129].

Lipopolysaccharides

Another surface-bound MAMP structure is LPS, which is a major constituent of the outer membrane of most Gram-negative bacteria. The LPS molecule consists of lipid A, a core oligosaccharide moiety and O-antigen repeats (Figure 5)[145]. LPS is probably the most well-known MAMP and is recognized by mammalian TLR4, working in synergy with several accessory components such as myeloid differentiation protein 2 (MD-2), LPS-binding protein (LBP) and CD14[127,133]. In mammals, LPS is capable of eliciting strong innate inflammatory responses, resulting in clinical disease and potential lethal effects[146]. Generally, the immunoreactivity of LPS is localized to the lipid A region[147]. In contrast to higher vertebrates, fish are resistant to endotoxic shock[148]. This may be explained by the absence of LPS-sensing TLR4 systems in fish. Although *tlr4* has been cloned and characterized in some fish species, accessory molecules *md-2* and *cd14* have never been identified in any fish genome examined[129,131]. Furthermore, TLR4 of zebra fish does not recognize LPS[149]. Nevertheless, LPS has been found to stimulate the production of cytokines and influence cellular and humoral immunity in several fish species[148]. Thus, fish cells may be able to sense LPS, but by the use of other receptors than TLR4[150].

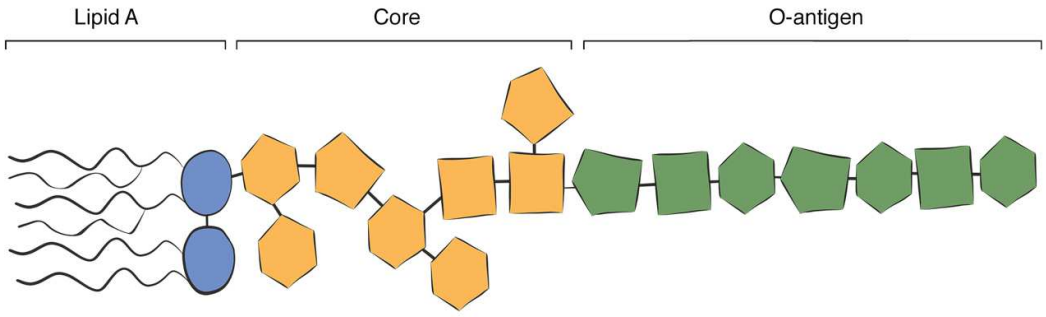

Figure 5: Schematic illustration of lipopolysaccharide.

Variations in LPS structure

In general, the O-antigen chains of LPS contribute to serological specificity, while the core oligosaccharides are highly conserved in structure. LPS, especially those of enterobacteria,

are traditionally characterized as rough or smooth type, depending on the length of their O-antigen chain[151]. Smooth LPS have long O-antigen chains, resultant colonies are smooth and entire, and bacteria do not autoagglutinate in saline. Bacteria with rough LPS have short or absent O-antigen chains, produce rough and irregular colonies and agglutinate in saline. In addition, rough type mutants of enterobacteria are generally attenuated in virulence[147].

Many bacteria residing on mucosal surfaces, such as members of the genera *Neisseria*, *Haemophilus* and *Bordetella,* have LPS structures that lack the O-antigen chains present in enteric organisms[151,152]. These structures have been described as lipooligosaccharides (LOS), although the use of this term is controversial and not universally applied[151]. In contrast to enterobacteria with rough type LPS, several mucosal organisms carrying LOS structures are pathogenic despite the lack of O-antigen groups[152]. Also, variation in the core phenotype of these glycolipid species, rather than O-antigen structure, seems to confer serological specificity[151]. Following this, LOS seem to differ slightly in function and features from enterobacterial rough type LPS[152].

Several *Vibrio* spp. have been observed to express rough LPS, resembling those of mucosal bacteria. *V. parahaemolyticus* displays LPS without repeating O-antigen chains, but still exhibit serological O-specificities and produce colonies that appear smooth and entire[153,154]. Likewise, the LPS of *Al. fischeri* and *V. cholerae* O139 appear to carry only one O-antigen unit[155–157].

MAMP signaling of *Aliivibrio fischeri*

A precise dialogue between host and symbiont facilitates the establishment of *Al. fischeri* as a light organ symbiont, and early communication between symbiont and host is mediated by MAMP signaling through bacterial excretion of surface products. By reacting to bacterial peptidoglycan through largely undetermined receptors, the squid facilitates colonization through induction of hemocyte trafficking and mucus secretion from the light organ epithelium[158–160]. The resultant mucus matrix promotes aggregation of *Al. fischeri*, which eventually outcompetes other bacteria and becomes the dominant species[161]. The symbiont then migrates through the pores, down ducts and into the crypts of the light organ[162].

Following colonization, the light organ undergoes a maturation process, loosing its ciliated epithelial surface and the production of mucus[163–165]. The maturation is also triggered by recognition of specific MAMP structures, being tracheal cytotoxin (TCT), a monomeric peptidoglycan fragment, working in synergy with the Lipid A portion of lipopolysaccharide (LPS)[164,166]. Furthermore, the bacterial signal promotes a reduction in the nitric oxide production of the host[167]. The O-antigen moiety of the LPS plays no part in signaling[168]. Nevertheless, an *Al. fischeri waaL* mutant lacking its O-antigen unit was attenuated in squid colonization[155].

Interestingly, motility seems to be involved in the deliverance of signals that trigger the light organ maturation process following colonization[169]. Rotation of the sheathed flagella of *Al. fischeri* has been shown to promote the release of outer-membrane vesicles (OMVs) containing LPS, which in turn trigger a developmental event associated with maturation of the light organ[168–170].

The LPS of *Aliivibrio salmonicida*

The LPS of *Al. salmonicida* has been described as rough type, being comprised of Lipid A and an oligosaccharide moiety[171,172]. Two serotypes have been described, and the LPS structure of both serotypes have been characterized[172,173]. The oligosaccharide structure of LPS of serotype C1, represented by type strain NCMB 2262, is shown in Figure 6. LPS of the C2 serotype differs from C1 only by the absence of the terminal Fuc*p*4NBA monosaccharide[173].

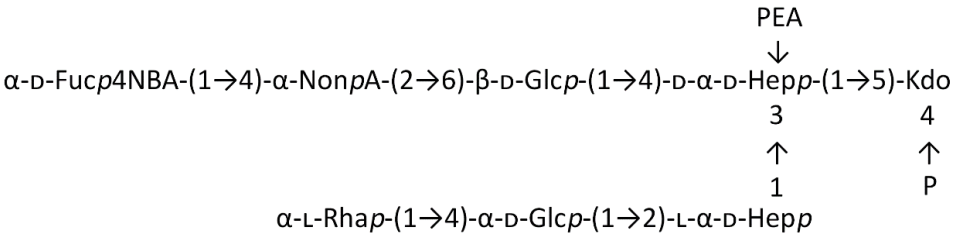

Figure 6: The oligosaccharide structure of *Al. salmonicida* NCMB 2262 lipopolysaccharide. α-D-Fuc*p*4N is 4-amino-4,6-dideoxy-α-D-galactopyranose, BA is (R)-3-hydroxybutanoyl, α-Non*p*A is 5-acetamidino-7-acetamido-3,5,7,9-tetrade-oxy-L-glycero-α-D-galacto-nonulosonic acid, β-D-Glc*p* is β-D-glucopyranose, D-α-D-Hep*p* is D-glycero-α-D-manno-heptopyranose, Kdo is 3-deoxy-α-D-manno-oct-2-ulosonic acid, L-Rha*p* is α-L-rhamnopyranose α-D-Glc*p* is α-D-glucopyranose, L-α-D-Hep*p* is L-glycero-α-D-manno-heptopyranose, and PEA is phosphoethanolamine[172].

Together with a protein moiety, LPS of *Al. salmonicida* is found as part of the hydrophobic surface layer product VS-P1[171] . VS-P1 is released during bacterial growth *in vitro* and in moribund fish, in what has been described as extremely high concentrations[62–64]. The complex has a molecular weight of 40 kDa, although aggregative forms ranging from 300 to more than 700 kDa are seen[63]. VS-P1 has been hypothesized to function in pathogenesis by binding effector molecules of the immune system, saving the bacterial cell from complement-mediated killing and phagocytosis[62,63].

VS-P1 is highly immunogenic to Atlantic salmon, and more than 90% of the antibodies raised in response to immunization with formalin-inactivated *Al. salmonicida* are directed towards this complex[62]. Furthermore, the immunogenic epitopes of the VS-P1 complex seems to be located to the LPS moiety[62,171,174]. However, immunization of Atlantic salmon using VS-P1 elicits a better protection than purified LPS, indicating a role in antigen presentation for the protein moiety[175]. It should also be noted that the correlation between the production of humoral antibodies and protective immunity is poor[176,177].

Strategies for evasion of microbially mediated damage

Host organisms have developed various strategies for evasion of damage caused by invading microbes. By altering behavior in response to specific olfactory and gustatory cues, an organism may reduce the risk of exposure to colonizers in the first place. In the case of invasion, resistance to disease is provided by the host immune system through detection, neutralization and destruction of pathogens. However, mechanisms of resistance commonly reduce host fitness[178]. The elimination of pathogens is often accompanied by collateral tissue damage, and inflammation may alter normal tissue function. In some cases, an inappropriate immune response is the main cause of tissue damage following an infection, a phenomenon commonly termed immunopathology[179]. As an alternative strategy, a host may increase its tolerance to disease by minimizing the negative impact of an infection on host fitness[178]. Rather than actively reducing the pathogen burden, measures that promote tissue protection and repair or restore homeostasis and normal tissue function are induced in order to reduce the vulnerability to damage.

The outcome of disease depends on properties of both microbe and host. A virulent microbe may be avirulent in a host with specific immunity, while microbes that are normally avirulent may cause disease in an immunocompromised host. Hence, virulence is a complex context-dependent phenomenon, encompassing both host and microbial factors. To include the impact of both host and pathogen-associated factors on the outcome of disease, Casadevall and Pirofski have proposed a classification of pathogens based on evaluation of host damage as a function of the host response raised[180]. By considering the relative contribution of pathogen-mediated and host-mediated damage, they argue that microbial pathogenesis is better understood.

Under such a scheme, nosocomial or opportunistic pathogens such as *Staphylococcus epidermidis*, associated with disease in individuals with impaired immune function, may be classified on the basis of their requirement for a weak immune response. Other pathogens are known to cause damage along the whole spectrum of immune responses. The release of toxins from classical toxigenic bacteria, such as *Corynebacterium diphteriae* and *V. cholerae*, generally elicit damage before an immune response has been raised, resulting in disease regardless of immune status. For other pathogens, damage is usually seen in normal hosts, but the degree of damage is enhanced in individuals both with impaired and strong immune responses. *Yersinia enterocolitica* is typically the cause of enterocolitis. However, immunocompromised hosts may develop septicemia, while individuals with an enhanced and inappropriate immune response may develop reactive polyarthritis.

By taking the interplay between host and microbe into consideration, one may identify the necessary medical intervention for reduction of host damage following an infection. Such an approach may be valuable in the design of novel, targeted vaccines, or as a guidance in prudent use of antimicrobial drugs.

Immunity of fish

The immune system of teleost fish is traditionally divided in the innate (non-specific) and the acquired (specific) system, although recent evidence suggests that these systems communicate and work together[181–183]. The mechanisms of acquired immunity require prior exposure to the microbes in question, while innate immunity relies on germline-encoded PRRs that recognize PAMPs[183]. The acquired immune response in fish is relatively slow and inefficient due to their poikilothermic nature and limited repertoire of antibodies,

and the innate immune system is considered to be an essential component in teleost defense against pathogenic organisms[181,182,184]. Innate immunity consists of mucosal and epithelial barriers that restrict microbial access, as well as both cellular and humoral mechanisms[183,185]. The cellular components include phagocytic cells such as granulocytes and monocytes/macrophages, non-specific cytotoxic cells, and epithelial and dendritic cells[181,183]. Humoral components include antibodies, the complement system, protease inhibitors, lytic enzymes, metal-binding proteins, lectins, pentraxins, antimicrobial peptides and opsonins[181–187].

Strategies for evasion of host immune defence factors

Bacterial resistance towards complement-mediated killing

The complement system enables killing of microbes by insertion of pores in their surface membranes and consists of around 35 soluble and membrane-bound proteins[188]. The system also participates in recruitment of phagocytic cells and stimulates phagocytosis through opsonization of pathogens[188]. Three pathways of complement activation have been described: the antibody-dependent classical pathway, the antibody-independent alternative pathway, and the lectin pathway involving mannose-binding lectins[182,188].

In some bacterial species including several pathogens of fish, resistance towards complement-mediated killing is an important virulence factor that enables *in vivo* growth[189–197]. Several mechanisms for complement resistance have been reported, and more mechanisms are probably left to be discovered. One of the described mechanisms is the variation of surface antigen expression, by which bacteria can evade recognition by antibodies and avoid activation of the classical complement system[189,198]. Alternatively, shedding of antigens, such as LPS, can provide a target for antibody binding out of reach from the bacterial membrane[199,200]. Another mechanism of complement resistance is the possession of LPS with long O-antigen chains, which provides sterical hindrance of complement factors and restricts access and consecutive damage to the bacterial membrane[189]. In *V. anguillarum*, a positive correlation has been observed between O-antigen length and complement resistance[194]. Also, the long O-antigen chains or other membrane structures may bind host-derived complement inhibitors or regulators and halt complement activation[189,201].

Some pathogens are able to synthesize or acquire sialic acids and use them in coating of surface-bound structures, such as flagella and LPS[202,203]. Sialic acids are a part of a larger family of nine carbon sugars (nonulosonic acids), and are found on the surface of eukaryotic cells and some prokaryotic cells[204]. Sialic acids have been proposed to constitute a form of "self-associated molecular pattern", recognized by self-PRRs such as sialic acid recognizing Ig-like lectins (Siglecs)[205]. Microbial decoration with sialic acids or other nonulosonic acids may function as a form of molecular mimicry that dampens innate immune responses, masks the microbial presence from the host immune system, or changes host cell specificity[203]. An example is the sialylated LPS of *Neisseria gonorrhoeae*, which contributes to complement resistance by binding of host factor H, which in turn leads to cleavage and inactivation of complement factor C3b[202,206]. Similarly, sialylation of the LPS of *Haemophilus influenzae* is protective against complement-mediated killing[201]. In *V. vulnificus*, a nonulosonic acid present in the LPS was shown to contribute to polymyxin B resistance and survival *in vivo*[207].

Interestingly, the LPS structure of *Al. salmonicida* contains a modified legionaminic acid (α-Non*p*A), a nonulosonic acid that structurally resembles sialic acids[204,208,209]. In addition to its production of legionaminic acid, *Al. salmonicida* is also capable of neuraminic acid synthesis[208]. Production of these sugars has been suggested to function in immune evasion strategies, but their roles in the pathogenesis of cold-water vibriosis have not been investigated[208].

Bacterial strategies for circumference of iron limitation

As bacteria require iron for growth due to its function as a co-factor in many enzyme systems, restriction of available iron is an efficient measure to keep infections at bay. In tissue fluids of vertebrates, the iron availability is extremely low due to its binding to transferrin, a high-affinity iron-binding protein[183,184]. Furthermore, the iron availability in fish injected with LPS has been shown to be reduced even further, possibly through the release of lactoferrin[184].

Nevertheless, several pathogens have high affinity systems for absorption of iron and are able to obtain sufficient iron for *in vivo* growth[184,210,211]. In *V. anguillarum*, the production of siderophores, diffusible iron chelating compounds, and associated membrane-bound receptors, has been identified as a virulence determinant[68,212–214]. *V. anguillarum* is also in

possession of siderophore-independent iron-acquisition systems, enabling the uptake of iron from heme and the heme-containing proteins hemoglobin and hemoglobin-haptoglobin[212]. Similarly, *Aeromonas salmonicida* is capable of iron acquisition by both siderophore-dependent and siderophore-independent mechanisms[185,215].

The genome of *Al. salmonicida* contains several iron acquisition systems[60]. Cultivation under iron-limited conditions has revealed the production of a hydroxamate siderophore and several iron-regulated outer membrane proteins (IROMPs)[65]. Proteins corresponding in size to these IROMPs has also been observed in bacteria cultured *in vivo*[119]. The highest *in vitro* production of siderophores was seen at temperatures below 10°C, harmonizing with the temperature range in which disease occurs[65]. In a later study, the dihydroxamate siderophore was characterized as bisucaberin[216].

Although iron is required for several metabolic functions, iron can also be a source of dangerous radicals under oxygen-rich conditions. Bacterial levels of intracellular iron is carefully monitored by ferric uptake regulator protein Fur, which regulates genes involved in iron sequestration and metabolism, oxidative-stress responses, and virulence[217]. The *fur* gene of *Al. salmonicida* has been characterized, and predicted Fur-binding sites (Fur-box) have been found in front of 60 single genes and 20 operons encoding 89 genes[218,219]. In a microarray analysis, 32 genes associated with predicted Fur-boxes were found to be up-regulated in response to iron-limitation, of which the majority of genes were predicted to be involved in iron homeostasis[220]. Like for *V. anguillarum* and *Aeromonas salmonicida*, the ability to adapt to iron-limited conditions through several iron acquisition systems is probably of uttermost importance for growth within the salmonid host.

Knowledge gaps

The pathogenesis of cold-water vibriosis is poorly understood. The endothelial damage seen in early disease development has been suggested to be caused by bacterial toxins or proteases[11,47,60,63,69]. The genome of *Al. salmonicida* encodes several protein secretion systems, proteases and hemolysins[60]. However, despite attempts made by several authors, no such toxins have ever been reported[63,66,68,69].

By using monoclonal antibodies specific for epitopes of VS-P1, this outer membrane complex has been identified in the blood of diseased fish, as well as in tissue of heart, spleen and head kidney[14,62–64]. Immunohistochemistry has rendered both focal and diffuse staining, and the authors linked this to the release of VS-P1 by the bacterium[14]. VS-P1 has been described as an important MAMP of *Al. salmonicida.* Furthermore, its release has been suggested to function as a "smoke screen", modulating host immunity and complicating the mounting of a targeted immune response[62,63]. Recently, a counterproductive immune response has been implied to be causative of the tissue damage seen[69]. The implications of VS-P1 for development of disease has never been functionally studied.

Bacterial production of flagellin, another well-known MAMP, has also been linked to pathogenesis through immunomodulation. *Al. salmonicida* is known to remain flagellated *in vivo*[44,47,119]. In challenge experiments with a spontaneous aflagellate mutant of *Al. salmonicida,* virulence was found to be reduced after immersion challenge and delayed after intraperitoneal challenge[44]. The observed delay was postulated to be related to either impediment of passage through the epithelial barriers into the blood stream and/or changes in immunomodulating properties. The specific roles of motility in initial host colonization, as well as for the port of entry, are not known. More knowledge is also needed on the role of the flagellum in later stages of disease.

Colquhoun and Sørum[119] identified iron-regulated outer membrane proteins of *Al. salmonicida* when grown *in vivo* or under iron-restricted conditions. This shows that an *in vitro* model is not necessarily relevant to the bacterial phenotype *in vivo*. These iron-regulated proteins were detected using one-dimensional SDS-PAGE. Because of the lack of sensitivity in that particular method, an *in vivo* phenotype could conceal additional bacterial components being immunogenic and/or relevant for virulence.

The bacterium has been shown to propagate in the circulation system of experimentally infected fish[44,69]. Thus, the bacteria must be able to survive in blood in the presence of immune factors of the fish, but little is known of the mechanisms employed for survival.

Aims of study

- To explore *in vivo* specific bacterial components in *Al. salmonicida* of importance for virulence and immunogenicity.

- To determine effects of motility and flagellation of *Al. salmonicida* on host colonization and development of cold-water vibriosis.

- To determine roles of LPS, being found as part of the outer membrane complex VS-P1 of *Al. salmonicida,* in the pathogenesis of cold-water vibriosis.

Summary of materials and methods

In vivo cultivation and proteomics

We have grown *Al. salmonicida* LFI1238 *in vivo* by surgically placing semi-permeable implants filled with bacterial cultures in the peritoneal cavity of Atlantic salmon. The protein composition of whole bacterial cells cultivated *in vivo* was compared to that of bacteria harvested from the same type of implants held in liquid broth. Proteomic analysis was conducted using nano-scale liquid chromatography coupled with tandem mass spectrometry, and peptide labelling with isobaric tags for relative and absolute quantitation (iTRAQ) enabled the relative quantification of peptide abundances in the compared samples.

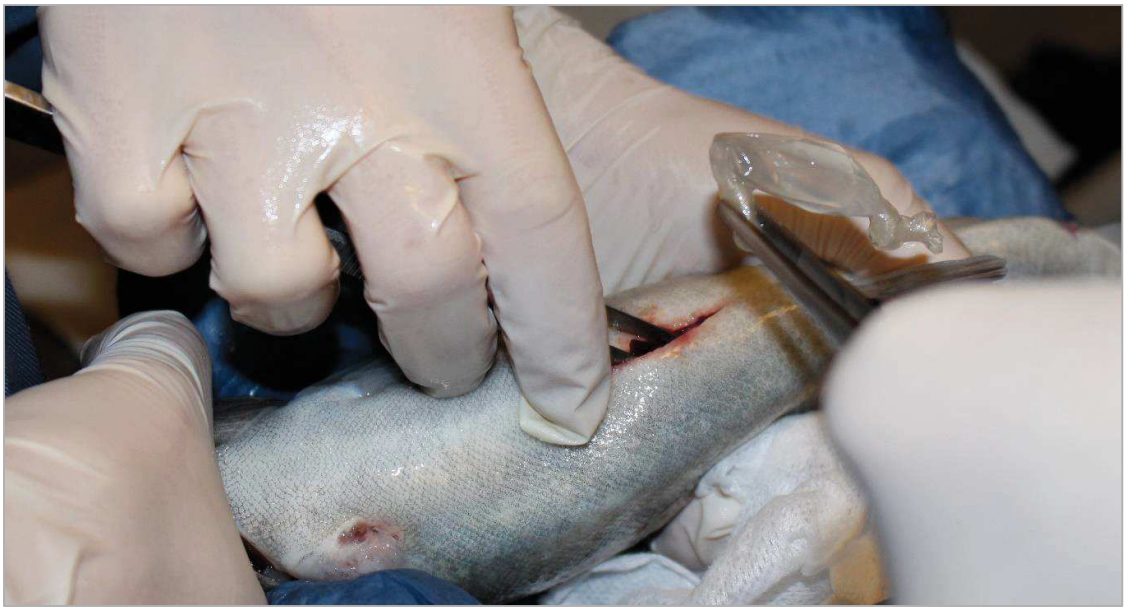

Figure 7: Surgical procedure for cultivation of bacteria within the peritoneal cavity of Atlantic salmon.

Construction of in-frame deletion mutants

In-frame deletion mutants were constructed as following: A deletion allele was created by overlap PCR, fusing together the regions flanking the gene of interest. This deletion allele was treated with endonucleases, ligated into R6K-origin suicide vector pDM4 and transformed into *E. coli* S17-1 by heat shocking. Next, pDM4 including the deletion allele

was introduced into *Al. salmonicida* LFI1238 by conjugation and integrated in its chromosome by allelic exchange. Successful transconjugants were identified by selecting for chloramphenicol resistance and sucrose sensitivity, before final verification was performed by PCR. Finally, the introduced pDM4 was coerced out by a second allelic exchange event, leaving only the deletion allele in the original locus.

In vitro assays

Motility assay

Motility of different strains was assessed by growth in semi-permeable agar. Growth zones were measured daily and plotted against time. Experiments were run under a series of different environmental conditions in order to investigate roles of flagellar components in adaption.

