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Novel aspects of pathogenicity of *Aliivibrio salmonicida*

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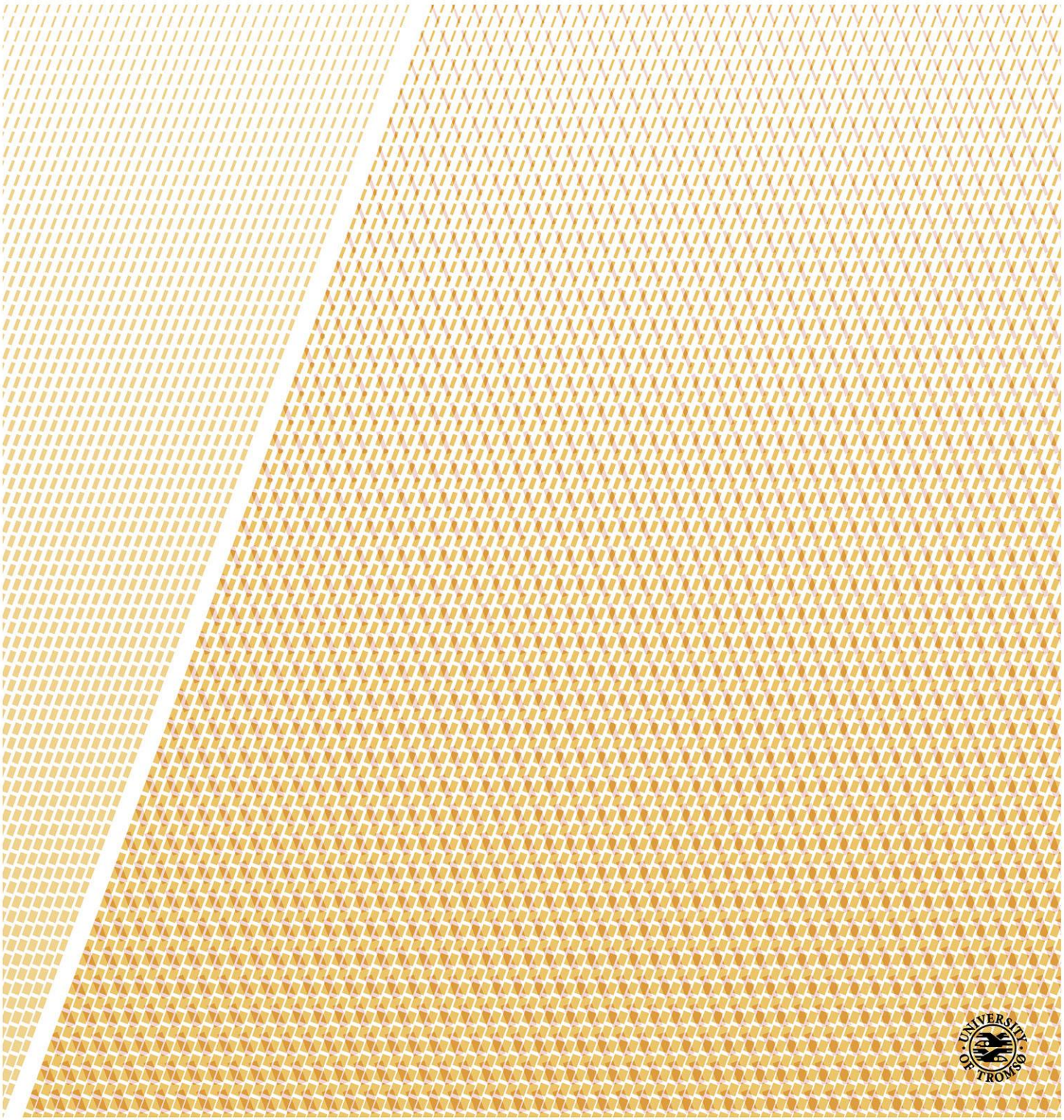
Alexander Kashulin

A dissertation for the degree of Philosophiae Doctor – March 2014



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ABBREVIATIONS

AHLs - acyl-homoserine lactones

CFU – colony forming units

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

CWV - cold water vibriosis

DR - direct target repeats

IM - intramuscular injections

IP - intraperitoneal injections

IR - terminal inverted repeats

IS – insertion sequence elements

LPS - lipopolysaccharides

MSHA - mannose sensitive-hemagglutinin pili

RC - Rolling-Cycle

S - serine

TCP - toxin co-regulated pili

TLR - Toll- like receptor

TNFα - tumour necrosis factor-alpha

UNG - Uracil-DNA N-glycosylase

Y - tyrosine

V. fischeri* - old taxonomic name used prior to December 2007

V. salmonicida* - old taxonomic name used prior to December 2007

V. logei* - old taxonomic name used prior to December 2007

V. wodanis* - old taxonomic name used prior to December 2007

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UNIVERSITY OF TROMSØ UiT

FACULTY OF SCIENCE AND TECHNOLOGY

Your reference:
Our reference:
Date: 13.03.2014

Statement of the author contribution

The intention of this letter is to describe the amount of work that PhD candidate **Alexander Kashulin** has contributed with to each of the three articles which are included in his thesis for the degree of Philosophiae Doctor, and provide a general description of the nature of his work. The three articles are listed below:

1. **Alexander Kashulin**, Henning Sørum. A novel in vivo model for rapid evaluation of *Aliivibrio salmonicida* infectivity in Atlantic salmon // Aquaculture. 15 January 2014., Volumes 420–421., Pages 112–118.

2. **Alexander Kashulin**, Henning Sørum. Early bacteremia in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) during immersion challenge with *Aliivibrio (Vibrio) salmonicida* // Submitted to the "Journal of Microbial Pathogenesis".

3. **Alexander Kashulin**, Henning Sørum, Erik Hjerde, Nils P. Willassen. IS Elements in Aliivibrio salmonicida LFI1238: Occurrence, Variability and Impact on Adaptability // Submitted to the "Gene".

Alexander Kashulin is the first author of all three papers. In all three papers he has, in collaboration with his supervisors, created the experimental design, performed all experimental work, analysed the results and made the draft manuscripts. **Alexander Kashulin** has completed all training that is required for a PhD degree, and has shown the ability to work independently.

Sincerely,

Nils Peder Willassen
Professor

Alexander Kashulin
PhD candidate

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ABSTRACT

Analysis of the fish health reports annually published online by the Norwegian Veterinary Institute (data available starting from 2005), have demonstrated a good epidemiological situation with Cold Water Vibriosis (CWV) in 2005-2010. During this 5 year period only 4 cases were detected in Norway. Starting from 2011 the situation began to change and only during 2011, 5 cases of CWV have been detected in Norway. In 2012 outbreaks of CWV occurred at 21 farms affecting vaccinated Atlantic salmon. In 2013, 13 outbreaks occurred. Regarded as a disease fully controlled by vaccination until 2011, CWV again became a considerable disease in the annual fish health reports. The motivation for this study originated from the hypothetical impact of mobile genetic elements on bacterial adaptation to pathogenicity in other host species than Atlantic salmon and the recent apparent inability of vaccination to provide acceptable protection against CWV. Analysis of nearly 90 research papers dedicated to CWC in 1975-2014 demonstrated a lack of important data on the early dynamics of the disease as well as a lack of clear information on the portal of CWV infection.

The aim of the current project was to fill in the gaps in the knowledge on early dynamics and portal of infection for Cold Water Vibriosis (CWV) as well as to characterize mobile genetic elements potentially driving adaptation of *Aliivibrio salmonicida* to pathogenicity. During the work on the project we have established a novel *in vivo* model for evaluation of Cold Water Vibriosis in Atlantic salmon (*Salmo salar*) and studied the role of different portals for initiation of CWV infection. The obtained results clearly indicated that skin is a major route of CWV infection. The experimental design reported in this study provides a new, rapid and cost-effective model for studying CWV and reveal new previously unknown characteristics of the pathogen. To further demonstrate the universal value of the model we present early dynamics of CWV infection in rainbow trout (*Oncorhynchus mykiss)* known to be less susceptible to CWV and hypothesize molecular prerequisites for efficient transfer of *A. salmonicida* into the blood of the two species. Complex analysis of IS elements (Vsa IS) present in the genome of *A. salmonicida* LFI1238 allowed us to propose a model of the spread of the Vsa IS elements over the genome of this microorganism. Evaluation of Vsa IS elements allowed us to hypothesize that differential transposition of Vsa IS elements might have an impact on the current epidemiological situation of Cold Water Vibriosis outbreaks caused by *A. salmonicida*.

INTRODUCTION

The change of the name

The initial name of the pathogen – *Vibrio salmonicida** was proposed by Egidius and colleagues in 1986 (Egidius *et al.*, 1986). In December 2007 the genus Vibrio was split in two and the bacterial species earlier designated as *Vibrio fischeri**, *Vibrio logei**, *Vibrio salmonicida** and *Vibrio wodanis** were renamed *Aliivibrio fischeri*, *Aliivibrio logei*, *Aliivibrio salmonicida* and *Aliivibrio wodanis*. Therefore all references published before December 2007 use the name *Vibrio salmonicida* (*V. salmonicida**) while most studies published later than 2007 use the suggested name *Aliivibrio salmonicida* (*A. salmonicida*) (Urbanczyk *et al.*, 2007).

General characteristics of the Vibrionaceae family

Members of the Vibrionaceae family are widely distributed and can be found in virtually all environmental niches where they often exist in association with various host species (Brito-Vega and Espinosa-Victoria, 2009; Chiavelli *et al.*, 2001; Colwell *et al.*, 1977; Nyholm *et al.*, 2009; Toyota and Kimura, 2000). Vibrionaceae currently includes 7 designated genera: *Vibrio, Aliivibrio, Catenococcus, Enterovibrio, Grimontia, Photobacterium, Salinivibrio* as well as a number of taxonomic groups under consideration. Members of the family include commensal, mutualistic and pathogenic species (Dryselius *et al.*, 2007; Lilburn *et al.*, 2010). While the involvement of Vibrionaceae bacteria in mutualistic relations in the light organs of marine invertebrates are relatively well understood and exists as a trade-off of the nutrients in exchange for controlled light burst, the role of the intestinal symbionts as well as zooplankton-associated species remains unknown (Boettcher and Ruby, 1990; Kaplan and Greenberg, 1985; Ruby *et al.*, 2005). Intestinal symbionts may be involved in prevention of host colonization by pathogens through their capability to perform competitive exclusion although other additional functions might be behind the symbiosis (Blaut and Clavel, 2007; Fjellheim *et al.*, 2007). Analysis of the spatiotemporal structures of populations of vibrios associated with surfaces of marine invertebrates showed their low host specificity. At the same time it has been demonstrated that some *Vibrio* species have stronger association with planktonic organisms rather than with mussels or crabs (Preheim *et al.*, 2011).

As well as the commensal bacteria of the Vibrionaceae family, pathogenic Vibrionaceae bacteria demonstrate a wide host range specificity causing infections of different severity. In humans they are primarily associated with ingestion of undercooked seafood or contaminated

water. Among those, infections caused by *Vibrio parahaemolyticus* (*V. parahaemolyticus*) are considered to be the least dangerous. The disease is usually self-limited and is accompanied with mild abdominal pain, watery diarrhea, and minor fever (Daniels and Shafaie, 2000). Another well-known and likely the most abundant infection in humans is Cholera. If treated, the disease caused by *Vibrio cholera* (*V. cholera)*, has a low fatality rate which is mainly associated with hypovolemic shock and metabolic acidosis (Daniels and Shafaie, 2000). The most lethal human pathogen is likely *Vibrio vulnificus*. This bacterium is capable of entering the host body via the wounds or ingestion of contaminated seafood and is causing cellulitis or septicaemia. Treated patients may have a total mortality of around 33 %, while the mortality can reach 60% or be even higher in patients with immuno-compromised states (Daniels and Shafaie, 2000). Non-human Vibrionaceae pathogens affect a variety of aquatic vertebrate as well as invertebrate species. While *V. salmonicida**, *Vibrio anguillarum* (*V. anguillarum*) and *V. wodanis** induce septicaemia, internal bleedings or ulceration of the fish skin surfaces (Benediktsdóttir *et al.*, 2000; Crosa, 1980; Egidius *et al.*, 1986), *Vibrio tubiashii (V. tubiashii)*, *Vibrio harveyi (V. harveyi)* and *Vibrio coralliilyticus (V. coralliilyticus)* affect a variety of shellfish, shrimp or coral species (Austin and Zhang, 2006; Ben-Haim *et al.*, 2003; Hada *et al.*, 1984).

Despite a huge biodiversity all species of Vibrionaceae are motile facultative anaerobes capable of fermentation (Ashiru *et al.*, 2012; Serratore *et al.*, 1999; Shieh *et al.*, 2004). Motility in low viscosity liquids in this group of microorganisms is mediated by Na^+ driven polar flagellas sharing significant homology among members of the family (Asai *et al.*, 1999; González *et al.*, 2010; McCarter, 2001). In addition to polar flagellas certain Vibrionaceae species synthetize H^+ driven lateral flagellas for swarming in the viscous secrets of epithelial surfaces (Atsumi *et al.*, 1996; Stewart and McCarter, 2003). The surface structures of vibrios are not limited to flagellas. In addition they synthesize two types of pili to a large extent determining their interaction with the host organisms. The genes encoding the first type of pili or toxin co-regulated pili (TCP) can mainly be found in *Vibrio* species and are used during colonization of the host intestine (Herrington *et al.*, 1988), the genes of the second type of pili (mannose sensitive-hemagglutinin pili - MSHA) are present in the genomes of all Vibrionaceae bacteria and is mainly involved in mutualistic interactions with the exoskeletons of marine invertebrates or other host surfaces (Chiavelli *et al.*, 2001; Thelin and Taylor, 1996). Both types of pili share no homology at the DNA level and are not recognizable in cross reactivity immunoassays (Ehara *et al.*, 1991). As well as genes of MSHA pili, genes of TCP are located on Chromosome I, however by being a part of the Vibrio Pathogenicity

Island the genes of TCP are horizontally transmissible (Ehara *et al.*, 1991; Jermyn and Boyd, 2005; O'Shea and Boyd, 2002; Rajanna *et al.*, 2003).

Besides motility and attachment, the virulence characteristics of Vibrionaceae include production of diverse protein toxins. The wide specificity pore-forming hemo- and cytolysins cause osmotic damage of the host cells and are probably the most common toxins produced by Vibrionaceae (De and Olson, 2011). In addition to the broad target range toxins, members of the family produce a large number of species specific toxins targeting particular cell structures and organ systems (Kimes *et al.*, 2011). The components of the toxin cocktails varies significantly and are specific to a particular host, however they generally include inductors for depolymerization of actin stress fibers (Ma and Mekalanos, 2010); increased secretion of Cl^- ions (Debellis *et al.*, 2009); modification of intercellular tight junctions (Guttman and Finlay, 2009) as well as toxins inducing increased water secretion by the host cells (Haan and Hirst, 2004). Along with toxins the secretory proteases have an important role in the pathogenicity of Vibrionaceae (Shinoda *et al.*, 1996). Experiments performed with *Vibrio vulnificus* (*V. vulnificus*) have shown that protease expression is regulated through the quorum-sensing system and thus is dependent on bacterial cell density (Kim *et al.*, 2003). Moreover, in addition to traditional host tissue digestion, they can activate host regulatory factors and thus influence the host cell function (Miyoshi *et al.*, 1994; Miyoshi, 2006; Wang *et al.*, 2008).

Overview of existing fish infection models

Fish models have become popular non-mammal alternatives for studies of both genetic and infectious diseases because fish are capable of creating an adaptive immune response and they often have a well-developed complement system (Boshra *et al.*, 2006). Fish models can provide researchers with possibilities of combining different models in one study for all-around evaluation of diseases which have significant budgetary and ethical advantages over mammalian models (O'Callaghan and Vergunst, 2010). Besides being important as substitutes of mammalian models, fish models are widely used for direct studies of fish diseases. Fish models are especially valuable when evaluation of the innate response to the pathogen is required because of lack of the adaptive immunity on the early stages of the development (Novoa and Figueras, 2012; van der Vaart *et al.*, 2012).

Fish models can be divided onto injection, immersion, intubation and cohabitation models by the method of administration of the pathogen. The selection of a particular type of an experimental fish model is largely dependent on the expected outcome of the experiment. In general, cumulative mortality rates are higher for injection experiments while immersion, cohabitation and intubation experiments produce lower mortality rates (Kondo *et al.*, 2001). Injection based administration of the pathogen has become a method of choice for various vaccination studies because it provides more predictable results while immersion, intubation or cohabitation are generally used for understanding natural ways of the pathogen transmission (Lunder *et al.*, 1995). The nature of the disease is also an important factor affecting the selection of the appropriate model. While for such systemic diseases as pasteurellosis, piscirickettsiosis or francisellosis the injection is often a method of choice (Colquhoun and Duodu, 2011; Toranzo *et al.*, 2005), for diseases primarily inducing ulcerated skin lesions, immersion studies are probably the most appropriate (Colquhoun and Duodu, 2011; Karlsen *et al.*, 2012).

Attention should be given to the selection of challenge approach in order to limit the potential effects of the technique on the outcome of experiments. By comparing the effects of intraperitoneal (IP) and intramuscular (IM) administration of inactivated *Aeromonas hydrophila* on the lymphocyte counts and serum proteins of the fish, Peyghan and colleagues have demonstrated that the method of injection significantly influences the immune response of common carp, (*Cyprinus carpio*) to vaccination. In particular, the authors have found that the relative lymphocyte counts in blood and kidney samples become higher after IM than IP injection (Peyghan *et al.*, 2010). Along with providing the most reliable delivery of the

pathogen, injection challenges are quite labour intensive and stressful for both the experimental animal and the personnel conducting the studies. Moreover, handling fish smaller then 20g is impractical and injection in general is associated with health risks for the researchers (Horne, 1996). The need of good immobilization requires deeper anesthetizing and thus compromises animal welfare conditions and increases mortalities among experimental animals.

Similarly to injections, intubation is labour intensive, time consuming and probably to an even greater extent compromises welfare of the experimental animals. Under normal feed delivery rates, the stomach of the fish are tightly filled with feed pellets and thus require 24-48 hours of starvation to provide suitable stomach emptiness required for delivery of the bacterial suspension (Handeland *et al.*, 2008). Evaluation of the pH cycles in the stomach of rainbow trout has demonstrated that intubation into an empty stomach is not only stressful for the animals, but significantly influences the outcome of the experiment due to its low pH values (Sugiura *et al.*, 2006). Influenced by high acidity of the stomach and antimicrobial properties of the bile salts in the fish intestine, the intubation experiments generally provide the lowest mortality rates when compared with injection or immersion administration of the pathogen (Smith *et al.*, 1999).

Immersion challenges are likely the simplest method for administration of the pathogens. Conducted by transfer of the fish from the holding tank into bacterial suspension for a certain period of time, immersion challenges provide the lowest stress levels for experimental animals. While it is easy to perform this type of experiments they require handling of large volumes of bacterial cultures (Bader *et al.*, 2003). In addition, in case of long immersions, additional aeration (Campos-perez *et al.*, 2000) as well as maintenance of the temperature (Rodkhum *et al.*, 2013) of the suspensions might be appropriate. The minor technical difficulties easily bypassed thorough planning and good laboratory practice, makes immersion challenges the most adequate model for studying host pathogen interactions. By entering the host via natural infection routes, the pathogens experience the full arsenal of host protective mechanisms (Martins *et al.*, 2013) and thus allow more thorough evaluation of the host-pathogen interactions.

Cohabitation studies are conducted by combined holding of the infected and healthy animals in the same holding facility and thus are primarily used for studies of the transmission of disease between individuals and the effects of vaccination on the transmission (De Decker

and Saulnier, 2011; Grove *et al.*, 2007; Munangandu *et al.*, 2012). Cohabitation challenges require induction of the disease in one group of experimental animals prior to cohabitation studies and thus stay slightly apart from the other three types of challenges. Infected via injection, immersion or intubation the group of animals infected initially are subsequently mixed with the healthy individuals and monitored for mortalities or signs of infection (Alcorn *et al.*, 2005; Murray *et al.*, 1992). To provide adequate evaluation of the results of cohabitation studies these experiments require addition of a control group challenged by non-cohabitation. Differences between cohabitation-challenged and non-cohabitation challenged groups are generally described as relative present survival, and mean days to death (Xu *et al.*, 2007).

As mentioned, the selection of the challenge models is largely dependent on the expected outcome of the experiment and the hypothesis tested in the study. In the course of the *V. salmonicida** research (1979-2006) and the *A. salmonicida* research (2007-present) all variety of injection, immersion, intubation and cohabitation models have been implemented to evaluate different aspects of the pathogenicity of the CWV disease agent. In particular administration of the pathogen by IP injection was used in the studies of Bjelland et al., Bøgwald et al., Eggset et al., Espelid et al., Hjeltnes et al., Karlsen et al., Melingen et al., Nordmo et al., Schrøder et al., Strømsheim et al., Valla et al. and Wiik et al. (Bjelland *et al.*, 2013; Bjelland *et al.*, 2012; Bøgwald *et al.*, 1992; Eggset *et al.*, 1997; Espelid *et al.*, 1987; Hjeltnes *et al.*, 1989; Karlsen *et al.*, 2008; Melingen (2) *et al.*, 1995; Nordmo *et al.*, 1998; Nordmo *et al.*, 1997; Schrøder *et al.*, 1992; Strømsheim *et al.*, 1994; Valla *et al.*, 1992; Wiik *et al.*, 1989). In addition to the above-mentioned, Brattgjerd and Evensen have used intravenous injection (Brattgjerd and Evensen, 1996). Administration of the pathogen by Immersion was used by Bjelland et al. (Bjelland *et al.*, 2012; Bjelland *et al.*, 2012). Cohabitation challenges are described by Dalmo et al. and Nordmo et al. (Dalmo *et al.*, 1998; Nordmo *et al.*, 1998). Administration of *V. salmonicida** by intubation is described by Bøgwald et al. (Bøgwald *et al.*, 1994) while cohabitation challenges are described by Dalmo et al. and Nordmo et al. (Dalmo *et al.*, 1998; Nordmo *et al.*, 1998).

