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Vibrio salmonicida pathogenesis analyzed by experimental challenge of Atlantic salmon (*Salmo salar*)

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ABSTRACT

Cold-water vibriosis (CV) is a bacterial septicemia of farmed salmonid fish and cod caused by the Gram-negative bacterium *Vibrio* (*Aliivibrio*) *salmonicida*. To study the pathogenesis of this marine pathogen, Atlantic salmon was experimentally infected by immersion challenge with wild type *V. salmonicida* and the bacterial distribution in different organs was investigated at different time points. *V. salmonicida* was identified in the blood as early as 2 h after challenge demonstrating a rapid establishment of bacteremia without an initial period of colonization of the host. Two days after immersion challenge, only a few *V. salmonicida* were identified in the intestines, but the amount increased with time. In prolonged CV cases, *V. salmonicida* was the dominating bacterium of the gut microbiota causing a release of the pathogen to the water. We hypothesize that *V. salmonicida* uses the blood volume for proliferation during the infection of the fish and the salmonid intestine as a reservoir that favors survival and transmission. In addition, a motility-deficient *V. salmonicida* strain led us to investigate the impact of motility in the CV pathogenesis by comparing the virulence properties of the mutant with the wild type LFI1238 strain in both i.p. and immersion challenge experiments. *V. salmonicida* was shown to be highly dependent on motility to gain access to the fish host. After invasion, motility was no longer required for virulence, but the absence of normal flagellation delayed the disease development.

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1. Introduction

Cold-water vibriosis (CV) is a bacterial septicemia of farmed salmonid fish [1–4] characterized by anemia and extended petechial hemorrhages, especially in the integument surrounding the internal organs of the fish, in the vent region and at the base of the pectoral, pelvic and anal fins. As its name indicates, the disease occurs mainly in late autumn, winter and early spring when the seawater temperature is below 10 °C [1]. Although affecting mainly farmed Atlantic salmon (*Salmo salar*), CV is identified in both farmed rainbow trout (*Oncorhynchus mykiss*) and wild caught captive and farmed Atlantic cod (*Gadus morhua*) [2,4].

The causal agent of CV is the cold- (psychrophilic) and salt-adapted (halophilic) bacterium *Vibrio* (*Aliivibrio*) *salmonicida*. This marine bacterium is a Gram-negative curved and motile rod which carries up to nine polar flagella [1]. *V. salmonicida* and its three closely related species *Vibrio fischeri*, *Vibrio logei* and *Vibrio wodanis* were recently proposed reclassified into a new genus, *Aliivibrio* gen. nov., resulting in the new name *A. salmonicida* [5]. The species designation *A. salmonicida* is, however, already occupied by the well-established abbreviation of *A. salmonicida*, the etiological agent of furunculosis in salmonids. To avoid possible nomenclature confusion, the name *V. salmonicida* will be used throughout this paper.

V. salmonicida shows a high potential for starvation and survival in the ocean environment and the numbers of *V. salmonicida* in fish farm seawater ranges from 12 to 43 bacteria/ml with concentrations being highest during the winter period when the total bacterial count in seawater generally is at the lowest [6–8]. The pathogen is suggested to be transmitted through seawater between salmonids either as bacterioplankton or on the surface of particles

Abbreviations: AFM, Atomic force microscopy; BA2.5, Blood agar with 5% ox blood and 2.5% NaCl; CFU, Colony forming units; CV, Cold-water vibriosis; LB1, Luria Bertani broth with 1% NaCl; LB3, Luria Bertani broth with 3% NaCl; OMVs, Outer membrane vesicles.

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[3,9,10]. *V. salmonicida* is detected in fish farms and fish farm sediments without a foregoing CV history, and in feces from fish that survived an experimental infection [6,8,11]. Based on these observations, an asymptomatic carrier state of the disease has been proposed, indicating that healthy salmon release bacteria into the environment. Additionally, a former study described non-pathogenic intestinal strains of *V. salmonicida* isolated in various amounts as parts of the ubiquitous intestinal microflora in Atlantic salmon, Atlantic cod, saithe (*Pollachius virens*), and Atlantic herring (*Clupea harengus*) [12].

Several studies have tried to uncover the pathogenicity of *V. salmonicida* [13–17]. The gills, skin and GI tractus have previously been suggested as the portal of entrance in *V. salmonicida* infections, but no clear conclusion has been made [11,12,18–20]. After challenge, but before the fish shows clinical signs of disease, *V. salmonicida* are only detected in the lumen of the capillaries. The first targets of *V. salmonicida* are reported to be the endothelial cells of the capillaries and leukocytes of the blood in which the bacteria are internalized. In the later stages of infection endothelial cells are completely disintegrated and actively proliferating bacteria can be detected in the extravascular space and in the surrounding tissue [16].

Flagellar motility helps the bacteria to reach the most favorable environments and to successfully compete with other microbes. These complex organelles also play an important role in adhesion to substrates and biofilm formation. Thus, motility is linked to colonization and virulence in several bacteria including species in the *Vibrio* group and attenuated virulence due to loss of motility have been thoroughly described [21–26]. Previous studies have shown that *V. salmonicida* motility is regulated by environmental factors such as osmolarity and temperature [27]. Although motility is suggested as a virulence factor of *V. salmonicida* [27], the direct impact of motility in the *V. salmonicida* pathogenesis has never been studied. In this work, we attempt to further elucidate the pathogenesis of *V. salmonicida* by including the contribution of motility to the bacterial virulence.