Transmission electron microscopy

For analysis of the flagellar phenotype of wild type bacteria and the constructed motility mutants, bacteria were visualized by transmission electron microscopy (TEM) after negative staining with uranyl acetate on carbon coated copper grids.

Serum resistance assay

To determine resistance towards the bactericidal action of salmon serum, bacterial cultures of different strains were added to serum or heat-inactivated serum isolated from Atlantic salmon smolt and kept at 8°C under gentle shaking. Before and at various time points following serum exposure, colony-forming units (CFU) were determined by serial dilution.

LPS visualization

LPS was isolated by water/phenol extraction and visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Two-step reverse transcription quantitative PCR (RT-qPCR)

For analysis of gene expression of flagellin genes in bacteria and immune genes in challenged fish, mRNA levels were assessed by two-step reverse transcription quantitative

PCR. To correct for differences in the amount of starting material, transcription of the gene in question was compared to a set of references genes through a comparative Cq approach (ΔΔCq).

Challenge studies

Intraperitoneal challenge

Atlantic salmon fry were anesthetized in water baths containing 0.0025 % benzocaine. Challenge was performed by injecting 0.1 ml bacterial suspension (~3 x 10^7 CFU) or PBS intraperitoneally. To differentiate between experimental groups, 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). After challenge, fish were mixed and transferred to holding tanks supplied with flow-through of carbon filtered fresh water. At three time points shortly after challenge, fish were euthanized and sampled for analysis of immune gene expression. For the duration of the experiment, fish were fed *ad lib,* and tanks were monitored for mortality twice daily.

Immersion challenge

Atlantic salmon smolt were divided into a number of experimental groups and one control group, and each group was kept in a separate tank supplied with flow-through of sea water at a temperature of 8°C and salinity of 35 ppm. Fish were challenged by immersion for 10 or 45 minutes by adding cultures of bacteria cultured in LB3. The control group was mock challenged by addition of sterile LB3. After challenge, the fish were fed *ad lib* and monitored for mortality over the course of the experiment. In one experiment, a number of fish were euthanized and sampled at time points shortly after challenge for determination of bacterial loads in blood.

Methods that are used in this thesis, but not included in the papers

Western blotting

For Western blot analysis, LPS was isolated and separated as described above and transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) using the

Trans-blot turbo transfer system running a Mixed MW program (Bio-Rad). The membrane was blocked for one hour at room temperature using PBS with 0.1% Tween 20 and 5% dry milk added, followed by incubation with primary antibody Mab 7F3 (specific for VS-P1) diluted 1:3000 over night at 4°C[64], and peroxidase-labelled sheep anti-mouse (Amersham ECL; GE Healthcare, Uppsala, Sweden) diluted 1:50 000 for one hour at room temperature. Immunoreactive bands were developed using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) according to the manufacturer's protocol and visualized in a ChemiDoc XRS (Bio-Rad).

LPS quantification

Extracellular LPS in spent culture was quantified to determine whether the flagellar apparatus is involved in LPS release. Cultures of wild type bacteria and motility-deficient mutants were cultivated to equal densities (OD_{600}: ~0.6) in triplicates. After two rounds of centrifugation for the removal of bacterial cells, the resultant supernatant was passed through a 0.22 µm filter. The cell-free supernatants were serially diluted in pyrogen-free water, and reactogenic LPS were quantified by the use of a ToxinSensor Chromogenic LAL Endotoxin Assay Kit (Genscript, Piscataway, New Jersey, USA) according to the manufacturer's protocol. Reactogenic LPS units for each sample were normalized against the OD_{600} of the initial cultures.

Summary of papers

Paper I:

Proteomic analysis of *Aliivibrio salmonicida* cultured *in vivo*
Simen Foyn Nørstebø, Michael Wade, Ane Mohn Bjelland, Henning Sørum and Pat Fidopiastis
Manuscript

Aliivibrio salmonicida is the cause of cold-water vibriosis, a hemorrhagic septicemia of salmonids. The mechanisms of host adaption and disease development are poorly understood. To identify *in vivo* specific components of importance for virulence and immunogenicity, we compared the proteome of *Al. salmonicida* cells implanted in the peritoneal cavity of Atlantic salmon and in LB broth. *Al. salmonicida* increased in numbers over the first day of *in vivo* cultivation, followed by a decline through the next four days that is suggestive of stationary growth. A disparity to the rapid proliferation expected during an infection suggests that this model does not fully replicate virulence properties of *Al. salmonicida.* However, the presence of host factors is likely to reveal features associated with *in vivo* adaption. Our results demonstrate major differences in metabolism between cells cultured *in vivo* and *in vitro*, including alterations in energy metabolism and a reduction in protein synthesis. After five days of cultivation, the majority of the 521 proteins observed to be differentially expressed *in vivo* and *in vitro* exhibited *in vivo* down-regulation. This general muting may be a strategy to hide from the host immune system, possibly related to the *in vivo* induction of several cold-shock proteins. Furthermore, all six flagellin subunits present in the *Al. salmonicida* genome were up-regulated *in vivo*. As most of the remaining proteins associated with motility showed no differential expression or were down-regulated *in vivo*, we postulate that the flagellins and/or flagellar filament function in a motility-independent manner. This study contributes to interpretation of the interplay between *Al. salmonicida* and its salmonid host. Our observations may serve as a foundation for future studies of virulence-associated properties of *Al. salmonicida*.

Paper II:

A unique role of flagellar function in *Aliivibrio salmonicida* pathogenicity not related to bacterial motility in aquatic environments
Simen Foyn Nørstebø, Erik Paulshus, Ane Mohn Bjelland and Henning Sørum
Microbial Pathogenesis 109 (2017) 263-273

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.

Paper III:

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

Simen Foyn Nørstebø, Leif Lotherington, Marius Landsverk, Ane Mohn Bjelland and Henning Sørum
Submitted to *Microbial Pathogenesis*

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 of importance 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 suggest that LPS is crucial for development of disease.

Results and discussion

Cold-water vibriosis has been recognized as a threat to farmed Atlantic salmon for almost four decades, but the pathogenesis of the disease is still largely unknown. The high challenge doses required for development of disease and the apparent lack of toxin activity bring forth *Al. salmonicida* as an interesting organism in terms of pathogenicity. Possibly, the damage to endothelial integrity observed in fish suffering from cold-water vibriosis may not be rooted in classical virulence determinants, but rather be the result of an unfortunate interplay between host and microbe. This work was initiated in order to expand the understanding of how *Al. salmonicida* interacts with its salmonid host, and ultimately to identify processes involved in the development of disease.

The capacity for invasion does not depend on motility

The list of Vibrionaceae species that require motility in host invasion and/or virulence is long and includes *Al. salmonicida*[44,83,96,98,103,114,221,222]. However, the means through which motility contributes to pathogenicity in *Al. salmonicida* is not completely understood. The inability of a motility-deficient mutant to cause disease after immersion challenge has been interpreted as a defect in invasion[44]. However, the same mutant also caused delayed mortality after i.p. challenge, which can hardly be attributed to the initial invasion phase. Furthermore, the flagellated state of *Al. salmonicida* observed by several authors *in vivo* implies a function for motility and/or the flagellum within the host[44,47,119].

In paper II, motility-deficient in-frame deletion mutants were constructed in order to determine roles of motility and flagellation in the pathogenesis of cold-water vibriosis. As *Al. salmonicida* is motile by the means of lophotrichous polar flagella, consisting of multiple flagellin subunits that are expressed simultaneously[97], the genes encoding flagellin subunits FlaA and/or FlaD were deleted. A substantial contribution to motility was found for FlaA, but not FlaD. The Δ*flaA* strain was markedly less motile than the wild type strain under all conditions tested, and the number and length of flagella were reduced relative to wild type. By complementation of Δ*flaA,* wild type motility was restored. In the Δ*flaD* strain, a minor reduction in motility was only seen after incubation in media containing 0.9% NaCl (physiological salinity), and no structural changes to the flagella were

observed. A Δ*flaA*Δ*flaD* mutant exhibited the same phenotype as Δ*flaA*, confirming the relative importance of FlaA. In addition, both of the introduced deletions affected the pattern of transcription of flagellar genes, suggesting that sensing of expression levels of individual flagellin genes influences flagellin gene regulation.

In *Vibrio* sp., energy for the propelling flagellar filament is provided by a Na^+-driven motor[93]. To obtain a mutant with defective, but intact flagella, the gene encoding motor component MotA was deleted. As expected, the resultant Δ*motA* strain appeared fully non-motile under the conditions tested. However, Δ*motA* was also found to be completely aflagellate, and transcriptional analysis revealed a muting of flagellin genes *flaCDE*, suggesting a regulatory role for MotA. 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[170,223,224]. Howbeit, as complemention of Δ*motA* was not performed, the observed aflagellate phenotype and down-regulation of flagellin genes may be results of polar effects on downstream genes.

The rapid host invasion rates observed for *Al. salmonicida* call for an efficient crossing of the fish integument[44,45]. To investigate whether motility is required for invasion, Atlantic salmon were challenged with the motility-deficient strains by immersion for ten minutes, followed by determination of CFU in blood sampled fifteen minutes after challenge. Surprisingly, all mutants constructed were able to enter the fish blood stream at rates similar to the wild type strain, showing that *Al. salmonicida* is capable of invasion in a motility-independent manner. Intriguingly, the CFU recovery rates for the non-motile Δ*motA* were higher than for the wild type strain, although the difference was not significant. A study comparing the invasion rates of different flagellation mutants of *V. anguillarum* led the authors to hypothesize that a nonfunctional flagellum was physically blocking internalization of the bacterium[115]. Similarly, the aflagellate state of Δ*motA* could be advantageous in invasion of the host.

Aliivibrio salmonicida down-regulates protein expression *in vivo*

After successful invasion, bacteria must adapt to the *in vivo* environment in order to maximize their potential for survival. In many bacterial species, metabolic cues are used to regulate and coordinate metabolism and the production of virulence determinants[73]. Due

to the complex and multi-factorial nature of the *in vivo* environment, artificial *in vitro* models may at best only partially duplicate the situation encountered by a microbe following infection[225]. Thus, artificial models may not necessarily allow the expression of virulence determinants required for development of disease. In paper I, *Al. salmonicida* was cultivated in sealed dialysis tubes within the peritoneal cavity of Atlantic salmon to explore how this organism adapts to the conditions met within a salmonid host. Bacteria were harvested from the tubes after one and five days of incubation, and global protein expression was compared to that of bacteria cultivated in LB broth *in vitro* by the use of quantitative proteomics (iTRAQ).

Lack of growth observed between day one and five suggests that the sample harvested at the later time point was in a stationary phase of growth, in contrast to the rapidly proliferating cells observed in the blood of infected fish[69]. Thus, the protein expression observed may not fully mimic that of the pathogen "in action". Nevertheless, the phenotype of the *in vivo*-cultivated bacteria is still likely to capture certain aspects of host adaptation. Also, the retrieval of bacteria from head kidney of the experimental fish and observations of pathology consistent with cold-water vibriosis suggest that the physiological state experienced by the bacteria resembles that of a true infection.

The highest number of differentially expressed proteins *in vivo* relative to *in vitro* was observed after five days of cultivation, and the majority (72.6%) of the 521 proteins showing differential expression were down-regulated *in vivo.* Functional classification revealed that a substantial number of the down-regulated proteins were involved in protein synthesis. This may be indicative of a general muting of bacterial metabolism and supports the assumption of Bjelland and co-workers that *Al. salmonicida* utilizes silencing of gene expression as a strategy to hide from the host immune system[69]. Also, the differential expression of proteins related to carbohydrate transport, glycolysis and the TCA cycle is consistent with a shift in nutrient source from LB broth to peritoneal transudate.

Interestingly, several cold-shock proteins, such as CspG and CspD, were up-regulated *in vivo* after five days of cultivation. Cold-shock proteins generally facilitate adaptation to stress associated with temperature down-shifts[226], but some cold-shock proteins are induced in response to other stressors such as osmotic stress and nutrition

starvation[226,227]. In *Escherichia coli*, cold-shock protein CspD was upregulated during stationary phase of growth and was found to inhibit DNA replication by binding to ssDNA[228]. The dominance of down-regulated proteins observed *in vivo* implies that inhibitory mechanisms are in place, and this observed inhibition may perhaps be associated with the induction of cold-shock proteins. In *Al. fischeri* expelled from the light organ of bobtail squid *E. scolopes*, three cold-shock proteins were found among the most abundant proteins[229]. Possibly, the cold-shock proteins identified in *Al. fischeri* and *Al. salmonicida* could be involved in the transition to a metabolic state that promotes *in vivo* survival.

The damage to endothelial integrity observed in fish suffering from cold-water vibriosis has brought several authors to hypothesize the involvement of extracellular toxins or proteases[11,47,60,63,69], but the activity of toxins or destructive enzymes has never been demonstrated in *Al. salmonicida*[63,66,68,69]. Nevertheless, the genome of *Al. salmonicida* harbors several protein secretion systems, putative hemolysins and proteases[60], and expression profiles of proteins related to these systems may provide clues to the pathogenesis. By iTRAQ analysis, components of two type 1 secretion systems (T1SS), one type two secretion system (T2SS) and two putative hemolysins were identified. However, neither the secretion systems nor the hemolysins appeared to be up-regulated in response to *in vivo* conditions. Even though the levels of proteins secreted by *Al. salmonicida* were not analyzed, the lack of *in vivo* induction of proteins associated with these secretion systems suggests that toxins do not participate in the pathogenesis of cold-water vibriosis. Nevertheless, systems for toxin secretion may be activated at a later stage of the pathogenesis not represented by the samples of this experiment.

Several outer membrane proteins (OMPs) were found among the proteins that were differentially expressed *in vivo* relative to *in vitro.* Being located at the host-bacterial interface, OMPs may be of interest as potential candidates for vaccine development[230]. Certain OMPs are also known to provide resistance towards antimicrobial peptides, complement-mediated killing, bile and antimicrobial drugs[231]. One of the up-regulated OMPs, OmpU, has been associated with host colonization and virulence in several *Vibrio* spp. by mediating adhesion, resistance to antimicrobial peptides and cytotoxicity[232–237].

Multiple iron acquisition systems are induced within the host

To defend themselves against bacterial infection, vertebrates may limit the availability of nutrients required for bacterial growth[211]. The best known example of such nutritional immunity is iron, for which the availability within vertebrates is extremely low due to intracellular storage and binding to high-affinity iron-binding proteins such as transferrin[211]. In a previous *in vivo* cultivation experiment, counter-measures to overcome iron limitation have been identified in *Al. salmonicida*[119]. Furthermore, *Al. salmonicida* has been shown to induce production of the hydroxamate siderophore bisucaberin under iron limitation[216,220]. In the present study, proteins associated with multiple iron acquisition systems were up-regulated *in vivo*, confirming previous reports on the ability of *Al. salmonicida* to grow under iron-limitation[119,216,220]. BibA and BibC, required for the synthesis of bisucaberin, showed similar abundance levels within and outside the host after one day of cultivation and were found to be down-regulated both *in vitro* and *in vivo* over the course of the experiment, although less so *in vivo*. In contrast, bisucaberin receptor BitA was highly up-regulated *in vivo* after five days, suggesting that utilization of bisucaberin is regulated through receptor expression. Besides BitA, four additional siderophore receptors were also identified, of which three exhibited up-regulation *in vivo.*

Active transport of iron-bound substrates across the outer membrane requires energy, which is provided by TonB energy-transduction systems[238]. Like several other Vibrionaceae species, *Al. salmonicida* possesses three TonB systems[60,238]. The TonB1 system contains a predicted pseudogene, and the failure of the current experiment in identification of associated proteins supports the assumption that this system is not active[60]. For each of the two remaining TonB systems (TonB2 and TonB3), the essential protein TtpC was identified. While TtpC2, belonging to the TonB2 system, was found up-regulated *in vivo*, TtpC3 of the TonB3 system showed no differential expression. Thus, the TonB2 system appears to be more important than the TonB3 system in *in vivo* iron acquisition. Similarly, *V. vulnificus* exhibited up-regulation of the TonB1 and TonB2 systems, but not the TonB3 system, in response to iron limitation[239]. In addition to siderophore-mediated systems, the *in vivo* up-regulation of several proteins associated with transporter systems for heme and iron ions was also observed, as well as additional proteins previously described to be iron-regulated.

In vivo adaption increases resistance to host immunity

In order to successfully proliferate and cause disease in a teleost host, *Al. salmonicida* must overcome the full repertoire of host defence mechanisms combating infections, including the complement system found in serum. In paper III, an increase in bacterial numbers retrieved from the blood of Atlantic salmon over the first two days following experimental challenge confirmed its resistance towards bactericidal factors present in serum. However, the same strain was found to be semi-sensitive towards naïve Atlantic salmon serum in an *in vitro* assay, as a tenfold reduction in viable cells was observed after 24 hours of incubation in serum. The discrepancy between *in vitro* serum sensitivity and retrieval rates from blood of challenged fish is suggestive of an *in vivo* adaptation, or that other factors are involved permitting *in vivo* growth.

Previously, serum resistance in *Al. salmonicida* has been postulated to be associated with outer membrane complex VS-P1, which is shed from the surface of the bacterium in high quantities[63,64]. As VS-P1 consists of LPS and a protein moiety, LPS modulation may constitute a strategy for *in vivo* adaptation, contributing to increased resistance towards the bactericidal action of fish serum. In paper I, 47 proteins associated with LPS metabolism were identified, including enzymes involved in sialic acid and rhamnose synthesis, both found as part of the oligosaccharide structure of LPS[172,173]. The majority of these proteins showed no differential expression between the *in vivo* and *in vitro* samples, although some differences were noted. RmlB and RmlD, two enzymes involved in rhamnose biosynthesis[240], were up-regulated *in vivo*, while four enzymes participating in Lipid A biosynthesis were down-regulated. As Lipid A anchors the LPS in the outer membrane-embedded Lipid A, it is tempting to relate the relative reduction in Lipid A-associated proteins to a reduction in LPS production associated with the stationary phase of growth. Similarly, the increase of rhamnose biosynthesis proteins implies an increase in rhamnose production, possibly involved in an *in vivo* alteration of the LPS structure.

O-antigen is required for virulence

In paper III, functional roles of the LPS structure in the cold-water vibriosis pathogenesis were determined by construction of in-frame deletion mutants lacking the O-antigen group of LPS. To achieve a phenotype absent of O-antigen, the putative O-antigen ligase

WaaL was targeted, predicted to be involved in ligation of the O-antigen group to the LPS core-oligosaccharide structure. As the *waaL* gene is found in two copies in the genome of *Al. salmonicida*, mutants defective of one or both copies were constructed. The deletion of both *waaL* copies was found to affect the LPS structure. While SDS-PAGE analysis of LPS isolated from the wild type and Δ*waaL* strains revealed one major band of 10 kDa, one band of 11.5 kDa and several slower migrating bands, the 11.5 kDa band was absent in LPS isolated from the Δ*waaL*Δ*waaL* strain. In several Vibrionaceae species, similar migration patterns have been described to represent the core oligosaccharide and the core oligosaccharide plus O-antigen, suggesting that the Δ*waaL*Δ*waaL* strain possesses a truncated LPS structure lacking the O-antigen group[153,155,156,241]. In addition, western blot analysis using a monoclonal antibody (Mab 7F3) previously shown to bind to LPS[171] reacted with an 18.5 kDa band of protein nature that was identified in all three strains (data not shown). This antibody has previously been shown to define an epitope recognized by sera of immunized Atlantic salmon[62], implying that the antigenicity of the Δ*waaL*Δ*waaL* strain is retained.

Following exposure to serum *in vitro*, both mutants exhibited a similar semi-sensitive resistance pattern. However, the loss of O-antigen seemed to compromise *in vivo* survival. By quantification of bacterial numbers in blood of Atlantic salmon over the first two days following experimental challenge, the retrieval rates for Δ*waaL* were found comparable to those of the wild type strain. In fish challenged with the Δ*waaL*Δ*waaL* strain, large variation was seen between replicates 24 and 48 hours after challenge, indicating a reduced survival potential for Δ*waaL*Δ*waaL* and denoting a role in survival for the O-antigen moiety.

Interestingly, the Δ*waaL*Δ*waaL* strain was found to be significantly less virulent than wild type bacteria after immersion and i.p. challenge of Atlantic salmon. 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. Hence, *Al. salmonicida* requires O-antigen for virulence in Atlantic salmon, but one copy of *waaL* seems to be sufficient. However, the challenge doses of these experiments are likely to exceed those associated with outbreaks of disease in a fish farm setting. A gene-dosage effect of the *waaL* duplication may have a greater impact on the virulence of

Al. salmonicida under doses associated with outbreaks of disease under real life conditions.

O-antigen modulates host immunity

Possibly, the reduced virulence of Δ*waaL*Δ*waaL* observed in both challenge trials may be explained in terms of reduced *in vivo* survival. Alternatively, it may relate to differences in host immunomodulation in response to the invading pathogen, or loss of other functions required for virulence. Bjelland *et al*. hypothesized that the cell damage observed in fish suffering from cold-water vibriosis is related to the inflammatory response raised against the invading pathogen[69].

To evaluate the host immune response of experimentally infected fish, a panel of immune parameters was analyzed 12, 24 and 48 hours after 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 hours after 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 relative reduction in immune gene transcription observed in fish challenged with Δ*waaL*Δ*waaL* could either be related to the loss of immunogenic properties, or a reduction in bacterial cell numbers at the time of sampling.

Considering that microbial decoration with sialic acids has been shown to modulate host immunity in several pathogenic species[203], it is tempting to hypothesize that the modified sialic acid moiety found in LPS of *Al. salmonicida* contributes to serum resistance in a similar manner as the sialylated LPS of gonococci[172,173,206]. Howbeit, this hypothesis requires additional experiments, targeting roles of sialic acids in the interplay between *Al. salmonicida* and its salmonid host. Furthermore, investigation of the relative protection obtained in fish immunized with the Δ*waaL*Δ*waaL* strain may enlighten the role of O-antigen in the pathogenesis.

In addition to its role in serum resistance and immunomodulation, a function of LPS in adhesion has been postulated for several bacterial species[242–245]. *Al. salmonicida* has been observed in intimate contact with endothelial cells under the progression of disease[47], 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 these data.

A complex requirement for flagellation

Even though paper I showed that motility is not involved in invasion, the results of paper I and II demonstrate a role for the flagellar filament in the pathogenesis of cold-water vibriosis. In paper I, all six flagellin subunits found in the *Al. salmonicida* genome were observed to be up-regulated under *in vivo* conditions. Intriguingly, the majority of the remaining proteins associated with flagellar function showed similar abundances *in vivo* and *in vitro*, or were down-regulated *in vivo* relative to *in vitro.* This is suggestive of a motility-independent role for the flagellar filament and/or monomeric flagellin. In paper II, flagellin mutants *ΔflaA,* Δ*flaA*Δ*flaD* and *ΔflaD,* as well as the aflagellar Δ*motA* mutant, were shown to be attenuated after immersion challenge. Moreover, the groups challenged i.p. with *ΔflaA* and Δ*flaA*Δ*flaD* exhibited a delay in disease development relative to the wild type group, even though the total mortality was similar 25 days after challenge. In contrast, no differences were seen between the mortality curves for fish challenged i.p. with wild type bacteria and Δ*motA* or Δ*flaD*. The lack of delay observed after challenge with Δ*motA* suggests that the cause of the delay was not the decreased motility. Previously, a similar delay in mortality has been observed after i.p. challenge of Atlantic salmon with a motility deficient mutant of *Al. salmonicida*[44].