In addition to all above-mentioned experiments the study of Colquhoun and Sørum (Colquhoun and Sørum, 1998) describes propagation of the bacteria in semipermeable capsules deposited surgically into the intraperitoneal cavity of Atlantic salmon. That study cannot be regarded as one using the fish as a disease model in a traditional sense since the parameters of infection were not evaluated. At the same time the study of Colquhoun and

Sørum has to be mentioned as one involving live fish individuals (Colquhoun and Sørum, 1998).

Bacterial insertion sequence elements and adaptation to pathogenicity

Members of the Vibrionaceae family are important human pathogens and pathogens of husbandry animals. Comparative genomics of different Vibrionaceae species performed by Lilburn and colleagues have demonstrated that similar pathogenic phenotypes of different Vibrionaceae species were obtained by different evolutionary pathways often intersecting each other and leading to exchange of genetic information. Based on the data of Keymer et al. (Keymer *et al.*, 2007) and Hunt et al. (Hunt *et al.*, 2008), Lilburn and colleagues hypothesize that the marine environment allows co-existence of different genotypes of one bacterial species. When conditions are favourable this co-existence of different genotypes provides a substrate for emergence of multiple epidemic strains driven by the horizontal exchange of genetic material (Lilburn *et al.*, 2010).

Analysis of marine metagenomic data has demonstrated the presence of virulence genes in up to 8% of the planktonic bacteria not known to be associated with diseases and thus constituting a passive reservoir of horizontally transmissible virulence factors (Persson *et al.*, 2009). According to the review of Hazen et al., a majority of the research on horizontal exchange of genetic information and its impact to bacterial pathogenicity are traditionally focused on phages and plasmids while the impact of other mobile genetic elements are studied insufficiently (Hazen *et al.*, 2010). At the same time a closer look at the genetic organization of particular pathogenic species demonstrates that in addition to the impact by horizontal exchange of genetic information with an external gene pool, certain features of the microorganisms are often influenced by mobile genetic elements serving as internal factors for diversification of bacterial populations (Boto and Martínez, 2011; Forde *et al.*, 2008; Yang *et al.*, 2011). Comparative study of *Vibrio splendidus* isolates have revealed remarkable genotypic diversity within this specie and have allowed identification of multiple strain-specific DNA regions: *Vibrio splendidus* (*V. splendidus*) strain 12B01 has been found to lack the chromosomal superintegron, typical for other Vibrios; *V. splendidus* strain LGP32 was found to contain a new dfrA cassette conferring resistance to trimethoprim and similar to those of clinical human isolates; *V. splendidus* strain Med222 was found to contain the Vibrio superintegron inactivated by an insertion of a mobile genetic element (Le Roux *et al.*, 2009).

Insertion sequence elements or IS elements are the smallest mobile genetic elements only encoding transposase genes required for their mobility (Mahillon *et al.*, 1985; Ooka *et al.*, 2009). Analysis of the sequence abundance performed on 10 million protein coding genes within bacterial, archaeal, eukaryotic, viral and metagenomes demonstrated that genes encoding transposases are the most prevalent genes in nature (Aziz *et al.*, 2010). While the sole movement of the IS elements most often leads to gene disruption (Mahillon and Chandler, 1998; Polard *et al.*, 1996), simultaneous transposition of two IS elements as parts of composite transposon promote relocation, inversion, excision, homologous recombination of large DNA fragments or might even lead to plasmid fusion (Downard, 1988; Hayes, 2003; Heritage and Bennett, 1985; Morita *et al.*, 1999). In addition they can promote integration of the DNA molecules received by acquisition from the environment or by horizontal gene transfer. These dramatic events can result in the assembly of new gene clusters providing multidrug resistance or encoding new metabolic pathways (Popa and Dagan, 2011; Thomas and Nielsen, 2005).

By date various molecular and structural studies have been conducted on the IS elements and a number of transposition mechanisms has been identified (Barabas *et al.*, 2008; Duval-Valentin *et al.*, 2004; Montaño and Rice, 2011; Steiniger-White *et al.*, 2004). As for the plasmids, multiple attempts on classification of the IS elements have been performed in the past and eventually evolved into one final stem based on the transposase proteins. Probably the most comprehensive review of different families of both pro- and eukaryotic IS elements as well as their detailed transposition mechanisms can be found in the excellent reviews by Curcio and Derbyshire as well as Cerveau and colleagues (Cerveau *et al.*, 2011; Curcio and Derbyshire, 2003). Judged by the results of transposition of all the known prokaryotic IS elements they utilize so-called replicative and conservative transposition mechanisms. During replicative transposition a copy of the IS element appears at the new site and one copy remains at the old locus thus doubling the amount of IS elements in the host genome. In the conservative pathway IS elements do not undergo replication. Elements simply excise and integrate into a new suitable site. Despite being the most prevalent in nature, all diversity of the insertion sequences relies on five catalytic mechanisms, four of which can be found in prokaryotes. The most numerous group relies on the so-called **DDE** catalytic motif and may or may not leave a copy of the IS element at the donor site after transposition (Berger and Haas, 2001; Brochet *et al.*, 2009; Mahillon *et al.*, 1999; Rousseau *et al.*, 2004). The second and the third family of bacterial IS elements require Tyrosine (**Y**) and Serine (**S**) residues in their catalytic sites and thus getting their respective names (Boocock and Rice, 2013; Nunvar

et al., 2010; Ton-Hoang *et al.*, 2012). Both **Y** and **S** transposases use the cut-and-paste mechanism and do not require intermediate replication steps. The last prokaryotic family of IS elements transposes via Rolling-Cycle (RC) transposition and is fully dependent on the intermediate replication step (IS elements containing **Y2-** transposases) (Garcillán-Barcia and Cruz, 2002; Mendiola and de la Cruz, 1992). The fifth family or the reverse transcription requiring family has so far not been detected in prokaryotes (Curcio and Derbyshire, 2003). IS elements are generally flanked by terminal inverted repeats (IR) which serve as a site for transposase binding. Upon transposition, many IS elements generate direct target repeats (DR) at the site of insertion. Generation of a DR is characteristic for the IS element families and reflects the mechanism of the transposition (for review see Mahillon and Chandler (Mahillon and Chandler, 1998)).

The mutation process is involved in generation of the occasional fitter mutants and thus increasing genetic variability in bacterial populations (Aras *et al.*, 2003). Similar to virtually irreversible mutations, the amount of IS elements determines the adaptation potential of the species however its impact on the host genotype is potentially reversible. While the replicative transposition mechanism, leaving a permanent copy of the mobile element in the target loci, might serve as an internal factor bringing gene inactivation out of a stochastic steady-state rate, the conservative mechanism can provide reversible advantages, beneficial to rapid transitions between environmental niches (Wagner, 2006). The genome annotation performed by Hjerde and colleagues (Hjerde *et al.*, 2008) have discovered 290 IS elements in the genome (4.6Mb) of *A. salmonicida* placing this organism among the bacteria with the highest IS element content when compared with all fully sequenced bacterial genomes (Cerveau *et al.*, 2011). According to the review of Siguier et al. analysing the impact of IS elements on the genome evolution and the emergence of pathogenicity, IS elements play an important role in the genome reduction thus allowing higher specialization of the pathogens Analysing multiple data available for the Bordetella species Siguier et al. found genomes of pathogenic isolates consisting of about 4.1-4.7 Mb and generally harbouring more than 260 IS elements while non-pathogenic strains have genomes of about 5.34 Mb and carry no insertion sequences. Further analysing the publications available for different bacterial species Siguier et al. conclude that the IS mediated genome reduction is a common tendency among Bacteria and Archaea where higher IS content is generally associated with adaptation to pathogenicity (Siguier *et al.*, 2006).

The history of the CWV and present status of the knowledge on *Aliivibrio salmonicida*

Pathogenesis

Despite many years of research a complete lifecycle of *A. salmonicida* is not known. We can assume that as for other related species it consists of free-living and facultative pathogenic phases. Most likely, as it has been demonstrated for *Vibrio cholerae* (Pruzzo *et al.*, 2008; Reidl and Klose, 2002), a free-living phase of the *A. salmonicida* life cycle is associated with marine invertebrates or their egg masses despite the fact that some of the strains of *A. salmonicida* might have a disrupted chitin utilization pathway (Hjerde *et al.*, 2008). As well as *V. cholerae, A. salmonicida* is known to produce biofilms that can provide a favourable microenvironment for its persistence in harsh marine conditions (Bjelland *et al.*, 2012; Hjerde *et al.*, 2008; Vu *et al.*, 2009). As a typical member of Vibrionaceae, *A. salmonicida* is motile in sea-water environment, but the motility is suppressed under late stages of the host colonization (Bjelland *et al.*, 2012; Karlsen *et al.*, 2008).

Salinity seems to have a great impact on the pathogenicity of *V. salmonicida**. *V. salmonicida** is a microorganism known to have poor resistance to low salinities (Hoff (2), 1989). Presence of a pro-phage in the genome of a microorganism might have great impact on its pathogenicity. By combining own experimental data with data on the long term survival of other Vibrio species, Hoff concluded that *V. salmonicida** had a potential ability to survive for more than one year in marine water suspended or attached to the surfaces of marine particles (Hoff (2), 1989).

Evaluation of survival of *V. salmonicida** in sediments from abandoned aquaculture sites demonstrated an important property of the microorganism. Using fluorescent monoclonal antibody labelling Husevaag and colleagues demonstrated the presence of *V. salmonicida** in the sediments of abandoned fish farms. Sampling in the area surrounding farms has indicated presence of *V. salmonicida** in a radius of 200–250 m from aquaculture sites and did not reveal *V. salmonicida** neither further away from the farming site nor in sediments taken at sites not affected by fish farming (Husevåg *et al.*, 1991). Moreover, a study of the seasonal occurrence of *V. salmonicida** in water samples taken at 12 Norwegian fish farms from October to June demonstrated the presence of *V. salmonicida** in all samples despite the fact that outbreaks of Cold Water Vibriosis were not reported at any of the locations (Enger *et al.*, 1991).

Description of the pathogenic properties of the microorganism will not be complete if it is not mentioned that already in 1986 Egidius et al. postulated that Atlantic salmon (*Salmo salar*) is more susceptible to CWV than rainbow trout (*Oncorhynchus mykiss*) (Egidius *et al.*, 1986). Moreover, Sørum and colleagues indicated the possibility of the transmission of Cold Water Vibriosis between Atlantic salmon and Atlantic Cod (*Gadus morhua*) (Sørum *et al.*, 1990).

Analysis of isolates of *V. salmonicida** collected in Norway, on Shetland, Faroe Islands, and in eastern Canada performed by Sørum et al. have demonstrated that disease outbreaks in all these areas are associated with a particular strain of *V. salmonicida** containing the pVS43, pVS54, and the epVS320 plasmids (Sørum *et al.*, 1993). At the same time the earlier study of Wiik and colleagues have demonstrated that the plasmid content had no impact on neither virulence nor the biochemical characteristics of the pathogen (Wiik *et al.*, 1989). By using plasmid-curing techniques Valla and colleagues were able to construct a plasmid free strain of *V. salmonicida** (Valla *et al.*, 1992). In addition, Valla et al. reported that extrachromosomal DNA is not required for the pathogenicity of *V. salmonicida** (Valla *et al.*, 1992) and thus we can hypothesize that plasmid content is important for environmental succession of *A. salmonicida* rather than its virulence.

Based on data of immunochemical staining Schrøder, Espelid and Jørgensen concluded that *V. salmonicida** have a high susceptibility to phagocytic digestion (Schrøder *et al.*, 1992). Similar findings were made by Brattgjerd and colleagues who regarded the presence of "grains smaller than bacteria located extracellularly" as a sign of intracellular inactivation of *V. salmonicida** (Brattgjerd *et al.*, 1995). Examination of the Atlantic salmon immune response after oral administration of formalin-killed and radioactively labelled *V. salmonicida** performed by Bøgwald and colleagues demonstrated the lack of uptake of *V. salmonicida** or further antigen presentation by the cells of the gastrointestinal tract of salmon (Bøgwald *et al.*, 1994). By using electron as well as light microscopy techniques, Brattgjerd and Evensen have demonstrated that antigens of *V. salmonicida** remain detectable in the phagosomes of head kidney macrophages of Atlantic salmon up to 27 h post infection (Brattgjerd and Evensen, 1996).

In vitro and *in vivo* studies performed by Mohn Bjelland et al. demonstrated increased motility, adhesion, cell-to-cell aggregation and biofilm formation by *A. salmonicida litR* mutants. At the same time, mutants produced decreased bioluminescence and were found to be inducing reduced mortality among experimental animals. The combination of such

observations allowed Mohn Bjelland et al. to hypothesize the importance of the LitR activity for maintenance of the suspended state of the microorganism more favourable for initiation of colonization of the host (Bjelland *et al.*, 2012).

Analysing bacterial presence in different organs of the Atlantic salmon, Mohn Bjelland et al. hypothesized that *A. salmonicida* uses the blood volume for proliferation while the fish intestine serves as a reservoir for survival and transmission of the bacterium. Additionally, the study confirmed that motility required for gaining access to the host is no longer needed for subsequent bacterial survival in the host tissues (Bjelland *et al.*, 2012). The results of another study by Bjelland et al. allowed the authors to hypothesize that *A. salmonicida* utilize general muting of gene expression to avoid the host immune system. The magnitude of the response made the authors to suggest that *A. salmonicida* might inhibit or resist the immune system of the host (Bjelland *et al.*, 2013).

Virulence factors

Despite the long history of experimentation with *A. salmonicida*, studies precisely pointing at evaluation, quantification and understanding the role of different virulence factors in the pathogenesis of *A. salmonicida* are relatively limited.

One of the studies is published by Colquhoun and Sørum. Comparing the outer membrane protein expression during *in vivo* cultivation of *V. salmonicida** the authors were able to demonstrate increased expression of 3 proteins of about 99, 90 and 78 kDa. Electron microscopy evaluation of the samples extracted after incubation in the peritoneal cavity of rainbow trout made it possible to suggest that *V. salmonicida** does not produce a capsule *in vivo* (Colquhoun and Sørum, 1998).

Studies of the bioluminescence properties of *V. salmonicida** performed by Nelson and colleagues with mutant and wild type strains of *V. salmonicida** demonstrated that the virulence of the *lux* operon mutants were attenuated up to 50-fold in comparison with the wild type strain (Nelson *et al.*, 2007).

Evaluation of the bacterial growth under different temperatures, as well as under iron limiting conditions made it possible to conclude that *V. salmonicida** is likely producing one or more iron acquisition systems. While the production of siderophores was found in a wide range of temperatures, putative components of a non-siderophore iron acquisition system were found

to be suppressed at temperatures above 15°C (Colquhoun and Sørum, 2001). Another important observation on the iron acquisition by *V. salmonicida** was made by Winkelmann and colleagues. The authors reported isolation and structural characterization of bisucaberin (siderophore) produced by *V. salmonicida** under iron-limited conditions. It was found that the production of siderophore was induced at temperatures below 10°C indicating a potential importance of the bisucaberin for virulence of *V. salmonicida** (Winkelmann *et al.*, 2002). Characterisation and phylogenetic analysis of the *fur* genes of *V. salmonicida** and *V. logei** performed by Colquhoun et al. have demonstrated that *fur* genes are conserved amongst different vibrios (Colquhoun and Sørum, 2002).

Examination of the effects of water temperature on bacterial growth, virulence and antigen expression in *V. salmonicida** have demonstrated that the highest rates of *V. salmonicida** cell division on solid surfaces occurred at 15°C while 10°C seems to be optimal for cultivation of this bacterium in liquid media. In addition, the authors reported that a novel 76-kDa OMP protein was produced by *V. salmonicida** in unshaken cultures. The protein was not found to stimulate a specific humoral response of Atlantic salmon (Colquhoun and Sørum, 2002).

Analysis of the effects of fish skin mucus on the soluble proteome of *V. salmonicida** was performed by Uttakleiv Ræder and colleagues and demonstrated increased expression of flagellins as well as proteins involved in oxidative stress and general stress responses (Uttakleiv Ræder *et al.*, 2007). In addition to Uttakleiv Ræder et al., Karlsen and colleagues reported the effects of salinity and temperature on the expression of flagellins by *A. salmonicida*. The study demonstrated important properties of *A. salmonicida*. In particular, *A. salmonicida* was demonstrated to be motile under sea water conditions while it is found to be non-motile at physiological salt concentrations. In addition it was demonstrated that six flagellin genes of *A. salmonicida* are located at two separate chromosomal loci similarly to those of *A. fischeri* (Karlsen *et al.*, 2008).

Treatment

The early data on the susceptibility of *V. Salmonicida** to antibiotics and bacteriostatic agents (available for reviewing from open sources) were published by Bruno et al., Egidius et al. and Hustvedt et al. Bruno et al. have reported treatment of CWV outbreaks with oxytetracycline in Scotland (Bruno *et al.*, 1985); Egidius et al. reported sensitivity of *V. salmonicida** to the vibriostatic agent 0/129 and its relative resistance to novobiocin (Egidius *et al.*, 1986).

Hustvedt et al. have reported a study testing success of the CWV treatment with oxolinic acid (Hustvedt *et al.*, 1992).

In the subsequent publication by Nordmo et al. (referring to the publications by other authors which are, however, not available for public access) it was reported that by 1998 *V. salmonicida** have developed resistance against quinolones, trimethoprim/sulphonamide combinations and tetracyclines. The same paper reports evaluation of florfenicol for treatment of CWV in Atlantic salmon (Nordmo *et al.*, 1998).

It might be appropriate to mention that detailed discussion on the peculiarities of the CWV therapy in Atlantic salmon goes beyond the frames of the current thesis. However if needed, a detailed overview of dosage and treatment regimens can be found in the book "Health Maintenance and Principal Microbial Diseases of Cultured Fishes" by Plumb and Hanson (Plumb and Hanson, 2011).

Here it might be appropriate to mention that Sørum and colleagues have performed detailed characterization of tetracycline resistance gene from *V. salmonicida**. (Sørum et al., 1992).

Vaccines

The successful vaccination of Atlantic salmon against Cold Water Vibriosis reported by Holm and Jørgensen became a breakthrough in the epidemiological situation of Cold Water Vibriosis. Protection against subsequent natural outbreaks of Cold Water Vibriosis was achieved with vaccination by immersion of Atlantic salmon parr in formalin treated bacterin (Holm and Jørgensen, 1987). The first production and partial characterization of the monoclonal antibodies against *V. salmonicida** was made by Espelid and colleagues. The authors were able to produce 17 hybridoma clones in total, 13 of which were strictly specific to the *V. salmonicida** surface antigen (VS-P1) and the 4 remaining clones had cross-reactivity with *V. anguillarum, Vibrio ordalii* as well as *V. fischeri** surfaces (Hjelmeland *et al.*, 1988). Almost simultaneously with the publication of Espelid et al., Hjelmeland and co-workers reported the isolation and partial characterization of the VS-P1 antigen (Hjelmeland *et al.*, 1988). The study of Espelid et al. reported the specificity of antibodies towards one particular epitope on the VS-P1 antigen and thus further refined the data of Hjelmeland and co-workers (Espelid *et al.*, 1987).

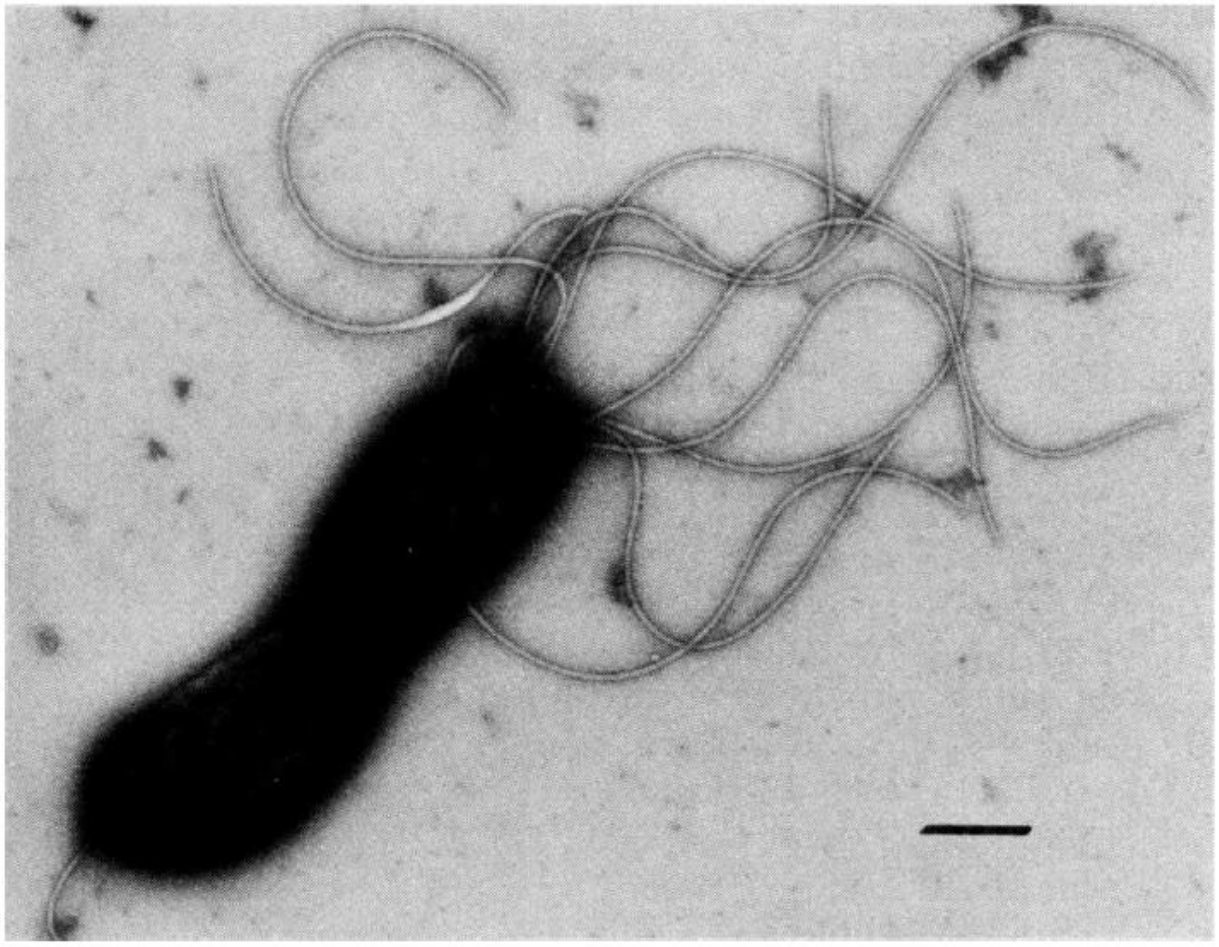

Figure 1. Scanning electron microscopy image of *V. Salmonicida**. Scale bar = 1 Fm. Image from publication "Vibrio salmonicida sp. nov., a New Fish Pathogen" by Egidius et al. Article containing image legally published in 1986 at the International Journal of Systematic Bacteriology (Egidius *et al.*, 1986). According to the Directive of European Union 93/98/EEC of 29 October 1993 harmonizing the term of protection of copyright and certain related rights (Article 4) and Norwegian Copyright Act (Chapter 4, § 41a), the duration of copyright protection for the image (25 years) expired in 2011.