2. Material and methods

2.1. Bacterial strains and culture conditions

Strains used in this study are listed in Table 1. The *V. salmonicida* wild type strains NCMB2262 (type strain) and LFI1238 (genomic sequenced strain) were originally isolated from the head kidney of a CV diseased Atlantic salmon and Atlantic cod, respectively [1,28]. The intestinal strains of *V. salmonicida* were originally isolated from Atlantic salmon (2A4h, T3B1, T3B2, TA22, TA30), Atlantic cod ($PT2^T$, T6) and Atlantic herring (T2S1) [12]. Bacteria were grown on blood agar base No. 2 (Oxoid, Cambridge, UK) supplemented with 5% ox blood and 2.5% NaCl (BA2.5) or in Luria Bertani broth with 3% NaCl (LB3) with agitation (200 rpm) at 8 °C for 2–4 days unless otherwise stated. *Escherichia coli* S17-1 cells were cultivated in Luria Bertani broth or agar at 37 °C [29,30]. For selection of *E. coli* transformants or *V. salmonicida* transconjugants a final concentration of 25 μg/ml or 2 μg/ml chloramphenicol were added to the medium, respectively.

2.2. DNA isolation, PCR and 16S rDNA sequencing

For 16S rDNA analysis, total nucleic acids were extracted either by using the Qiagen DNAeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) or the MoleStrips DNA Blood Kit (Mole Genetics AS, Lysaker, Norway) according to the manufacturer's instructions. When using the MoleStrips DNA Blood Kit and before the samples were processed by the GeneMole® automatic nucleic acid extractor, the samples were first incubated with 180 μl lysis buffer (20 mM Tris HCl pH 8.0, 2 mM sodium EDTA, 1.2% Triton® X-100 and 20 mg/ml lysozym) at 37 °C for 30 min, following the addition of 100 μl of the solution from well 2 of the MoleStrips and 20 μl Proteinase K (Qiagen) with further incubation at 56 °C for 30 min. Amplification of 16S rDNA was performed using a bacterium-specific forward primer B27F and a universal reverse primer 1492R DNA obtained from Medprobe (Eurogentec, Liege, Belgium) [31,32]. The PCR reagents were obtained from Fermentas (Thermo Fischer Scientific, Waltham, MA, USA) and the PCR reactions were performed using standard protocols [33]. The primers used for sequencing are listed in Table 1.

Table 1
Bacterial strains, plasmids and primers used in this study.

Strain or primer	Description or sequence	Source or reference
Strains		
V. salmonicida		
LFI1238	Wild type; isolated from Atlantic cod, sequenced strain	[28]
NCMB2262	Wild type; isolated from Atlantic salmon, type strain	[1]
Δ*qrr*	LFI1238 containing an in-frame deletion in *qrr*	This study
MOT-	Δ*qrr* complemented with the LFI1238 *qrr* gene; Cmr	This study
2A4h	Isolated from head kidney of CV diseased Atlantic salmon	[12]
T2S1	Intestinal isolate from Atlantic herring	[12]
T3B1	Intestinal isolate from Atlantic salmon	[12]
T3B2	Intestinal isolate from Atlantic salmon	[12]
TA22	Intestinal isolate from Atlantic salmon	[12]
TA30	Intestinal isolate from Atlantic salmon	[12]
$PT2^T$	Intestinal isolate from Atlantic cod	[12]
T6	Intestinal isolate from Atlantic cod	[12]
E. coli		
S17-1	Donor strain for conjugation, λ-pir	[30]
Plasmids		
pDM4	Cm^r; suicide vector with an R6K origin (λ-pir requiring) and sacBR	[22]
pNQ705	Cm^r; suicide vector with an R6K origin (λ-pir requiring)	[22]
pDM4Δqrr	pDM4 containing a fragment of *qrr* harboring an internal deletion	This study
pNQ705qrr	pNQ705 containing wild type *qrr* and flanking sequences	This study
Primers (5′–3′)		
16S rDNA sequencing primers for identification of intestinal strains and verification of V. salmonicida		
B27F	AGAGTTTGATCATGGCTCAGA	[32]
U1492R	GGTTACCTTGTTACGACTTC	[31]
*Primers used for construction and verification of Δ*qrr *and MOT-:*		
Qrr A-F	TAACTCGAGCGATAAAGCGCAGCAACA	This study
Qrr B-R	AACCGTAATATACCGCCTTTGGCTTAAAGGGTC	This study
Qrr C-F	CGGTATATTACGGTTGGCTTC	This study
Qrr D-R	CGAACTAGTAAGAAGGAGCGAGTTATCAATC	This study
Qrr E-F	GGCAACATCAATAGAACCAT	This study
Qrr F-R	GGCTGATATTCTTGAATTGG	This study
Pnq-F	TAACGGCAAAAGCACCGCCGGACATCA	*
Pnq-R	TGTACACCTTAACACTCGCCTATTGTT	*

* Kindly provided by Professor Debra Milton, Umeå University, Sweden.

2.3. Construction of a motility-deficient strain of V. salmonicida LFI1238

During mutant construction experiments in *V. salmonicida* LFI1238, a motility-deficient Δ*qrr* mutant was observed. The *qrr* mutation was complemented by the insertion of a functional *qrr* gene into the original locus. This rescue mutant (MOT-) maintained the motility-deficient phenotype. The protocols for mutant

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