Considering that host invasion appears 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. Nordmo and co-workers found a dose-effect relationship between the challenge dose and onset of disease after i.p. challenge, while immersion challenge revealed a dose-effect relationship between dose and total mortality[246]. Thus, the different mortality patterns observed in fish challenged by immersion and i.p. injection with Δ*flaA* and Δ*flaA*Δ*flaD* may reflect the host immune system, and not properties of the mutants.

Probing flagellar function

The role of flagellins in the pathogenesis may be explained in various ways. In addition to their role in motility, flagella are known to function in immune modulation, secretion and adhesion[81]. As flagellin is known as a potent MAMP contributing to inflammation, differences in flagellar composition and integrity may influence the abundance and/or nature of flagellins available and influence immunomodulation. Toll-like receptor TLR5 recognizes specific sites on the conserved D1 domain of monomeric flagellins[142], which are found as part of the secretome in *Vibrio* spp. including *Al. salmonicida, Al. fischeri* and *V. cholerae*[170,247,248]. 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[247]. To evaluate the immune response raised towards the motility-deficient mutants and wild type bacteria, the expression of IL-1β, TNFα and TLR5S was analyzed in fish sampled 12, 24 and 72 hours post challenge. However, a similar response was observed for all groups in the study. This included the TLR5S response seen in fish challenged with the aflagellate Δ*motA* strain, being comparable to what was seen for wild type bacteria and indicative of a maintained flagellin production in this strain, but without flagellar assembly. Nevertheless, large internal variation was seen in expression of all genes assayed, possibly masking minor differences between the groups. In addition, measured parameters were restricted to transcription of three immune genes, and transcription was only measured in i.p.-challenged fish. Evaluation of additional immunity parameters and the inclusion of fish challenged by immersion may have further elucidated roles of the flagella in immunomodulation and development of disease.

Recently, *Al. fischeri* was shown to release LPS in the form of OMVs by rotation of its sheathed flagella[168,170]. Together with peptidoglycan, LPS of *Al. fischeri* is involved in

immunomodulation and establishment of symbiosis with its squid host[159,164]. Possibly, the flagella of *Al. salmonicida* could be involved in VS-P1 release in a manner similar to that observed in *Al. fischeri*. The mechanisms for VS-P1 release are unknown, but several authors have noticed blebbing of the outer membrane consistent with OMV release[44,47]. In the wild type and mutant strains examined in paper II, membrane blebbing and formation of sheath-like tubular projections were observed, similar to what has been described by Baumann and Allen[249]. To investigate the role of flagella in release of VS-P1, reactogenic LPS units present in the cell-free supernatants of wild type bacteria and motility mutants were quantified (Figure 8). Although a non-significant decrease was seen in the level of LPS units present in the supernatant of the Δ*motA* strain relative to that of wild type, differential LPS release did not correlate with the observed differences in mortality of the conducted challenge studies.

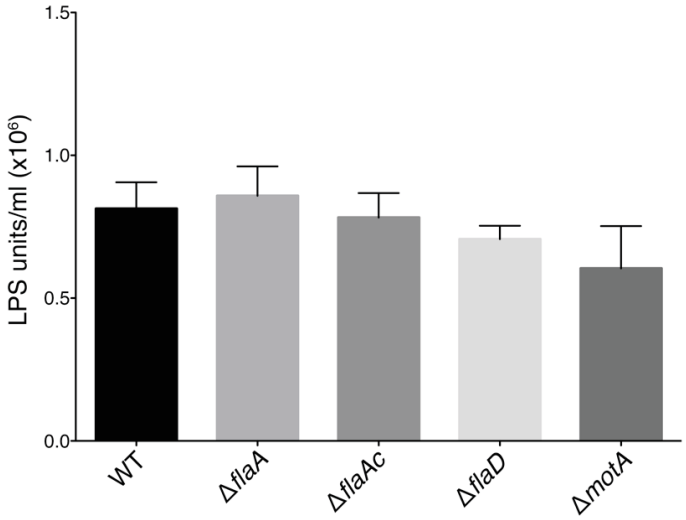

Figure 8: Levels of reactogenic LPS units in supernatants of mid-log cultures motility-deficient strains and wild type bacteria (mean ± SEM).

Compromised adhesive properties could provide an alternative explanation for attenuated virulence observed in the motility-deficient mutants. In *Al. fischeri,* disruption of *flaA* resulted in delayed and reduced colonization of the deep crypts of the squid light organ, as well as a decreased ability to remain in the host[96]. A defect in attachment to host cells was postulated, although no differences were seen in attachment to host mucus or glass slides relative to wild type. Some strains of *V. anguillarum* have been reported to attach to CHSE cells and mucus-coated glass slides[250], but Santos and co-workers found no correlation between measured adhesive properties of *V. anguillarum* and virulence in fish[251]. In *Al.*

salmonicida, adherence to tissue sections of mucosal surfaces from Atlantic salmon has been demonstrated, although in a nonspecific manner[252]. To the knowledge of the author, the target cells for *Al. salmonicida* have not been identified. However, the difference between the delayed mortality of fish challenged i.p. with Δ*flaA*, carrying compromised flagella, and the wild-type virulence observed after i.p. challenge with Δ*motA*, being aflagellate, indicates that the attenuation is not related to adhesion.

Future perspectives

Due to the extensive disease problems caused by cold-water vibriosis in the early years of large-scale Norwegian aquaculture, *Al. salmonicida* was a topic of high research activity during the eighties and nineties. Following the introduction of vaccines, cold-water vibriosis does no longer constitute an immediate threat to farmed fish. Consequently, focus has been shifted towards more imminent challenges such as salmon lice and viral diseases, leaving little attention to *Al. salmonicida.* However, the pathogenesis of cold-water vibriosis is still not fully understood. The outbreaks observed between 2011 and 2013, although modest in numbers, demonstrate that changes in farming practices and/or bacterial properties may reanimate *Al. salmonicida* as a relevant pathogen. Also, the landscape of emerging fish diseases is constantly changing. Increased knowledge on the *Al. salmonicida* pathogenesis may not only expand our understanding of this particular bug, but also benefit perception of the interplay between fish and bacteria in general.

The aquatic environment facilitates intimate contact between bacterial species and potential hosts. In light of the rapid uptake of *Al. salmonicida* in blood of salmonid fish, it is interesting to find that neither motility nor LPS is required for invasion. Future studies should address the mechanisms involved in invasion and the prevalence of bacteria in blood of healthy salmon.

The findings of this thesis suggest a role for flagellins or the flagellar filament in the pathogenesis of cold-water vibriosis, but their functions remain unknown. The construction of a mutant fully devoid of flagellin production may elucidate roles of flagellins in interactions with the host. In addition, analysis of a more comprehensive set of immunity parameters may reveal effects of flagellation on host immunity.

Challenge studies with a mutant strain deficient of O-antigen demonstrated a requirement for O-antigen in virulence. Immunization of salmon with this particular strain followed by challenge with wild type bacteria may illustrate the contribution of LPS to protective immunity. Also, experimental challenge with lower challenge doses of the mutants lacking one or two copies of the *waaL* gene may reveal a beneficial role for the gene duplication. By increasing our understanding of the role of LPS for survival and immunomodulation, the pathogenesis of cold-water vibriosis may be better understood.

Methodological considerations

Strain selection

For studies conducted throughout this thesis, the *Al. salmonicida* strain LFI1238 has been used due to its sequenced and annotated genome. LFI1238 was originally isolated from a diseased Atlantic cod. The LPS structure of cod strains, generally serotyped as C2, has been shown to lack the terminal Fuc*p*4NBA monosaccharide found in LPS isolated from salmonids. Differences between strains may have implications for the applicability of the results of this work. However, *Al. salmonicida* is regarded as a highly homogenous species, and LFI1238 have been shown to be virulent in Atlantic salmon in this work and by other authors.

Proteomic analysis of bacteria cultivated in vivo

In paper I, *Al. salmonicida* was cultivated in semi-permeable implants placed in the peritoneal cavity of Atlantic salmon in order to explore changes in phenotype associated with host-dependent factors. We reasoned that the *in vivo* environment may influence the production of virulence determinants not necessarily seen *in vitro.* All the same, the validity of the peritoneal growth chamber as a representative for the conditions met by *Al. salmonicida* during an infection should be questioned. Also, the samples analyzed may at best be regarded as snap shots of the bacterial protein composition at the time of sampling, whereas the phenotype is largely dependent on the various phases of growth. CFU counts of bacteria retrieved from the implants suggested a stationary state for the samples harvested after five days of cultivation, contrary to the proliferation previously seen in blood of experimentally infected fish[69]. In contrast to our observations, Colquhoun

and Sørum reported exponential growth for the first four days of *in vivo* cultivation of *Al. salmonicida,* plateauing at a level of approximately 10^{10} CFU between day four and seven[119]. Possibly, the discrepancy with our results relate to differences in experimental procedures. As a higher initial inoculum was used by Colquhoun and Sørum, cell density may be of importance for the ability of rapid *in vivo* growth. Nevertheless, the observations in this study represents *one* phenotype of *Al. salmonicida* influenced by growth in an *in vivo* environment.

Proteomic analysis of bacteria retrieved from blood of infected salmonids may have provided a more accurate picture of the pathogen in action. However, the requirement for sufficient material for analysis as well as the need to separate bacterial proteins from a background of host proteins call for means of separation. Preparatory steps, such as density gradient centrifugation, immunomagnetic separation and fluorescence-activated cell sorting, have previously been used in proteomic analysis of bacteria *in vivo*[253]. Howbeit, such measures prolong the period between bacterial retrieval and analysis, potentially influencing the proteome composition and increasing the risk for preparation bias.

Prior to analysis, biological replicates were pooled in order to avoid culture variations and increase the amount of sample material available. Although this process results in biological averaging of protein abundances, it eliminates the estimation of variance among the pooled samples and the identification of outliers, and influences data analysis. Also, no steps for pre-fractionation of the sample (e.g. cytoplasmic proteins, membrane proteins, cell wall proteins) were carried out prior to or in connection with protein extraction. Separate analyses of different sample fractions may have increased the number of proteins identified.

Quantitative proteomics using iTRAQ enables the identification and quantification of thousands of peptides in complex samples over two orders of magnitude, and allows for simultaneous analysis of multiple samples[254]. However, iTRAQ analysis is known to suffer from variance heterogeneity, where low signal data show higher relative variability[255]. Also, peptide abundance ratios derived from iTRAQ data have been described to be underestimated, leading to the report of artificially low protein ratios[255].

In spite of the described pitfalls, paper I presents the identification of numerous proteins differing in expression between samples cultivated in peritoneal implants and samples cultivated in conventional LB broth. Similar trends for proteins predicted to participate in similar processes suggest that the data are biologically sound. Several of these proteins represent interesting targets for further studies.

Construction of in-frame deletion mutants

In paper II and III, in-frame deletion mutants were constructed for functional analysis of a number of genes of interest. By designing deletions that maintained the original translational reading frame of the targeted genes, the risk of introducing polar effects that affect transcription of downstream genes should be minimized. However, the risk posed by unintentional effects of the mutagenesis procedure should still be assessed, as polar effects on surrounding genes or introduction of secondary mutations elsewhere in the genome will preclude experimental interpretation. Generally, the effect of an introduced mutation is analyzed by complementation of the mutant strain, restoring the original gene *in cis* by reinsertion of the gene into its original locus, or *in trans* by providing the gene with its promoter on a plasmid vector. In paper II, mutant strain Δ*flaA* was complemented *in cis*, restoring the original function of the encoded flagellin subunit FlaA. For the remaining mutants constructed, complementation was not performed. Thus, a scenario in which the observed mutant phenotypes are associated with unintentional secondary effects must be considered. For the Δ*motA* mutant constructed in paper I, the observed phenotype differed from previous observations of *Vibrio* spp. mutants defective for the orthologous gene. Following this, a complementation analysis should be performed for verification of the Δ*motA* phenotype. While the experiments assessing phenotypes of the remaining mutants showed expected results, complementation of these strains would strengthen the data in the same manner.

In vitro *assays*

In vitro assays are conducted under controlled conditions that do not fully reproduce the environment experienced by the bacterium when it interacts with its host. It is likely that the phenotypic traits observed throughout these experiments do not represent its “true” *in vivo* state. Thus, the experimental data may lead to erroneous conclusions of minor or

major character. Following this, all *in vitro* results should ultimately be interpreted in light of *in vivo* experiments.

Challenge experiments

To determine if the introduced mutations affected virulence, Atlantic salmon were infected with deletion mutants or the isogenic parent strain through intraperitoneal (i.p.) or immersion challenge. Atlantic salmon is the natural host of *Al. salmonicida*, benefitting interpretation of data as no extrapolation between species is required. However, the genetic makeup and the rearing conditions of the fish should be considered, as well as the conditions under which the experiments were run. In both i.p. and immersion experiments, the challenge doses utilized are likely to exceed the doses encountered by fish in a fish farm setting. Also, while challenge by immersion is thought to resemble a natural route of infection, i.p. injection enables the instantaneous access of high numbers of bacteria to the host. Following this, i.p. challenge is commonly found to result in more rapid disease progression than challenge by immersion.

Physiologic differences between the experimental fish of the i.p. and immersion challenge experiments should be kept in mind when comparing the two experiments. As the i.p. challenge experiment involved parr kept in fresh water at 11°C and the immersion challenge experiment was run using smolts kept in sea water at 8°C, factors related to physiological development and environmental temperature may explain some of the differences observed between the experiments.

Furthermore, in the immersion challenge conducted in paper II and paper III, several fish developed winter ulcer disease over the course of the experiment. Presumably, *Moritella viscosa* and *Al. wodanis* were introduced in the experimental tanks through intake of sea water, affecting all tanks in an equal manner. Although the impact of this unintentional co-infection is difficult to determine, it is likely to have contributed to fish stress, and may have increased the susceptibility to cold-water vibriosis.

Importantly, *in vivo* challenge models involve stress and suffering for the experimental animals. Thus, ethical considerations imply that such experiments should be replaced with *in vitro* models whenever possible. Due to the lack of knowledge on the pathogenesis of cold-water vibriosis, some extent of experimental use of animals is required to obtain

reliable results. When such experiments were deemed necessary, the number of experimental animals were reduced to a minimum, while attempts were made to minimize suffering and increase fish welfare.

RT-qPCR

qPCR allows the detection and relative quantification of extremely small amounts of DNA by measuring the exponential amplification of DNA fragments. For detection of RNA, the RNA sample must be reverse transcribed to cDNA prior to qPCR analysis (RT-qPCR). In the analyses conducted in paper II and III, relative gene expression of a number of genes were calculated by comparison of target gene expression with the expression of a set of reference genes that were assumed to be stably expressed in the samples. Each step of the analysis process is associated with the risk of introducing technical bias, including variability in RNA quality, differences in reverse transcriptase efficiencies, differences in PCR efficiencies, unspecific binding of qPCR reporter dye, and variations in expression of reference genes between samples. Although measures were taken to minimize the introduction of technical errors, effects on precision and bias of the assays should be considered. Also, the analysis of immune gene expression in paper II and III showed large biological variation, and an increased number of biological replicates may have better shown differences in gene expression.

Main conclusions

Al. salmonicida does not require motility or LPS O-antigen for invasion of Atlantic salmon.

The flagellar structure and/or motility of *Al. salmonicida* are involved in the pathogenesis of cold-water vibriosis through other means than invasion. *In vivo* up-regulation of six flagellin subunits, but not other flagellar components, is suggestive of a motility-independent role for the flagellar filament and/or monomeric flagellin.

LPS O-antigen is essential for the virulence of *Al. salmonicida*.

Flagellin subunit FlaA is of major importance for motility and flagellation in *Al. salmonicida, while* FlaD only plays a minor role in motility under certain environmental conditions.

WaaL of *Al. salmonicida* functions in ligation of O-antigen to the core oligosaccharide moiety of LPS.

Al. salmonicida cultured *in vivo* shows a tendency to a general muting of protein expression.

Our results also indicate that:

Toxins are not involved in interactions between Atlantic salmon and *Al. salmonicida* during stationary phase of growth.

Al. salmonicida utilize multiple iron acquisition systems within the host.

Cold-shock proteins are involved in a stress response raised towards the host environment, and may be associated with the *in vivo* down-regulation of proteins involved in biosynthesis of proteins.

Transcription of flagellin genes shows a regulatory coupling to the levels of flagellins produced.

LPS O-antigen contributes to *in vivo* survival and modulates the magnitude of the host immune response following infection with *Al. salmonicida*.

At low challenge doses, the two copies of the gene encoding O-antigen ligase gene *waaL* contribute to virulence in a gene dosage-dependent manner. At high challenge doses, one copy of *waaL* is sufficient for development of cold-water vibriosis.

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Proteomic analysis of *Aliivibrio salmonicida* cultured *in vivo*

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Abstract

Aliivibrio salmonicida is the cause of cold-water vibriosis, a hemorrhagic septicemia of salmonids. The mechanisms of host adaption and disease development are poorly understood. To identify *in vivo* specific components of importance for virulence and immunogenicity, we compared the proteome of *Al. salmonicida* cells implanted in the peritoneal cavity of Atlantic salmon and in LB broth. *Al. salmonicida* increased in numbers over the first day of *in vivo* cultivation, followed by a decline through the next four days that is suggestive of stationary growth. A disparity to the rapid proliferation expected during an infection suggests that this model does not fully replicate virulence properties of *Al. salmonicida.* However, the presence of host factors is likely to reveal features associated with *in vivo* adaption. Our results demonstrate major differences in metabolism between cells cultured *in vivo* and *in vitro*, including alterations in energy metabolism and a reduction in protein synthesis. After five days of cultivation, the majority of the 521 proteins observed to be differentially expressed *in vivo* and *in vitro* exhibited *in vivo* down-regulation. This general muting may be a strategy to hide from the host immune system, possibly related to the *in vivo* induction of several cold-shock proteins. Furthermore, all

six flagellin subunits present in the *Al. salmonicida* genome were up-regulated *in vivo*. As most of the remaining proteins associated with motility showed no differential expression or were down-regulated *in vivo*, we postulate that the flagellins and/or flagellar filament function in a motility-independent manner. This study contributes to interpretation of the interplay between *Al. salmonicida* and its salmonid host. Our observations may serve as a foundation for future studies of virulence-associated properties of *Al. salmonicida*.

1. Introduction

Aliivibrio salmonicida is the causative agent of cold-water vibriosis, a bacterial septicemia of farmed Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhyncus mykiss*) and Atlantic cod (*Gadus morhua* L.). The disease was the cause of great loss in Norwegian aquaculture during the eighties, but today it is considered to be well controlled by the use of oil-adjuvanted injection vaccines [1]. However, the pathogenesis of cold-water vibriosis is poorly understood, and only a few virulence factors of *Al. salmonicida* have been identified. The damage to endothelial integrity observed in fish suffering from cold-water vibriosis has brought several authors to hypothesize the involvement of extracellular toxins or proteases [2–6]. Although the genome of *Al. salmonicida* harbors several protein secretion systems, putative hemolysins and proteases [3], toxin activity has not been identified in *Al. salmonicida* [5–8].

The surface layer product VS-P1, consisting of lipopolysaccharides (LPS) and a protein moiety, is hypothesized to provide resistance towards the host immune system [6,9–11]. Several iron acquisition systems allows the acquisition of iron within fish tissues where iron availability is extremely low [12,13]. The iron-scavenging siderophore bisucaberin is produced at the highest levels below 10°C, corresponding well with the temperature range in which disease is known to occur [12,14]. In addition, the genome of *Al. salmonicida* encodes three TonB systems associated with energy-dependent transport of iron-siderophore compounds, one heme uptake system and additional systems for uptake of free iron [3]. Also, the polar flagella of *Al. salmonicida* have been shown to be of importance for the pathogenesis [15,16]. Recently, we demonstrated that motility is not required for invasion of Atlantic salmon, but that the flagellar filament is involved in virulence through still unknown mechanisms [16]. Finally, quorum sensing and the production of hydrogen peroxide has been suggested as possible virulence factors [8,17,18].

Experiments addressing pathogenicity are often conducted using *in vitro* models. However, artificial models may not necessarily allow the expression of virulence determinants required for development of disease [19]. By cultivation of bacteria within growth chambers placed in the peritoneal cavity of fish, several authors have reported the expression of iron acquisition systems, novel antigens, cytotoxic extracellular products and protective components [13,20–24]. Some of the properties were also identified in bacteria grown in media composed to mimic *in vivo* conditions, while other properties seem to relate to host signals that are not as easily targeted and reproduced. In *Al. salmonicida, in vivo* cultivation has revealed the expression of three high molecular weight bands not found in *in vitro*-cultured bacteria (as shown by SDS-PAGE), as well as the absence or decrease in expression of several minor bands [13]. The same high molecular weight bands were also expressed in cells cultured under iron-restricted conditions, but the relative abundance between the three bands seemed to differ.

In another *in vivo* experiment, the transcription of genes encoding putative extracellular toxins, secretion systems and adhesion factors was analyzed in *Al. salmonicida* retrieved from the blood of experimentally infected fish [5]. However, all genes analyzed showed the highest transcription levels *in vitro*, leading the authors to hypothesize a general muting of gene expression as a strategy for avoidance of the host immune system.

In order to expand our understanding of the processes associated with *in vivo* growth in *Al. salmonicida*, we have grown bacteria in implants placed in the peritoneal cavity of Atlantic salmon and compared the global proteome to that of bacteria cultured *in vitro* through a quantitative proteomic approach.

2. Materials and methods

2.1 *In vivo* cultivation

Aliivibrio salmonicida LFI1238, originally isolated from head kidney of a diseased cod (*Gadus morhua* L.) [3], was cultivated in Luria Bertani broth supplemented with 0.9% NaCl (LB0.9) and incubated at 10°C with shaking to OD_{600}: 0.8.

For *in vivo* cultivation, semi-permeable tubes containing cultures of *Al. salmonicida* were surgically placed in the abdominal cavity of 11 Atlantic salmon (*Salmo salar* L.) at an average weight of 434 g as previously described [13]. Dialysis tubing (Molecular weight cut-off: 12-14 kDa, Spectra/Por 4; Spectrum Laboratories Inc., LA, Ca, USA) was cut in lengths of 20 cm, sealed at one end and autoclaved in PBS. Subsequently, the tubes were inoculated with 3 ml of LFI1238 culture (2,2 x 10^8 CFU/ml) or PBS and closed by knotting twice. The knots were dipped in 70% EtOH, and the exterior of the tubes was rinsed with PBS. Prepared implants were stored in LB0.9 until the start of the surgical procedure.

Atlantic salmon undergoing surgery were anesthetized in a water bath of 0.005% benzocaine (Benzoak VET; ACD Pharmaceuticals, Leknes, Norway). For fixation and anesthesia during surgery, the fish was placed dorsally in a holding tray, exposing the gills to benzocaine solution (0.0001%). The ventral surface of the salmon was disinfected with chlorhexidine, before an incision of 1 cm was made craniomedially for the ventral fins. An implant was carefully placed inside the peritoneal cavity, and the incision was finally closed with two interrupted sutures (PDS II 2-0; Ethicon, Johnson & Johnson Medical GmbH, Norderstedt, Germany). Control salmon, carrying PBS implants, were marked by fin clipping. After surgery, the fish were moved to a tank

supplied with sea water (8°C), and all individuals displayed normal behavior within 5-10 minutes. The experiment was approved by the Norwegian Research Animal Authorities (ID: 7393) and was carried out in compliance with relevant Norwegian laws. For the control samples, dialysis tubes were inoculated with bacterial culture or PBS as described and placed in LB0.9. The control samples were incubated at 8°C with shaking.