Hjeltnes and colleagues demonstrated that single bath vaccination with a vaccine made by whole cell culture of *V. salmonicida** inactivated with 0.5% formalin did not provide reliable protection against CWV while repetitive revaccination might give some improvements. Similar results were obtained after injection of inactivated *V. salmonicida** cells. A single injection provided relatively low protection against CWV however combination of the injection with revaccination by bath administration or a second injection of the vaccine gave best protection (Hjeltnes *et al.*, 1989).

The initial data of Holm and Jørgensen (Holm and Jørgensen, 1987) as well as a subsequent study of Hjeltnes and colleagues (Hjeltnes *et al.*, 1989) demonstrated the principle possibility of vaccination of Atlantic salmon against CWV and already in January 1990 the first data from a large scale field vaccination trial against the disease were reported. The experiment

was performed on 25 different farms each keeping around 4000 fish vaccinated by immersion. The vaccination experiment resulted in a relative survival of 92% in the groups of the vaccinated animals. After evaluation of the results of the field trials Lillehaug made a conclusion that water temperature is insignificant for development of protection against Cold Water Vibriosis (Lillehaug, 1990). Further experimentation with the intraperitoneal injection of formalin inactivated *V. salmonicida** followed by evaluation of the antibody titres allowed Håvarstein and colleagues to confirm that protection against *V. salmonicida** is mediated by the humoral immune system of Atlantic salmon. The humoral immune system was found to be capable of discrimination between related bacterial antigens (Håvarstein *et al.*, 1990). In addition Lillehaug et al. demonstrated cross-protection of immunization of Atlantic salmon against different strains of *V. salmonicida** (Lillehaug *et al.*, 1990).

During subsequent studies of the microorganism Bøgwald and colleagues demonstrated that the major surface antigen, VS-P1 from *V. salmonicida** is a complex of both protein and LPS molecules (Bøgwald *et al.*, 1990). Robertsen et al. demonstrated that glucans from *Saccharomyces cerevisiae* enhance non-specific disease resistance of Atlantic salmon to bacterial infections including CWV (Robertsen *et al.*, 1990). Edebrink and colleagues further performed evaluation of the oligosaccharide part of the rough LPS from *V. salmonicida** which was found to have branched structure and was attached to the phosphorylated Kdo residue as an antigen (Edebrink *et al.*, 1996).

The publication by Bøgwald et al. reported a comparative study of salmon polyclonal antibody specificities against *V. salmonicida** and *V. anguillarum* and confirmed earlier data on the cross-recognition of both species (Bøgwald *et al.*, 1991).

Despite great benefits from vaccination against CWV, the procedure was found to have adverse effects of the salmon health. Evaluation of the duration of protection and impact of vaccination on growth rates of Atlantic salmon showed that protection provided by the vaccine declines with time. In addition it was found that vaccinated fish had a lower weight than fish in unvaccinated groups (Lillehaug, 1991).

The first report on the immunological properties, as well as vaccination experiments, in cod was published by Schrøder et al (Schrøder *et al.*, 1992). By using a panel of monoclonal antibodies the existence of two distinct serotypes of *V. salmonicida** was demonstrated. In addition it was shown that *V. salmonicida** is more pathogenic in salmon than in cod

(Schrøder *et al.*, 1992). Immunization of salmon with particulated LPS from *V. salmonicida** was found to provide low protection during subsequent IP challenges while the LPS / VS-P1 antigen complex was found to be highly immunogenic and provide better protection (Bøgwald *et al.*, 1992).

The two publications by Melingen et al. demonstrated a need of early vaccination (before smolting) as well as relatively high water temperature for efficient production of antibodies in Atlantic salmon (Melingen (1) *et al.*, 1995; Melingen (2) *et al.*, 1995).

Fjalestad and colleagues published a large scale study on the genetic variations in antibody response to *V. anguillarum* and *V. salmonicida** by Atlantic salmon. The experiments conducted on 1200 fish confirmed the previous knowledge and demonstrated that the concentration of antibodies in Atlantic salmon increases significantly after the second immunisation while the first immunization only results in a mild response to pathogens (Fjalestad *et al.*, 1996).

Eggset and colleagues demonstrated the importance of temperature for development of immunity against CWV. The results indicated that both aqueous and oil-adjuvanted vaccines gave full protection against CWV however the antibody response was delayed or strongly suppressed by low temperatures. In addition it was demonstrated that protection provided by the aqueous vaccines tends to be reduced after 18 weeks from immunization while oil adjuvanted vaccine kept its immunomodulating properties (Eggset *et al.*, 1997). Steine and colleagues published a refined production of antibodies towards LPS of *V. salmonicida** during smolting and early post-smolting periods (Steine *et al.*, 2001).

In 2011 Karlsen et al published an article on identification and cloning of the immunogenic *A. salmonicida* Pal-like protein and suggested it as a potential candidate for future vaccine production (Karlsen *et al.*, 2011).

etc

Disease caused by *V. salmonicida** originally appeared at Norwegian salmon farms near Hitra island in 1979 (Egidius *et al.*, 1986), however have likely been persisting in and around the commercial aquaculture facilities for years before. A study of survival of Atlantic salmon under different feeding regimes indicated strong correlation of survival with optimal feeding showing that CWV is tightly associated with fish farming (Damsgård *et al.*, 2004). CWV

significantly affected the aquaculture economy in Norway, but in addition influenced the environment surrounding the fish farms because of the antibiotic treatment that was necessary to control the CWV outbreaks. On top of this, the public opinion related to fish farming was turned into a negative mode that still to some extent has an impact on the industry.

The first DNA hybridization analysis of different strains of *V. salmonicida** collected along the Norwegian coast performed by Wiik and Egidius found remarkable homogeneity in the hybridization results among different isolates of the microorganism. Surprisingly *V. salmonicida** was found to be substantially distinct from other known *Vibrio* species (Wiik and Egidius, 1986). The homogeneity of the *V. salmonicida** isolates correlates with the high host specificity of the pathogen (Wiik and Egidius, 1986). All known to date natural isolates of *V. salmonicida** contain plasmids [Henning Sørum, personal communications]. Sørum and colleagues described and analysed 341 isolates of *V. salmonicida** collected during natural outbreaks of Cold Water Vibriosis along the coast of Norway during 1982-1984 and 1986-1987. In total Sørum et al. identified 11 different plasmid profiles that were stable over the 6 years of sampling. The isolates were collected at sites located along more than 2000 km of the Norwegian coast and their analysis gave good initial insight into the genetic organization of the *V. salmonicida** (Søorum *et al.*, 1988). Other important studies of the genetic organization of the microorganism were made by Hjeltnes et al., Nelson et al. and Nordmo et al. (Hjeltnes *et al.*, 1987; Nelson *et al.*, 2007; Nordmo *et al.*, 1997). The most comprehensive study of the genetic organization of the microorganism was made by Hjerde and colleagues (Hjerde *et al.*, 2008). By performing full genome sequencing it was possible to demonstrate that functions of the chromosomes in *A. salmonicida* are similar to those of other Vibrionaceae species. The large chromosome (chromosome I) harbours essential genes while accessory genes are located on chromosome II. The study also demonstrated that the genome of *A. salmonicida* contains 4286 predicted protein coding sequences spread over 4.6 Mb of DNA. Analysis of the CDSs indicated at least 370 inactive genes suggesting reduced metabolic capacity and thus a restricted range of potential environmental carriers of the microorganism (Hjerde *et al.*, 2008).

Along with Hjerde et al. important discoveries related to the genetic organization of *A. salmonicida* were made by Manukhov et al. (Manukhov *et al.*, 2011), Hansen et al. (Hansen *et al.*, 2012), and Ahmad et al. (Ahmad *et al.*, 2012). A comparative study of the *lux* operons of *A. logei* KCh11 and *A. salmonicida* LFI1238 performed by Manukhov et al. demonstrated that the reduced production of bioluminescence in *A. salmonicida* is most likely associated with a specific mutation in the *luxD* gene (Manukhov *et al.*, 2011). Hansen and colleagues described

the role of Spot 42, the small RNA regulator, for regulation of the central metabolism of *A. salmonicida* and found that Spot 42 of *A. salmonicida* share 84% identity with Spot 42 of *E. coli*. Besides directly affecting several key cellular processes, Spot 42 of *A. salmonicida* was found to mediate an on/off expression pattern of the pirin-like protein thus indicating its importance for switching between respiration and a fermentative type of metabolism. In addition, Hansen et al. have identified sRNA with an expression profile opposite to Spot 42 and thus potentially involved in regulation of activity or expression of Spot 42 (Hansen *et al.*, 2012). Ahmad et al. have published results of a microarray study of the transcriptome of *A. salmonicida.* The analysis identified 252 new potential ncRNAs 12 of which were verified by Northern blot analysis (Ahmad *et al.*, 2012).

A study performed by Strømsheim et al. have demonstrated a need of salmon selection for further commercial production (Strømsheim *et al.*, 1994). Similar data were obtained in by Gjedrem and Gjøen studying the genetic variation in susceptibility of Atlantic salmon to Cold Water Vibriosis, furunculosis, and salmon bacterial kidney disease (Lund *et al.*, 1995). A more detailed study of the impact of genetic variation on survival of Atlantic salmon after challenges with a broader range of pathogens was reported (Lund *et al.*, 1995).

Experimentation with vaccination, natural susceptibility of Atlantic salmon as well as other challenge studies of the CWV using live animal models has demonstrated significant differences in the outcome of the challenges. The first comparative study of different experimental infection models was published by Nordmo and colleagues. Comparison of the outcomes of 20 injections, 9 immersions and one cohabitation study made it possible to conclude that IP injection provided the best reliability for vaccine testing challenges leading to the highest mortality rates in the groups of control animals (Nordmo *et al.*, 1997).

In general the interest to CWV started to decrease in 1998, and thus several papers published after 1998 were focused on refinement of previously demonstrated data such as: immunological cross reactions between *Aeromonas salmonicida* and *V. salmonicida** (Hoel *et al.*, 1998); antibody response to a T-dependent antigen in rainbow trout mediated by *V. salmonicida** bacterin (Hoel *et al.*, 1998); prophylactic effects of β(1,3)-D-glucan (laminaran) against experimental infections by *Aeromonas salmonicida* and *V. salmonicida** were shown (Dalmo *et al.*, 1998).

New data were reported by Fidopiastis and colleagues. The authors demonstrated the ability of *V. salmonicida** to produce light in culture. Importantly, the light was produced only when the major *V. fischeri** autoinducer of the *lux* genes was added to the culture of *V. salmonicida**. Extract of spent medium from *V. salmonicida** cultures led to an induction of luminescence in *V. fischeri** indicating possibility of active intercellular communication between these two species (Fidopiastis *et al.*, 1999).

A new wave of interest to *V. salmonicida** started from 2006. Lorentzen and colleagues have reported a comparative study of catalase from *V. salmonicida** and later Riise et al. published preliminary X-ray diffraction analysis of this enzyme. Data in both studies indicated that catalase from *V. salmonicida** is a typical cold-adapted enzyme and is a good candidate for a number of biotechnological applications (Lorentzen *et al.*, 2006; Riise *et al.*, 2005). Another structural study was dedicated to the O-antigenic oligosaccharide of *V. salmonicida** isolated from Atlantic cod. Bøgwald and Hoffman reported the chemical structure of the oligosaccharide established by NMR spectroscopy and mass spectrometry and demonstrated that the α-d-Fucp4NBA residue is not present in the LPS extracted from the cod serotype of *V. salmonicida** (Bøgwald and Hoffman, 2006).

The complete structure of the cold-active catalase from *V. salmonicida** was also published. The reduced thermal stability of the protein was found to be associated with a reduced number of ion-pair networks. The reported crystal structure was the first crystal structure of a psychrophilic heme-containing catalase (Riise *et al.*, 2007) and this thus not only contributed to an understanding the adaptation of the microorganism to low temperatures, but had an impact on the understanding of the properties of this family of enzymes in general.

Comparative studies of EndA secretory endonucleases from *V. salmonicida** and *V. cholerae* reported by Altermark and colleagues demonstrated that EndA from *V. salmonicida** has a lower unfolding temperature, lower temperature optimum, and higher specific activity in comparison with the respective *V. cholerae* protein. Optimal catalytic conditions for the enzyme were found to correspond with temperature requirements of *V. salmonicida**, at the same time demonstrating highest activity at physiological salinities (Altermark *et al.*, 2007). In addition Niiranen and Altermark published two articles describing effects of salt concentration on kinetics and structural adaptation of the protein (Altermark *et al.*, 2008; Niiranen *et al.*, 2008).

Ræder and colleagues introduced a study of the third enzyme from *A. salmonicida*. As well as the previously characterized proteins, the Uracil-DNA N-glycosylase (UNG) had typical cold-adapted features and was found to have increased substrate affinity when compared with the respective protein from *V. cholerae* (Ræder *et al.*, 2008).

Another experimentally and computationally characterized protein of *A. salmonicida* is ferric uptake regulator (Pedersen *et al.*, 2010). The global responses of *A. salmonicida* to hydrogen peroxide was also reported by Pedersen and colleagues and further contributed to understanding of physiological properties of the microorganism (Pedersen *et al.*, 2010; Pedersen *et al.*, 2010).

Gurung and colleagues reported characterization of the sialic acid synthase from *A. salmonicida* and suggested a novel pathway for bacterial synthesis of 7-O-acetylated sialic acids (Gurung *et al.*, 2013). Purohit and colleagues reported characterization and quantification of the acyl-homoserine lactones (AHLs) in the members of the Vibrionaceae family. The study of Purohit et al. have screened 57 strains from genera *Aliivibrio* (including *A. salmonicida*), *Photobacterium* and *Vibrio* and demonstrated that most of the isolates produced multiple AHLs suitable for broad intercellular communication between the members of the family. In particular, *A. salmonicida* LFI1238 was found to have common AHL profiles with potentially pathogenic *A. wodanis* and non-pathogenic *A. logei* (Purohit *et al.*, 2013).

Research gaps

The retrospective review of the 30 years of research of CWV makes it possible to identify knowledge gaps related to understanding of the pathogenesis of *A. salmonicida*. According to the data available for other vibrio species, the environmental part of the lifecycle of these microorganisms is associated with chitinous surfaces of marine invertebrates (Chiavelli *et al.*, 2001; Pruzzo *et al.*, 2008). The full genome sequencing performed by Hjerde et al. in 2008 have demonstrated that major components of the chitinolytic cascade of *A. salmonicida* LFI1238 are compromised by point mutation or insertion of mobile genetic elements (Hjerde *et al.*, 2008). Disruption of chitinolytic cascade correlates well with the inability of the bacterium to utilize chitin as a nutrient source [Lorentzen, personal communications]. Disruption of the key proteins mediating attachment to chitin by insertion of the IS elements rises an important question related to the environmental carrier of the bacterium. While number of other bacterial species found to be associated with marine particles (Zhang *et al.*, 2007), algal communities (Burke *et al.*, 2009), or jellyfish (Delannoy *et al.*, 2011) the carrier of *A. salmonicida* remains undiscovered. Successful implementation of CWV vaccines turned identification of the portal of CWV infection from an urgent goal into a low priority target with little practical value. To our opinion the missing knowledge on the portal of CWV infection restrains further evaluation of the lifecycle of *A. salmonicida* and requires immediate attention. Despite the number of studies clearly indicating the importance of mobile genetic elements for bacterial adaptation, little respect have been paid to the 290 IS elements described in the genome of *A. salmonicida* LFI1238. Emergence of new virulent isolates of *A. salmonicida* retaining the plasmid characteristics of the LFI1238 strain sequenced by Hjerde et al. (Hjerde *et al.*, 2008) in the recent outbreaks of CWV in vaccinated Atlantic salmon indicates that virulence properties of the new isolates are not associated with newly acquired plasmids [Kashulin, Sørum 2012., unpublished data]. Understanding of new virulence properties either acquired via horizontal gene transfer or obtained through restoration of previously inactivated virulence pathways require detailed characterization of the IS elements known to be deeply involved into both processes.

Materials and Methods used in the study

Fish work

During the work on the project we have established a novel *in vivo* model for studying the first steps of *A. salmonicida* infectivity in Atlantic salmon. To avoid unnecessary duplication, the current section will give only general description of the methods since each challenge had variable parameters. Detailed information on the experimental setups can be found in the articles I and II.

Identification of the portals of infection requires step by step analysis of carrier agents and to even greater extent should consider morphology of the studied species. The earliest studies on the pathogenicity of *V. Salmonicida** performed by Egidius and colleagues gave results that made it possible to suggest that the gills were the most probable portal of CWV infection in Atlantic salmon (Egidius *et al.*, 1986). A subsequent study by Bjelland and colleagues have also hypothesized that the gills or the gastrointestinal tract are putative portals of infection (Bjelland *et al.*, 2012).

In order to complement the knowledge on the portal of CWV infection we have designed series of experiments where different parts of the experimental animals were exposed to the suspension of *A. salmonicida*. Taking into account that *A. salmonicida* can be detected in the lumen of capillaries hours after experimental challenge and that colonization of other tissues develops much slower (Bjelland *et al.*, 2012), we have proposed quantification of the pathogen in the blood as a tool for determination of the invasion as well as colonization dynamics. According to the study by Løvoll et al. performed for evaluation of invasion of *Moritella viscosa* into Atlantic salmon, plate counts provide the same accuracy as real-time PCR studies (Løvoll *et al.*, 2009). Moreover, evaluation of direct plate counts is a method of choice for a number of bacterial competitive fitness studies (Bhatter *et al.*, 2012; Guo *et al.*, 2012; Yates *et al.*, 2006). In addition, such techniques are widely used in routine diagnostics in human and veterinary medicine (Fecteau *et al.*, 1997; Isaacman *et al.*, 1996; Mermel and Maki, 1993; Wenz *et al.*, 2001).

Unvaccinated Atlantic salmon fry weighing 30-50 g were received from Sørsmolt AS, Sannidal, Norway, and young freshwater going rainbow trout (200 g) were obtained from the aquarium of the Norwegian University of Life Sciences. Having no possibility to use the fish of similar sizes we however have chosen an advanced statistical approach which allowed us to compare post-invasion thrive of the pathogen invariably of its numerical values. Similar

approach is widely used for analysis of gene expression where the parameters of expression may vary, but the manner or the dynamics of the change remains the same (Criel and Tsiporkova, 2006). While additional information on the statistical method used for data analysis and its implementation in biological studies can be found elsewhere, it is necessary to indicate that conducted experiments were performed assuming that susceptibility of the experimental animals is not dependent of other factors than the size of the skin surface. In other words, we assumed that the fish skin permeability is not changing with age. In addition it is necessary to indicate that the paper describing differences in the susceptibility of Atlantic salmon and rainbow trout does not compare "how many" bacteria entered the hosts, it compares "what had happened" with the bacteria after they entered. Thus we have performed comparison of the relative parameters of the post-invasion thrive. Under standard experimental designs relying on the methods of parametric statistics, comparison of fish having such great differences in sizes could not be regarded as a correct approach.

The fish was collected at the same time of the year, at the same day and transported to the experimental facility with the same car. Where relevant the challenges with Atlantic salmon and rainbow trout were performed at the same day, same place and using immersion solutions prepared from the same bacterial culture. Dilution with marine water was made from the same 50L canister right before the experiment. All procedures we performed by the same people and the fish was kept at the similar tanks connected to the same water supply, aeration and waste water treatment systems.

The fish used in the experiments were received from commercial suppliers, thus the health status of the experimental animals was checked by the producers according to existing industrial standards. In addition, the health status was controlled in our laboratory. Prior to experiments, blood samples obtained from randomly selected individuals of both species were plated on blood agar and observed for bacterial growth. The control manipulations were made similarly to the blood samplings performed after the challenges to ensure the lack of *A. salmonicida* in the blood of the experimental animals prior to experiments.

Control and experimental blood samples were taken aseptically from the tail vein of the experimental animal anesthetized in a bath containing benzocaine (0.03 mg/l) (Benzoak® 200 mg/ml ACD Pharmaceuticals AS, Leknes, Norway). Evacuated blood collection tubes with added heparin (Venoject® Terumo Europe N.V, Leuven, Belgium) were used for sampling blood. After blood sampling, the experimental animals were immediately euthanized by a

sharp blow to the head. The collected blood samples were transferred to the laboratory where 10 and 100 µl aliquots of each sample were plated on blood agar and incubated at 12ºC until visible growth. The identification of the colonies was performed visually based on the morphological characteristics. In addition single colonies were checked microscopically for the presence of the characteristic rod shaped Gram-negative bacterial cells.