2.1.1 Sampling

One and five days after implantation, three and six fish, respectively, were euthanized by an overdose of benzocaine and dissected for removal of the implants. The implants were rinsed with PBS, and the contents of the implants was removed with an 18G cannula and stored on ice. A similar number of control implants incubated in LB0.9 were also harvested. For quantification of bacterial load, 100 µl of bacterial suspension was serially diluted and plated onto blood agar plates (Blood agar base no. 2; Oxoid, Cambridge, UK) supplemented with 2.5% NaCl (BA2.5). In addition, head kidney samples from all fish were plated on BA2.5.

Immediately after removal, the harvested bacterial suspensions were centrifuged at 4000 rpm for 20 min at 4°C (Sorvall RC-5B refridgerated superspeed centrifuge; DuPont Instruments, Newtown, CT, USA). The supernatants were removed, and the bacterial pellets were stored at -80°C until analysis.

2.2 Proteomic analysis by nano LC-MS/MS

2.2.1 Protein extraction

The samples were processed for proteomic analysis as described by Tomanek and Zuzow [25]. Bacterial pellets were re-suspended and lysed in homogenization buffer [7 M urea, 2 M thiourea, 1% ASB-14 (amidosulfobetaine-14), 40 mM Tris-base, 0.5% immobilized pH 4–7 gradient (IPG) buffer (GE Healthcare, Piscataway, NJ, USA) and 40 mM dithiothreitol] using a

pre-chilled glass homogenizer for mechanical agitation, and pooled together for each treatment (*in vivo*/*in vitro*) and time point (incubation for one/five days). Six volumes of pre-chilled (-20°C) acetone was added to each sample, and proteins were precipitated overnight at -20°C. After centrifugation at 8000 g for 10 minutes at 4°C, the acetone was decanted and the resultant pellet was resuspended in 100 µl of 100 mM Triethylammonium bicarbonate buffer (TEAB, pH 8.5+/-0.1; Sigma, St. Louis, MO, USA). Protein concentrations were determined by the use of Pierce BCA Protein Assay Kit (Thermo Fisher Science) according to the manufacturer's instructions.

2.2.2 Protein digestion

Samples were reduced by addition of 10 mM dithiothreitol (DTT; Sigma) and incubation at 56˚C for 1 h, and alkylated by addition of 20 mM iodoacetamide (IAA; Sigma) and incubation at room temperature in the dark for 1 hour. Digestion with trypsin (from bovine pancreas; Promega, Madison, WI, USA) was performed at a 1:50 ratio (enzyme:protein) at 37˚C overnight. Following trypsin digestion, digested peptides were lyophilized to near dryness and re-dissolved with 100 mM TEAB.

2.2.3 iTRAQ labeling

For each sample, 100 µg peptides were diluted in 50 mM TEAB to a final volume of 100 µl and labeled with the iTRAQ reagents of a TMT10plex Isobaric Label Reagent Set (Thermo Fischer Scientific, Waltham, MA, USA) for 1 h at room temperature as recommended by the manufacturer. After labeling, samples were combined.

2.2.4 Sample fractionation

The combined sample was separated using a Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Spin columns were conditioned by washing twice with acetonitrile (ACN; Sigma), followed by two additional washes with 0.1% trifluoroacetic acid solution (TFA; Sigma). Digested samples were dissolved in 300 µl of 0.1% TFA solution and applied to the column. Following elution of a flow through fraction and unreacted TMT reagent, the sample was eluted in six fractions employing a gradient of ACN in 0.1% triethylamine (5%, 10%, 12.5%, 15%, 20%, 50%). After fractionation, the liquid contents of the sample were evaporated by the use of a vacuum centrifuge.

2.2.5 Nano LC-MS/MS analysis

Separation and analysis was conducted using a Dionex Ultimate 3000 Nano LC system coupled with an Orbitrap Fusion ™ Tribrid™ mass spectrometer (Thermo Fisher Scientific) with an ESI nanospray source. The sample was loaded onto a Nanocolumn□100 µm x 10 cm in-house made column packed with a reversed-phase ReproSil-Pur C18-AQ resin (3 µm, 120 Å, Dr. Maisch GmbH, Germany) with a sample injection volume set at 5 µl. Peptide separation was achieved at a flow rate of 600 nL/min as following: from 6% to 9% B for 15 min, from 9% to 14% B for 20 min, from 14% to 30% B for 60 min, from 30% to 40% B for 15 min and from 40 to 95% B for 3 min, and elution with 95% B for 7 min. The phase composition of phase A and B were 0.1% formic acid in acetonitrile and 0.1% formic acid in acetonitrile, respectively. Mass spectrometer settings included an ion spray voltage of 2.2 kV, a heated capillary temperature of 320˚C and a normalized collision energy setting of 35 eV for collision-induced dissociation. Data-dependent acquisition was performed for 250 ms over a mass (m/z) range of 300 – 1650. For ions with a

charge state of +5 that exceeded 500 counts per second, up to 15 of the most intense peptide ions were collected.

2.2.6 Data analysis

The six raw MS/MS files were analyzed and searched against a protein database based on the sequenced genome of *Al. salmonicida* LFI1238 using Proteome Discoverer 2.0 (ThermoFisher Scientific). Searching parameters were set as following: the protein modifications were carbamidomethylation (C) (fixed), oxidation (M) (variable); the enzyme specificity was set to trypsin; the maximum missed cleavages were set to 2; the precursor ion mass tolerance was set to 10 ppm, and MS/MS tolerance was 0.6 Da. Only peptides identified with high confidence, as defined by Proteome Discoverer 2.0, were chosen for downstream protein identification analysis. A quantitative ratio over 1.5 was considered up-regulation, while a quantitative ratio less than 0.67 was considered as down-regulation [26].

3. Results

To study how *Al. salmonicida* adapts to the conditions met within a salmonid host, we cultivated bacteria in sealed dialysis tubes within the peritoneal cavity of Atlantic salmon. Bacteria were harvested from the tubes after one and five days of incubation, and global protein expression was compared to that of bacteria cultivated *in vitro* by the use of quantitative proteomics (iTRAQ).

3.1 *In vivo* cultivation

After one day of incubation, the three experimental fish showed no pathology except for local erythema in the region of the incision, and no bacteria were found in either head kidney or blood (<10 CFU/ml). The implants held 1-3 ml of bacterial suspension containing 2.1 x 10^9 CFU/ml. After five days of incubation, *Al. salmonicida* was isolated from the head kidney of one of six experimental fish sampled, and from the blood (>10^4 CFU/ml) of all individuals. At this time point, five fish showed local peritonitis in the incision area, and peritoneal petechiae were observed in two individuals. After removal of the implants, 1.8 – 4 ml of bacterial suspension containing 6.8 x 10^8 CFU/ml was retrieved. In the implants incubated in LB broth for five days, the bacterial load was 8.0 x 10^8 CFU/ml.

3.2 Quantitative analysis of global protein profiles *in vivo* and *in vitro*

By the use of iTRAQ, we identified 1585 proteins from 32 711 MS/MS spectra and 12 425 peptides, for which 1278 proteins were recognized by at least two peptides. For each protein, differences in abundance between samples were calculated, and ratios above 1.5 and below 0.67 were regarded as up- and down-regulation, respectively. The numbers of differentially expressed proteins between samples are summarized in Table 1. In order to obtain further information about biological processes represented by our data, the proteins that were differentially expressed *in vivo* and *in vitro* were functionally

195 classified according to the Clusters of Orthologous Groups of proteins (COG) database (Figure 1) and
196 subjected to Gene Ontology over-representation analysis [27,28].

197 After one day of cultivation, proteins required for transcriptional regulation, RNA processing, stress
198 responses, iron ion binding and organic acid transport were up-regulated *in vivo* relative to *in vitro*.
199 Down-regulated proteins were predominantly related to metabolism of vitamins and cofactors, motility,
200 metal ion binding and nucleotide binding.

201 After five days of cultivation, *in vivo*-grown cells of *Al. salmonicida* showed significant up-regulation of
202 proteins required for iron ion transport, siderophore-mediated iron transport, carbohydrate transport,
203 glycolysis, cold shock proteins, transcriptional regulation, and flagellar assembly. The list of down-
204 regulated proteins was dominated by proteins needed for translation and protein synthesis, amino acid
205 synthesis, two-component signal transduction systems and transcriptional regulation, TCA cycle and
206 organic acid catabolism, and cell redox homeostasis. As the main focus of this study was host-pathogen
207 interactions and identification of bacterial proteins with putative roles in pathogenicity, we have
208 concentrated our analysis on the expression of proteins that are hypothesized to be involved in host-
209 pathogen interactions in *Al. salmonicida* or related species.

210 3.3 Motility and flagellation

211 Previously, flagellation has been demonstrated to be of importance for the virulence of *Al. salmonicida*
212 [15]. Here, we identified 52 proteins with putative functions in the flagellar basal body, Na+-driven
213 motor, switch components, hook and flagellin, as well as proteins required for flagellin export and
214 assembly, chemotaxis and flagellar regulation (Supplementary Table S1). After one day of cultivation, the
215 expression profiles of motility-related proteins seen in the *in vivo* and *in vitro* samples were largely
216 similar. Two proteins (transcriptional activator FlrA and flagellin FlaA) were down-regulated, and one
217 protein (flagellar hook-associated protein HAP2) was up-regulated *in vivo* compared to *in vitro* (Table 2).

After five days of cultivation, most proteins identified showed similar abundances *in vivo* and *in vitro*, or were down-regulated *in vivo* relative to *in vitro.* For four of the six proteins identified with putative functions in flagellar regulation, decreased abundances were observed *in vivo* relative to *in vitro* (Table 2). However, for all six flagellin subunits produced by *Al. salmonicida,* increases in abundance were observed *in vivo* (Table 2). Interestingly, flagellin subunits FlaE and FlaC were among the fifty most abundant proteins identified *in vivo* at day 5 (data not shown). In addition, two putative chaperones involved in flagellin export and assembly, FliS and FlgN, showed differential expression *in vivo* and *in vitro* (Table 2).

In addition to the proteins with putative functions in flagellar motility, we also identified four proteins associated with the production of mannose-sensitive hemagglutinin (Type IV pilus MSHA), four proteins associated with Flp pilus production, and two proteins (PilT and PilU) required for pili-associated twitching motility (Supplementary Table S1). The majority of these proteins exhibited a similar expression pattern *in vivo* and *in vitro*, although differential expression was noted for one MSHA protein (MshB) after one day of cultivation, and one MSHA protein (MshA) and two Type IV pilus assembly proteins (PilU and a FimV-like protein) after five days of cultivation (Table 2).

3.4 Iron acquisition

As the iron availability within vertebrates is limited due to high-affinity iron-binding proteins such as transferrin, bacteria require systems for iron sequestration in order to obtain iron and maintain growth *in vivo* [29]. Previously, an increase in iron-regulated outer membrane proteins has been observed in *Al. salmonicida* cultivated *in vivo* [13]. Likewise, we observed an increase *in vivo* in abundance of several proteins related to iron acquisition (Table 3). The majority of the 22 iron-related genes identified did not differ between *in vivo* and *in vitro* cultures after one day of cultivation. After five days, up-regulation of heme transporter HuvB, ferrous iron transporter FeoB, bisucaberin synthesis proteins BibA and BibC,

four siderophore receptors, one siderophore-binding periplasmic protein, TtpC2 of the TonB2 energy transduction system, three proteins associated with iron ion ABC transporter systems, and four additional iron-regulated proteins was seen *in vivo* relative to *in vitro*. Also, ferric uptake regulator Fur, acting as a transcriptional repressor of iron uptake genes in the presence of intracellular Fe2+, increased in abundance between day 1 and day 5 *in vitro*, whereas a minor reduction was seen *in vivo* over the same time span.

Interestingly, proteins BibA and BibC showed the highest abundance after one day of cultivation, with similar proteins levels observed under both conditions. After five days, a decrease in abundance was seen both *in vivo* and *in vitro*, although less so *in vivo*. However, the putative bisucaberin receptor BitA demonstrated *in vivo* up-regulation at this time point.

3.5 Stress responses

Among the most abundant proteins identified *in vivo* at day 5, we found several cold shock proteins. In total, six cold shock proteins were identified (Supplementary Table S1). Five of these proteins exhibited differential expression *in vivo* and *in vitro*, four being up-regulated and one down-regulated *in vivo* relative to *in vitro* after five days of cultivation (Table 4). We also identified 18 additional proteins associated with stress responses, including several heat shock proteins, proteases, antioxidants, and proteins associated with cell redox homeostasis. The majority of these proteins showed a similar expression pattern *in vivo* and *in vitro*, or were down-regulated *in vivo* (Table 4).

3.6 Lipopolysaccharide biosynthesis

The lipopolysaccharide (LPS) of *Al. salmonicida* is, in addition to being an important constituent of the outer membrane, found as part of the outer membrane complex VS-P1 [11]. This complex has been found in high quantities in blood, tissue and tissue fluids of moribund fish and is hypothesized to contribute to the cold-water vibriosis pathogenesis [6,9,10,30]. By analysis of expression patterns of

proteins related to LPS biosynthesis, we wanted to determine whether LPS production was modulated qualitatively or quantitatively under *in vivo* growth.

In all samples combined, 47 proteins associated with LPS metabolism were identified (Supplementary Table S1). These included proteins with putative roles in Lipid A and core oligosaccharide biosynthesis, LPS export and assembly, as well as enzymes involved in the synthesis of neuraminic acid, legionaminic acid (both sialic acids) and L-rhamnose. Both legionaminic acid and L-rhamnose have been reported to be part of the *Al. salmonicida* LPS [31,32]. For most of the LPS-related proteins identified, no change was seen in protein abundance between the *in vivo* and *in vitro* samples. However, for four of the eight proteins related to Lipid A biosynthesis, the abundance levels were lower *in vivo* than *in vitro* after five days of cultivation (Table 5). Similarly, four proteins associated with core oligosaccharide or exopolysaccharide modification were down-regulated *in vivo* at day 5, including two enzymes with putative functions in sialic acid biosynthesis (Table 5). In contrast, two enzymes of the L-rhamnose biosynthesis pathway showed up-regulation in *vivo* at the same time point (Table 5).

3.7 Outer membrane proteins

Bacterial outer membrane proteins, being localized in the cell envelope, may facilitate interactions between host and microbe, such as bacterial adhesion to host cells and immune recognition. We identified several outer membrane proteins that were differentially expressed *in vivo* and *in vitro* (Table 6). Proteins that were up-regulated *in vivo* relative to *in vitro* include OmpU, an OmpA-like protein, three additional putative outer membrane proteins, two outer membrane transport proteins, and an outer membrane efflux protein. Proteins that were down-regulated *in vivo* relative to *in vitro* include outer membrane protein assembly factors BamA, BamC and BamD, a membrane-associated sulfatase, a protein-export membrane protein (SecF), a component of an efflux system, and ten putative outer membrane proteins.

3.8 Secretion

The genome of *Al. salmonicida* LFI1238 encodes several secretion systems and putative hemolysins and proteases. We identified components of two type 1 and one type 2 secretion systems, but the identified proteins exhibited similar expression profiles (Supplementary Table S1), or were down-regulated in the *in vivo* samples relative to the *in vitro* samples (Table 7). We also found identified two putative hemolysins: hemolysin Vah2, which is similar to a hemolysin of *V. anguillarum* and was found in lower abundance *in vivo,* and a hemolysin-type calcium-binding protein which was found in similar levels throughout all samples.

4. Discussion

We compared the proteome of *Al. salmonicida* cells implanted in the peritoneal cavity of Atlantic salmon and in LB broth. This approach allowed us to identify proteins that might be required for initial and late-phase infection of host tissue. While LB broth is a complex medium commonly used for *in vitro* growth of *Al. salmonicida*, the implants constitute a composition of nutrients and host components of salmonid origin. Although we have not analyzed the contents of peritoneal fluid in the salmon of this experiment, some assumptions can be made regarding its properties. The semi-permeable nature of the dialysis bags (12-14 kDa) limits the communication between host and microbe, but the identification of bacteria in head kidney and blood of experimental fish shows that the tubes are not entirely leak-proof. Similarly, a previous publication reports the isolation of bacteria from peritoneal fluid and kidney tissue after seven days of implant incubation, as well as the presence of a limited number of macrophages within the implants [13]. Furthermore, MS analysis of the extracellular fractions of peritoneal implants prepared in a similar manner revealed several host factors with a molecular mass up to 184 kDa, including complement components, transferrin and immunoglobulin (unpublished results). The peritonitis and peritoneal petechiae observed in salmon of this experiment suggest an influx of inflammatory cells to the peritoneal cavity, and these cells may have influenced the environment experienced by the implanted bacteria.

We observed an increase in viable cell numbers after one day of *in vivo* cultivation, followed by a drop in cell count which indicates that the *in vivo* cultures harvested at day five were in a stationary growth phase. In contrast to our observations, Colquhoun and Sørum reported exponential growth for the first four days of *in vivo* cultivation of *Al. salmonicida,* plateauing at a level of approximately 10^{10} CFU between day four and seven [13]. Possibly, the discrepancy with our results relate to differences in experimental procedures. As a higher initial inoculum was used by Colquhoun and Sørum, cell density may be of importance for the ability of rapid *in vivo* growth. Previously, quorum sensing has been postulated as a virulence factor in *Al.*

318 *salmonicida* [8].

319 Garduño and co-workers found that *Aeromonas salmonicida* cultivated *in vivo* within peritoneal implants
320 in rainbow trout was killed due to bactericidal activity of the peritoneal fluid [33]. Also, limited growth has
321 been reported for *Aeromonas hydrophila*, *Photobacterium damsela* ssp. *piscicida* and *Flavobacterium*
322 *psychrophilum* cultivated *in vivo* [20,23,24]. Although differences in bacterial species, host species and
323 experimental procedures hamper a direct comparison to our results, these studies suggest that the
324 peritoneal cavity of teleosts is a hostile environment.

325 Challenge by intraperitoneal (i.p.) injection and immersion are the two most common infection models for
326 evaluating *Al. salmonicida* virulence in Atlantic salmon. Immersion challenge mimics the natural route of
327 transmission and produces typical signs of disease that resemble those seen under a natural infection,
328 while i.p. challenge is an artificial model that involves internal delivery of large numbers of bacteria and
329 expedites the onset of disease (Nordmo 1997). The physiological conditions experienced by bacteria in
330 this experiment may be comparable to the i.p. model, and the phenotype observed in this study may not
331 necessarily be representative for the rapidly proliferating bacteria seen in blood of infected fish [5].
332 Nevertheless, our findings represent *one* phenotype of *Al. salmonicida* influenced by growth in an *in vivo*
333 environment.

334 The highest number of differentially expressed proteins *in vivo* relative to *in vitro* was observed after five
335 days of cultivation, and the majority (72.6%) of the 521 proteins showing differential expression were
336 down-regulated *in vivo.* Functional classification revealed that a substantial number of the down-regulated
337 proteins were involved in protein synthesis. This finding supports the assumption of Bjelland and co-
338 workers that *Al. salmonicida* utilize a general muting of gene expression as a strategy to hide from the host
339 immune system [5]. Also, the differential expression of proteins related to carbohydrate transport,
340 glycolysis and the TCA cycle is consistent with a shift in nutrient source from LB broth to peritoneal fluid.

4.1 Secretion systems

The tissue damage observed in moribund fish has been suggested to be related to extracellular toxin activity, but no such components have previously been identified. In our data set, we identified components of two type 1 secretion systems (T1SS) and one type two secretion system (T2SS), as well as two putative hemolysins. However, neither the secretion systems nor the hemolysins appeared to be up-regulated in response to *in vivo* conditions. TolC, associated with one of the T1SS, increased equally in abundance through the experiment both *in vitro* and *in vitro.* In contrast, the identified component of the remaining T1SS was down-regulated between day 1 and day 5. For the majority of the proteins associated with the T2SS, similar levels of abundance were detected *in vitro* and *in vivo.* Likewise, the two hemolysins identified did not show up-regulation *in vivo*, confirming the previous observations by Bjelland et al. [5]. However, we have not analyzed the levels of proteins secreted by *Al. salmonicida*. Nevertheless, as we failed to detect the induction of secretion systems in *in vivo*-grown bacteria, our data suggest that toxins do not participate in the pathogenesis of cold-water vibriosis.

4.2 Flagellation

Previously, we have implicated that flagellation is involved in the pathogenesis of cold-water vibriosis [15,16]. Interestingly, our observations show that components of the flagellar filament are upregulated in response to unknown factors of the salmonid host. Although *Al. salmonicida* has been found to downregulate transcription of flagellar proteins *in vitro* at physiologic salinity compared to sea water-like salinity [34], flagellated bacteria are commonly isolated from diseased fish [4,13,15]. Also, a similar increase in abundance of flagellin subunits has previously been observed in *Al. salmonicida* after exposure to mucus of Atlantic salmon [35].

The discrepancy between the observed expression patterns of flagellin subunits and other flagellar components is suggestive of a motility-independent role for the flagellar filament and/or monomeric

flagellin. Mutagenesis experiments strongly suggest that the flagellins are involved in the pathogenesis of cold-water vibriosis, although the mechanisms involved are not known [16]. Intriguingly, chaperone FliS, for which the *Salmonella* Typhimurium orthologue is involved in inhibition of flagellin polymerization prior to export and assembly [36,37], was found down-regulated *in vivo*. Possibly, the identified flagellins could have accumulated in the cytoplasm due to premature polymerization. However, such a scenario would be highly wasteful and detrimental to cell viability. Alternatively, the flagellins may contribute to host immunomodulation, as flagellin is known to be highly immunogenic [38,39]. Flagellins have previously been identified as part of the *Al. salmonicida* secretome [34], and the high abundance of flagellins detected *in vivo* in the present study suggests that flagellins may be delivered to the host in high quantities.

4.3 Iron acquisition

Iron acquisition systems are well described in a wide range of bacterial pathogens and enable *in vivo* growth in spite of nutritional immunity strategies that limit iron availability within the host [40]. Systems for iron acquisition include siderophores, which are scavenging molecules with high affinity for iron, heme acquisition systems, transferrin/lactoferrin receptors, and energy-driven membrane transporters for translocation of iron [40,41].

Previous *in vivo* cultivation studies have identified an increase in iron-regulated outer membrane proteins in *Al. salmonicida* [13], as well as in other fish pathogens such as *Aeromonas salmonicida ssp. salmonicida* and *Flavobacterium psychrophilum* [21,23]. Also, *Al. salmonicida* has been shown to induce production of the hydroxamate siderophore bisucaberin under iron limitation [14,42].

In this study, we have identified bisucaberin biosynthesis proteins BibA and BibC, and one TonB-dependent receptor protein (BitA) associated with bisucaberin uptake. In a microarray study, Thode and co-workers observed the up-regulation of 32 genes of *Al. salmonicida* in response to iron limitation, of which *bibA* and *bibB* exhibited the largest fold change of all differentially expressed genes identified [42].

To our surprise, we found BibA and BibC to exhibit the highest abundance at day 1. Protein abundance levels were reduced after five days both *in vivo* and *in vitro*, although less so *in vivo*. The requirement for siderophore production may relate to cell density and reflect the reduction in cell numbers observed after five days of *in vivo* cultivation. The *in vivo* up-regulation of bisucaberin receptor BitA observed at day five indicates that bisucaberin is utilized for iron acquisition *in vivo*.

We also identified four additional siderophore receptors, of which three exhibited up-regulation *in vivo.* The gene encoding one of these receptors, a TonB-dependent receptor, is located adjacent to the genes encoding a TonB2-system. TonB energy-transduction systems provide energy for active transport of iron-bound substrates across the outer membrane, and *Vibrio* spp. are generally in possession of two or three TonB systems [43]. *Al. salmonicida* has three TonB systems, although one of these, the TonB1 system, contains a predicted pseudogene and is probably not active [3]. In support of this assumption, no proteins related to the TonB1 system were detected. For each of the two remaining TonB systems (TonB2 and TonB3), we identified the essential protein TtpC. TtpC2, belonging to the TonB2 system, was found up-regulated *in vivo*, while TtpC3 of the TonB3 system showed no differential expression. Thus, the TonB2 system appears to be of higher importance in iron acquisition *in vivo* than the TonB3 system. Similarly*, Vibrio vulnificus* exhibited up-regulation of the TonB1 and TonB2 systems, but not the TonB3 system, in response to iron limitation [44]. We also observed the *in vivo* up-regulation of several proteins associated with transporter systems for heme and iron ions, as well as additional proteins previously described to be iron-regulated. Overall, our results confirm that *Al. salmonicida* induces multiple iron acquisition systems under *in vivo* growth.

4.4 Stress responses

After five days of cultivation, four cold-shock proteins were up-regulated *in vivo*, of which two were among the hundred most abundant proteins identified. Cold-shock proteins generally facilitate adaption

410 to stress associated with temperature down-shifts, such as decreases in membrane fluidity and inhibition
411 of ribosomal translation [45]. However, some cold-shock proteins are induced in response to other
412 stressors, and are shown to function during stationary phase or in response to osmotic stress and
413 nutrition starvation [45,46]. As the cultivation temperature did not differ between the *in vivo* and *in*
414 *vitro* cultures of this study, it is likely that the up-regulation of cold-shock proteins relate to stress
415 associated with the host environment rather than temperature down-shift. In *Escherichia coli*, cold-shock
416 protein CspD was upregulated during stationary phase of growth and was found to inhibit DNA
417 replication by binding to ssDNA [47]. The dominance of down-regulated proteins observed *in vivo* implies
418 that inhibitory mechanisms are in place, and this observed inhibition may perhaps be associated with the
419 induction of cold-shock proteins. Interestingly, three cold-shock proteins were found among the most
420 abundant proteins of *Aliivibrio fischeri* expelled from the light organ of bobtail squid *Euprymna scolopes*
421 [48]. Possibly, the cold-shock proteins identified in *Al. fischeri* and *Al. salmonicida* could be involved in
422 the transition to a metabolic state that promotes *in vivo* survival.

423 We also identified numerous heat-shock proteins and proteins associated with cell redox homeostasis.
424 The majority of these proteins were less abundant *in vivo* than *in vitro*, although superoxide dismutase,
425 an ATP-dependent Clp protease, one heat-shock protein and an anti-oxidant co-factor exhibited up-
426 regulation *in vivo*. The relative reduction in abundance of proteins related to cell redox homeostasis may
427 relate to the absence of host immune cells within the implants. Phagocytic cells kill bacteria by
428 production of reactive oxygen species (ROS) during a respiratory burst [49], and proteins involved in ROS
429 neutralization are required for bacterial defense. Cell redox homeostasis processes may be involved in
430 certain aspects of the cold-water vibriosis pathogenesis. However, *Al. salmonicida* has been reported to
431 be rapidly engulfed and degraded by Atlantic salmon phagocytes [50,51].

4.5 LPS biosynthesis

The septicemic nature of cold-water vibriosis suggests that *Al. salmonicida* is able to resist the bactericidal action of fish serum during an infection. Several strategies for serum resistance are described in bacterial pathogens, including steric hindrance by capsules or long O-antigen chains, binding of inhibitory host components, or shedding of surface antigens [52]. Although the production of a capsule has not been identified in *Al. salmonicida* grown within or outside the host [13], shedded VS-P1 has been shown to form aggregates in the proximity of the bacteria, and has been postulated to be involved in serum resistance [6,10]. Previously, we have shown that *Al. salmonicida* is partially sensitive to the bactericidal activity of fish serum *in vitro* (submitted elsewhere). Following this, we were wondering whether *Al. salmonicida* modulates activities associated with LPS biosynthesis in response to *in vivo* conditions in order to increase its resistance to the bactericidal action of fish serum. However, our results suggest that the LPS biosynthesis apparatus is largely similar *in vivo* and *in vitro*, as the majority (72.9%) of the identified proteins with putative functions in LPS biosynthesis showed no differential expression between the samples. Nevertheless, RmlB and RmlD, two enzymes involved in rhamnose biosynthesis [53], were up-regulated *in vivo*, while four enzymes participating in Lipid A biosynthesis were down-regulated. As LPS consists of an outer membrane-embedded Lipid A, an oligosaccharide core and an O-antigenic side-chain, it is tempting to relate the relative reduction in Lipid A-associated proteins to a reduction in LPS production associated with the stationary phase of growth. Similarly, the increase of rhamnose biosynthesis proteins implies an increase in rhamnose production, possibly embedded as part of the LPS oligosaccharide moiety. However, further experimental data are needed to probe the biological significance, if any, of these findings. In *V. anguillarum*, the loss of RmlD resulted in the loss of the O-antigen side chain, leading to reduced serum resistance and defective siderophore transport [54].

4.6 Outer membrane proteins

The outer membrane of Gram-negative bacteria contains numerous proteins in the form of integral membrane proteins or lipoproteins, constituting about 50% of the outer membrane mass [55]. The expression of outer membrane proteins (OMPs) is influenced by environmental conditions and promotes adaption and survival [55,56]. For instance, OMPs are known to provide resistance towards antimicrobial peptides, complement-mediated killing, bile and antimicrobial drugs [55]. Also, as they are located at the host-bacterial interface, OMPs may be of interest as potential candidates for vaccine development [57].

In addition to OMPs involved in processes already described, we identified at least 23 OMPs differentially expressed *in vivo* and *in vivo*, of which 8 were up-regulated and 15 were down-regulated *in vivo*. One of the up-regulated OMPs, OmpU, has been associated with host colonization and virulence in several *Vibrio* spp. by mediating adhesion, resistance to antimicrobial peptides and cytotoxicity [58–63]. OmpU has previously been shown to be up-regulated in *Al. salmonicida* cultivated at 8°C relative to 15°C [64]. Interestingly, OmpU and an additional OMP (UniProt accession no: B6EJ86) that was up-regulated *in vivo* were among the fifty most abundant proteins in the *in vivo* proteome. Although the properties for most OMPs identified remain concealed, the list of differentially expressed OMPs may be of use for further identification of proteins related to virulence or candidates for vaccine development.

4.7 Conclusion

We have analyzed the *in vivo* proteome of *Al. salmonicida* and identified proteins that were differentially expressed as compared to under *in vitro* conditions. Cells harvested after five days of *in vivo* cultivation were reduced in numbers compared to the samples harvested after one day, indicative of stationary growth. A disparity to the rapid proliferation expected during an infection suggests that this model does not fully replicate virulence properties of *Al. salmonicida.* However, the presence of host components is likely to reveal features associated with *in vivo* adaption. Our results demonstrate major differences in

metabolism between cells cultured *in vivo* and *in vitro*, including alterations in energy metabolism and a reduction in protein synthesis. The majority of the 521 proteins observed to be differentially expressed *in vivo* and *in vitro* exhibited *in vivo* down-regulation. This general muting may be a strategy to hide from the host immune system, possibly related to the *in vivo* induction of several cold-shock proteins.

Proteins associated with multiple iron acquisition systems were found to be up-regulated *in vivo*, confirming previous reports on the ability of *Al. salmonicida* to grow under iron-limitation. Furthermore, all six flagellin subunits present in the *Al. salmonicida* genome were up-regulated *in vivo*. As most of the remaining proteins associated with motility showed no differential expression or were down-regulated *in vivo*, we postulate that the flagellins and/or flagellar filament function in a motility-independent manner.

We also identified proteins associated with type 1 and type 2 secretion systems, as well as two putative hemolysins. However, the lack of differential expression *in vivo* and *in vitro* suggests that toxin secretion is not induced in response to the *in vivo* environment. Likewise, for most proteins associated with LPS biosynthesis, the abundance levels were similar between the *in vivo* and *in vitro* samples, implying that the characterized LPS structure of *in vitro*-grown *Al. salmonicida* is representative for an *in vivo* phenotype.

This study contributes to interpretation of the interplay between *Al. salmonicida* and its salmonid host. Our observations may serve as a foundation for future studies of virulence-associated properties of this organism.

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675

676 Table 1: Number of differentially expressed genes between samples.

Comparison	Number of up-regulated genes *(ratio > 1.5)*	Number of down-regulated genes *(ratio < 0.67)*
In vitro: day 1 vs day 5	323	316
In vivo: day 1 vs day 5	273	423
Day 1: *In vivo* vs *in vitro*	80	56
Day 5: *In vivo* vs *in vitro*	143	378

677

678 Table 2: Differentially expressed proteins related to motility and type IV pilus.

Function	UniProt Accession	Protein name	iTRAQ ratio *in vivo/in vitro* Day 1		Day 5	
Flagellar motility						
Basal body	B6EJD2	Polar flagellar protein FliL	1,138		0,528	↓
	B6EJH1	Flagella basal body P-ring formation protein FlgA	1,206		1,694	↑
Na+-motor	B6ENQ4	Sodium-type flagellar protein MotY	0,909		0,654	↓
Hook	B6EJE5	Flagellar hook-associated protein 2	1,637	↑	0,713	
Filament	B6EJF7	Flagellin flaA	0,573	↓	1,739	↑
	B6EJF5	Flagellin flaB	1,073		1,759	↑
	B6EJE9	Flagellin flaC	0,787		4,232	↑
	B6EJE8	Flagellin flaD	1,253		2,838	↑
	B6EJE7	Flagellin flaE	0,971		4,942	↑
	B6EKM1	Flagellin flaF	0,770		3,283	↑
Flagellin export and assembly	B6EJE3	Flagellar protein FliS	1,206		0,378	↓
	B6EJH3	Polar flagellar FlgN, putative chaperone	0,871		1,859	↑
Chemotaxis	B6EJB3	Chemotaxis protein CheW	0,912		0,659	↓
	B6EJB8	Chemotaxis protein CheZ	0,841		0,391	↓
	B6EJB9	Chemotaxis protein CheY	1,220		0,507	↓
Switch	B6EJD7	Flagellar motor switch protein FliG	1,020		0,371	↓
	B6EJD1	Flagellar motor switch protein FliM	0,771		0,521	↓
Regulation	B6EMA2	RNA polymerase sigma-54 factor (rpoN)	1,033		0,499	↓
	B6EJE0	Sigma-54 dependent response regulator (flrC)	0,893		0,392	↓
	B6EJE2	Sigma-54 dependent transcription regulator (flrA)	0,633	↓	0,540	↓
	B6EJC2	Flagellar biosynthesis protein FlhF	1,055		0,635	↓
Type IV pilus						
MSHA	B6EM79	Type IV pilus, prepilin-like protein (MshB)	1,707	↑	1,263	
	B6EM80	Type IV pilus, mannose-sensitive hemagglutinin A	0,954		2,006	↑
Type IV pilus assembly	B6EMV1	Twitching motility protein PilU	1,002		2,390	↑
	B6EIW5	Protein of FimV superfamily (Tfp pilus assembly)	0,752		0,313	↓

679 Table 3: Differentially expressed proteins related to iron acquisition.

Function	UniProt Accession	Protein name	iTRAQ ratio *in vivo/in vitro*			
			Day 1		*Day 5*	
Heme uptake	B6EN28	Heme transporter protein HuvB, putative periplasmic binding protein	1,472		4,610	↑
Siderophore synthesis/uptake	B6EP92	Bisucaberin siderophore biosynthesis protein A	1,030		2,737	↑
	B6EP94	Bisucaberin siderophore biosynthesis protein C	1,154		2,451	↑
	B6EP95	TonB-dependent bisucaberin receptor BitA	0,979		4,519	↑
	B6EQQ5	Ferric aerobactin receptor	1,053		1,427	
	B6ESH4	Ferrioxamine B receptor	0,975		3,059	↑
	B6EQD3	Ferrichrome-binding periplasmic protein	1,055		2,771	↑
TtpC2-TonB2	B6EQ93	TonB dependent receptor	1,131		5,784	↑
	B6EQ95	TonB2 complex-associated transport protein C TtpC2	0,767		2,835	↑
Iron transport	B6EJ17	Ferrous iron transport protein B	0,641	↓	2,188	↑
	B6EL18	Iron(III) ABC transporter, ATP-binding protein	0,896		1,750	↑
	B6EL20	Iron(III) ABC transporter, periplasmic iron-compound-binding protein	0,930		5,550	↑
	B6ET71	Uncharacterized protein	1,714	↑	0,639	↓
	B6ET68	Iron ion ABC transporter, periplasmic component	0,850		5,039	↑
Regulation	B6EHL8	Ferric uptake regulation protein Fur	1,408		0,575	↓
Iron-regulated proteins	B6EN59	Putative exported protein	0,936		1,546	↑
	B6EN60	Putative iron-regulated protein	1,412		6,604	↑
	B6EQ60	Membrane protein	0,553	↓	3,513	↑
	B6ESD5	Putative lipoprotein	1,072		3,193	↑

680

681 Table 4: Differentially expressed proteins involved in stress responses.

Function	UniProt Accession	Protein name	iTRAQ ratio *in vivo/in vitro*			
			Day 1		*Day 5*	
Cold shock proteins	B6EP01	Cold shock protein	1,028		3,003	↑
	B6EL71	Cold-shock protein	1,521	↑	1,651	↑
	B6EIX2	Cold shock-like protein CspD	1,912	↑	6,406	↑
	B6EMP8	Ribonuclease R	1,128		0,426	↓
	B6ESU0	Cold shock-like protein CspG	0,902		2,418	↑
Cell redox homeostasis	B6ELF5	Dihydrolipoyl dehydrogenase	0,866		0,536	↓
	B6ENN6	Thioredoxin 2	2,216	↑	1,350	
	B6EMJ5	Glutaredoxin 3	1,112		0,621	↓
	B6ENQ0	Glutaredoxin	0,838		0,657	↓
	B6EH64	Glutaredoxin 2	0,751		0,651	↓
	B6EHQ4	Glutaredoxin 1	0,661	↓	1,468	
	B6EJ33	Thiol:disulfide interchange protein DsbE	1,103		0,593	↓
	B6EN77	Thiol:disulfide interchange protein DsbD	1,015		0,607	↓
	B6ENT7	Soluble pyridine nucleotide transhydrogenase	0,863		0,594	↓

	B6ES84	Putative glutaredoxin	0,998		0,458	↓
Antioxidants	Q3LSM1	Catalase	1,031		0,608	↓
	B6ERD2	Superoxide dismutase	1,224		2,409	↑
Heat shock proteins and proteases	B6EKA0	Chaperone protein DnaJ	0,961		0,617	↓
	B6EMG9	Chaperone protein DnaK	1,305		0,537	↓
	B6EGD6	Chaperone protein ClpB	1,844	↑	0,653	↓
	B6EHJ9	Chaperone protein HtpG	0,926		0,508	↓
	B6EGS9	Small heat shock protein	1,325		0,251	↓
	B6EMG8	Small heat shock protein IbpA	1,089		0,327	↓
	B6EI33	Heat shock protein	0,657	↓	2,268	↑
	B6EKY5	Autonomous glycyl radical cofactor	0,858		4,305	↑
	B6EMQ7	RNA-binding protein Hfq	1,448		0,662	↓
	B6EGV0	Universal stress protein	1,008		0,347	↓
	B6EKC3	Protease HtpX	0,638	↓	0,828	
	B6EHK3	ATP-dependent Clp protease	0,852		1,967	↑
	B6ELY8	Exported serine protease	0,966		0,379	↓

682

683 Table 5: Differentially expressed proteins involved in LPS biosynthesis.

Function	**UniProt Accession**	**Protein name**	**iTRAQ ratio *in vivo/in vitro***			
			Day 1		*Day 5*	
Lipid A	B6EJW8	Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase	0,946		0,607	↓
	B6EK47	UDP-3-O-acylglucosamine N-acyltransferase	0,975		0,491	↓
	B6EJW9	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	0,875		0,457	↓
	B6ES05	Lipid A export ATP-binding/permease protein MsbA	0,943		0,633	↓
Core oligosaccharide	B6EPB8	ADP-heptose-LPS heptosyltransferase II	0,870		0,424	↓
Exopolysaccharide biosynthesis	B6EHE0	Polysaccharide biosynthesis protein, putative epimerase/dehydratase	1,020		0,494	↓
	B6EHD8	O-antigen biosynthetic gene WbjF	0,610	↓	1,397	
	B6EPH8	Exported protein	1,056		1,788	↑
	B6EHC6	dTDP-glucose 4,6-dehydratase (rmlB)	1,107		1,535	↑
	B6EHC8	dTDP-6-deoxy-l-mannose-dehydrogenase (rmlD)	1,161		2,717	↑
	B6EHC0	N-acylneuraminate cytidylyltransferase	0,818		0,560	↓
	B6EPC9	Capsular polysaccharide biosynthesis protein	1,109		0,542	↓
LPS export and assembly	B6EMA5	Lipopolysaccharide export system protein LptC	1,454		1,598	↑
	B6EL51	LPS-assembly protein LptD	1,050		0,629	↓

684

685 Table 6: Differentially expressed outer membrane proteins.

	UniProt Accession	**Protein name**	**iTRAQ ratio *in vivo/in vitro***			
			Day 1		*Day 5*	
Up-regulated	B6EQ60	Membrane protein	0,553	↓	3,513	↑

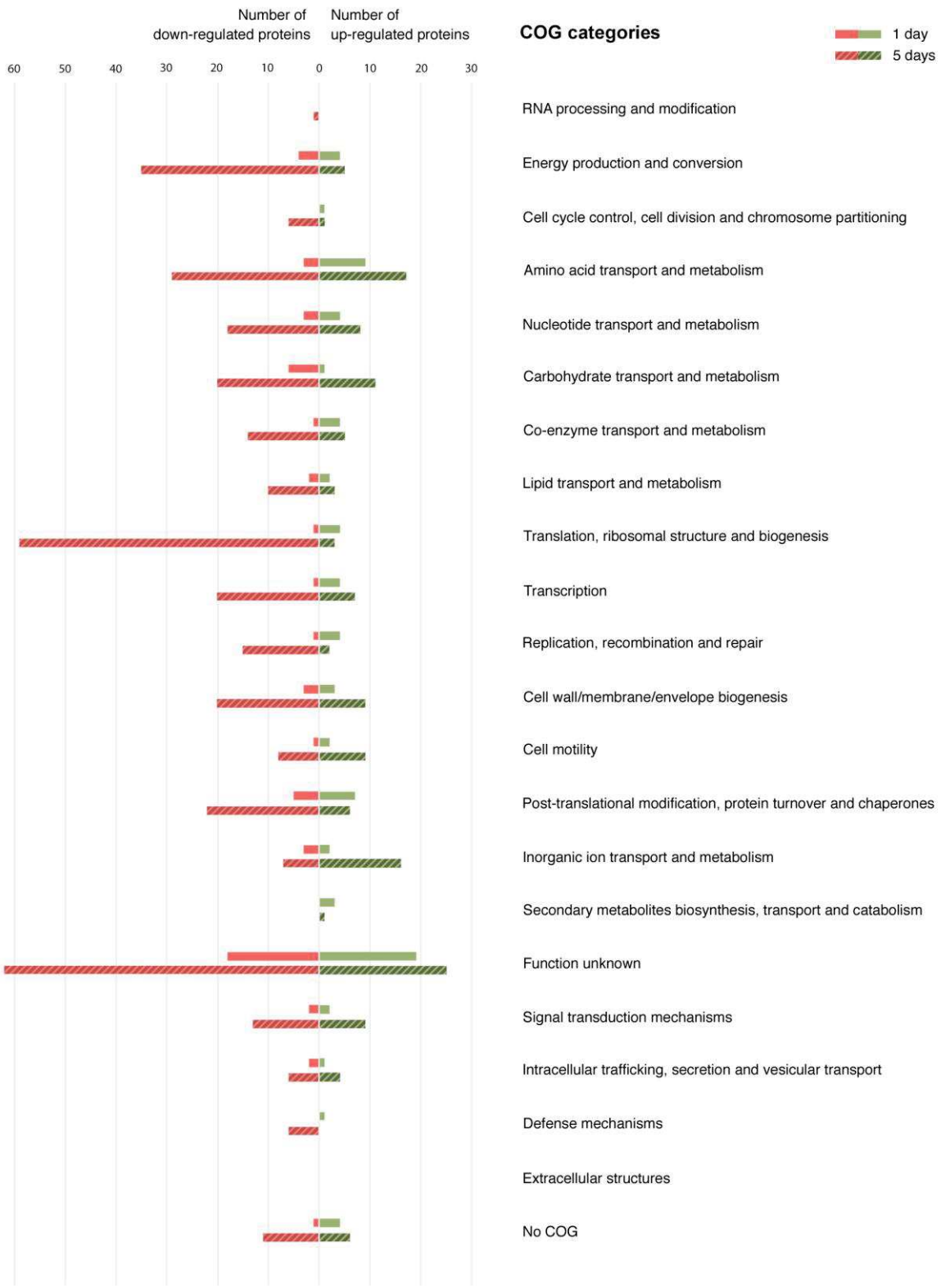
	B6EQU8	Putative membrane protein	1,152	3,082	↑
	B6EJ86	Putative outer membrane protein	1,000	2,411	↑
	B6EQ11	Outer membrane efflux protein	1,117	2,115	↑
	B6END2	Major outer membrane protein OmpU	1,032	2,180	↑
	B6EQ10	Outer membrane protein OmpA family	1,210	1,699	↑
	B6EM04	Putative membrane protein	1,200	1,613	↑
	B6EJ27	Outer membrane protein transport protein	1,137	1,561	↑
Down-regulated	B6EK62	Protein-export membrane protein SecF	1,097	0,661	↓
	B6ELY7	Putative membrane protein	1,286	0,652	↓
	B6EPD8	Membrane associated sulfatase	1,312	0,599	↓
	B6ERE5	Putative membrane protein	1,010	0,577	↓
	B6EP35	Putative integral membrane component of multidrug efflux system	0,912	0,571	↓
	B6ERE3	Putative membrane protein	0,938	0,536	↓
	B6EK49	Outer membrane protein assembly factor BamA	1,075	0,527	↓
	B6EJT1	Outer membrane protein assembly factor BamC	1,074	0,506	↓
	B6EGD3	Outer membrane protein assembly factor BamD	1,452	0,437	↓
	B6ERE4	Putative membrane protein	0,553 ↓	0,498	↓
	B6EJY9	Putative membrane protein	1,067	0,468	↓
	B6ENJ0	Putative membrane protein	1,200	0,460	↓
	B6EJB2	Putative membrane protein	0,845	0,439	↓
	B6ENL7	Putative membrane protein	1,026	0,348	↓
	B6ERA8	Membrane protein	1,460	0,341	↓

686

687 Table 7: Differentially expressed proteins involved in secretion.

Function	**UniProt Accession**	**Protein name**	**iTRAQ ratio *in vivo/in vitro***		
			Day 1	Day 5	
T2SS1	B6EGP4	General secretion pathway protein C	0,5858 ↓	0,959	
Exotoxin	B6EIN5	Hemolysin Vah2	0,663 ↓	0,767	
Protein export	B6EN68	Protein-export protein SecB	0,677	1,533	↑

688



689

690 Figure 1: Numbers of proteins differentially expressed in vivo relative to in vitro after
691 cultivation for one day (plain bars) and five days (striped bars). Proteins are classified
692 according to COG categories, and green bars represent up-regulated proteins while red
693 bars represent down-regulated proteins.