As mentioned, prior to the control blood sampling, the experimental animals were anesthetized in water containing benzocaine. For challenges the fish was anesthetised in a similar manner. Following signs of anesthesia, up to 5 fish were placed in the holding position allowing differential exposure of a defined body area. To avoid dehydration and provide acceptable breathing conditions, open fish surfaces were constantly hydrated by spraying with clean fresh water. The marine water used in the experiments was collected at a random location of the inner Oslofjord, Norway one day before the experiments. The collected water was stored at 4ºC and was heated to the appropriate temperature immediately before the experiments.

Earlier studies demonstrated that *A. salmonicida* does not tolerate fresh water conditions, and thus water transmission of the disease in freshwater is avoided. In these experiments, incubation in fresh water for 15 min was performed to kill any *A. salmonicida* on the fish surface prior to blood sampling. During the course of all experiments the fish were fed with commercial feed pellets and regularly observed. The experimental groups were marked by fin piercing in a manner not affecting surfaces exposed to the bacterial suspension.

Computer work

To characterize IS elements persisting in the genome of *A. salmonicida* we have implemented staged strategy for identification of potential integration sites; distribution of the IS elements over the genome of the microorganism and identification of the similar IS elements in other bacterial classes.

The genomic data mining was performed using Blast-p, Blast-n, Pfam, and UniProt search algorithms with default parameters. The prediction of chromosomal *ori*, *ter*, and *dif* sites was performed using a manual homology search with *Vibrio cholerae*, *Escherichia coli* and *Bacillus subtilis* as references. In addition we have used prediction functions and data of the DoriC 5.0 database of bacterial and archaeal *oriC* regions. The identification of potential

integration sites was performed using the MEME motif-based sequence analysis tool combined with manual curation according to procedures described in paper III.

Distribution of the IS elements in the genome of *A. salmonicida* LFI1238 was performed according to the pipeline specifically designed for the purpose of the study by the student. The strategy implemented for the assessment of the distribution of IS elements is described in detail in the respective section of paper III. Here we provide only brief description of the pipeline: grouping of IS elements sharing identity of their transposase CDSs → clustering of the IS elements based on the identity of the whole IS element DNA sequences → evaluation of the transposition success of particular variances within each individual family of IS elements.

Survey of related IS elements in other bacterial genomes was performed manually using the NCBI protein BLAST search for matches with parameters that were originally proposed by Mijenendonckx et al. for the examination of insertion sequences in the genome of *Cupriavidus metallidurans* CH34 (Mijnendonckx *et al.*, 2011). To provide reader-friendly data the occurrence of each corresponding family was represented as a normalised value calculated as described in the paper III. The numerical data on the occurrence of the IS elements in other bacterial classes (phylum) were mapped onto the cladogram constructed using 16s RNA sequences characteristic for the respective taxonomic groups.

AIMS AND OBJECTIVES

Analysis of the fish health reports annually published online by the Norwegian Veterinary Institute (data available starting from 2005) have demonstrated a good epidemiological situation with Cold Water Vibriosis in 2005-2010. During this 5 year period only 4 cases were detected in Norway. Starting from 2011 the situation began to change and only during 2011, 5 cases of CWV have been detected in Norway. In 2012 outbreaks of CWV occurred at 21 farms affecting vaccinated Atlantic salmon. In 2013, 13 outbreaks occurred. Regarded as a disease fully controlled by vaccination until 2011, CWV again became a considerable disease in the annual fish health reports. The motivation for this study originated from the hypothetical impact of mobile genetic elements on bacterial adaptation to pathogenicity in other host species than Atlantic salmon and the recent apparent inability of vaccination to provide acceptable protection against CWV. Analysis of nearly 90 research papers dedicated to CWC in 1975-2014 demonstrated a lack of important data on the early dynamics of the disease as well as a lack of clear information on the portal of CWV infection.

The **aim** of the current project is to fill in the gaps in the knowledge on early dynamics and portal of infection for Cold Water Vibriosis as well as to characterize mobile genetic elements potentially driving adaptation of the microorganism to pathogenicity.

To solve the assigned aim the following **objectives** were formulated:

1) Outline and further examine the role of gills, skin, fin blood vessels and gastrointestinal tract as portals for CWV infection.
2) Optimize and standardize the experimental procedures for comparative evaluation of impacts of different factors on the initiation of CWV infection in susceptible species.
3) Study effects of temperature as well as bacterial concentration on the efficiency of bacterial entry into the blood of Atlantic salmon.
4) Perform characterization of the mobile genetic elements (IS elements) in the genome of *A. salmonicida* LFI1238.
5) Examine the potential role of IS elements as factors driving adaptation of *A. salmonicida* to pathogenicity.

ETHICAL CONSIDERATIONS

The use of animal experimental models in general is still important despite recent methodological developments in non-animal models. After a certain stage, *in vitro* experiments can no longer provide sufficient and reliable data that can be extrapolated for treatment of humans or diseased animals. Implementation of modern statistical approaches along with sophisticated experimental designs nowadays brought the use of experimental animals to historical minimums (FDU, 2011). Known for decades, research involving experimental animal models has been brought to a large scale only at the 20th century when the scientific community collected sufficient amount of data on the mechanisms and prerequisites for disease development. The scientific revolution of the 20th century led to a dramatic increase in the amount of experimental animals involved into research and already in the middle of the 1970's, beginning of the 1980's only in US up to 20 million of laboratory animals has been involved into experimentation each year (Caplan, 1986; Taylor *et al.*, 2008).

Ethical considerations of animal use evolved side by side with the evolution of mankind. Prerequisites for ethical dilemmas date back to the 4th century BC when on the pages of "Historia Animalium" Aristotle (384-322 BC) argues that "animals have memory, and are capable of instruction at the same time in contrast to humans they cannot recall the past at will" (Thompson, 1910). Further observing differences between animals, Aristotle postulate that some "animals, like plants, simply procreate their own species at definite seasons; other animals busy themselves also in procuring food for their young, and after they are reared, quit them and have no further dealings with them" a third group of animals "is more intelligent and endowed with memory, and they live with their offspring for a longer period and on a more social footing" (Thompson, 1910).

Aristotelian concepts of animal gradation and subordination to human being possessed its remarkable viability and got further development in the postulates of René Descartes (1569-1650). Equating soul with mind in his famous "Cogito ergo sum" (Moriarty, 2008), Descartes defines non-human animals as machines acting in a situationally appropriate manner (Hall, 2003). Largely due to efforts of vivisectionists, predominating for another two centuries the mechanistic concept of animal essence existed, but was no longer accepted by society by the end of 18th century.

In 1789 Jeremy Bentham postulated his fundamental principles that changed the course of all animal experimentation. "Can they *reason*? nor, Can they *talk*? but, Can they *suffer*?" (Hart, 1996). Through the depth of this phrase modern ethical standards of animal experimentation are based on the principles of the best possible care and avoidance of unnecessary pain or suffering. In 1959 the initial concept of Bentham was further shaped by Russell and Burch. In the publication "The principles of humane experimental technique" they evaluated unethical and inhumane aspects of animal experimentation and proposed approaches for their diminishing and removal (Russell *et al.*, 1959). Today the *leitmotiv* of three R's proposed by them has become a standard of animal experimentation.

The principles of Replacement, Reduction and Refinement can be found in both legislative documents regulating animal use in Norway – The Norwegian Animal Welfare Act (LOV, 2009) and in the Norwegian Regulation on Animal Experimentation (dyr, 1996) complying with a European Directive EC 86/609 issued in 1986 (86/609/EEC, 1986). Both documents provide legal and ethical standards for experimentation with mammals, birds, reptiles, amphibians, fish, decapods, honey bees and Cephalopoda. According to §13, to deal with animal experimentation both the institution and a person responsible for experimentation must be approved by the control authority. In addition, approval of the experimental protocol cannot be achieved without compliance with the ethical norms and if results can be achieved without use of animals. Besides that, the number of animals used shall be restricted to the number necessary and animals should be subjected to least possible strain. While the Norwegian Animal Welfare Act has a broad orientation and oriented on all members of the society, the Regulation on Animal Experimentation specifically covers activities held by research institutions. To ensure the best possible treatment for animals, the regulation enforces the use of tranquillizing, sedative or analgesic agents. Paragraph 12 of the Animal Experimentation regulation determines care and supervision of animals under experimentation.

According to the requirements of both the EU directive and national legislation, experiments performed in the current study have been conducted after all necessary training and examination. Experiments were performed at a certified animal research facility. National animal research authority approved the study design. Implementation of anaesthesia in combination with improved study design ensured minimal stress levels for experimental animals at the same time allowing us to obtain the best possible data.

SUMMARY OF PAPERS

Paper I

A novel in vivo model for rapid evaluation of *Aliivibrio salmonicida* infectivity in Atlantic salmon. Alexander Kashulin, Henning Sørum // Aquaculture., 15 January 2014., Volumes 420–421., Pages 112–118.

Due to wide implementation of apparently effective vaccines in all farmed Atlantic salmon (*S. salar*) the phenomenon of Cold Water Vibirosis gradually went out of research focus. Since CWV emerged as a recognized infection in the early 1980s, several attempts have been undertaken to identify the initial steps of the pathogenesis of CWV, however, no final explanation to how *A. salmonicida* enters the host has been reported. In this study, we present a novel and simple model for analysing the initial steps of CWV. To identify the initial pathogenic steps in CWV, Atlantic salmon fry were differentially immersed in a suspension of *A. salmonicida* and the number of bacteria entering the host was measured. Analysis of CFU recovery rates from the blood of the experimental animals have demonstrated that *A. salmonicida* enters the host much faster than was anticipated. In particular, 15 minutes of immersion into a bacterial suspension was found to be enough to repeatedly recover the pathogen from the blood of Atlantic salmon. Bacterial counts were obtained from freshly collected blood samples, thus representing immediate snap-shots of the early stages of host invasion. The putative roles of the gills, skin, rectal and oral routes as well as the role of the fin blood vessels as portals of infection were investigated. The results clearly indicated that skin was a major route of infection. The experimental design reported in this study provides a new, rapid and cost-effective model for studying CWV.

Paper II

Early bacteremia in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) during immersion challenge with *Aliivibrio (Vibrio) salmonicida*. Alexander Kashulin, Henning Sørum // submitted to Microbial Pathogenesis. Manuscript number: YMPAT-D-14-00042.

Despite a high number of bacteria in blood that would have caused critical systemic disease in other species, Atlantic salmon fry demonstrate normal behaviour at early stages of Cold Water Vibriosis. In the current study we have further evaluated the phenomenon and tested impacts of temperature as well as bacterial concentration on the efficiency of bacterial entry

in the blood of Atlantic salmon. In addition we reported detailed dynamics of *A. salmonicida* CFU recovery rates from the blood of Atlantic salmon up to 22 days post challenge. To further demonstrate the universal value of the model and its applicability for studies of CWV in other susceptible species, the early dynamics of CWV infection has been studied in rainbow trout (*Oncorhynchus mykiss)* known to be less susceptible to cold water vibriosis. Transfer of the pathogen into the blood stream of Atlantic salmon was observed for suspensions with concentrations above 3×10^3 CFU x ml^{-1} during 10 minute challenges. The level as well as the duration of the bacteremia was found to be temperature dependent and dependent on the initial dose of the pathogen. Rainbow trout demonstrated lower level of as well as a more protracted bacteremia by *A. salmonicida* in comparison with Atlantic salmon. Comparison of the CFU recovery dynamics from the blood of both species allowed us to hypothesize the characteristics of the TLR receptor systems as the most probable reasons for the differences in susceptibility of Atlantic salmon and rainbow trout to Cold Water Vibriosis.

Paper III

IS Elements in Aliivibrio salmonicida LFI1238: Occurrence, Variability and Impact on Adaptability. Alexander Kashulin, Henning Sørum, Erik Hjerde, Nils P. Willassen // submitted to Gene. Manuscript Number: GENE-D-14-00038.

In the current study, we performed a complex analysis of IS elements (Vsa IS) in the previously sequenced genome of *A. salmonicida* LFI1238 and proposed a model of the spread of the Vsa IS elements over the genome of this microorganism. Along with the prediction of the integration sites for Vsa IS elements, the current study provided an overview of the properties of *A. salmonicida* IS elements, as well as reported information regarding their occurrence in different bacterial classes. An analysis of individual alleles of the IS elements has allowed us to depict a history of the accumulation of mutations and to describe distinctive microevolution lines for actively transposing Vsa IS elements. The accumulation of mutations generally has negative effects on the fitness of IS elements, eliminating these elements from further transposition processes and creating dead-end IS element offsprings. The differential transposition of IS elements from distinct chromosomal loci under a variety of environmental conditions contributes to the creation of bacterial subpopulations with different arrays of inactivated genes and, thus, different fitness. Increased rates of bacterial evolution that is mediated by the differential transposition of IS elements correlate well with epidemiological observations of Cold Water Vibriosis outbreaks caused by *A. salmonicida*.

RESULTS AND DISCUSSION

The current section represents a general condensed discussion of the combined results of all three papers of the study and corresponds to the aims and objectives of the study. A more specific and detailed discussion additionally complemented with findings by other authors can be found in the respective papers.

The animal model general

Fish subjected to experimental procedures showed behaviour typical of salmon juveniles (Vaz-Serrano *et al.*, 2011) already four minutes after being placed into the tank containing clean fresh water. The feeding habits of the experimental animals did not differ significantly from those of unexposed individuals which serve as an important indication of the well-being of the fish (Folkedal *et al.*, 2012; Pankhurst *et al.*, 2008). By mplementing the model in the research it is necessary to remember its limitations significant for some types of studies while irrelevant for other types of experiments (Hartung, 2008). In particular, determination of whether anesthetized salmon have similar susceptibility to pathogens to that of non-anesthetized fish is required (Ferreira *et al.*, 1981; Iwama *et al.*, 1989). Additionally it is necessary to compare (Eggset *et al.*, 1997) the susceptibility of salmon fry (not adapted to marine water through a natural smoltification process) with smoltified individuals which have undergone a complete cycle of physiological challenges (Piironen *et al.*, 2013; Yamauchi *et al.*, 1985). In general the model has proven its universal value and applicability to other fish species. In our second paper the model has been successfully applied for evaluation of the susceptibility of rainbow trout to Cold Water Vibriosis. The obtained data correlates well with the data of paper I and allow us to conclude the applicability of the model not only for Atlantic salmon but of other fish species as well.

According to current European legislation, fish experimentation requires approval by the national animal research authority (86/609/EEC, 1986). The proposed experimental model not only offers a less laborious and more rapid design, but minimizes the risks associated with negative decisions made by the animal research authority. Our study clearly demonstrated that the challenge time can be decreased from 1-2 hours to 3-10 minutes followed by immediate blood sampling. This significantly reduced the duration of the experiments which can be performed under full anesthesia. Due to the terminal manner of the studies, application for experiments based on our model will unlikely receive negative comments from a competent body reducing the time required for coordination of the experiments. In addition, the model

decreased the magnitude of the nonspecific physiological responses in the experimental animals and thus provided more reliable and reproducible data. Implementation of our model into research protocols may potentially be used to reduce the number of research animals needed for routine vaccine batch studies, thus reducing costs and improving animal welfare (Russell *et al.*, 1959). Despite the fact that further comparison of the method reported in this study with a traditional disease to death models (Purcell *et al.*, 2008) is still required, the impact of this model at several levels of the vaccine testing process is potentially of high significance.

The fish work

Continuing discussion of the peculiarities of the experiments performed in frames of the current study we would like to emphasize that potential weakness of our study related to estimation of bacterial counts by direct cultivation should not be considered as weakness. Having the whole arsenal of modern techniques available in our laboratories we have intentionally chosen the classical, easiest and the most elegant way for determination of bacterial concentration. By reporting the direct CFU counts we would like to demonstrate that even the simplest techniques can still provide excellent, previously unknown data when the experiments are planned correctly. Especially taking into consideration that comparative study by Løvoll et al. have demonstrated that plate counts provide the same accuracy as real-time PCR studies (Løvoll *et al.*, 2009).

Implementation of the qPCR (Horváth *et al.*, 2013), immunochemistry (Álvarez-Barrientos *et al.*, 2000; Boye *et al.*, 1983) or even mass spectrometry techniques (Ballabio *et al.*, 2014; Ho and Reddy, 2010; Thorn *et al.*, 2011) will definitely create a more spectacular and impressively looking publication. At the same time, significantly increasing the costs of the study, implementation of such techniques will not increase the value of the obtained scientific data. In addition such techniques have their own limitations and thus can to even greater extent incorporate the error into estimated bacterial counts. In particular, the qPCR is highly dependent on the selected markers as well as standards used for quantification (Mackay, 2004). Examination of the genome of the *A. salmonicida* have demonstrated that a majority of the genes traditionally used in qPCR studies are multiplied and exists in several copies. For example, the 16s RNA gene (which use as a marker is highly disputable in itself) exists in 16 copies and its use as a marker will require additional normalization incorporating even larger errors to the CFU estimations. Due to peculiarities of the genome organization of *A. salmonicida* (duplications, disruption by insertion of IS elements, gene loss) an accepted

(standard) panel of the qPCR primers for quantitative studies of this particular microorganism does not exist. Such circumstances implementation of any custom selected primer set creates an even more disputable situation. The similar aspects apply for immunochemical (Mackay, 2004) and to even larger extent mass-spectrometry techniques (Ho and Reddy, 2010). Implementation of these methods to blood samples requires intermediate separation/extraction/preparation steps (Álvarez-Barrientos *et al.*, 2000; Ho and Reddy, 2010; Horváth *et al.*, 2013) and as it was already mentioned will introduce even greater error when compared with CFU estimation by direct blood plating.

Likelly the most contradictory aspect of our work is related to the involvement of the fish of different sizes into the same study. The study was performed assuming that susceptibility of the fish to the pathogen is not dependent on the size of the fish, when the volume of the bacterial suspension is excessive. In other words, the amount of bacteria entering the fish of the particular size will be 10 times less than the amount of bacteria entering the fish of the same species which is 10 times bigger. Despite the fact that fish of the different sizes will "absorb" different amount of bacteria, the concentration of the bacteria in the blood of both species will be the same after exposure of the same duration. It might be appropriate to mention that several studies have demonstrated that salmonid susceptibility to at least two bacterial pathogens is dependent on the age of the fish (Fujihara *et al.*, 1971; Perez *et al.*, 1996). At the same time, no information was found for size dependent susceptibility of Atlantic salmon and rainbow trout to CWV (likelly because it was not discovered – negative results often remaining unpublished). Unfortunately, the assumption that susceptibility to the pathogen is not dependent on the size of the fish was not verified experimentally in our study and thus we cannot conclude that the observed differences are significant until additional studies will be conducted.

It is necessary to indicate that the comparison of the susceptibilities of the two salmonid species was not the main objective of paper II.

The computer work

Looking at the methods used in paper III we have to conclude that the main limitations of the study are not associated with the implemented methods. The chosen methodology allowed us to reliably describe the majority of the families of the IS elements present in the genome of *A. salmonicida*. Lack of a detailed description of several IS families is not associated with the methodology of the study, but is determined by the available raw data and available limits to the length of publication. Few a IS families present in the genome of *A. salmonicida* LFI1238

they exist in such low numbers that are not sufficient to make reliable conclusions on their integration sites. The problem of the detailed characterisation of such low copy number IS elements cannot be bypassed methodologically in the frames of the purely *in silico* study. The problem however might be solved by increasing the number of the genomes involved into the study (Ali *et al.*, 2013). We can assume that implementation of the cumulative data sets combining IS elements from different isolates will allow to obtain a new data similarly to the data obtained in other studies after integration of multiple data sets (Binnewies *et al.*, 2006).

Peculiarities of the TLR system as factors mediating transfer of the pathogen

After about 500 million years of evolution, the complexity of the immune responses varies between higher organisms however they all rely on conservative mechanisms of initial recognition of pathogens via a variety of Toll-like receptors (TLRs) (Litman *et al.*, 2010). Studies by Hayashi and colleagues indicated that activation of TLR receptors stimulates production of tumour necrosis factor-alpha (TNFα) as well as other co-stimulatory cytokine molecules (Hayashi *et al.*, 2001).

A recent study performed on Atlantic salmon and published in 2011 by Hynes et al. reveals an immune response in Atlantic salmon to recombinant flagellin of *V. anguillarum*. According to the authors Atlantic salmon possesses typical responses to *V. anguillarum* flagellin resulting in induction of inflammatory cytokines, chemokines, interferons and up-regulation of co-stimulatory molecules and in particular increased expression of TNFα (Hynes *et al.*, 2011).

While the immune reaction of Atlantic salmon on purified bacterial products are staying in line with the general knowledge about the responses of vertebrate organisms to bacterial invasion, analysis of induction of the immune response of Atlantic salmon against intact cells of certain pathogen species seems to be a different story. According to the most recent publication of Kvamme et al., describing the responses of Atlantic salmon to a water-based *V. anguillarum* vaccine, no responses were detected for TNFα known to be among the first responders with a rapid turnover (Kvamme *et al.*, 2012).

Absence of the immediate increase in production of TNFα as a response to the *V. anguillarum* vaccine described, but not explained by Kvamme et al. led the author of the current thesis to propose the potential ignorance of the surface antigens of *V. anguillarum* by salmon TLRs. Taking into consideration a close similarity of the pathogens (cross-reactivity of antibodies), such a hypothesis might be directly extended to *A. salmonicida*. Lack of the immediate TLR

recognition correlates well with data obtained in our study. In absence of the immediate activation of the TLRs *A. salmonicida* efficiently enters the host and thrives in the blood of the experimental animals.

The results of the challenges performed in our study as well as earlier studies related to the occurrence of *A. salmonicida* in the surroundings of the Atlantic salmon farms support the hypothesis of the existence of an asymptomatic state of the disease (initially proposed by Enger and colleagues in 1989, (Enger *et al.*, 1989; Enger *et al.*, 1991)) and indicate a delicate interplay between the environmental form of *A. salmonicida*, temperature as well as the innate immune system of the host.

Exposure to *A. salmonicida* at very low or very high temperature not always leads to CWV while interaction with a pathogen at 8-9ºC will likely ends up as a clinical infection. Freely entering the host at 8-9ºC *A. salmonicida* seems to inhibit its division. Not dividing, and not recognized as a pathogen, *A. salmonicida* is to a certain extent eliminated by the host macrophages without subsequent triggering of antigen presentation and activation of the immune response. Persisting in the host for the period required for adaptation, the pathogen initiates cell division which is observed as an increase of CFU recovery rates. Entering the host at certain optimal cell density allows *A. salmonicida* to shorten the time required for switching into a pathogenic mode from 120 to 48 hours.

Despite being relatively closely related, Atlantic salmon and rainbow trout species seem to contain genetic differences in the organization of the TLR system (Rebl *et al.*, 2007). A phylogenetic reconstruction of relations between 4 different classes of TLRs in teleost fish performed by Rebl and colleagues has demonstrated clear differences between Atlantic salmon and rainbow trout TLR5, TLR3, TLR7 and TLR11 receptors (Rebl *et al.*, 2010). A closer examination of the TLR22s in both species performed by the same authors has showed only 82% similarity in the amino acid sequences of the TLR22s (Rebl *et al.*, 2007). Since the complete genomes of Atlantic salmon and rainbow trout are unavailable, comprehensive analysis or at least full scale functional studies of the TLRs in both species have not been conducted. Therefore the discussion on the role of TLRs in the different susceptibility of the species to *A. salmonicida* will remain speculative. At the same time, the provided hypothesis correlates well with data on early dynamics of *A. salmonicida* CFU recovery rates from the blood of Atlantic salmon and rainbow trout obtained in the current study.

The role of IS elements as factors driving adaptation of *A. salmonicida* to pathogenicity

Rapid and efficient invasion of *A. salmonicida* into the host blood stream not only require tight collaboration with the host defence mechanisms, but in the first place relays on its effective adaptation to pathogenicity. Apparent domination of *A. salmonicida* isolates similar to *A. salmonicida* LFI1238 among the isolates associated with recent CWV outbreaks motivated us to take a closer look on the genome of this strain and evaluate potential drivers of the phenomenon. A more detailed overview of the insertion sequence elements present in the genome of *A. salmonicida* LFI1238 allowed us to reveal distinctive microevolution lines of the IS elements, not only inactivating the host genes, but also mediating inactivation of other IS elements and thus reducing the total inactivation potential of the mobilome.

Accumulation of mutations in the lines of actively transposing IS elements highlighted an important fundamental aspect of the involvement of host polymerases into transposition. Where relevant, synthesis of the complementary DNA strand of the IS element is mediated by a host polymerase with similar expected level of accuracy as for other host processes involving replication. Identification of numerous single copy IS offsprings containing mutations in the transposase SDSs clearly indicates the increased rates of introduced errors into those sequences. Mutated transposases originally responsible of recognition and transfer of the strands of the IS elements to new sites, are likely incapable of further activity thus creating a silent copy of the element.

The characterization of the insertion sequence alleles in this study is novel and no similar studies have yet been described. A clear explanation of the increased mutation accumulation rates in the transposase genes in *A. salmonicida* will remain speculative until further studies of the phenomenon have been conducted. An increased accumulation of mutations in the actively transposing IS elements correlate well with the existing knowledge on the bacterial CRISPR system. While Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system provides resistance to exogenous genetic sequences silencing their transcripts, the yet unknown pathway might provide a mechanism for directed introduction of mutations into those sequences. Complementing the CRISPR system, this yet uncharacterized pathway might potentially play a significant role in the cascade of bacterial intracellular immunity.

Characterization of the IS elements in the genome of *A. salmonicida* LFI1238 allowed us to describe important features of its DNA apparatus likely mediating the manner of distribution

of IS elements over the genome of this microorganism. Examination of potential mechanisms of termination of replication of the chromosomes (further designated as Chr_I and Chr_II) of *A. salmonicida* does not reveal the presence of the *Ter-Tus* system in the *A. salmonicida* LFI1238 thus indicating that termination of replication of its chromosomes likely occur by a conservative mechanism of a fork collision.

Search for the characteristic *dif* sites responsible for chromosome dimer resolution performed in our study demonstrated that while the Chr_I *dif* site is located in the area opposite to the *ori* and thus in the vicinity of the *ter* region, on Chr_II the *dif* site is shifted clockwise by approximately 85 kbp from the putative *ter* region of the chromosome. Despite the minor GC skew inconsistency likely associated with recombination events (Hjerde *et al.*, 2008) the major GC skew shift points on Chr_I of *A. salmonicida* LFI1238 are linked with *ori* and *ter* regions. The situation is different for the Chr_II where the GC skew shift points are associated with *ori* and *dif* sites. Such obvious discrepancy in organization as well as replication mechanisms might stay beneath the manner of localization of Vsa_IS elements on the chromosomes of *A. salmonicida* LFI1238. While multi-copy insertion sequences are uniformly spread over the genome, low copy IS elements (1-3 copies) tend to be grouped in the vicinity of the *ori* or the *ter* regions.

The concentration of the low copy number IS elements in the vicinity of *ori* and *ter* suggests that these regions acts as entry points for newly acquired insertion sequences into the genome of *A. salmonicida*. After entering the host, IS elements initially transpose into a number of loci and then further evolve independently creating their own microevolution lines within the particular host population. Similar explanation is likely applicable to the plasmids of *A. salmonicida* which seem to replicate by a Rolling-cycle mechanism (pVS43 and, pVS54) as well as *Theta* mechanisms (pVS320 and pVS840). An apparent ability of differentially transposing IS elements to drive the diversification of the host population (Wiens *et al.*, 2008) motivated us for a series of the experiments aimed on further evaluation of the phenomenon and determined direction of our research for the next few years.

CONCLUSIONS

The proposed experimental model for conducting CWV challenges in Atlantic salmon can shorten the duration of animal experimentation from the traditional 20-24 days to just 20-25 minutes. Blood sampling using sterile vacuum containers allows not only rapid evaluation and comparison of CFU counts, but also freeze-storage of primary samples important for subsequent vaccination quality control or studies on the effects of host-pathogen interactions on the metabolism and genetic organization of *A. salmonicida.*

The evaluation of the early bacteremia in Atlantic salmon and rainbow trout performed in our study has demonstrated outstanding properties of *A. salmonicida* as a pathogen of salmonid species. Entering the blood stream of the host at extremely high numbers, CFU counts undergo a significant drop within the first hours followed by a steady state period with extremely low, if any, bacterial recovery rates. Our studies have demonstrated that an immersion concentration of 3×10^3 CFU x ml^{-1} is enough to recover viable bacteria from the blood of experimental animals after 10 minutes of challenge. Known to be less susceptible to CWV (Brattgjerd *et al.*, 1995; Egidius *et al.*, 1986), rainbow trout demonstrated a lower level of *A. salmonicida* CFU recovery as well as a more protracted manner of the steady state period of low, if any, bacterial recovery rates. The reasons for the differences between Atlantic salmon and rainbow trout are likely hidden in the characteristics of TLR systems of the two species and require further evaluation.

According to the hypothesis tested in the third part of the current study, after entering the host, IS elements initially transpose into a number of loci and then further evolve independently creating their own microevolution lines within the particular host population. Increased accumulation of mutations by actively transposing IS elements might be an indication of the existence of a specific bacterial mechanism for directed introduction of mutations into xenogeneic sequences and thus might be of interest for a broad range of molecular biologists. Differential transposition of IS elements from various chromosomal loci under changing environmental conditions potentially contribute to creation of bacterial subpopulations with different arrays of inactivated genes and thus different fitness. Such a hypothesis correlates well with epidemiological observations of recent Cold Water Vibriosis outbreaks caused exclusively by *A. salmonicida* isolates similar to *A. salmonicida* LFI1238 and indicate the importance of further studies of the impact of IS elements on adaptation of *A. salmonicida* to pathogenicity.

FUTURE PROSPECTIVES

To further investigate the impact of IS elements on adaptation of *A. salmonicida* to pathogenicity in Atlantic salmon we have picked up and completely sequenced 20 isolates of *A. salmonicida* LFI1238 passaged in Atlantic salmon during experimentally induced Cold Water Vibriosis. Total DNA of the 20 isolates has been sequenced using Illumina MiSeq Gene & Small Genome Sequencer. The genomes of all 20 isolates are already assembled and will be annotated. A comparative study of the genomes of various isolates will allow us to understand the speed of diversification of the sub-populations of *A. salmonicida* LFI1238 and predict the expected number of host passages required for establishment of relatively distinct populations of the pathogen.

The reported model for rapid evaluation of infectivity of *A. salmonicida* in Atlantic salmon allowed us to significantly reduce the time of experimentation as well as considerably minimize the labour required in performing such experiments. Legerity of the model motivated us to further experiment with the parameters affecting transfer of *A. salmonicida* into the blood of Atlantic salmon and rainbow trout and such studies as evaluation of impact of vaccination, dietary conditions and stress levels will be or are already partially conducted.

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Paper I

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A novel in vivo model for rapid evaluation of *Aliivibrio salmonicida* infectivity in Atlantic salmon

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Skin as a major port of infection

ABSTRACT

Cold-Water Vibriosis (CWV) is a well-known disease which significantly influences the aquaculture industry of the North Atlantic coasts (Egidius et al., 1981). Due to wide implementation of apparently effective vaccines in all farmed Atlantic salmon (*Salmo salar*) the phenomenon of CWV was not a research focus for nearly two decades (Lillehaug, 1990, 1991). Although prevented by vaccination since the 1980s, CWV was again reported in farmed, vaccinated Atlantic salmon in 2012 (Johansen, 2012). Since CWV emerged as a recognized infection in the early 1980s (Egidius et al., 1981; Sørum et al., 1990), several attempts have been undertaken to identify the initial steps of the pathogenesis of CWV. However, no final explanation to how *Aliivibrio* (*Vibrio*) *salmonicida* enters the host has been reported. In this study, we present a novel and simple model for analyzing the initial steps of CWV. Our results demonstrate that initiation of CWV is more complex than was previously thought. In particular, *A.* (*V.*) *salmonicida* enters the host much faster than was anticipated. To identify the initial pathogenic steps in CWV, Atlantic salmon fry were differentially immersed in a suspension of *A.* (*V.*) *salmonicida* and the number of bacteria entering the host was measured. The putative roles of the gills, skin, rectal and oral routes as well as the role of the fin blood vessels as portals of infection were investigated. Bacterial counts were obtained from freshly collected blood samples, thus representing immediate snapshots of the early stages of host invasion. The results clearly indicated that skin was a major route of infection. The experimental design reported in this study provides a new, rapid and cost-effective model for studying CWV.

1. Introduction

The routes of infection for many pathogenic bacterial species are still largely unknown. Identification of the portals of infection requires step by step analysis of carrier agents as well as an understanding of molecular prerequisites for the initial steps of colonization. The uniqueness of the aquatic environment along with the morphology of gills, skin, mucosal membranes and other fish organs which are continuously in contact with water makes identification of the portal of bacterial infection challenging (Elliott, 2011; Fast et al., 2002; Suzuki et al., 2003; Wilson and Laurent, 2002). Bacterial invasion through the skin and gill epithelium, via fin blood vessels, intake through the gastrointestinal tract or a combination of these appears to play a critical role in the development of water-borne bacterial infections (Buras et al., 1985; Trust, 1986). Under normal conditions, various fish species demonstrate high potencies in defending themselves against invasive microorganisms, however, compromised immunity, stress or surface damage makes fish vulnerable to adhesion and subsequent invasion of pathogens (Ashley, 2007; Hansen and Olafsen, 1999; Toranzo et al., 2005).

The protective role of the skin should not be underestimated. In both aquatic and terrestrial species it serves as an interface with the environment helping to maintain the water balance of internal tissues and organs (Madison, 2003; Proksch et al., 2008). The skin of terrestrial species participates in the maintenance of body temperature, sensing, and defense against microorganisms and plays a role in protecting against harmful substances. The skin of aquatic species performs similar functions (Glover et al., 2013; Lee et al., 2006). The uppermost layer of the skin in terrestrial animals is composed of dead protein-rich cells and intercellular lipid domains. This two-component structure provides water impermeability properties similar to those of plastic films with similar thicknesses (Denda, 2000). In contrast to the dead outer stratum of the skin in terrestrial animals, the surface of fish, including the fins, is covered by live epithelium enriched with goblet cells responsible for mucus secretion (Lee et al., 2006). Along with the significant areal size of the fish skin surface its exposure to various mechanical injuries makes it an excellent candidate for pathogen invasion. To avoid bacterial colonization through the skin, aquatic animals utilize complex systems of defense measures. A central role in this defense line is surface mucus (Shephard, 1993, 1994; Svendsen et al., 1999). Constant renewal of mucus not only results in the physical removal of attached

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microorganisms, but also creates a thick high viscosity shield enriched with various antibacterial peptides, proteases, lectins and lysozymes (Narvaez et al., 2010; Palaksha et al., 2008; Subramanian et al., 2008). In addition to chemical factors in the innate immune system, the surface of fish skin is protected by various cellular defense mechanisms. Of these mechanisms, the most specialized ones are motile keratocytes that internalize bacteria thus removing them from the skin. In addition, keratocytes can actively migrate to surface wounds and form a new protective layer covering the damaged area (Aasbakk and Dalmo, 1998; Åsbakk, 2001; Karlsen et al., 2012).

Regarded as structurally similar to the skin of terrestrial species, fish skin is considered to have similar impermeability properties, and thus traditionally believed to be impenetrable to pathogens, which are generally richer in aquatic environments compared with the aerial habitats of terrestrial species. However, a study by Masson and colleagues demonstrated significant differences in the permeability of fish skin compared with the skin of terrestrial organisms (Másson et al., 2002). In particular, these authors indicated that drug flux through fish skin was more than 1×10^2 times faster than that through hairless mouse skin and more than 1×10^4 times faster than that through snake skin. The authors concluded that molecules are transported through fish skin in aqueous channels. Additional electron microscopy examination performed by the same authors revealed that the structure of mouse skin is much denser than the structure of catfish skin. In particular, the intercellular spaces in catfish skin were found to be larger (Másson et al., 2002).

Similar to skin, the respiratory organs of fish are continuously hydrated and thus subjected to suspended microbial agents. Active breathing accompanied by intensified water passage makes the gills even more vulnerable to infections. Reaching an area of several thousand square centimeters (Palzenberger and Pohla, 1992), the non-keratinized surface of the gills provides an excellent attachment site for bacterial pathogens (Løvoll et al., 2009). Due to extensive exposure to potential pathogens, gills like the skin are equipped with a vast arsenal of defensive measures including antimicrobial peptides, acute phase reactants, complement components as well as components of the adaptive immune response (Caipang et al., 2010). Of these, the most substantial role is that of the so-called undifferentiated epithelial cells (Monteiro et al., 2010a,b). These cells can fuse secondary lamellae entrapping pathogenic microorganisms which are further destroyed by migrating leucocytes. In some fish species, leucocytes express major histocompatibility complex class IIß molecules and are involved in further antigen presentation (Morrison et al., 2006; Star et al., 2011). In addition, novel intraepithelial lymphoid tissue, as part of the local immune system, has been described in the gill bows of Atlantic salmon (Haugarvoll et al., 2008).

The third major route of infection is by oral intake of contaminated water or food. It is generally believed that the acidic environment in the stomach significantly affects the probability of infection by decreasing the viability of microorganisms entering the gastrointestinal tract. Detailed studies of the pH cycles of the stomach in different fish species have demonstrated high variability in pH. The acidity of an empty fish stomach is relatively high and was found to be approximately 2.8 in rainbow trout (corresponding values in mammals are typically <2 (Gardner et al., 2002)). Experiments by Sugiura and colleagues demonstrated that the pH of chyme in the stomach of rainbow trout was around 4.0 even 9 h after feeding on commercial feed pellets. Intestinal pH measured within 24 h after feeding ranged from 7.15 to 8.65, depending on fish size and the time of measurement (Sugiura et al., 2006). Moreover, depending on the buffering capacity of the feed pellets or diet of wild fish stocks, the pH of a full stomach may be even more neutral and thus contribute to the survivability of microbial pathogens. For *vibrios* this has been demonstrated on *Vibrio anguillarum*, which under experimental conditions was able to survive in an acidic environment (Larsen, 1984). After passage through the stomach, microbial pathogens are subjected to bile salts in the intestine and numerous components of the cellular immune system associated with the intestinal epithelium including intraepithelial lymphocytes, macrophages and eosinophilic granular cells situated in the lamina propria (Dalmo et al., 1997). In addition, the gastrointestinal tract is covered with constantly renewed mucus which complicates the adhesion of microorganisms and their subsequent penetration into the epithelial layer, underlying tissues and blood vessels.

The earliest studies on the pathogenicity of *A.* (*V.*) *salmonicida* in Atlantic salmon were performed by Egidius and colleagues. These studies suggested that the gills were the most probable portal of infection (Egidius et al., 1981; Egidius et al., 1986; Hjeltnes et al., 1987; Sørum et al., 1990). In addition, it was demonstrated that surface wounds seem to enhance the probability of infection. Several subsequent studies have also hypothesized that the gills or the gastrointestinal tract are putative portals of infection (Bjelland et al., 2012). Unfortunately, no final conclusions have been made and the portal of infection for *A.* (*V.*) *salmonicida* in the development of CWV remains unknown. It has been demonstrated that the infectious process is rapid. *A.* (*V.*) *salmonicida* can be detected in the lumen of capillaries hours after experimental challenge and the colonization of other tissues develops much slower and requires days for disease development (Bjelland et al., 2012).

In the present study, we determined the major portal of CWV infection and report a novel experimental model for Cold Water Vibriosis. The role of the gills, skin, fin blood vessels and the gastrointestinal tract were evaluated for the invasion of *A.* (*V.*) *salmonicida* in Atlantic salmon.

2. Materials and methods

2.1. Bacterial strains and culturing conditions

All experiments in this study were performed using *A.* (*V.*) *salmonicida* LFI1238 originally isolated from a diseased farmed cod at the Norwegian School of Fishery Science, Tromsø, Norway (Hjerde et al., 2008). The bacterium was grown on a blood agar plate (agar base no 2, Oxoid CM271, supplemented with 5% whole bovine blood and 2.5% (w/v) NaCl) at 12 °C. Single colonies from the plates were then expanded in pre-culture using Luria-Bertani broth containing 2.5% NaCl at 12 °C, and incubated overnight with shaking at 220 rpm. After overnight incubation, the pre-cultures were expanded in larger culture volumes to the mid-log phase ($OD_{600} = 0.70$) under the same conditions and used in the experiments.

Cells of *A.* (*V.*) *salmonicida* were isolated from blood samples taken aseptically from the tail vein of salmon fry anesthetized in a bath containing benzocaine (0.03 mg/l) (Benzoak® 200 mg/ml ACD Pharmaceuticals AS, Leknes, Norway). Evacuated blood collection tubes with added heparin (Venoject® Terumo Europe N.V, Leuven, Belgium) were used for sampling blood. After blood sampling, the experimental animals were immediately euthanized by a sharp blow to the head. The collected blood samples were immediately transferred to the laboratory where 10 and 100 µl aliquots of each sample were plated on blood agar and incubated at 12 °C until visible growth.

2.2. Experimental animals and invasive procedures

This study was performed using unvaccinated Atlantic salmon fry (weight 30–50 g, from Sørsmolt AS, Sannidal, Norway kindly provided by Stein Helge Skjelde). The fish were kept in fresh water at the animal research facility of the Norwegian School of Veterinary Science (Oslo, Norway). Prior to the challenge with bacteria, the experimental animals were anesthetized in water containing benzocaine. Following signs of anesthesia, up to 5 fish were placed in the holding position allowing differential exposure of a defined body area and exposed to the bacterial suspension added in 0.03 mg/l benzocaine. To avoid dehydration and provide acceptable breathing conditions, open fish surfaces were

constantly hydrated by spraying with clean fresh water. The challenge with *A.* (*V.*) *salmonicida* was performed in marine water collected at a random location of the inner Oslofjord, Norway one day before the experiments. The collected water was stored at 4 °C and was heated to the appropriate temperature immediately before the experiments.

Earlier studies demonstrated that *A.* (*V.*) *salmonicida* does not tolerate fresh water conditions, and thus water transmission of the disease in freshwater is avoided. In these experiments, incubation in fresh water for 15 min was performed to kill any *A.* (*V.*) *salmonicida* on the fish surface prior to blood sampling. During the course of all the experiments the fish were fed with commercial feed pellets and regularly observed. The experimental groups were marked by fin piercing in a manner which did not affect exposed surfaces (hole 1 mm in diameter between fin spines).

2.3. Challenge procedures

All experiments described in this study were performed according to existing ethical standards for animal experimentation (EU Directive 2010/63/EU for animal experiments) and were approved by the National Animal Research Committee.

To outline and then further examine putative portals of CWV infection, experimental animals were differentially immersed in a bacterial suspension containing 3×10^7 CFU $\times$ ml^{-1} *A.* (*V.*) *salmonicida* LFI1238. To ensure comparable conditions for the various experimental groups, post-challenge holding was performed in the same holding tanks supplied with fresh running water. Experimental animals were randomly assigned to the following immersion groups: head and gills (group 1); tail excluding the anal opening (group 2); tail including the anal opening and the skin area immediate above it (group 3); the most distal end of the tail (group 4); and full body immersion served as a control (group 5). Such stratification allowed us to examine the role of the gills, skin, rectum, tail blood vessels and full body immersion and to compare their impact on initiation of CWV infection.

To outline the putative role of the portals for initiation of CWV and evaluate the reproducibility of the results, the study was performed as a series of experiments consisting of 4 independent challenges. During challenge 1, which was used to collect pilot data, the role of the most distal end of the tail fin was not studied. The experiment was performed using 38 fish (10 in the experimental groups and 8 in the control) which were differentially immersed in the bacterial suspension containing 3×10^7 CFU $\times$ ml^{-1} *A.* (*V.*) *salmonicida* LFI1238 for 10 min. During immersion, the temperature of the suspension was maintained at 10 °C. After immersion the exposed fish were placed into a 200 l holding tank supplied with flow-through freshwater (9 °C) and kept there for 24 h. The next day, blood samples were collected and processed. The presence of *A.* (*V.*) *salmonicida* in each blood sample was graded as "no growth", "occasional", and "rich" growth. To control potential swallowing of the bacterial suspension during head and full body immersion, one fish was immersed in bacterial suspension stained with Bromophenol blue for 10 min. After immersion, the content of the stomach of this fish was examined for coloration.