694

695 Supplementary Table S1: Differential expression of proteins related to flagellar motility,
696 pilus formation, iron acquisition, stress responses, LPS biosynthesis, outer membrane
697 proteins and secretion systems in samples cultivated in vivo and in vitro.

UniProt Accession no.	Description	iTRAQ ratio				Peptides identified
		In vitro: day 5/day 1	*In vivo:* day 5/day 1	Day 1: *in vivo/in vitro*	Day 5: *in vivo/in vitro*	
Flagellar motility						
B6EJG1	Flagellar P-ring protein (Basal body P-ring protein)	1,213	0,971	0,842	0,674	16
B6EJG2	Flagellar L-ring protein (Basal body L-ring protein)	1,191	1,220	1,154	1,182	7
B6EJG6	Basal-body rod modification protein FlgD	2,350	2,356	1,022	1,025	17
B6EJD2	Polar flagellar protein FliL	2,636	1,223	1,138	0,528	3
B6EJG3	Flagellar basal-body rod protein FlgG (Distal rod protein)	1,022	1,312	0,839	1,077	2
B6EJH1	Flagella basal body P-ring formation protein FlgA	0,920	1,293	1,206	1,694	3
B6EIB0	Sodium-driven polar flagellar protein MotA	1,067	0,685	1,222	0,784	7
B6EIB1	Sodium-driven polar flagellar protein MotB	1,066	0,836	0,880	0,691	7
B6ENQ4	Sodium-type flagellar protein MotY	0,733	0,527	0,909	0,654	3
B6EJG5	Flagellar hook protein FlgE	2,155	2,288	0,873	0,927	14
B6EJE5	Flagellar hook-associated protein 2 (HAP2)	2,660	1,159	1,637	0,713	8
B6EJD3	Polar flagellar hook-length control protein FliK	1,472	1,983	0,869	1,171	17
B6EJF8	Flagellar hook-associated protein type 3 FlgL	1,806	1,089	1,383	0,834	2
B6EJF7	Flagellin flaA	0,754	2,291	0,573	1,739	20
B6EJF5	Flagellin flaB	0,768	1,259	1,073	1,759	13
B6EJE9	Flagellin flaC	0,497	2,670	0,787	4,232	26
B6EJE8	Flagellin flaD	0,509	1,153	1,253	2,838	22
B6EJE7	Flagellin flaE	0,583	2,969	0,971	4,942	29
B6EKM1	Flagellin flaF	0,417	1,777	0,770	3,283	21
B6EJD5	Polar flagellum-specific ATP synthase FliI	0,966	0,625	1,477	0,956	10
B6EJD6	Polar flagellar assembly protein FliH	1,184	0,901	1,114	0,848	7
B6EJE3	Flagellar protein FliS	5,122	1,604	1,206	0,378	2
B6EJH3	Polar flagellar FlgN, putative chaperone	0,565	1,205	0,871	1,859	3
B6EJB7	Chemotaxis protein CheA	1,522	1,360	1,001	0,895	22
B6EJB3	Chemotaxis protein CheW	0,663	0,479	0,912	0,659	7
B6EJB6	Chemotaxis response regulator protein-glutamate methylesterase	1,112	0,819	1,040	0,767	19
B6EJB8	Protein phosphatase CheZ	1,334	0,620	0,841	0,391	6
B6EJB9	Chemotaxis protein CheY	0,866	0,360	1,220	0,507	3
B6EJG9	Chemotaxis protein methyltransferase CheR	1,282	0,903	1,483	1,045	6
B6EJH0	Chemotaxis protein methyltransferase CheV	0,885	0,501	1,419	0,803	10
B6EHI4	Methyl-accepting chemotaxis protein	0,592	1,006	0,773	1,314	8
B6EI76	Methyl-accepting chemotaxis protein	0,945	0,774	1,140	0,934	13
B6EIK8	Methyl-accepting chemotaxis protein	0,495	0,474	1,248	1,195	12
B6EK29	Methyl-accepting chemotaxis protein	0,718	0,454	1,217	0,769	5
B6ELZ9	Methyl-accepting chemotaxis protein	1,535	1,484	0,971	0,939	15
B6EMD7	Methyl-accepting chemotaxis protein	0,832	1,177	0,859	1,215	21
B6EQF2	Methyl-accepting chemotaxis protein	0,930	1,025	1,003	1,105	16
B6ES26	Methyl-accepting chemotaxis protein	0,911	0,519	1,313	0,748	8
B6ESS8	Methyl-accepting chemotaxis protein	0,955	1,092	1,058	1,209	20

B6EJG9	Chemotaxis protein methyltransferase CheR	1,282	0,903	1,483	1,045	6
B6EI94	Nitrate and nitrite sensing methyl-accepting chemotaxis protein	0,687	0,652	0,988	0,938	4
B6EH54	Methyl-accepting chemotaxis protein	0,472	0,594	0,961	1,209	3
B6ERY2	Methyl-accepting chemotaxis citrate transducer	0,935	1,604	0,830	1,424	3
B6EJD7	Flagellar motor switch protein FliG	2,135	0,777	1,020	0,371	15
B6EJD0	Flagellar motor switch protein FliN	1,272	1,224	1,144	1,100	4
B6EJD1	Flagellar motor switch protein FliM	0,766	0,518	0,771	0,521	4
B6EMA2	RNA polymerase sigma-54 factor (rpoN)	0,729	0,352	1,033	0,499	7
B6EJE0	Sigma-54 dependent response regulator (flrC)	1,306	0,574	0,893	0,392	7
B6EJE1	Histidine kinase (flrB)	0,900	0,629	1,004	0,702	9
B6EJE2	Sigma 54 dependent transcription regulator (flrA)	0,354	0,302	0,633	0,540	3
B6EJH2	Negative regulator of flagellin synthesis FlgM, anti-sigma28 factor	2,900	2,095	1,162	0,839	10
B6EJC2	Flagellar biosynthesis protein FlhF	1,075	0,646	1,055	0,635	19
Pili						
B6EM73	Type IV pilus, mannose-sensitive hemagglutinin D (MSHD)	1,819	1,429	1,024	0,804	13
B6EM79	Type IV pilus, prepilin-like protein (MSHB)	1,049	0,776	1,707	1,263	7
B6EM80	Type IV pilus, mannose-sensitive hemagglutinin A	1,018	2,140	0,954	2,006	9
B6EM81	Type IV pilus, mannose-sensitive hemagglutinin A	1,880	3,509	0,709	1,324	9
B6EMV0	Twitching motility protein PilT, type II/IV secretion system protein	0,862	0,733	0,908	0,772	4
B6EMV1	Twitching motility protein PilU, type II/IV secretion system protein	0,066	0,158	1,002	2,390	2
B6EIW5	Protein of FimV superfamily (Tfp pilus assembly)	0,855	0,356	0,752	0,313	12
B6EQZ2	Putative Flp pilus assembly protein	0,621	0,655	1,056	1,113	8
B6EQZ3	Type II/III secretion system protein	0,861	0,619	0,954	0,685	9
B6EQZ4	Putative lipoprotein	0,920	0,642	1,462	1,020	11
B6ER02	Membrane associated secretion system protein	0,721	0,970	1,058	1,424	8
B6EJ95	V10 pilin	0,652	0,792	0,857	1,042	3
Iron acquisition						
B6EN28	Heme transporter protein HuvB, putative periplasmic binding protein	0,866	2,713	1,472	4,610	15
B6EP92	Bisucaberin siderophore biosynthesis protein A	0,196	0,521	1,030	2,737	10
B6EP94	Bisucaberin siderophore biosynthesis protein C	0,266	0,565	1,154	2,451	3
B6EP95	TonB-dependent iron-siderophore receptor	0,323	1,491	0,979	4,519	19
B6EQQ5	Ferric aerobactin receptor	0,572	0,775	1,053	1,427	2
B6ESH4	Ferrioxamine B receptor	0,423	1,327	0,975	3,059	11
B6EQD3	Ferrichrome-binding periplasmic protein	0,577	1,516	1,055	2,771	4
B6EQ93	TonB dependent receptor	0,383	1,958	1,131	5,784	26
B6EQ95	TonB2 complex-associated transport protein C TtpC2/TolrR2	0,539	1,992	0,767	2,835	5
B6ESG9	TonB3 complex-associated transport protein C TtpC3/TolrR3	0,755	0,530	1,245	0,873	3
B6EJ17	Ferrous iron transport protein B	0,232	0,794	0,641	2,188	9
B6EL18	Iron(III) ABC transporter, ATP-binding protein	1,082	2,112	0,896	1,750	5
B6EL20	Iron(III) ABC transporter, periplasmic iron-compound-binding protein	0,568	3,388	0,930	5,550	17
B6EPW7	Putative iron transporter, ATP-binding protein	0,634	0,526	0,903	0,749	2
B6ET70	Iron ion ABC transporter ATP-binding protein	1,439	2,109	0,952	1,395	4

B6ET71	Uncharacterized protein	0,877	0,327	1,714	0,639	2
B6ET68	Iron ion ABC transporter, periplasmic component	0,473	2,803	0,850	5,039	18
B6EHL8	Ferric uptake regulation protein	2,024	0,827	1,408	0,575	6
B6EN59	Putative exported protein	0,416	0,687	0,936	1,546	6
B6EN60	Putative iron-regulated protein	0,723	3,380	1,412	6,604	11
B6EQ60	Membrane protein	0,378	2,398	0,553	3,513	3
B6ESD5	Putative lipoprotein	0,778	2,316	1,072	3,193	9
Stress response						
B6EGP6	33 kDa chaperonin (Heat shock protein 33 homolog)	0,919	0,698	1,170	0,889	10
B6EML9	ATP-dependent protease ATPase subunit HslU	0,602	0,440	0,947	0,693	15
B6EKA0	Chaperone protein DnaJ	0,286	0,183	0,961	0,617	6
B6EJZ3	Chaperone protein DnaJ	0,298	0,311	0,861	0,897	5
B6EKC3	Protease HtpX (Heat shock protein)	0,502	0,652	0,638	0,828	2
B6EKA3	Protein GrpE (HSP-70 cofactor)	2,214	3,252	0,791	1,162	11
B6EMQ7	RNA-binding protein Hfq	1,052	0,481	1,448	0,662	4
B6EGD6	Chaperone protein ClpB	2,036	0,721	1,844	0,653	5
B6EHJ9	Chaperone protein HtpG (Heat shock protein)	0,529	0,290	0,926	0,508	33
B6EN38	Putative heat shock protein	0,578	0,586	1,050	1,064	9
B6EI33	Heat shock protein	2,211	7,627	0,657	2,268	3
B6EKA1	Chaperone protein DnaK (HSP70)	2,338	2,442	0,904	0,944	59
B6EMG9	Chaperone protein DnaK (HSP70)	2,139	0,880	1,305	0,537	49
B6EMM0	ATP-dependent protease subunit HslV	1,052	1,143	0,900	0,979	5
B6EGS9	Small heat shock protein	2,871	0,543	1,325	0,251	7
B6EMG8	Small heat shock protein IbpA	2,631	0,789	1,089	0,327	2
B6EHK6	Lon protease (ATP-dependent protease La)	0,234	0,227	1,002	0,971	12
B6EHK3	ATP-dependent Clp protease proteolytic subunit	1,966	4,541	0,852	1,967	9
B6EGV0	Universal stress protein	1,438	0,496	1,008	0,347	5
B6ELY8	Exported serine protease	1,971	0,773	0,966	0,379	19
B6EKY5	Autonomous glycyl radical cofactor	0,848	4,255	0,858	4,305	11
B6EP01	Cold-shock protein	0,759	2,217	1,028	3,003	11
B6EL71	Cold-shock protein	0,654	0,710	1,521	1,651	2
B6EIX2	Cold shock-like protein CspD	0,552	1,850	1,912	6,406	2
B6EMP8	Ribonuclease R	0,408	0,154	1,128	0,426	7
B6ERN5	Cold shock-like protein	2,345	2,651	0,992	1,122	11
B6ESU0	Cold shock-like protein CspG	0,606	1,624	0,902	2,418	11
B6ELF5	Dihydrolipoyl dehydrogenase	0,920	0,570	0,866	0,536	24
B6ENZ4	Thiol:disulfide interchange protein	2,423	1,972	0,867	0,706	10
B6EP79	Glutathione reductase	0,805	0,689	0,915	0,783	13
B6EPA5	Thioredoxin	2,417	2,961	1,124	1,378	6
B6EMW9	Thiol:disulfide interchange protein	2,833	2,012	0,987	0,701	15
B6EHI9	Thioredoxin	2,361	1,715	1,147	0,833	2
B6ELB1	Putative glutaredoxin	1,504	1,444	1,070	1,028	9
B6EMJ5	Glutaredoxin-3 (Grx3)	0,853	0,476	1,112	0,621	2
B6ENN6	Thioredoxin 2	0,995	0,606	2,216	1,350	2
B6ENQ0	Glutaredoxin	2,720	2,132	0,838	0,657	4
B6EH64	Glutaredoxin 2	0,699	0,606	0,751	0,651	5
B6EHQ4	Glutaredoxin 1	2,417	5,369	0,661	1,468	7
B6EJ33	Thiol:disulfide interchange protein DsbE	1,346	0,724	1,103	0,593	4
B6EK71	Putative peroxidase/antioxidant	1,660	2,331	0,936	1,314	17
B6EM33	Dihydrolipoyl dehydrogenase	1,016	0,804	1,148	0,909	17

B6EM34	Hybrid peroxiredoxin (Thioredoxin reductase)	1,354	2,655	0,734	1,438	18
B6EN77	Thiol:disulfide interchange protein DsbD	1,768	1,058	1,015	0,607	10
B6ENT7	Soluble pyridine nucleotide transhydrogenase	0,791	0,545	0,863	0,594	6
B6ES84	Putative glutaredoxin	0,779	0,357	0,998	0,458	2
Q3LSM1	Catalase	0,429	0,253	1,031	0,608	25
B6ENP9	Superoxide dismutase	1,207	1,235	0,790	0,808	6
B6ERD2	Superoxide dismutase (Cu-Zn)	0,928	1,827	1,224	2,409	7
B6ENM9	Thioredoxin reductase	1,085	1,092	1,160	1,167	10
B6EJR3	Putative Dyp-type peroxidase	0,764	0,998	0,895	1,169	6
LPS biosynthesis						
B6EJW8	UDP-N-acetylglucosamine acyltransferase	1,234	0,792	0,946	0,607	7
B6ELH0	UDP-3-O-acyl-GlcNAc deacetylase	0,574	0,430	1,337	1,003	8
B6EK47	UDP-3-O-acylglucosamine N-acyltransferase	1,788	0,901	0,975	0,491	11
B6ES04	Tetraacyldisaccharide 4'-kinase	1,685	1,248	0,981	0,727	7
B6EPB9	3-deoxy-D-manno-octulosonic-acid transferase	0,506	0,624	0,853	1,052	2
B6EJW9	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	2,982	1,558	0,875	0,457	6
B6ES05	Lipid A export ATP-binding/permease protein MsbA	0,789	0,530	0,943	0,633	14
B6EMA7	Arabinose 5-phosphate isomerase	1,287	1,375	1,148	1,226	10
B6EHH9	3-deoxy-D-manno-octulosonic acid 8-phosphate synthase	2,398	1,573	1,109	0,727	15
B6EMA6	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase KdsC	1,554	1,849	1,077	1,282	10
B6ES02	3-deoxy-manno-octulosonate cytidylyltransferase	0,872	1,543	0,749	1,326	14
B6ELZ7	Bifunctional protein HldE	0,947	0,992	0,947	0,992	2
B6EPB6	ADP-L-glycero-D-manno-heptose-6-epimerase	1,303	1,503	1,000	1,154	16
B6EPB8	ADP-heptose-LPS heptosyltransferase II	1,877	0,915	0,870	0,424	6
B6EPF4	Glycosyl tranferase	0,571	0,408	1,138	0,814	3
B6EHD6	Putative NAD dependent epimerase/dehydratase	0,983	1,477	0,800	1,201	20
B6EHE0	Polysaccharide biosynthesis protein, putative epimerase/dehydratase	0,278	0,135	1,020	0,494	2
B6EPI2	Periplasmic protein involved in polysaccharide export	0,635	0,556	0,909	0,795	23
B6EHD8	O-antigen biosynthetic gene WbjF	1,211	2,770	0,610	1,397	2
B6EPH7	Putative outer membrane protein	0,871	0,878	1,152	1,161	13
B6EPH8	Exported protein	0,879	1,488	1,056	1,788	3
B6EPH9	Putative lipoprotein	0,691	0,731	1,129	1,194	3
B6EHD9	Galactosyl transferase, capsular polysaccharide synthesis enzyme	0,495	0,464	1,129	1,059	2
B6EPI1	Putative membrane protein	0,607	1,039	0,507	0,869	4
B6EHC6	dTDP-glucose 4,6-dehydratase (rmlB)	1,509	2,093	1,107	1,535	19
B6EHC7	Glucose-1-phosphate thymidylyltransferase (rmlA)	1,506	1,827	0,943	1,143	12
B6EPD6	Glucose-1-phosphate thymidylyltransferase (rmlA)	1,961	1,170	1,212	0,723	11
B6EPD7	dTDP-glucose 4,6-dehydratase (rmlB)	1,951	1,581	1,034	0,838	15
B6EHC8	dTDP-6-deoxy-l-mannose-dehydrogenase (rmlD)	0,669	1,567	1,161	2,717	9
B6EHC9	dTDP-6-deoxy-D-xylo-4-hexulose-3,5-epimerase (rmlC)	0,498	0,556	1,002	1,118	7
B6EPD4	dTDP-4-dehydrorhamnose 3,5-epimerase (rmlC)	1,544	1,024	1,340	0,889	7
B6EHB8	Putative capsular polysaccharide biosynthesis protein NeuD	1,226	0,929	1,083	0,820	7
B6EHB9	Putative sialic acid synthase NeuB	1,261	1,022	1,042	0,844	18
B6EHC0	N-acylneuraminate cytidylyltransferase	0,524	0,359	0,818	0,560	2
B6EPC2	Putative outer membrane protein	0,371	0,396	0,885	0,946	16
B6EPC4	Nucleotidyl transferase	1,710	1,318	1,420	1,095	12

B6EPC5	Putative acetyltransferase	0,866	0,908	0,954	1,000	3
B6EPC6	N-acetylneuraminic acid synthase	1,287	1,452	0,772	0,870	14
B6EPC7	UDP-N-acetylglucosamine 2-epimerase	1,815	1,124	1,371	0,849	14
B6EPC8	DegT/DnrJ/EryC1/StrS aminotransferase	1,584	1,090	1,017	0,700	13
B6EPC9	Capsular polysaccharide biosynthesis protein	1,219	0,596	1,109	0,542	15
B6EPB7	O-antigen ligase WaaL	1,289	0,821	1,184	0,755	5
B6EPI3	O-antigen length determinant protein	0,811	0,831	1,100	1,127	9
B6EMA4	Lipopolysaccharide export system protein LptA	0,991	1,539	0,887	1,378	7
B6EMA5	Lipopolysaccharide export system protein LptC	1,106	1,215	1,454	1,598	5
B6EL51	LPS-assembly protein LptD	1,326	0,794	1,050	0,629	10
B6EIN1	LPS-assembly lipoprotein LptE	1,884	1,275	1,270	0,860	8
Outer membrane proteins						
B6EJ86	Putative outer membrane protein	0,298	0,717	1,000	2,411	15
B6END2	Major outer membrane protein OmpU	1,194	2,522	1,032	2,180	14
B6ELZ6	Outer membrane protein TolC	1,802	2,330	1,015	1,313	15
B6ERE3	Putative membrane protein	2,196	1,255	0,938	0,536	20
B6EHE3	Putative outer membrane protein	1,382	2,171	0,738	1,159	3
B6EGZ0	Outer membrane protein assembly factor BamB	1,463	1,259	1,086	0,935	14
B6EP35	Putative integral membrane component of multidrug efflux system	1,212	0,760	0,912	0,571	21
B6EPC2	Putative outer membrane protein	0,371	0,396	0,885	0,946	16
B6EJT1	Outer membrane protein assembly factor BamC	2,518	1,187	1,074	0,506	9
B6EK49	Outer membrane protein assembly factor BamA	1,149	0,564	1,075	0,527	20
B6EPD8	Membrane associated sulfatase	1,189	0,543	1,312	0,599	14
B6EGY9	Putative outer membrane protein	1,433	1,167	0,994	0,809	13
B6EL61	Outer membrane protein	1,287	1,262	0,988	0,968	6
B6EQ11	Outer membrane efflux protein	0,943	1,784	1,117	2,115	16
B6EQ10	Outer membrane protein OmpA family	1,311	1,841	1,210	1,699	11
B6EJY9	Putative membrane protein	2,984	1,310	1,067	0,468	11
B6EK62	Protein-export membrane protein SecF	0,677	0,408	1,097	0,661	3
B6EK48	Chaperone protein skp	1,411	1,218	1,226	1,058	7
B6EJ27	Outer membrane protein transport protein	0,690	0,947	1,137	1,561	7
B6ERE5	Putative membrane protein	1,458	0,833	1,010	0,577	10
B6ELY7	Putative membrane protein	1,529	0,775	1,286	0,652	5
B6ES23	Outer membrane protein, OmpA family	1,382	1,055	1,154	0,881	6
B6EM04	Putative membrane protein	0,396	0,532	1,200	1,613	5
B6EQU8	Putative membrane protein	0,380	1,016	1,152	3,082	5
B6EPH7	Putative outer membrane protein	0,871	0,878	1,152	1,161	13
B6ENL7	Putative membrane protein	1,047	0,354	1,026	0,348	5
B6ERA8	Membrane protein	1,700	0,397	1,460	0,341	2
B6EPJ0	Outer membrane protein	1,149	0,537	1,279	0,598	5
B6EQ60	Putative heavy-metal transporter	0,378	2,398	0,553	3,513	3
B6EPV6	Thiamine-binding periplasmic protein	1,006	1,923	0,702	1,342	3
B6EGD3	Outer membrane protein assembly factor BamD	3,025	0,911	1,452	0,437	5
B6ENJ0	Putative membrane protein	1,624	0,623	1,200	0,460	3
B6EJB2	Putative membrane protein	1,135	0,590	0,845	0,439	4
B6ERE4	Putative membrane protein	0,627	0,565	0,553	0,498	4
Secretion systems						
B6ELZ6	Outer membrane protein	1,802	2,330	1,015	1,313	15

B6EPY7	Membrane signal transduction protein	0,441	0,593	0,853	1,147	2
B6EGP0	General secretion pathway protein G	1,455	1,942	1,041	1,389	3
B6EGP3	General secretion pathway protein D	0,677	0,540	0,856	0,683	9
B6EGP4	General secretion pathway protein C	0,786	1,287	0,586	0,959	4
B6EQ12	Hemolysin-type calcium-binding protein	1,311	1,041	1,023	0,812	3
B6EIN5	Hemolysin	0,583	0,674	0,663	0,767	2
B6EN68	Protein-export protein	1,263	2,862	0,677	1,533	7

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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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ABSTRACT

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%

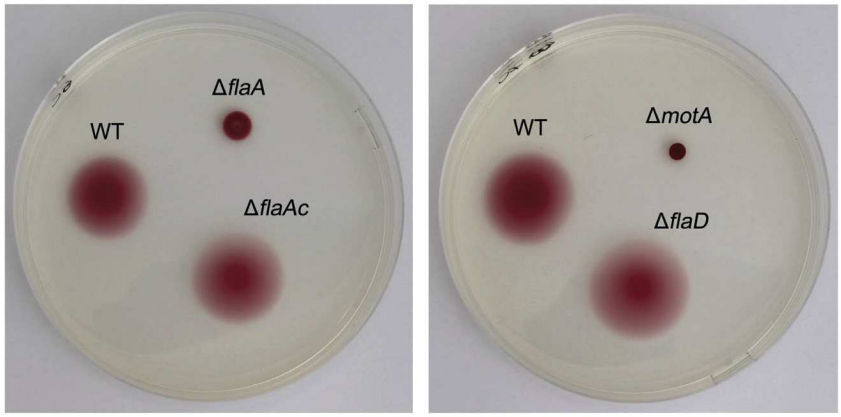

Fig. 1. Motility assay showing zonal expansion of strains grown in 2.5% NaCl at 8 °C for five days. Compared to wild type, Δ*flaA* displayed reduced motility, while Δ*motA* appeared non-motile. Δ*flaD* and Δ*flaAc* did not differ from wild type.