In the second challenge, 48 fish were assigned to 4 experimental groups (12 fish in each group) in the manner mentioned earlier (first challenge). As before, the animals were challenged with a bacterial suspension containing 3×10^7 CFU $\times$ ml^{-1} *A.* (*V.*) *salmonicida* FI1238 for 10 min at 10 °C. After exposure, all salmon fry were returned to the 200 l tank with running fresh water for post-challenge holding. After 72 h the fry was anesthetized, and blood was sampled using evacuated blood collection tubes containing heparin.

During the third challenge, experimental animals were immersed in 3×10^7 CFU $\times$ ml^{-1} bacterial suspension for 10 min at 10 °C. The experiment was performed using 48 animals assigned to groups as described in the first challenge. After the challenge, all the salmon fry were marked with 1 mm holes in the fins as described and placed into the same 200 l tank with running fresh water for 1 h to eliminate all residual *A.* (*V.*) *salmonicida* from the surface of the fish. After 1 h in fresh water the fish were anesthetized, and blood was sampled using vacuumed tubes containing heparin as anticoagulant. As in all previous experiments, the blood samples were transferred to the laboratory and plated on blood agar with 2.5% NaCl. The plates were incubated at 12 °C until observable growth and then used for CFU $\times$ ml^{-1} determination. After blood sampling, the salmon fry was kept in the benzocaine solution and euthanized by a sharp blow to the head.

The first three challenges were used for evaluation and optimization of the experimental conditions, and challenge 4 was used to validate the reproducibility of the results and to demonstrate the efficacy of the model. Challenge 4 was performed with 18 experimental animals allocated into 3 groups containing 6 fish each (thus forming 3 independent parallel studies in one experiment). The salmon fry were immersed in 3×10^7 CFU $\times$ ml^{-1} bacterial suspension for 3 min at 10 °C and blood was sampled after 15 minute incubation in fresh water.

2.3.1. Data analysis and representation

Taking into account the wide distribution of the CFU recovery rates within the experimental groups, we decided to represent our data graphically in the form of box-and-whisker diagrams. This method of data representation allowed us to exclude skewness risks arising from the differential susceptibility of the experimental animals to infection caused by variations in health status or stress levels associated with the hierarchal position of the animals within the population and to minimize the effects of small sample sizes. Qualitative data from challenge 1 used for initial screening were evaluated by the Mann–Whitney test for independent variables at a level of significance $\alpha = 5\%$ ($p < 0.05$). To test the null hypothesis, the observed growth rates were ranked as 1, 2 and 3 for "no growth", "moderate growth" and "rich growth" and treated accordingly. Quantitative data from subsequent experiments were evaluated by the Student's *t*-test for independent variables at the same level of significance. Large CFU counts were log-transformed and parametric statistical methods were used to draw conclusions. The "Zero problem" was solved by using $\log_{10}(1 + X)$ transformation rather than common logarithm transformation ($\log_{10}X$).

3. Results

3.1. Experimental model

The combination of anesthesia and immobilization in the holding rack ensured minimal stress levels in the experimental animals. Additional aeration of the bacterial suspension as well as constant hydration of the body areas exposed to the air resulted in no mortalities during the experiments and the post-experimental holding period. Moreover, fish subjected to the experimental procedures showed behavior typical of salmon juveniles 4 min after being placed in the tank containing clean fresh water. The feeding habits of the experimental animals did not differ significantly from those of unexposed individuals. Examination of gastrointestinal content performed in one fish revealed no signs of stomach or intestine coloration after head-gills exposure to the stained bacterial suspension.

Challenge 1 demonstrated that the identification of portals for CWV is more complex than previously reported by other authors. Bacteria were recovered from all examined blood samples (Table 1). Statistical evaluation of the data demonstrated the significance of the null hypothesis for full body immersion compared with immersion of the fish tail including rectal sphincter and the area above it ($p < 0.05$). Thus similar rate of invasion of *A.* (*V.*) *salmonicida* into the host via these areas was demonstrated. Immersion of the head or tail below the rectal sphincter showed no similarities in invasion rates when compared with full body immersion.

Initial screening demonstrated that this qualitative approach did not allow reliable identification of the portals for CWV infection. Based on the quantitative data obtained in the second challenge we can conclude

Table 1
CFU recovery rates (CFU × ml^{-1}) from the blood samples obtained after challenge 1. Experiment performed using immersion into suspension of *A.* (*V.*) *salmonicida* LFI1238 containing 3×10^7 CFU × ml^{-1} for 10 min. Following ranks have been used: 3 — corresponds to rich bacterial growth, 2 — indicates occasional and 1 — no bacterial growth. Full immersion used as a control.

Fish no	Head	Tail including anal opening	Tail excluding anal opening	Full immersion
1	2	3	1	3
2	2	2	2	3
3	3	3	1	3
4	3	2	2	3
5	1	2	3	2
6	1	2	2	3
7	3	2	2	3
8	1	2	1	3
9	1	3	2	
10	3	2	1	

that infection developed more frequently in fry in the control group. Infection in other experimental animals seemed to occur occasionally at a level of significance of $\alpha = 5\%$ ($p > 0.05$) when compared to the control group. The group with the tail tip immersed in the bacterial suspension showed no growth (Table 2, Fig. 1).

Final CFU counts recovered from the blood of experimental animals after immersion in the bacterial suspension during challenge 3 are presented in Table 3. Strong growth was observed in the animals which were fully immersed. Exposure of the tail tip resulted in a single case of relatively low growth (Fig. 2). Exposure of the tail including the rectal sphincter as well as head exposure demonstrated low, but stable CFU recovery which was probably associated with minor skin exposure to the bacterial suspension in these experimental groups. A statistical comparison demonstrated significant differences in all experimental groups when compared with the control ($p < 0.05$).

In challenge 4, CFU recovery was observed in all 3 experimental groups (Table 4). A statistical comparison of the CFU counts demonstrated the same origin of the variables ($p > 0.05$) (Fig. 3).

4. Discussion

The purpose of this study was to establish a new experimental model suitable for reproducible and comparable studies on the portal of infection for *A.* (*V.*) *salmonicida* which causes Cold-Water Vibriosis in Atlantic salmon. Previously reported challenge experiments gave no clear answers regarding the route of CWV infection (Bjelland et al., 2012; Hjeltnes et al., 1987; Nordmo et al., 1997). In addition, the design of these studies was time consuming and cost-ineffective. Previously reported exposure and post-exposure holding time resulted in disputable welfare conditions for the model animals. In our experiments we examined all three putative portals of infection using uniform conditions and demonstrated their individual contribution to disease development. In addition, our data indicate that the initial stage of the infectious process is much faster than was previously hypothesized. In particular, 3 min of exposure to the bacterial suspension was sufficient to detect microorganisms in the blood of the experimental animals.

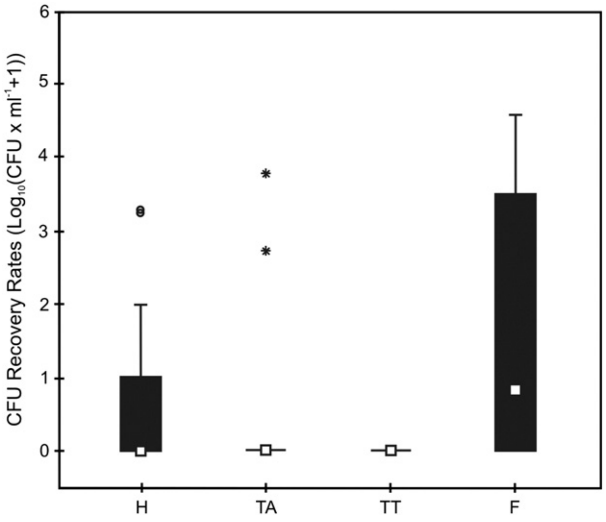

Fig. 1. CFU recovery rates from the blood samples obtained after challenge 2. Experiment performed using immersion into suspension of *A.* (*V.*) *salmonicida* LFI1238 containing 3×10^7 CFU × ml^{-1} for 10 min. H, head. TA, tail including anus opening. TT, tail tip. F, full body immersion. Full body immersion used as a control. Data represented as $\log_{10}$(CFU × ml^{-1} + 1) and expressed as median box: 25%–75%; whiskers: non-outlier range; outliers: ○; extremes: *.

Our results clearly demonstrate that *A.* (*V.*) *salmonicida* not only requires a short invasion time, but also enters the host in extremely high numbers reaching concentrations up to 1×10^6 CFU × ml^{-1} of blood after 3 min of exposure. From previous experimentation on CWV it is known that immersion in a suspension of *A.* (*V.*) *salmonicida* even for long periods (1–2 h) does not always lead to the development of CWV infection. Healthy individuals have been repeatedly recorded among diseased animals. In longer challenges, when the role of secondary bacterial transmission by marine water is feasible, the fate of these healthy individuals has been defined and they become diseased due to co-habitation and constitute a distal part of the mortality curves (Nordmo et al., 1997). However, in fresh water challenges, they successfully thrive for weeks without bacteria in the blood. A detailed study of such natural resistance is beyond the scope of the experiments reported here.

In a recent publication on the pathogenicity of *A.* (*V.*) *salmonicida*, Mohn and colleagues discuss the putative course of the initial steps of CWV (Bjelland et al., 2012). The authors hypothesize that unlike *Moritella viscosa*, *A.* (*V.*) *salmonicida* does not possess anti-keratocyte

Table 2
CFU recovery rates (CFU × ml^{-1}) from the blood samples obtained after challenge 2. Experiment performed by immersion into suspension of *A.* (*V.*) *salmonicida* LFI1238 containing 3×10^7 CFU × ml^{-1} for 10 min. Full immersion used as a control.

Fish no	Head	Tail including anal opening	Tail tip	Full immersion
1	0	0	0	0
2	0	0	0	2.0×10^2
3	0	0	0	0
4	0	0	0	3.5×10^4
5	1.6×10^3	0	0	4.0×10^1
6	0	4.0×10^2	0	3.3×10^3
7	0	0	0	0
8	0	4.1×10^3	0	0
9	0	0	0	3.7×10^3
10	1.5×10^3	0	0	0
11	0	0	0	0
12	1.0×10^2	0	0	2.7×10^3

Table 3
CFU recovery rates (CFU × ml^{-1}) from the blood samples obtained after challenge 3. Experiment performed by immersion into suspension of *A.* (*V.*) *salmonicida* LFI1238 containing 3×10^7 CFU × ml^{-1} for 10 min. Full immersion used as a control.

Fish	Head	Tail including anal opening	Tail tip	Full immersion
1	1.5×10^2	0	0	0
2	0	6×10^2	0	3.5×10^4
3	1×10^1	4×10^2	0	1.2×10^4
4	$1.7\ 10^3$	4×10^1	0	4.4×10^4
5	0	1.9×10^3	5×10^1	0
6	4.0×10^1	9.7×10^2	0	1.4×10^4
7	4.0×10^1	5×10^1	0	1.0×10^2
8	1.0×10^1	5×10^1	0	9.10×10^3
9	0	0	0	3.8×10^4
10	2.0×10^1	1.1×10^1	0	1.7×10^3
11	4.0×10^1	0	0	$1. \times 10^2$
12	7.0×10^1	5×10^1	0	2.3×10^4

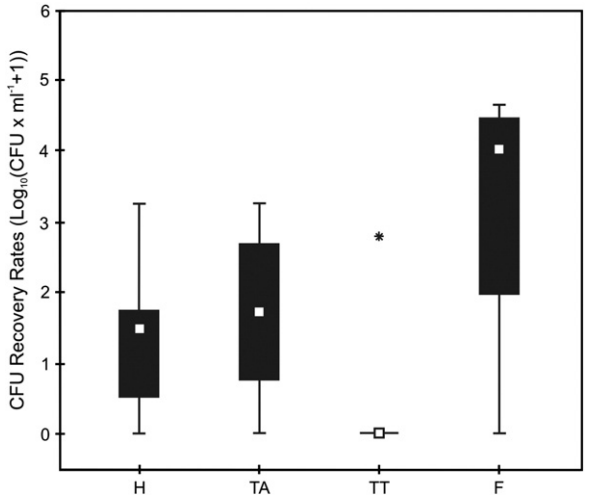

Fig. 2. CFU recovery rates from the blood samples obtained after challenge 3. Experiment performed using immersion into suspension of *A.* (*V.*) *salmonicida* LFI1238 containing 3×10^7 CFU $\times$ ml^{-1} for 10 min. H, head. TA, tail including anus opening. TT, tail tip. F, full body immersion. Full body immersion used as a control. Data represented as $\log_{10}$(CFU $\times$ ml^{-1} + 1) and expressed as median box: 25%–75%; whiskers: non-outlier range; outliers: ○; extremes: ⋆.

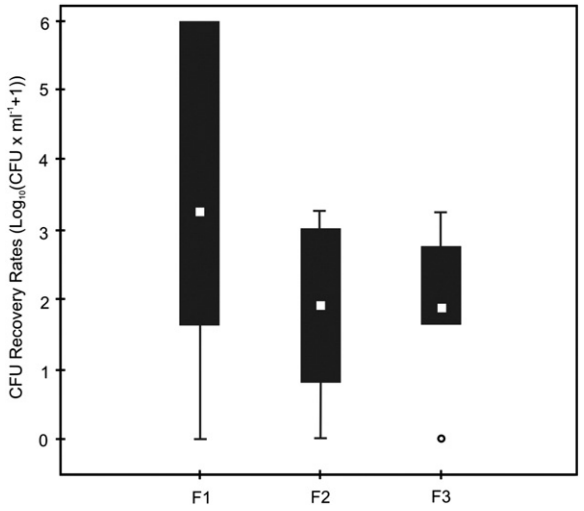

Fig. 3. CFU recovery rates from the blood samples obtained after challenge 4. Experiment performed using immersion into suspension of *A.* (*V.*) *salmonicida* LFI1238 containing 3×10^7 CFU $\times$ ml^{-1} for 3 min. F1–F3 full immersion; number corresponds to number of the experimental group. Data represented as $\log_{10}$(CFU $\times$ ml^{-1} + 1) and expressed as median box: 25%–75%; whiskers: non-outlier range; outliers: ○; extremes: ⋆.

properties and intact fish skin provides a non-penetrable barrier to the microorganism. The same study in addition to a number of previously published studies refuted the gastrointestinal tract as a portal of CWV infection and regarded the gills as the most probable portal. Despite a number of associated cellular and humoral defense mechanisms, the morphological structure of fish respiratory organs makes them excellent candidates for microorganism invasion. The size of the gill surface and swimming habits of the Atlantic salmon only exacerbate this situation. A morphological comparison of the gills of fast-swimming pelagic and sluggish benthic fish illustrated that during evolution fast-swimming species developed larger gas-exchange gill surfaces. In addition, the size of the blood to water distance in the respiratory regions of these species is about 0.533 μm (Wilson and Laurent, 2002). Compared to the size of *A.* (*V.*) *salmonicida* cells of approximately 0.5 to 3 μm (Egidius et al., 1986) this provides almost immediate penetration into blood vessels. Despite the apparent minimal water-blood distance in the gills of fast swimming fish species, our experiments have demonstrated that infection via the gills has low or no relevance in the initiation of CWV infection. Infection via head exposure is likely associated with co-exposure of significant skin area during head dipping in the bacterial suspension. In support of this conclusion, dipping the opposite side of the fish body had a similar effect on the initiation of CWV infection. Immersion of the fish tail results in sporadic cases of CFU recovery.

In addition to fish skin, the wall of the gastrointestinal tract provides a much greater physical barrier between the outer environment and the internal tissues and organs of the fish (Løkka et al., 2013). However, despite the actual thickness of the gastric or intestinal wall, the existence of specialized antigen presenting cells in the gut wall of teleost fish can result in rapid translocation of the intact bacteria directly to blood vessels or macrophages (Bassity and Clark, 2012). In macrophages, *A.* (*V.*) *salmonicida* can successfully propagate and is transported to the liver. Moreover, selective endocytosis of pathogenic microflora has been demonstrated not only in specialized, but also in regular epithelial cells of many salmonid species (Ringø et al., 2001). In addition, it was hypothesized by Mohn Bjelland and colleagues that *A.* (*V.*) *salmonicida* can cause disruption of the tight junctions typical for *Salmonella* species, and thus overcome epithelial layers (Bjelland et al., 2012). A similar phenomenon was recently described for *Aeromonas salmonicida* (Jutfelt et al., 2006). A number of secretary proteases have been annotated in the recently sequenced genome of *A.* (*V.*) *salmonicida* (Hjerde et al., 2008), however, detailed studies on their role and mechanism of action have yet to be performed. Translocation over the gastrointestinal tract wall most likely takes place in the posterior intestine which can be accessed by bacteria either through ingestion or bacterial translocation through the anal opening. To determine if ingestion of the bacterial suspension used in the experiments occurred, the stomach of one fish was examined to assess the presence of bacterial suspension using Bromophenol blue staining. Following exposure to *A.* (*V.*) *salmonicida* no coloration was observed in the stomach. Experimental animals were fed to satiety prior to the experiments. The examined stomach had an exaggerated shape, and was full of feed pellets. Taking into consideration that all fish were kept under the same conditions and were selected from the same stock, we can extrapolate this observation to all the groups and exclude ingestion of the bacterial suspension as an important factor affecting the outcome of the challenge experiments.

The impact of the gastrointestinal tract on the initiation of CWV was tested by exposing the posterior part of the fish body including the anal opening and an area of 5 mm to the bacterial suspension. Exposure of the terminal part of the gastrointestinal system along with the tail did not lead to an increase in CFUs recovered from blood samples compared to exposure of the skin behind the anal opening. A slight increase in the CFU recovery rate from the blood of animals after immersion of the tail to the rectal sphincter and slightly above was probably associated with the increased area of skin exposed to the bacterial suspension. Dipping of the tail end which is enriched with blood vessels did not lead to host invasion at rates detectable by the methods used in our study. Such an observation not only supports the role of the skin in the infectious process, but also indicates that a short physical distance between the environment and blood stream (as in the gills) is not highly important for

Table 4
CFU recovery rates (CFU $\times$ ml^{-1}) from the blood samples obtained after challenge 4. Experiment performed by immersion into suspension of *A.* (*V.*) *salmonicida* LFI1238 containing 3×10^7 CFU $\times$ ml^{-1} for 3 min.

Fish	Full immersion	Full immersion	Full immersion
1	6.7×10^5	2.8×10^3	2.8×10^3
2	1.0×10^6	1.4×10^3	8.0×10^1
3	1.0×10^6	8.0×10^1	1.3×10^2
4	0	1.2×10^2	7.8×10^2
5	1×10^2	1.0×10^1	0
6	1×10^2	1.0×10^1	6.0×10^1

initiation of CWV infection. Thus, translocation over the rectal sphincter has no or minimal impact on the initiation of CWV under the chosen experimental conditions.

It should be emphasized that the majority of the studies on CWV have been conducted using rich bacterial suspensions under artificial laboratory conditions. The cell densities used in these studies can never be reached in nutrient-limited ecological niches of the open marine environment. While use of a rich bacterial culture in the challenge increased the probability of initiation of infection and increased the chances of identifying the true portals for CWV infection, experimentation with low doses of the infectious agent is also of significant interest.

The experimental model used in the present study resembles natural infection conditions where multiple routes can contribute to the initiation of infection. In this study, the role of ingestion of contaminated food or consumption of contaminated water was not investigated. Nevertheless it can be hypothesized that these factors would have significant impact only in wild salmon stocks whose diet, and thus chances of infection, are directly correlated with seasonal feeding behavior and migration patterns. In contrast to wild salmon populations, animals in aquaculture are fed regularly and excessively. Moreover, commercial dry feed pellets are supplied in a presterilized form, and thus are not contaminated with *A.* (*V.*) *salmonicida*. In all the experimental challenges performed in this study, we clearly demonstrated the role of the skin as a major portal of CWV infection. In challenge 1, we demonstrated that an increase in the skin area exposed to the bacterial suspension leads to increased CFU recovery rates in the blood of experimental animals. Subsequent quantitative evaluation of the host invasion rates showed that the invasion process was extremely rapid, and thus allowed alteration of the experimental model from a long laborious process to an elegant and simple routine.

When implementing the model in daily practice it is important to remember its focus on the earliest stages of the infectious process, that is, the transfer of *A.* (*V.*) *salmonicida* from marine water to the blood of salmon. Furthermore, it is necessary to determine whether anesthetized salmon have similar susceptibility to pathogens to that of non-anesthetized fish. Finally, the development of a model requires a comparative study of the susceptibility of salmon fry (not adapted to marine water through a natural smoltification process) with smoltified individuals which have undergone a complete cycle of physiological challenges.

While the focus on *A.* (*V.*) *salmonicida* pathogenesis has been limited over the past two decades, the interest in CWV research is expected to increase from 2012, when 21 outbreaks of CWV were reported in vaccinated stocks of farmed Norwegian Atlantic salmon (Lillehaug, 1990, 1991; Johansen, 2012). An increase in demand for vaccination trials as well as the need for basic studies has demonstrated low conformity of the existing CWV models in high-throughput infectious experimentation. Requiring approximately 1 to 2 h of immersion, existing models are not only time-consuming, but result in significant stress levels in the experimental animals, thus influencing the outcome of the experiments. Long intervention may significantly influence experimental animal welfare conditions and is therefore suboptimal. Moreover, traditional models based on long post-experimental holding, where clinical symptoms or mortality is expected, are generally considered objectionable by national animal research authorities.

According to current European legislation, fish experimentation requires approval by the national animal research authority (EU Directive 2010/63/EU for animal experiments). The proposed experimental model not only offers a less laborious and more rapid design, but also minimizes the risks associated with negative decisions made by the animal research authority. Our study clearly demonstrated that the challenge time can be decreased from 1–2 h to 3–10 min followed by immediate blood sampling. This significantly reduced the duration of the experiments which can be performed under full anesthesia in a manner which does not compromise animal welfare conditions. Terminal studies, where all manipulations and subsequent euthanasia are conducted on fully anesthetized animals are regarded as humane and are unlikely to receive negative decision from a competent body. In addition, the model decreased the magnitude of nonspecific physiological responses in the experimental animals and thus provided more reliable and reproducible data.

Our novel in vivo model for testing the infectivity of *A.* (*V.*) *salmonicida* may potentially be used to reduce the number of research animals needed for routine vaccine batch studies, thus reducing costs. Despite the fact that further comparison of the method reported in this study with a traditional disease to death models (required by the regulatory authorities) is still required, the impact of this model at several levels of the vaccine testing process is potentially of high significance.

5. Conclusions

In summary, the outlined experimental approach can shorten the duration of animal experimentation from the traditional 20–24 days to just 20–25 min when blood samples have been collected and experimental animals euthanized while unconscious. Shortening the challenge time to approximately 10 min not only results in a reduction in resources required to conduct large scale experiments to study the infectivity of *A.* (*V.*) *salmonicida*, but also significantly contributes to improved fish welfare as the fish can be anesthetized during the experiment. According to our observations, when planning further studies researchers should consider the following time intervals during freshwater CWV challenges: administration of anesthesia — 5 min; exposure to bacterial suspension (3×10^7 CFU ml^{-1}, 8–9 °C) — 3 min; and incubation in freshwater — 5 min. These time periods are not absolute and should be adjusted for each individual stock of fish taking into consideration their susceptibility to the chosen anesthetic, stress level, etc. In addition, in contrast to the traditional approach when bacteria are recovered from the internal organs of experimental animals by direct streaking on solid microbiological media (mostly head kidney), blood sampling using sterile vacuum containers allows not only rapid evaluation and comparison of CFU counts, but also freeze-storage of the primary samples. Preservation of the blood containing the pathogen in its original state and quantity may be important for subsequent vaccination quality control. Besides providing excellent reference possibilities, frozen primary samples will allow studies on the effects of host–pathogen interactions on the metabolism and genetic organization of *A.* (*V.*) *salmonicida*.

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Paper II

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ABSTRACT

In a recent study, we presented a novel *in vivo* model for the evaluation of cold water vibriosis (CWV) in Atlantic salmon (*Salmo salar*) and studied the role of different portals for the initiation of CWV infection. Our data have demonstrated that, unlike most bacterial pathogens, *Aliivibrio (Vibrio) salmonicida* enters the host at extremely high numbers within 15 min after challenge. The bacterial concentration can reach up to 10^7 CFU x ml^{-1} of blood. Despite a high number of bacteria in the blood that would have caused critical systemic disease in other species, Atlantic salmon fry demonstrated normal behaviour identical to that of healthy fish. In the current study, we have further evaluated the phenomenon and tested impacts of temperature, as well as impacts of the bacterial concentration, on the efficiency of bacterial entry in the blood of Atlantic salmon. In addition, we report detailed dynamics of *A. (V.) salmonicida* CFU recovery rates from the blood of Atlantic salmon up to 22 days post challenge. To further demonstrate the universal value of the model, we present early dynamics of CWV infection in rainbow trout (*Oncorhynchus mykiss)*, which are known to be less susceptible to CWV infection, and hypothesise molecular prerequisites for the efficient transfer of *A. (V.) salmonicida* into the blood of the two species. Our studies have demonstrated the complex dynamics of the bacterial recovery from the blood of both species. The transfer of the pathogen into the blood stream of Atlantic salmon has been observed for suspensions with concentrations above 3 x 10^3 CFU x ml^{-1} during 10-min challenges. The level and the duration of the bacteraemia were found to be dependent on temperature and on the initial dose of the pathogen. Rainbow trout demonstrated a lower level and a more protracted bacteraemia by *A. (V.) salmonicida* in comparison with *S. salar*.

1. INTRODUCTION

After several recent reclassification events, the Vibrionaceae family is currently composed of seven designated genera along with number of taxonomic groups under consideration. Designated genera include: *Vibrio, Aliivibrio, Catenococcus, Enterovibrio, Grimontia, Photobacterium* and *Salinivibrio* (Dryselius *et al.*, 2007; Lilburn *et al.*, 2010; Munoz *et al.*, 2011). Due to a broad ecological distribution the family has been extensively studied (Reen *et al.*, 2006). Vibrionaceae is tightly associated with important human food borne diseases and in addition includes numerous endosymbiotic species inhabiting intestinal tracts or specialised light organs of different aquatic

organisms (Colwell *et al.*, 1977; Davis *et al.*, 2004; Heidelberg *et al.*, 2000; Yeung and Boor, 2004). Furthermore, several vibrios have been identified in the gut of soil invertebrates, indicating an even broader role of this family in the ecosystem (Jolly *et al.*, 1993; Toyota and Kimura, 2000).

Well equipped with efficient virulence factors, the members of the Vibrionaceae family are successful pathogens of human and aquatic animals, causing massive disease outbreaks in the marine biotopes from cold arctic to equatorial waters. Important non-human pathogens in the northern hemisphere include *Aliivibrio (Vibrio) salmonicida* (Egidius *et al.*, 1986) and *Vibrio anguillarum* (Crosa, 1980). The two species affect wild and farmed fish stocks, causing septicemia and internal bleeding in salmonid species. In tropical and subtropical waters, vibrio pathogens predominantly affect invertebrate marine organisms, such as shellfish (*Vibrio tubiashii*) (Hada *et al.*, 1984), shrimps (*Vibrio harveyi*) (Austin and Zhang, 2006) or corals (*Vibrio coralliilyticus*) (Ben-Haim *et al.*, 2003).

Bacteremia or the presence of viable bacteria in the blood of an organism, establishes when a pathogen manages to escape the host defence mechanisms at the sites of infection or in the blood stream (Christaki and Giamarellos-Bourboulis, 2013; Vu *et al.*, 2012; Wang *et al.*, 2006). Although well studied in humans and partly in mammalian husbandry species, bacteremia in fish has not been well studied scientifically, and thus, no special efforts have been made towards an understanding of molecular prerequisites for its development. The most recent review of bacteremia in humans by Christaki and colleagues in 2013 put a special emphasis on the host genetic signatures that increase the risks of the development of bacteremia. In the review, the authors describe a series of single nucleotide polymorphisms at specific regions of Tumour necrosis factor, Toll-like receptors (TLR), Interleukins and other components of the immune response system, which increasing the risks of the development of bacteremia by up to 66.7% in humans (Christaki and Giamarellos-Bourboulis, 2013).

In our previous study, we presented a novel and simple model for analysing the initial steps of cold water vibriosis infection (CWV) caused by *A. (V.) salmonicida* in Atlantic salmon (*Salmo salar*) and demonstrated the extreme transfer efficacy of *A. (V.) salmonicida* into the blood stream of this species (Kashulin and Sørum, 2014). As a further presentation of the model, the current study was designed to provide information concerning the impacts of temperature and bacterial concentration on the initiation of CWV infection in Atlantic salmon, as well as the model's applicability for infectious studies in other fish species. The study was performed as a series of independent challenges, each of which started with a full immersion of experimental animals into suspensions of

A. (V.) salmonicida and were followed by the evaluation of the bacterial concentration in the blood of the animals. The valuation of the early dynamics of bacteremia during experimentally induced cold water vibriosis has been performed in Atlantic salmon and in rainbow trout (*Oncorhynchus mykiss*), known to have different susceptibility to CWV infection (Brattgjerd *et al.*, 1995; Egidius *et al.*, 1986).

2. MATERIALS AND METHODS

2.1. Bacterial strains and experimental animals

All experiments in this study were performed using the *A. (V.) salmonicida* LFI1238 strain isolated from a diseased farmed cod at the Norwegian School of Fishery Science, Tromsø, Norway (Hjerde *et al.*, 2008). The complete genome of the isolate LFI1238 was among the first fully sequenced and annotated bacterial genomes available via NCBI (Hjerde *et al.*, 2008). Prior to experiments the virulence potential of the isolate was checked via control bath challenge in Atlantic salmon. Offspring bacteria isolated from the diseased salmon were preserved in glycerol stock and the same stock was used to conduct the described as well as number of other studies. Purity of the glycerol stock as well as cultures used in the challenges was confirmed microscopically. In addition the 16s RNA of the isolates was sequenced and compared with reference sequences published by Hjerde et al (Hjerde *et al.*, 2008). Unvaccinated Atlantic salmon fry weighing 30-50 g were received from Sørsmolt AS, Sannidal, Norway, and young rainbow trout (200 g) were obtained from the aquarium of the Norwegian University of Life Sciences, which is held in freshwater. The fish used in the experiments was received from commercial suppliers, thus the health status of the experimental animals was checked by the producers according to existing industrial standard. In addition, the health status was controlled in our laboratory. Prior to experiments, blood samples obtained from randomly selected individuals were plated on blood agar and observed for bacterial growth. The quantification of the cells of *A. (V.) salmonicida* in the blood samples obtained from anesthetised fish was performed by cultivation on blood agar plates, as earlier described by Kashulin and Sørum (Kashulin and Sørum, 2014). After blood sampling, the experimental animals were immediately euthanised by a sharp blow to the head.

2.2. Challenge procedures

All procedures described in this study were performed according to existing ethical standards for animal experimentation (EU Directive 2010/63/EU for animal experiments) and were approved by the National Research Animal Committee.

During **challenge 1,** which was used to determine the impact of the temperature on the initiation of CWV infection in Atlantic salmon, three groups containing 12 fish each were immersed into a bacterial suspension in marine water containing 3×10^7 CFU x ml^{-1} at 2-3^oC, 8-9^oC and 15-17^oC. To ensure the initiation of infection under sub-optimal temperature conditions, the immersion time was increased to 10 min (our previous data demonstrated the sufficiency of 3 min exposure at 8-9^oC for the initiation of the infection). In addition, one extra group consisting of 12 fish was immersed in 3×10^7 CFU x ml^{-1} at 8-9^oC for 6 min. After immersion, each fish was marked by a one mm hole

in the fins using a mouse ear marker and placed into the same holding tank to avoid a tank effect on the outcome of the experiment. Blood sampling was performed 15 min, 24 h, 48 h and 120 h (5 days) after immersion and involved three fish from each experimental group.

A study concerning the impact of the initial concentration of *A. (V.) salmonicida* on the success of invasion into the host was performed using 48 Atlantic salmon fry randomly placed into four groups (**challenge 2**). All 12 individuals from each experimental group were simultaneously immersed into bacterial suspensions containing 3×10^1, 3×10^3, 3×10^5 and 3×10^7 CFU x ml^{-1} of *A. (V.) salmonicida* in marine water for 10 min at 8-9^oC, marked and transferred into a freshwater tank to neutralise the residual *A. (V) salmonicida* cells from the fish surface. After 15 min in freshwater, three fish from each group were anesthetised and sampled for blood. Additional blood sampling was performed 24, 48 and 72 h after immersion.

To study the dynamics of the establishment of *A. (V.) salmonicida* LFI1238 in the blood stream of the infected Atlantic salmon and to allow a comparison with challenges 1 and 2, salmon fry were immersed for 10 min into the 3×10^7 CFU x ml^{-1} bacterial suspension in marine water at 8-9^oC. The fish were sampled for blood with regular time intervals (**challenge 3**). In total, the experiment included 54 animals treated nine at a time. After exposure, each fish was placed into the same 200 L holding tank and sampled for blood for the next three (22 days) weeks. The first blood samples were collected from 12 randomly chosen individuals after 15 min in fresh water. During subsequent samplings, blood was collected from three individuals at a time. Samplings were performed 1, 2, 3, 5, 8, 10, 12, 15, 17, 19 and 22 days after immersion.

The last challenge (**challenge 4**) was used to demonstrate the applicability of the model for studies of CWV infection in other fish species. In addition, this challenge allowed us to determine the survival dynamics of *A. (V) salmonicida* LFI1238 in rainbow trout known to be less susceptible to CWV infection (Brattgjerd *et al.*, 1995; Egidius *et al.*, 1986). Challenge 4 was performed at 15-17^oC with 21 rainbow trout individuals (average weight 200 g) kept in fresh water until the experiment began. After immersion into a bacterial suspension in marine water containing 3×10^7 CFU x ml^{-1} of *A. (V.) salmonicida* LFI1238 for 10 min, the trout were transferred back into fresh water holding tanks and sampled for blood each 24 h for the next seven days. The first sampling was performed after 15 min in fresh water. Blood was collected from three randomly chosen individuals during each sampling.

2.2.3. Data Analysis

To allow better graphical visualisation of the data, CFU counts were transformed using $\log_{10}(1+X)$ transformation. A comparison of the CFU recovery profiles was performed using the GenTχWarper software package (template matching mode), which allows the comparison of data time series based on a dynamic time warping algorithm (Menges *et al.*, 2003; Sakoe and Chiba, 1978). Where relevant, the CFU recovery profile obtained after immersion into the suspension containing 3×10^7 CFU x ml^{-1} *A. (V.) salmonicida* cells was regarded as a template profile and compared with other CFU recovery profiles.

4. RESULTS

The **first challenge**, which was used to evaluate the effects of temperature on the initiation of CWV infection in Atlantic salmon, demonstrated the ability of *A. (V.) salmonicida* to effectively enter the host within the 2-17^{o}C range. CFU counts were recovered from most of the blood samples collected 15 min after the challenge (Table 1). Samples collected 24 and 48 h after the challenges performed for 10 min within 2-17^{o}C temperature range demonstrated a gradual drop, which was followed by a minor increase in the CFU counts. A comparison of the various CFU recovery profiles obtained after 10 min of exposure indicated moderate (2-3^{o}C/10 min and 8-9^{o}C/10 min, fit-score 0.19) and relatively low similarity (15-17^{o}C/10 min and 8-9^{o}C/10 min, fit-score 0.32). A comparison of the CFU recovery profiles obtained after 6 and 10 min of exposure at 8-9^{o}C indicated that these two profiles are likely dissimilar (fit-score 0.55) (Figure 1). The involvement of the extra experimental group immersed at 8-9^{o}C for 6 min revealed a clear dependence of the magnitude of the CFU drop on the initial dose of the pathogen. Performed under optimal temperature conditions (8-9^{o}C), a shorter immersion and, thus, a smaller dose of the pathogen decreased the time from the challenge to the CFU drop from 120 to 48 h. Fit-scores of CFU recovery profiles obtained after immersion at 2-3^{o}C and 15-17^{o}C differ by almost 1.7-fold (0.19 and 0.32, respectively). A closer examination of the actual CFU recovery values obtained at 120 h post-challenge (Table 1) indicates that the groups immersed at 2-3^{o}C and at 8-9^{o}C for 10 min already demonstrated an initial CFU increase, whereas the group immersed at 15-17^{o}C had no indication of a potential CFU increase.

The evaluation of the impact of the initial bacterial concentration on the ability of *A. (V.) salmonicida* to enter the host (**second challenge**) demonstrated that a concentration of $3x10^{3}$ CFU x ml^{-1} is sufficient for the bacteria to systematically enter the blood stream of experimental animals. Although *A. (V.) salmonicida* was repeatedly recovered from the groups immersed into suspensions with bacterial concentration above this critical value, no bacterial colonies were obtained from the blood of the fish immersed into the suspension containing 1 $x10^{1}$ CFU x ml^{-1} of *A. (V.) salmonicida* LFI1238 (Table 2). Subsequent bacterial survival in the blood of the experimental animals was found to be dependent on the initial concentration of the bacteria in the suspension used for the challenge. Although *A. (V.) salmonicida* was found in the blood during the next 96 h after immersion into $3x10^{7}$CFU x ml^{-1} of *A. (V.) salmonicida*, immersion into suspensions with lower concentrations gave no signs of the presence of bacteria in blood sampled 24 h after the challenge (Figure 2).

The results of the **third challenge** fully supported the observations made during the first challenge. The efficient early entry of *A. (V.) salmonicida* into the host at relatively high numbers was replaced

by a noticeable decrease in the amount of bacteria present in the blood of the fish 48-72 h after the immersion (Table 3). Sampling five days after immersion demonstrated a clear increase in the CFU recovery rates and within eight days after immersion, plating of 10 µl of undiluted, freshly collected blood produced an even carpet of bacterial growth (Figure 3). The subsequent plating of 10 µl of blood collected 10 days, 12 days, 15 days, 17 days, 19 days and 22 days after immersion repeatedly produced a carpet of bacterial growth, indicating the permanent establishment of CWV infection in the experimental animals.

Known to be less susceptible to CWV (Brattgjerd *et al.*, 1995; Egidius *et al.*, 1986), rainbow trout provided (to our opinion) substantially lower CFU recovery rates in comparison with Atlantic salmon during the **fourth challenge** (Table 4) at the same time, since age/size-dependent susceptibility of the rainbow trout to CWV was not tested in our study, such conclusion remains subjective and disputable. As for salmon, the CFU recovery rates from the blood of the rainbow trout had a characteristic lag, which, however, occurred earlier (24 h after immersion) and lasted longer than that in Atlantic salmon (Table 4). A dynamic time warp comparison (Figure 4) of the selected part of the CFU recovery profiles (0-48 h) was performed due to substantial differences in the discreetness of the data, indicating low similarity of selected parts of the profiles (fit-score 0.65).

4. DISCUSSION

Having the whole arsenal of the modern techniques available in our laboratory we have intentionally chosen the classical, easiest and the most elegant way of determination of bacterial concentration. According to the study by Løvoll et al. performed for evaluation of invasion of *Moritella viscosa* into Atlantic salmon, plate counts provide the same accuracy as real-time PCR studies however at significantly lower cost (Løvoll *et al.*, 2009). Use of more advanced techniques - qPCR (Horváth *et al.*, 2013), immunochemistry (Álvarez-Barrientos *et al.*, 2000; Boye *et al.*, 1983), mass spectrometry techniques (Ballabio *et al.*, 2014; Ho and Reddy, 2010; Thorn *et al.*, 2011) for determination of bacterial concentration might have certain advantages over the implemented approach. The use of other methods, however, will significantly increase the costs of the study, not increasing the value of the obtained data. In addition such techniques will incorporate errors into the bacterial counts estimations due to intermediate sample preparation steps (Álvarez-Barrientos *et al.*, 2000; Ho and Reddy, 2010; Horváth *et al.*, 2013). The genome of *A. salmonicida* contains large amount of duplications as well as number of genes disruption by insertion of IS elements and thus the panel of standard qPCR primers for quantitative studies of this particular microorganism does not exist. Similar limitations could be identified for mass-spectrometry techniques (Ho and Reddy, 2010) and immunochemical methods (Mackay, 2004). In such circumstances authors found direct plate counting to be the most rational method of the study. Elegance of the technique allows its implementation at any laboratory while other methods will require advanced equipment (MS) or production of antibodies which are not available commercially (or their availability is limited).

The observed difference in the dynamics of CFU recovery from the blood of Atlantic salmon after 6 and 10 min of immersion into a 3 x 10^7 CFU x ml^{-1} suspension of *A. (V.) salmonicida* resembles natural field conditions of the CWV outbreaks that generally occur during the winter-spring period. Outbreaks occur most typically between 8 and 10^oC (Colquhoun *et al.*, 2002) and are accompanied by the extensive presence of *A. (V.) salmonicida* in the waters surrounding fish farms. According to Enger et al., water concentrations of *A. (V.) salmonicida* vary between 4 x 10^4 and 9 x 10^5 CFU x ml^{-1}, with the lowest values observed during the winter period (Enger *et al.*, 1991). The same authors have reported the persistence of 1 x 10^4 and up to 1 x 10^7 of *A. (V.) salmonicida* cells per gram of the sediments under salmon farms that experienced outbreaks of CWV. The combination of these facts allowed Enger and colleagues, in 1989, to suggest the existence of an asymptomatic carrier state of the disease (Enger *et al.*, 1989; Enger *et al.*, 1991).

The results of the challenges performed in our study, as well as in earlier studies related to the occurrence of *A. (V.) salmonicida* in the surroundings of the Atlantic salmon farms, support the

hypothesis of the existence of an asymptomatic state of the disease and indicate a delicate interplay among the environmental form of *A. (V.) salmonicida*, temperature and the innate immune system of the host. Exposure to *A. (V.) salmonicida* at extremely low or extremely high temperatures does not always lead to CWV whereas an interaction with the pathogen at 8-9^{o}C will most likely result in a clinical infection. Carrying the environmental type of microbe associated molecular patterns (MAMPs), which are likelly not recognizable by TLR receptor system of Atlantic salmon, *A. (V.) salmonicida* seems to freely enter the host and inhibit its division at the early stages of colonisation. With no division and unrecognised as a pathogen, *A. (V.) salmonicida* is, to a certain extent, eliminated by the host macrophages without the subsequent triggering of antigen presentation and the activation of the immune response. Persisting in the host for the period required for adaptation, the pathogen initiates cell division, which is observed as an increase in CFU recovery rates. Entering the host at a certain optimal cell density allows *A. (V.) salmonicida* to shorten the time required for switching into a pathogenic mode from 120 to 48 h.

Our yet unpublished data obtained on smolts of Atlantic salmon vaccinated using a commercially available vaccine clearly demonstrate the ability of *A. (V.) salmonicida* to enter the vaccinated host in a manner similar to what is described in the current study. The combination of systematic CFU recovery from the blood of vaccinated experimental animals with the data reported in the current study allow us to assume the high plausibility of the ignorance of the environmental MAMP of *A. (V.) salmonicida* by the TLR receptor system of Atlantic salmon. Despite being relatively closely related, Atlantic salmon and rainbow trout species seem to contain genetic differences in the organisation of the TLR receptor system. A phylogenetic reconstruction of relations between four different classes of TLR receptors in teleost fish that was performed by Rebl and colleagues has demonstrated clear differences between Atlantic salmon and rainbow trout TLR5, TLR3, TLR7 and TLR11 receptors (Rebl *et al.*, 2010). A closer examination of the TLR22 receptors in both species, which was performed by the same authors, has showed only 82% similarity in the amino acid sequences of the TLR22 receptors (Rebl *et al.*, 2010). Because the complete genomes of Atlantic salmon and rainbow trout are unavailable, a comprehensive analysis or at least full-scale functional studies of the TLR receptors in both species have not been conducted. Therefore, the discussion concerning the role of TLR receptors in the different susceptibilities of these species to *A. (V.) salmonicida* will remain speculative. Simultaneously, the provided hypothesis correlates well with data concerning the early dynamics of *A. (V.) salmonicida* CFU recovery rates from the blood of Atlantic salmon and rainbow trout obtained in the current study.