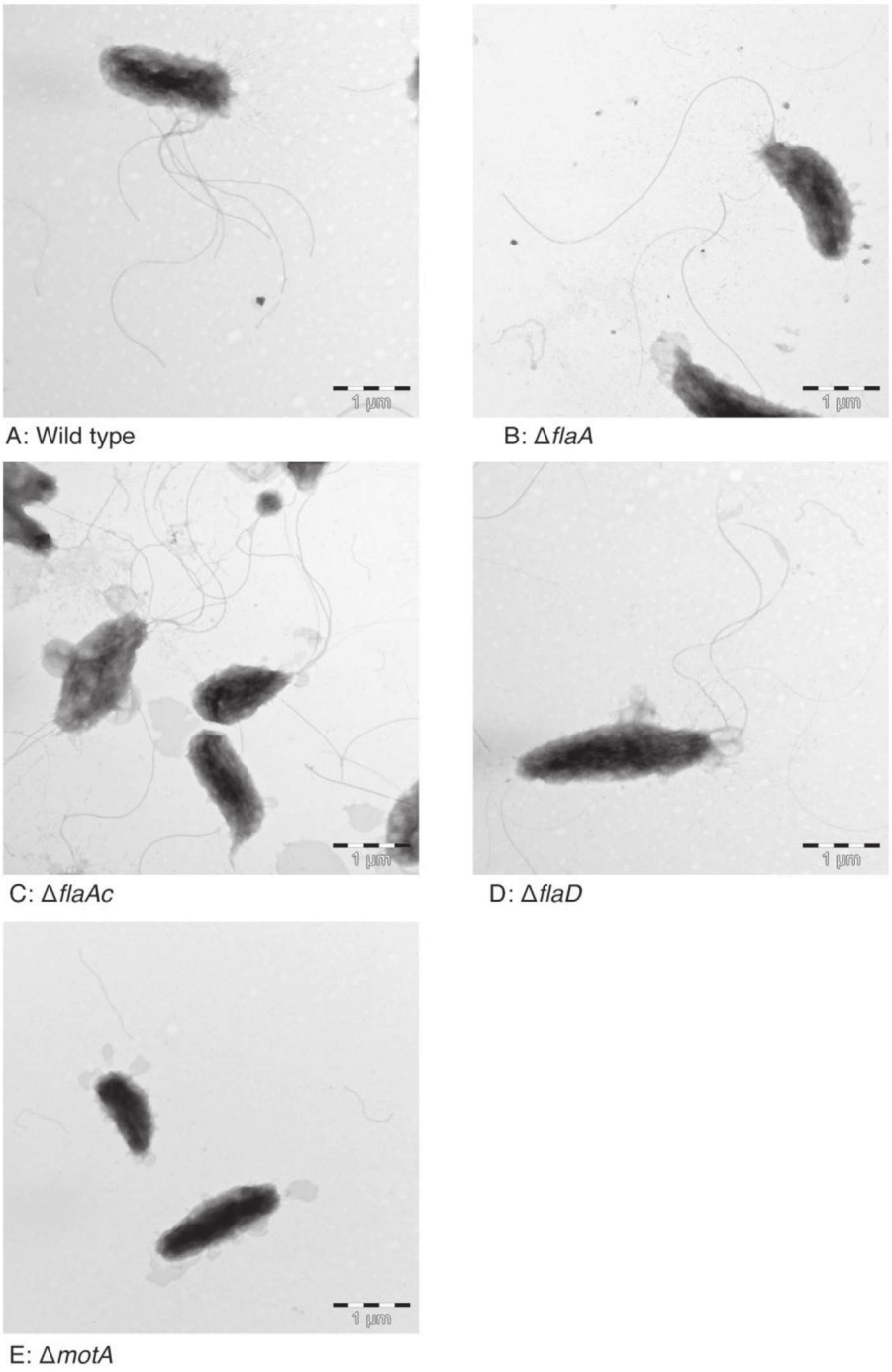

Fig. 2. Transmission electron micrographs showing flagellar structures of *Al. salmonicida* LFI1238 wild type (A), Δ*flaA* (B), Δ*flaAc* (C), Δ*flaD* (D) and Δ*motA* (E) strains. All cells were grown in LB3 at 8 °C (150 rpm) overnight before imaging.

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

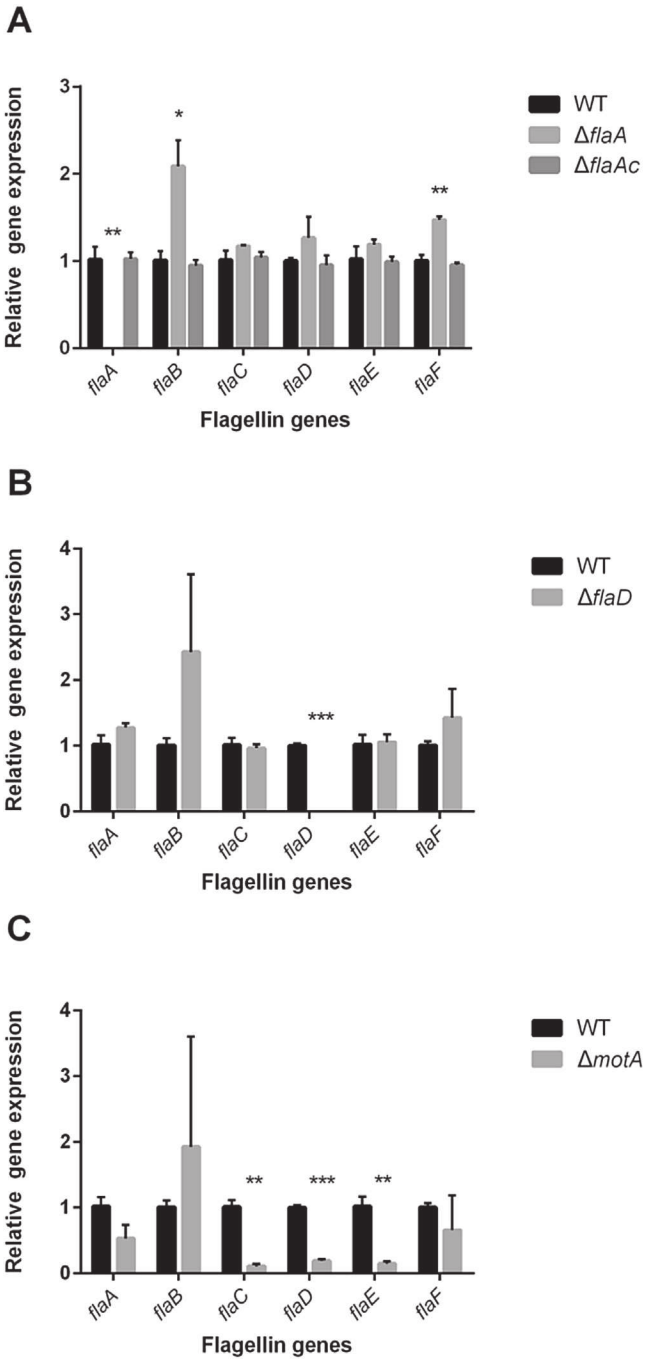

Fig. 3. Transcription of flagellin genes *flaABCDEF* in strains Δ*flaA*, Δ*flaAc* (A), Δ*flaD* (B) and Δ*motA* (C) relative to wild type. Statistical analysis was performed using Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), comparing transcription levels of each flagellin gene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

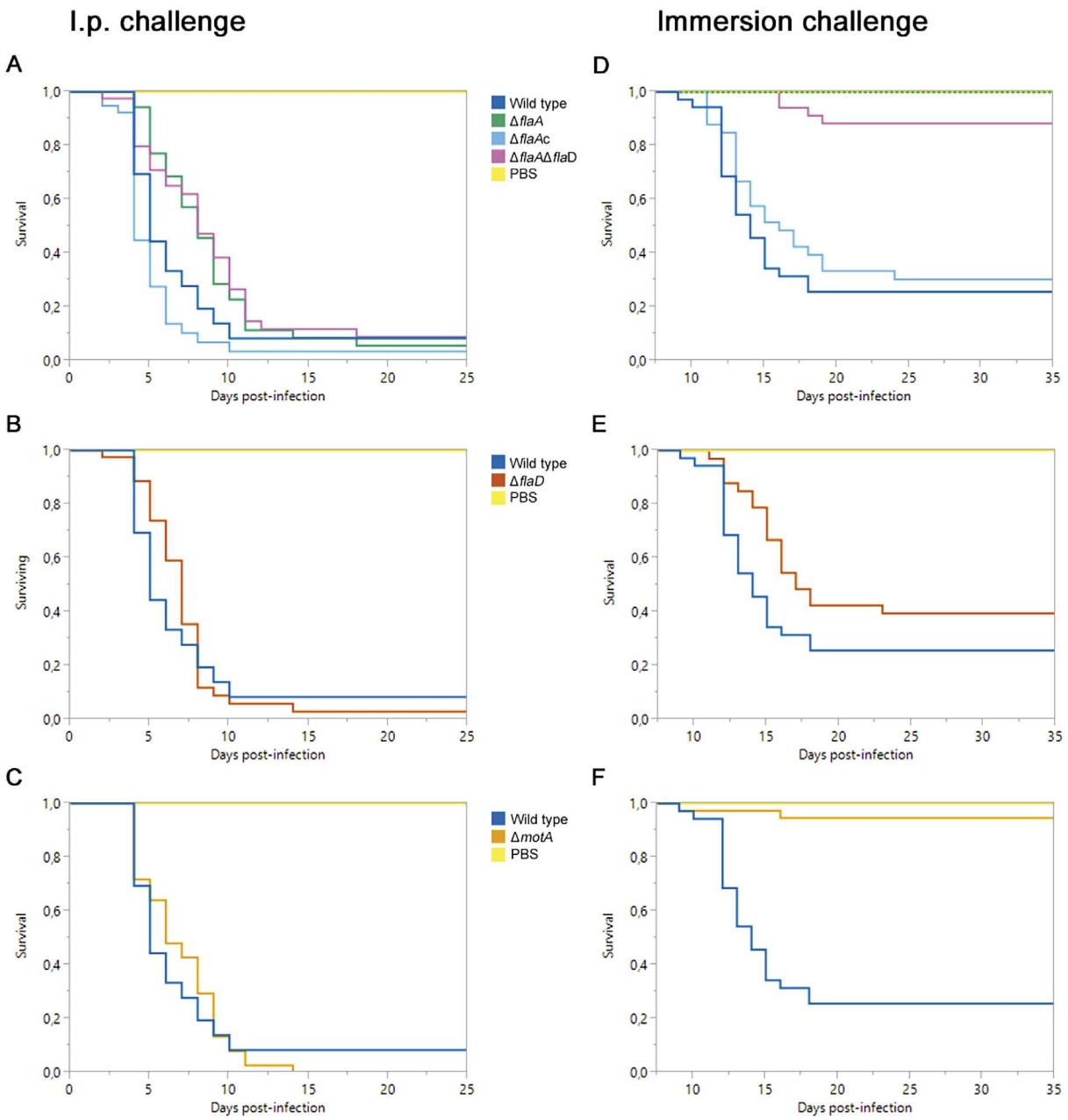

Fig. 4. Survival plots after challenge of Atlantic salmon by i.p. injection (A, B, C) and immersion (D, E, F) with *Al. salmonicida* LFI1238 wild type (blue), Δ*flaA* (green), Δ*flaAc* (light blue), Δ*flaA*Δ*flaD* (purple), Δ*flaD* (red) and Δ*motA* (orange). (For interpretation of the references to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

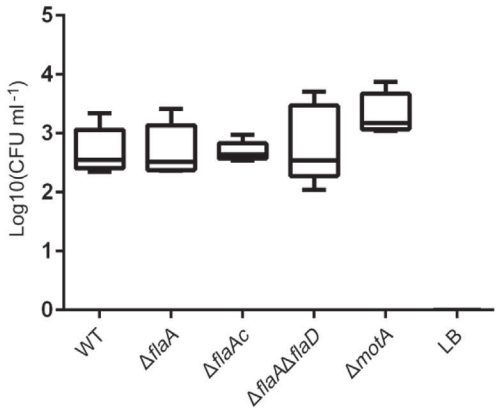

Fig. 5. Bacteria isolated from blood of fish challenged by immersion for ten minutes in bacterial suspension of wild type (WT), Δ*flaA*, Δ*flaAc*, Δ*flaA*Δ*flaD* or Δ*motA*. Blood was sampled fifteen minutes after challenge. Data are shown as box-and-whiskers plots representing log-transformed colony-forming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

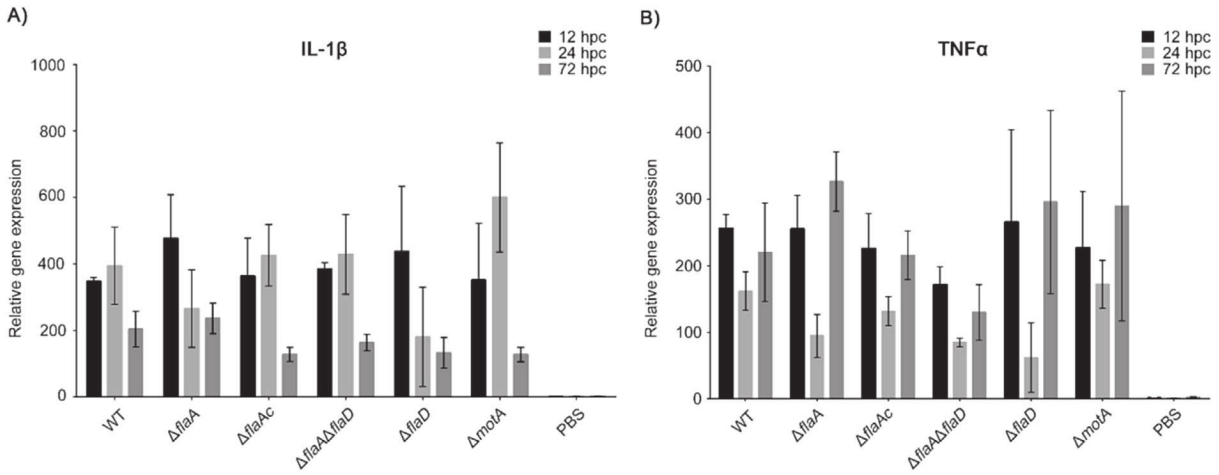

Fig. 6. Relative expression of pro-inflammatory cytokines IL-1β (A) and TNFα (B) in fish challenged with *Al. salmonicida* LFI1238 wild type (WT), Δ*flaA*, Δ*flaAc*, Δ*flaA*Δ*flaD*, Δ*flaD*, Δ*motA* and PBS. Fish were sampled 12, 24 and 72 h post challenge (hpc), and expression data are shown as mRNA 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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Aliivibrio salmonicida requires O-antigen for optimal virulence in Atlantic salmon (*Salmo salar* L.)

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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 of importance 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 suggest that LPS is crucial for development of disease.

Highlights:

- WaaL of *Aliivibrio salmonicida* is an O-antigen ligase.
- LPS O-antigen is essential for the virulence of *Al. salmonicida*.
- Invasion of Atlantic salmon does not depend on LPS.
- LPS contributes to the immune response raised towards *Al. salmonicida.*

Key words: *Aliivibrio salmonicida*, Atlantic salmon, pathogenesis, cold-water vibriosis, O-antigen, lipopolysaccharide

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 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]. After

The pathogenesis of cold-water vibriosis is poorly understood, and few virulence factors have been described that can explain the tissue damage observed in moribund fish. One of the described virulence factors is VS-P1, a highly immunogenic outer-membrane complex consisting of LPS and a protein moiety, which is released both *in vitro* and *in vivo* [5–7]. Extracellular VS-P1 has been postulated to bind effector components of the host immune system and provide protection from complement-mediated killing and phagocytosis [5,6]. Although the mechanism of 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, 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].

66 *Al. salmonicida* harbors a short-chain LPS resembling that of rough-type bacteria [10,11]. The
67 organism is described as serologically homogenous [7,12,13], and two serotypes are
68 recognized. Serotype C1 has predominantly been isolated from Atlantic salmon, and serotype
69 C2 has been isolated from diseased cod [14]. The LPS structures of the two serotypes are
70 closely related, and C2 differs from C1 only by the absence of a 4-amino-4,6-dideoxy-a-D-
71 galacto-pyranose (Fuc*p*4NBA) residue [15]. Although the alteration in LPS structure affects the
72 antigenicity of the bacterium, both serotypes are capable of causing disease [14]. Interestingly,
73 the LPS structure of *Al. salmonicida* contains a modified legionaminic acid (α-Non*p*A), a
74 nonulosonic acid that structurally resembles sialic acids [16–18]. In several pathogenic bacteria,
75 microbial decoration with sialic acids or other nonulosonic acids may function as a form of
76 molecular mimicry that dampens innate immune responses, masks the microbial presence from
77 the host immune system, or changes host cell specificity [19]. However, a role for legionaminic
78 acid in the pathogenesis of cold-water vibriosis has not been described.

79 In contrast to higher vertebrates, fish are resistant to endotoxic shock [20]. Nevertheless, LPS
80 has been found to stimulate the production of cytokines and influence cellular and humoral
81 immunity in several fish species [20]. LPS injected in fish is mainly taken up by the anterior and
82 posterior kidney and spleen [20]. After intravenous (i.v.) administration of *Al. salmonicida* LPS in
83 Atlantic cod (*Gadus morhua* L.), Seternes and co-workers found an accumulation of LPS in heart
84 tissue, which they believed was endocytosed by endocardial endothelial cells through a
85 scavenger-receptor-mediated mechanism [21]. Also, LPS of fish-pathogenic bacteria is reported
86 to participate in resistance to complement-mediated killing, phagocytosis, and in adhesion [22–
87 25].

The sequenced genome of *Al. salmonicida* LFI1238 contains a 29 kb perfect duplication encoding 27 genes [26]. The majority of these genes are predicted to encode products involved in biosynthesis of LPS. Previously, Hjerde and co-workers have postulated a gene-dosage effect for the duplicated genes, leading to an increase in LPS production [26]. To investigate the role of LPS and the gene duplication in the pathogenesis of cold-water vibriosis, we constructed in-frame deletion mutants lacking one or two copies of *waaL* (Δ*waaL* and Δ*waaL*Δ*waaL*), an O-antigen ligase found in the duplicated region that is predicted to be involved in LPS biosynthesis. In general, O-antigen ligases participate in LPS biosynthesis, binding O-antigen to the core oligosaccharide-lipid A complex [27]. By experimental challenge of Atlantic salmon with these mutants, we found that the LPS structure of *Al. salmonicida* is of major importance for virulence.

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), 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 or 2 µg/ml 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 hours. Growth curve experiments were performed in duplicates.

2.2 Mutagenesis

Mutagenesis was performed as earlier described [28,29]. 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

120 original locus of the gene. As *waaL* is present in two copies in the LFI1238 genome, a nested
121 approach was utilized in order to target both copies of the gene in the constructed double
122 mutant.

123 The deletion allele was constructed by overlap PCR. For LFI1238Δ*waaL*, segment waaL-A1A2
124 (247 nt) immediately upstream of *waaL* was amplified using primers waaL-A1 and waaL-A2.
125 Segment waaL-A3A4 (259 bp), consisting of the last 47 bp of *waaL* and the downstream
126 sequence, was amplified using primers waaL-A3 and waaL-A4. Restriction sites were included in
127 the 5' end of waaL-A1 (*SpeI*) and waaL-A4 (*XhoI*). The 5' end of waaL-A2 included a 15 bp
128 sequence complementary to waaL-A3. Fusion PCR creating segment waaL-A1A4 was performed
129 in a two-step manner. First, a PCR reaction was conducted with no added primers using
130 waaLA1A2 and waaLA3A4 as template and the following temperature settings: Denaturation at
131 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.
132 Immediately after, primers waaL-A1 and waaL-A4 were added, and an additional program was
133 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
134 min. The resultant construct *waaLA* and vector pDM4 were digested using restriction enzymes
135 *XhoI* and *SpeI* (New England Biolabs, Ipswich, MA, USA), and cut products were ligated (T4 DNA
136 ligase; Invitrogen) creating pDM4Δ*waaLA*. Purification of plasmids and gel extraction of DNA
137 segments were performed using QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit
138 respectively (both Qiagen, Hilden, Germany), according to the manufacturer's instructions.
139 Following ligation, pDM4Δ*waaLA* was introduced in *E. coli* S17-1 λpir by transformation.
140 Potential transformants were plated on LA1 (25CAM) and CAM-resistant colonies were verified
141 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 hours and 12°C overnight. Next, spotted cells were resuspended in 2 ml LB2.5 (no antibiotics) and incubated at 12°C for 24 hours. For selection of transconjugates, volumes of 30-100 µl were plated on LA2.5 (2CAM) and incubated at 12°C for 5 days. Potential transconjugates 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 hours. 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 phenol-water extraction procedure [30]. Cells harvested after growth on BA2.5 at 8°C for five days were suspended in PBS to OD_{600}: 0.6, washed once in PBS with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and resuspended in distilled water. Next, the solution was mixed with phenol (Sigma-Aldrich) (70°C) and incubated at 70°C for 15 minutes while vortexing frequently. After cooling on ice, the solution was centrifuged at 8500 g for 15 min (4°C). 100-200 µl of 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 sodium acetate (Sigma-Aldrich) was added to a final concentration of 0.5 M. The solution was then mixed with ten volumes of ethanol and precipitated over night at -20°C. Ethanol precipitation was repeated, and the precipitated LPS was resuspended in 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) and boiled for 5 min. Extracted LPS were resolved by SDS-PAGE using a 4-12% Criterion XT Bis-Tris gel (Bio-Rad) with the XT MES buffer system (Bio-Rad) ran at 200 V. Bands were visualized

185 by staining with the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes,
186 Inc., Eugene, OR, USA) according the manufacturer's instructions.

187 2.4 Serum assay

188 Strains were exposed to serum of Atlantic salmon (*Salmo salar* L.) to investigate serum
189 resistance. Serum was collected from 17 Atlantic salmon smolt previously not exposed to *Al.*
190 *salmonicida* and pooled together. As a control, serum was heat-inactivated at 44°C for 20 min
191 to inactivate complement activity [31]. Strains were grown over night in LB0.9 at 8°C (200 rpm),
192 washed once in cold PBS and resuspended in the same buffer to OD_{600}: 0.2. For each strain, 25
193 µl of bacterial suspension and 75 µl untreated serum, heat-inactivated serum or LB0.9 were
194 mixed and incubated at 8 °C (50 rpm). After 0, 2, 24, 48 and 72 hours, colony-forming units
195 (CFU) were determined by serial dilution followed by plating on BA2.5. The experiment was
196 performed in triplicates and the results are presented relative to the starting amount as means
197 ± standard error of the mean (SEM).

198 2.5 Challenge experiments

199 Virulence of the Δ*waaL*, Δ*waaL*Δ*waaL* and wild type strains were determined in challenge
200 experiments by challenging Atlantic salmon (*Salmo salar* L.) through immersion or
201 intraperitoneal injection (i.p.) of bacterial suspension. Prior to challenge, the strains were
202 passaged in Atlantic salmon to avoid loss of pathogenicity due to passage on artificial substrates
203 [13]. Challenge doses were based on experience from earlier experiments (Nordmo 1997).
204 Experimental fish were kindly provided by Sørsmolt (Sørsmolt AS, Sannidal, Norway). Ahead of
205 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 minutes. 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 x 10^7 CFU ml^{-1}, Δ*waaL*Δ*waaL*: 4.87 x 10^6 CFU ml^{-1} and wild type: 4.97 x 10^6 CFU ml^{-1} sea water. After challenge, the volume of the tanks 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 x 10^7 CFU, Δ*waaL*Δ*waaL*: 2.59 x 10^7 CFU and wild type: 2.85 x 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 hours 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.*

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 ten minutes, fish were moved to 150 L holding tanks supplied with flow-through of sea water (8°C). At time points 15 minutes, 24 hours and 48 hours 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 ten minutes and homogenized in a Tissuelyser II (Qiagen) at 25 Hz for five minutes. After homogenization, samples were briefly incubated at room temperature, mixed with 200 µl chloroform and separated by centrifugation at 11 400 rpm for 20 minutes 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, 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 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 [33]. For each gene assayed, amplification efficiency of the qPCR reactions was calculated using LinRegPCR (version: September 2014) [34]. 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 twelve hours 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 the role of LPS in virulence, we constructed in-frame deletion mutants for a putative *waaL* O-antigen ligase, containing a Wzy_C superfamily domain (pfam04932) known to be involved in synthesis of O-antigen. 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 conducted mutagenesis 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 (10 kDa; Figure 1, open arrow), a second band of 11.5 kDa (Figure 1, filled arrow), in addition to several slower migrating bands. No differences were seen between the migration patterns of wild type and Δ*waaL*. For LPS of Δ*waaL*Δ*waaL*, the 11.5 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 (Figure 2). For the wild type strain, a minor increase in CFU was seen after two hours of serum exposure, followed by a reduction of approximately one log per 24 hours of incubation in serum. Cells incubated in heat-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 two hours 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 (Figure 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, 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 (Figure 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 minutes, 24 hours and 48 hours 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 (Figure 4). An increase was observed from 24 to 48 hours, 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 minutes and 24 hours after challenge, followed by an increase at 48 hours. However, in one fish sampled 24 hours after challenge and one fish sampled 48 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. 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 [33]. 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 (Figure 5A-C). For genes encoding IL-8 and complement component C3, a gradual increase in expression was seen (Figure 5D and 5E). 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 hours post challenge (hpc) was lower than in fish challenged with wild type due to high IL-6 expression in one individual of the wild type group. Also, the increases in C3 expression observed 24 and 72 hours post challenge (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 (Hjelmeland 1988; Espelid 1988). As LPS is found as part of the VS-P1 complex, we constructed in-frame deletion mutants lacking one or two copies of O-antigen ligase *waaL* in order to obtain an O-antigen deficient phenotype and increase the understanding of how VS-P1 is involved in virulence.

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 10 kDa, one band of 11.5 kDa and several slower migrating bands. A similar pattern has been observed in other *Vibrionaceae* spp. and is described to represent the core oligosaccharide, the core oligosaccharide plus O-antigen, and aggregative LPS forms seen as slower migrating bands [35–38]. In LPS isolated from the Δ*waaL*Δ*waaL* strain, the 11.5 kDa band was absent, indicative of a truncated structure.

Aliivibrio fischeri possesses one copy of an *waaL* O-antigen ligase [35], which exhibits 26% similarity with *waaL* of *Al. salmonicida* and also contains a Wzy_C superfamily domain. An *Al. fischeri* Δ*waaL* mutant produced a similar, truncated structure, interpreted by the authors as lacking the O-antigen group of its LPS [35]. Similarly, our results suggest that *waaL* of *Al. salmonicida* is an O-antigen ligase involved in ligation of the O-antigen group to the core-oligosaccharide structure.

Through experimental challenge of Atlantic salmon by immersion and i.p. injection, we found the Δ*waaL*Δ*waaL* strain to be less virulent than wild type bacteria, 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* was 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 defence mechanisms combating infections, including the complement system found in serum. In several known fish pathogens, LPS has been shown to provide serum resistance [39–41]. 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 [39,40,42], we found approximately 12% of the initial inoculum to still be viable after 24 hours 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 [43]. Thus, the lack of capsule and repeating O-antigen units in *Al. salmonicida* may explain the inability of growth in serum [10,44]. However, the slow rate of reduction is suggestive of some means of protection. Possibly, the sialic acid content of *Al. salmonicida* LPS may contribute to serum resistance in a similar manner to the sialylated LPS of gonococci [11,15,45], but the evidence for this is lacking. Alternatively, shedding of VS-P1/LPS may provide the complement system with a target without putting the cell envelope at risk.

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 minutes, 24 and 48 hours after 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

hours after challenge suggests that these strains are able to overcome the bactericidal factors present in serum 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* [46].

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 hours after challenge. This may indicate a reduced survival potential for Δ*waaL*Δ*waaL*, denoting a role in survival for the O-antigen moiety. 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* [47]. Furthermore, 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 [48]. The requirement for a high dose to overcome the defence 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 hours after 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 hours after 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 [49–51]. In the enteric pathogens *V. mimicus* and *V. cholerae*, the polysaccharide moiety of LPS is involved in hemagglutination [52]. 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 antigenic properties of *Al. salmonicida* LPS in challenge experiments following immunization with the O-antigen deficient mutant strain.

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662 extrahepatic expression of complement factor C3 in Atlantic salmon (*Salmo salar*), Fish Shellfish
663 Immunol. 23 (2007) 542–552. doi:10.1016/j.fsi.2007.01.002.

664

665

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

Strain or plasmid	Description	Reference
Aliivibrio salmonicida LFI1238	Wild type strain	[26]
Escherichia coli S17-1 λpir	Donor strain for conjugation	[53]
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*	[28]
pDM4Δ*waaLA*	pDM4 containing Δ*waaL* allele	*
pDM4Δ*waaLB*	pDM4 containing nested Δ*waaL* allele	*

667

668 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 *SpeI* 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 *XhoI* restriction site	
Primers for construction of LFI1238Δ*waaL* Δ*waaL:* 861 bp deletion targeting a region of *waaL* inside the deleted fragment of LFI1238Δ*waaL*	waaL-B1	ATCTCGAGGCGATTGTTAATAAGTAAAGCTC	5' end contains a *XhoI* restriction site	284 bp
	waaL-B2	CCACGTAAGAGTCAGGATAAATAATAGG		
	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 *SpeI* 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		

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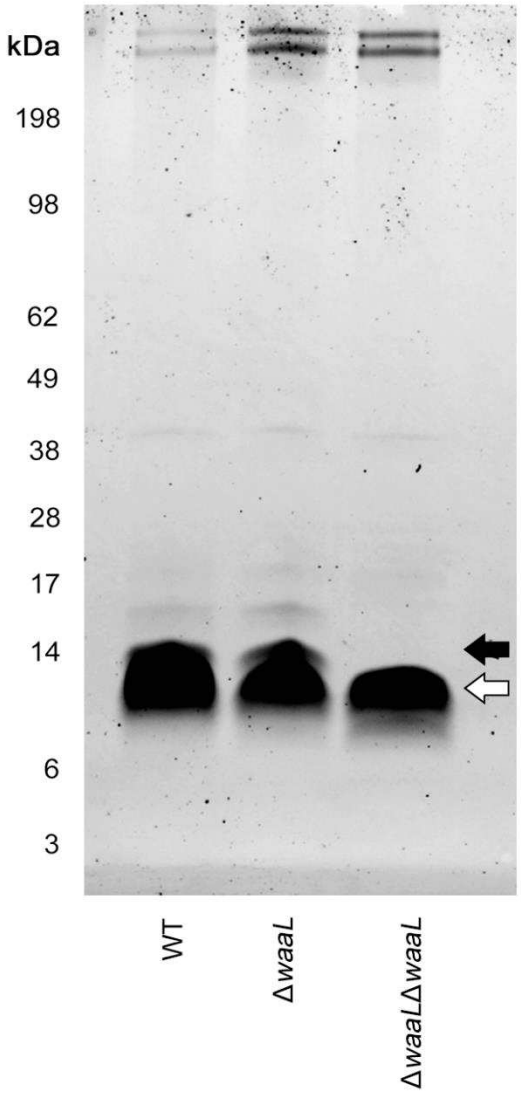
672

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

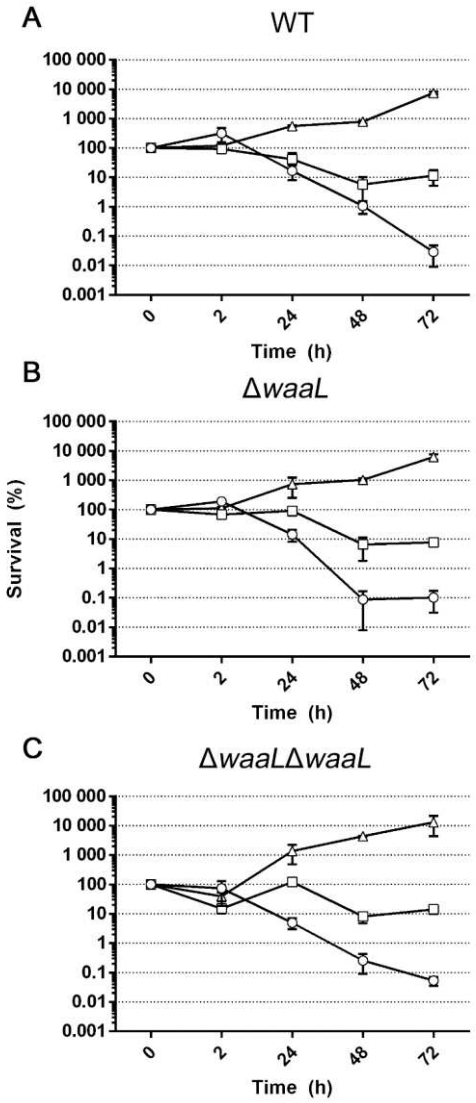
Description:	Primers:	Sequence (5' – 3'):	Construct size:	Ref.
Elongation factor 1Aa (AF321836.1)	EF1Aa-F	CCCCTCCAGGACGTTTACAAA	57 bp	[33]
	EF1Aa-R	CACACGGCCCACAGGTACA		
Elongation factor 1Ab (BG933853.1)	EF1Ab-F	TGCCCCTCCAGGATGTCTAC	57 bp	[33]
	EF1Ab-R	CACGGCCCACAGGTACTG		
β-actin (BG933897.1)	B-actin-F	CCAAAGCCAACAGGGAGAAG	91 bp	[33]
	B-actin-R	AGGGACAACACTGCCTGGAT		
Interleukin 1-β (AY617117.1)	IL-1b-F	GCTGGAGAGTGCTGTGGAAGA	73 bp	[54]
	IL-1b-R	TGCTTCCCTCCTGCTCGTAG		
Tumor necrosis factor α (NM_001123589.1)	TNFa-F	AGGTTGGCTATGGAGGCTGT	173 bp	[54]
	TNFa-R	TCTGCTTCAATGTATGGTGGG		
Interleukin 6 (XM_014143031.1)	IL-6-F	ACCAACAGTTTGTGGAGGAGTT	105 bp	[54]
	IL-6-R	AGCAAAGAGTCTTGGAGAGGTG		
Interleukin 8 (CXCL8) (XM_014187025.1)	IL-8-F	ATTGAGACGGAAAGCAGACG	136 bp	[54]
	IL-8-R	CGCTGACATCCAGACAAATCT		
Complement component 3 (XM_014186867.1)	C3-F	TCCCTGGTGGTCACCAGTACAC	157 bp	[55]
	C3-R	ATGATGGTGGACTGTGTGGATC		

674



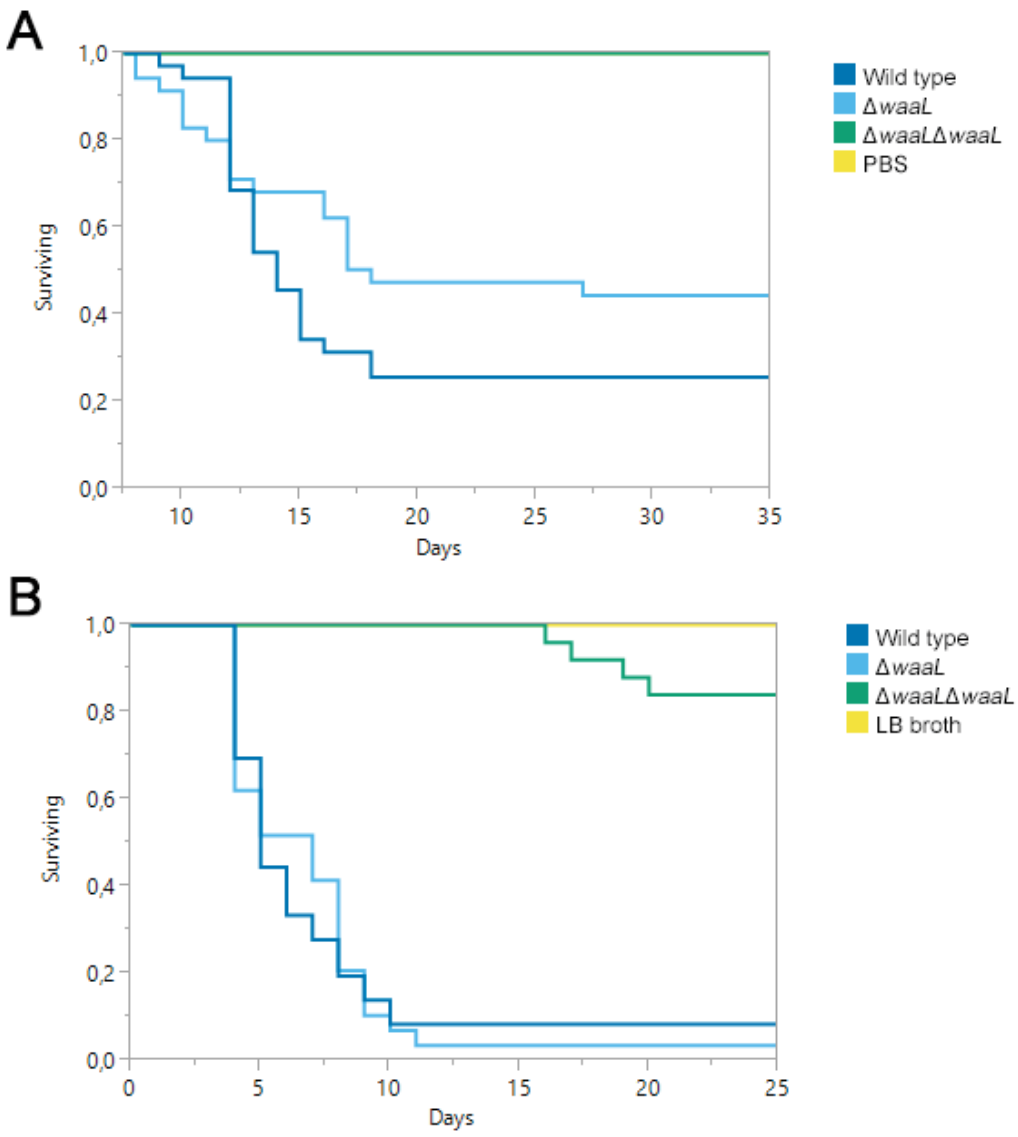
675

676 Figure 1. SDS-PAGE showing LPS structures of WT, Δ*waaL* and Δ*waaL*Δ*waaL*, extracted by
677 a phenol-water method. Arrows indicate a faster migrating high density band (open
678 arrow) and slower migrating low density band (filled arrow), of which the latter is absent
679 in the Δ*waaL*Δ*waaL* strain.



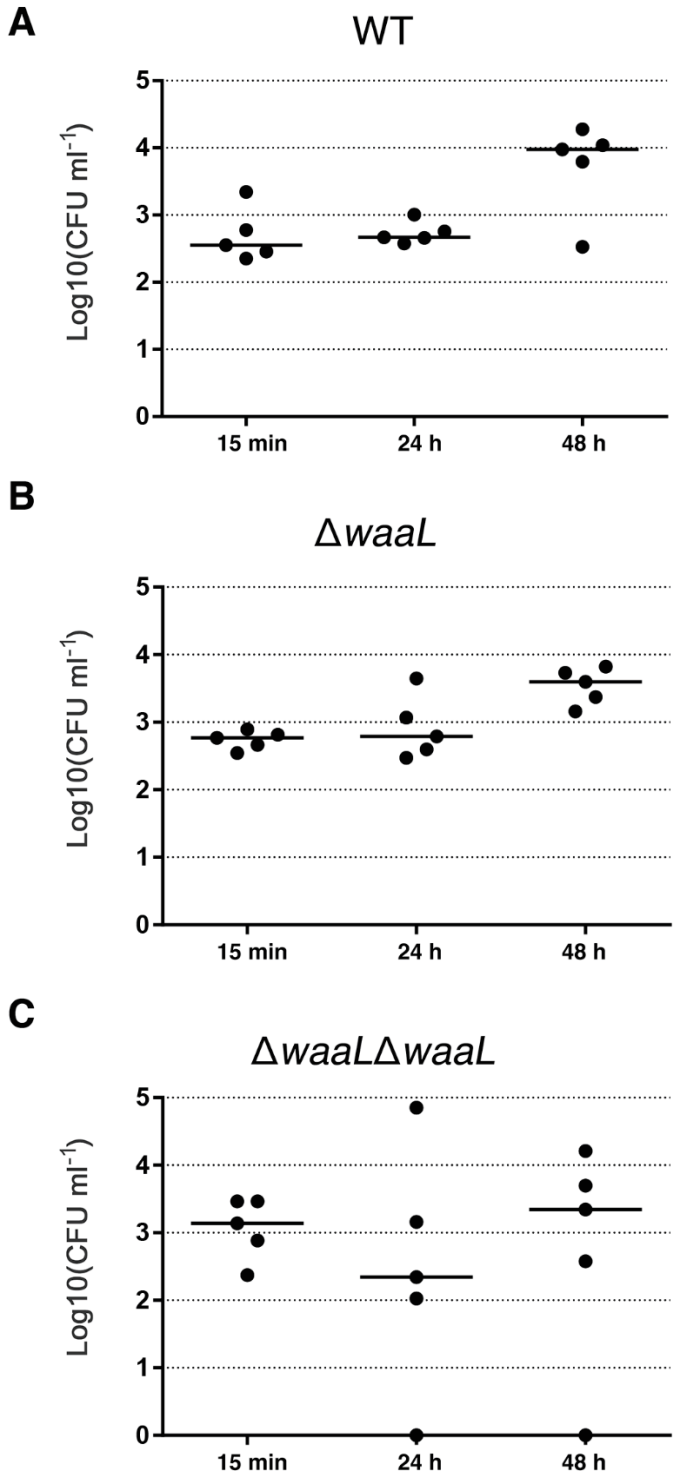
680

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



684

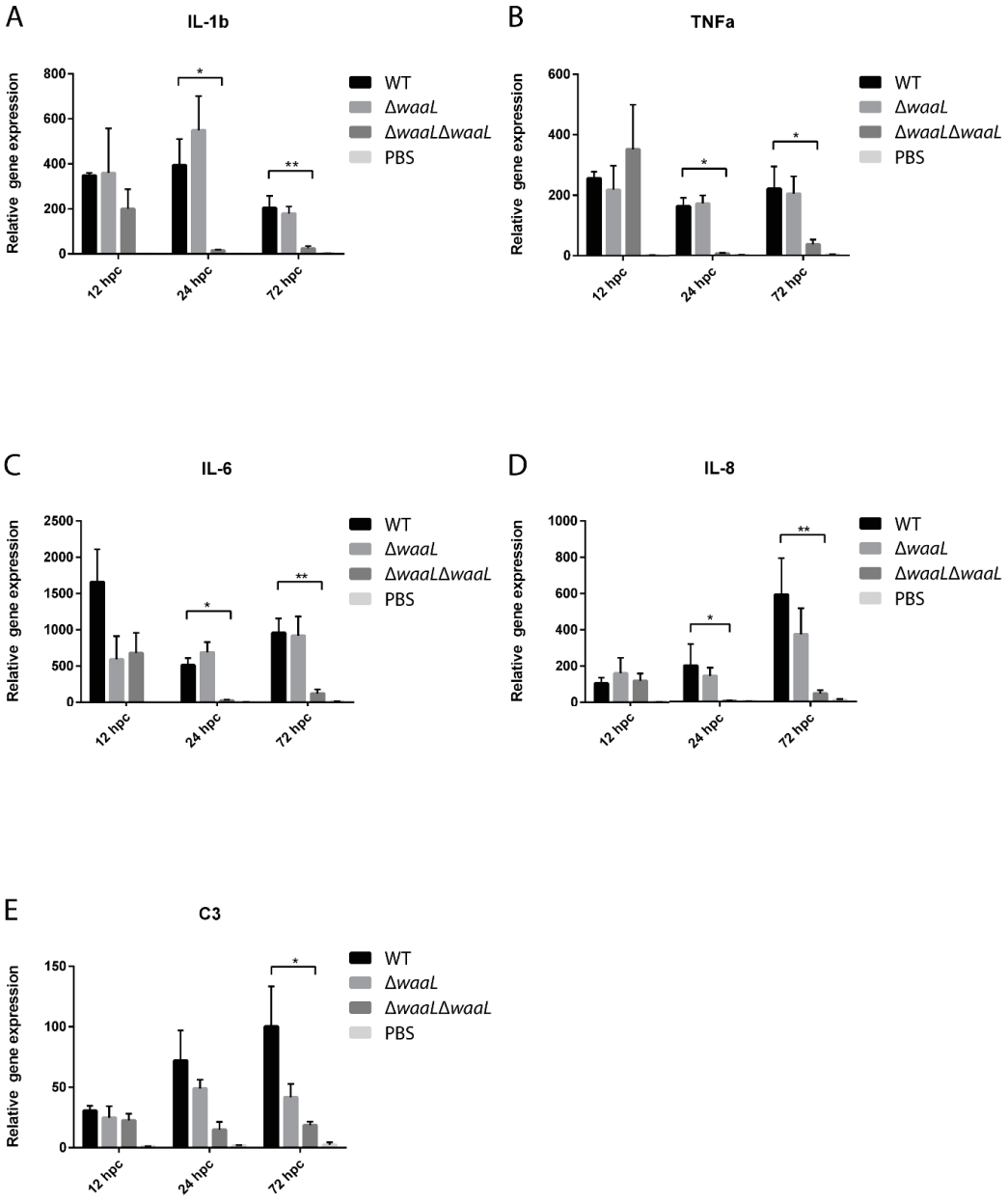
685 Figure 3: Survival plots for immersion (A) and intraperitoneal (B) challenge of Atlantic
686 salmon with the wild type (dark blue), Δ*waaL* (light blue) and Δ*waaL*Δ*waaL* (green)
687 strains. As a negative control, fish were mock challenged with PBS or LB broth (yellow).



688

689 Figure 4: Log-transformed median values for CFU ml^{-1} blood of fish challenged with the
690 wild type (WT; A), Δ*waaL* (B) and Δ*waaL*Δ*waaL* (C) strains. Blood was sampled 15 minutes,
691 24 and 48 hours after challenge. Bars represent range of values.

692



693

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