Likelly the most contradictory aspect of our work is related to the involvement of the fish of different sizes into the one publication. The study was designed taking into account assumption that susceptibility to *A. salmonicida* is not dependent on the age/size of the fish. We have not found any published data describing age/size dependent susceptibility of the rainbow trout or Atlantic salmon to CWV and not verified such susceptibility experimentally. In such circumstances we cannot conclude that observed differences are significant until additional studies will be conducted.

5. CONCLUSIONS

The evaluation of the early bacteremia in Atlantic salmon and rainbow trout performed in our study has demonstrated outstanding properties of *A. (V.) salmonicida* as a pathogen of salmonid species. Entering the blood stream of the host at extremely high numbers, CFU counts undergo a significant drop within the first hours, followed by a steady state period with extremely low, if any, bacterial recovery rates. The magnitude of the drop and the duration of the steady state period were found to be dependent on the bacterial dose and on the temperature used during the challenge. Our studies have demonstrated that an immersion concentration of 3×10^3 CFU x ml^{-1} is sufficient to recover viable bacteria from the blood of experimental animals after 10 min of challenge. In addition, the current study has demonstrated the remarkable difference in the entry dynamics of *A. (V.) salmonicida* into Atlantic salmon and into rainbow trout. Known to be less susceptible to CWV (Brattgjerd *et al.*, 1995; Egidius *et al.*, 1986), rainbow trout had relatively low level of *A. (V.) salmonicida* and a more protracted period with the occurrence of detectable *A. (V.) salmonicida* in blood. Although further functional studies are required to uncover the detailed molecular prerequisites of this phenomenon, we hypothesise the characteristics of the TLR receptor systems as the most probable reasons for the susceptibility of Atlantic salmon and rainbow trout to cold water vibriosis.

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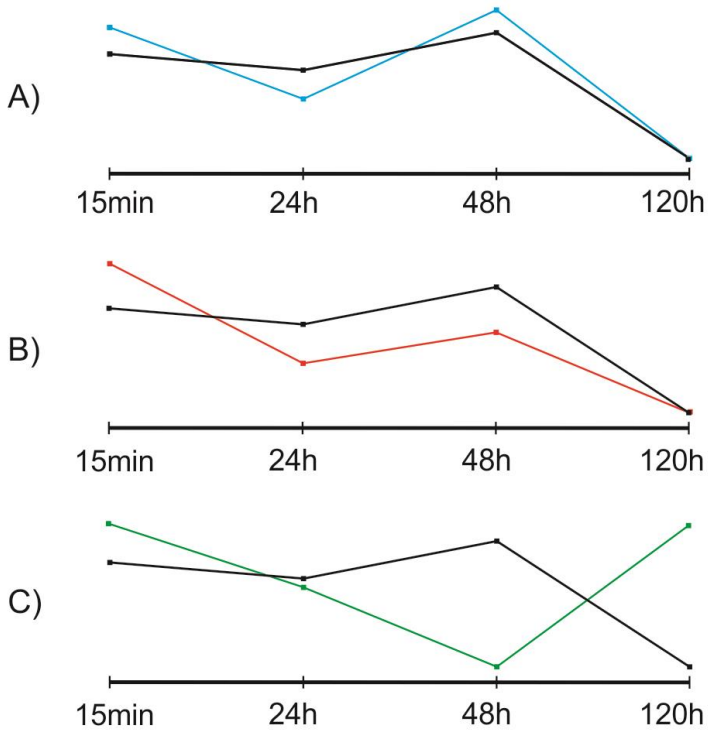

Figure 1. Comparison of the CFU recovery time series for the blood sampled after challenge 1. Nodes of the curves represent median values for each time point. The challenge performed with a 3 x 10^7 CFU x ml^{-1} suspension of *A. (V.) salmonicida* in marine water. A comparison of the time series provided the following dynamic time warping fit-scores: A) 2-3^{o}C/10 min (blue) and 8-9^{o}C/10 min (black), fit-score 0.19; B) 15-17^{o}C/10 min (red) and 8-9^{o}C/10 min (black), fit-score 0.32; C) 8-9^{o}C/6 min (green) and 8-9^{o}C/10 min (black), 0.55.

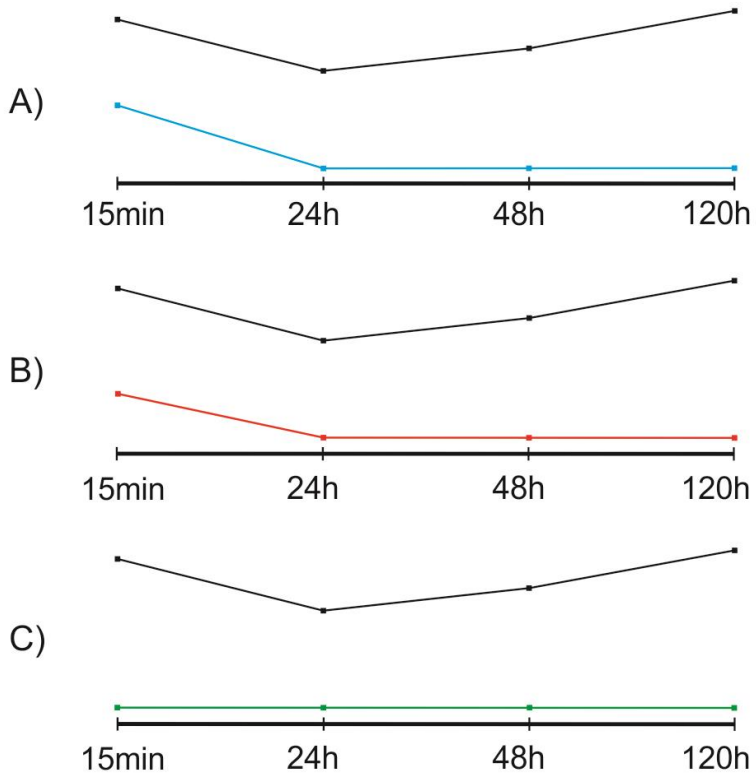

Figure 2. Comparison of the CFU recovery time series for the blood sampled after challenge 2. Nodes of the curves represent median values for each time point. The challenge performed with 3 x 10^1 - 3 x 10^7 CFU x ml^{-1} suspensions of *A. (V.) salmonicida* in marine water at 8-9^{o}C. A comparison of the time series provided the following dynamic time warping fit-scores: A) 3 x 10^5 CFU x ml^{-1} (blue) and 3 x 10^7 CFU x ml^{-1} (black), fit-score 1.33; B) 3 x 10^3 CFU x ml^{-1} (red) and 3 x 10^7 CFU x ml^{-1} (black), fit-score 1.39; C) 3 x 10^1 CFU x ml^{-1} (green) and 3 x 10^7 CFU x ml^{-1} (black), fit-score 1.52.

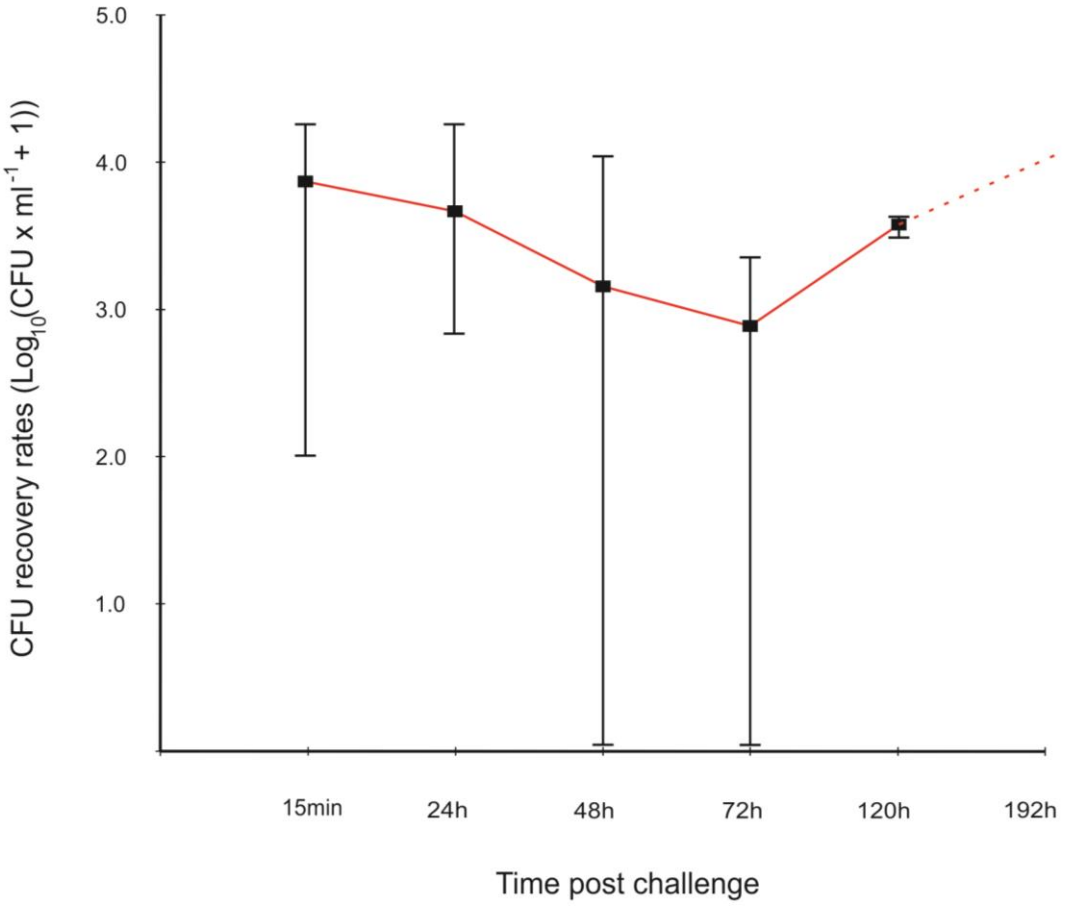

Figure 3. Distribution of CFU recovery rates obtained after challenge 3. The experiment was performed using immersion into a suspension of *A. (V.) salmonicida* LFI1238 containing 3 x 10^7 CFU x ml^{-1} for 10 min at at 8-9°C. The data are log_{10}(CFU x ml^{-1}+1) transformed and are expressed as median values with non-outlier range. The dotted line indicates carpet bacterial growth.

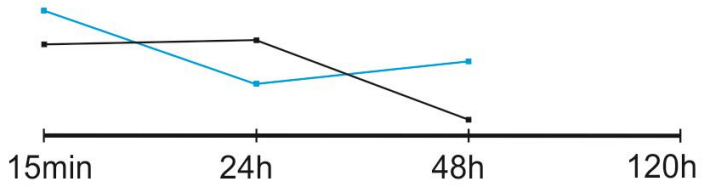

Figure 4. Comparison of the selected parts of a CFU recovery time series (0-48 h) for the blood sampled after challenge 4 with the respective data from challenge 1. Nodes of the curves represent median values for each time point for rainbow trout (black) and Atlantic salmon (blue). The challenge was performed with a 3 x 10^7 CFU x ml^{-1} suspension of *A. (V.) salmonicida* in marine water at 8-9^oC (Atlantic salmon) and 15-17^oC (rainbow trout). A comparison of the time series demonstrates their relatively low similarity (fit-score 0.65).

Table 1. CFU recovery rates (CFU x ml^{-1}) from the blood of Atlantic salmon sampled after challenge 1. The challenge was performed with a 3 x 10^7 CFU x ml^{-1} suspension of *A. (V.) salmonicida* in marine water.

Sampling	fish	2-3°C/10min	8-9°C/10min	8-9°C/6min	15-17°C/10min
15 min	1	$4.4x10^2$	$2.8x10^3$	0	$2.8x10^3$
	2	$5.4x10^3$	$8.0x10^1$	$2.6x10^2$	$1.4x10^3$
	3	$8.0x10^1$	$1.3x10^2$	$1.6x10^3$	$8.0x10^1$
24 hours	1	$1.5x10^1$	$7.8x10^2$	$2.0x10^2$	$1.2x10^2$
	2	$1.1x10^2$	0	$2.0x10^1$	$1.0x10^1$
	3	0	$6.0x10^1$	0	$1.0x10^1$
48 hours	1	$9.6x10^2$	$1.7x10^3$	0	$1.4x10^3$
	2	$2.4x10^3$	$3.4x10^2$	0	0
	3	0	0	0	$5.0x10^1$
120 hours	1	$6.4x10^2$	$5.0x10^4$	$2.7x10^2$	0
	2	0	0	$2.0x10^1$	0
	3	0	0	$4.0x10^3$	0

Table 2. CFU recovery rates (CFU x ml^{-1}) from the blood of Atlantic salmon sampled after challenge 2. The challenge was performed with 3 x 10^1 - 3 x 10^7 CFU x ml^{-1} suspensions of *A. (V.) salmonicida* in marine water at 8-9^oC.

Sampling	fish	$3x10^1$	$3x10^3$	$3x10^5$	$3x10^7$
15 min	1	0	$1.0 x10^1$	$2.0 x10^1$	$3.8 x10^4$
	2	0	$2.0 x10^1$	$3.0 x10^1$	$3.0 x10^2$
	3	0	$1.0 x10^1$	$5.2 x10^3$	$3.0 x10^3$
24 hours	1	0	0	0	$1.0 x10^3$
	2	0	0	0	$2.0 x10^2$
	3	0	0	0	$1.0 x10^1$
48 hours	1	0	0	0	$6.0 x10^2$
	2	0	0	0	$8.0 x10^3$
	3	0	0	0	$2.0 x10^2$
96 hours	1	0	0	0	$4.4 x10^3$
	2	0	0	0	$6.0 x10^3$
	3	0	0	0	$4.0 x10^3$

Table 3. CFU recovery rates (CFU x ml^{-1}) from the blood of Atlantic salmon sampled after challenge 3. The challenge was performed with a 3 x 10^7 CFU x ml^{-1} suspension of *A. (V.) salmonicida* in marine water at 8-9^{o}C.

	Fish1	Fish2	Fish3	Fish4	Fish5	Fish6	Fish7	Fish8	Fish9	Fish10	Fish11	Fish12	Fish13
15 min	$1.0x10^2$	$2.0x10^4$	$3.4x10^3$	$1.6x10^3$	$1.5x10^4$	$8.0x10^3$	$8.0x10^3$	$8.0x10^3$	0	$1.5x10^4$	$1.0x10^3$	$1.2x10^4$	$1.8x10^4$
24 hours	$5.0x10^3$	$2.0x10^4$	$7.0x10^2$										
48 hours	$1.2x10^4$	0	$1.5x10^3$										
72 hours	$2.4x10^3$	$8.0x10^2$	0										
120 hours	$4.0x10^3$	$4.6x10^3$	$3.3x10^3$										
192 hours	carpet growth												

Table 4. CFU recovery rates (CFU x ml^{-1}) from the blood sampled from rainbow trout. The challenge was performed with a 3 x 10^7 CFU x ml^{-1} suspension of *A. (V.) salmonicida* in marine water at 15-17^{o}C.

fish	15 min	24 hours	48 hours	60 hours	72 hours	96 hours	120 hours
1	$9.0x10^1$	$5.7x10^2$	$4.0x10^1$	$1.8x10^2$	0	0	$3.6x10^2$
2	$1.5x10^2$	$2.0x10^2$	0	$2.0x10^1$	0	0	0
3	$1.8x10^3$	$2.0x10^1$	0	0	$6.0x10^2$	$2.0x10^1$	0

Paper III

IS Elements in Aliivibrio salmonicida LFI1238: Occurrence, Variability and Impact on Adaptability. Alexander Kashulin, Henning Sørum, Erik Hjerde, Nils P. Willassen // submitted to Gene. Manuscript Number: GENE-D-14-00038.

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SUMMARY

Insertion sequence (IS) elements are short, self-replicating DNA sequences that are capable of efficiently spreading over the host genome. Possessing varied integration specificity IS elements are capable of the irreversible inactivation of genes, which diversifies the pool of intact genetic determinants in host populations. In the current study, we performed a complex analysis of IS elements (Vsa IS) in the previously sequenced genome of *A. salmonicida* LFI1238 and proposed a model of the spread of the Vsa IS elements over the genome of this microorganism. Along with the prediction of the integration sites for Vsa IS elements, the current study provides an overview of the properties of *A. salmonicida* IS elements, as well as information regarding their occurrence in different bacterial classes. An analysis of individual alleles of the IS elements has allowed us to depict a history of the accumulation of mutations and to describe distinctive microevolution lines for actively transposing Vsa IS elements. Our results demonstrate the high importance of the dead end microevolution of actively transposing Vsa IS elements for inactivation of genes in *A. salmonicida* LFI1238.

1. INTRODUCTION

Outbreaks of cold-water vibriosis (CWV), which are caused by *A. salmonicida*, occur during the winter-spring period. Before vaccination against CWV began in Norway, an average of 100-450 g x t^{-1} of different antibiotic substances were used, which resulted in the consumption of up to 49 tons of such compounds per year (Lillehaug *et al.*, 2003; Seymour and Bergheim, 1991). The situation changed dramatically when the first formalin-inactivated vaccines against CWV were reported (Lillehaug, 1990; Schrøder *et al.*, 1992). In 2008, the genome of *A. salmonicida* was sequenced and annotated by Hjerde et al. (Hjerde *et al.*, 2008). The genome of *A. salmonicida* consists of two chromosomes and several plasmids (4.3 kb, 5.4 kb, 8.3 kb, 11.5 kb, 32 kb and 92 kb), which form at least 11 observed plasmid profiles (Søorum *et al.*, 1988). Despite such plasmid diversity, no correlation between the plasmid content and the pathogenicity of the microorganism has been discovered to date (Wiik *et al.*, 1989). In particular, a plasmid-cured *A. salmonicida* strain was found to have similar virulence properties and phenotypic features compared with wild-type *A. salmonicida* strains (Valla *et al.*, 1992). According to the initial analysis, none of the 111 predicted plasmid encoded CDSs are homologous to known virulence determinants (Hjerde *et al.*, 2008). Although the role of these plasmids in the lifestyle of *A. salmonicida* remains unclear, the functions of the chromosomes in this pathogen generally resemble those functions that have been observed in other Gammaproteobacteria (Heidelberg *et al.*, 2000). Despite the lower G+C content (38.9%), the genome of *A. salmonicida* has similar architecture compared with the genomes of other vibrios (G+C = 42-48%), where the small chromosome (Chr II) predominantly contains species-specific genes (Jha *et al.*, 2012; Rasmussen *et al.*, 2007). The majority of the genes that are located on Chromosome I (Chr_I) are involved in replication, transcription, cell division or other core functions. The smaller chromosome contains more accessory genes and, thus, is primarily involved in environmental responses and adaptability. Another interesting feature of the *A. salmonicida* genome is the huge anomaly in GC bias that was detected in the replication origin (*ori*) and in the terminus regions (*ter*) of Chr II. In addition, both chromosomes of *A. salmonicida* carry traces of large intra-chromosomal rearrangements when compared with other related species (Hjerde *et al.*, 2008). The genomic and plasmid DNA in *A. salmonicida* are extremely enriched by mobile genetic elements. Although phage gene sequences are relatively rare and no regions with homology to characterised transposons can be found, insertion sequence (IS) elements persist in numbers. A prediction study discovered 290 IS elements in the genome of *A. salmonicida*, which places it among the bacteria with the highest IS element content (Cerveau *et al.*, 2011; Hjerde *et al.*, 2008). As mentioned, the genomic sequence of *A. salmonicida* maintains traces of large intra-chromosomal rearrangements. With no exceptions, those regions are flanked by IS elements (Mahillon *et al.*, 1985; Ooka *et al.*, 2009). While the simultaneous transposition of IS elements quite often promotes

the relocation, inversion, or excision of large DNA regions or might even lead to plasmid fusion (Downard, 1988; Hayes, 2003; Heritage and Bennett, 1985; Morita *et al.*, 1999), the individual movement of IS elements might not have an impact on the host or lead to gene knockout or to an alteration of the gene expression level (Mahillon and Chandler, 1998; Polard *et al.*, 1996; Turlan *et al.*, 2000).

Despite great variability, all types of insertion sequences rely on 5 catalytic mechanisms, 4 of which can be found in prokaryotes. The most numerous group consists of the IS elements, whose transposases contain the so-called **DDE** catalytic motif and may or may not leave a copy of the IS element in the donor site after the transposition (Berger and Haas, 2001; Brochet *et al.*, 2009; Mahillon *et al.*, 1999; Rousseau *et al.*, 2004). The second and the third family of bacterial IS elements rely on tyrosine (**Y**) and serine (**S**) residues in their catalytic sites, thus obtaining their respective names (Boocock and Rice, 2013; Nunvar *et al.*, 2010; Ton-Hoang *et al.*, 2012). Both **Y** and **S** transposases use the cut and paste mechanism and do not require intermediate replication steps. The last prokaryotic family of IS elements transpose via rolling-cycle (RC) transposition and is fully dependent on intermediate replication (IS elements containing **Y2-** transposases) (Garcillán-Barcia and Cruz, 2002; Mendiola and De La Cruz, 1992). The fifth family, or the reverse transcription-requiring family, has not been detected in prokaryotes thus far (Curcio and Derbyshire, 2003). As well as sharing common chemistry, the insertion sequences have similar architecture. Generally, IS sequences encode one CDS (transposase) and might rarely carry 1-2 extra CDSs that are responsible for recombinase or other generally unknown activities. Despite the yet undiscovered roles, all CDSs that are encoded by IS elements are essential for their transposition (Mahillon and Chandler, 1998). Functional studies have demonstrated that a deletion in any of the CDSs leads to the inability of the elements to transpose into new sites (Han *et al.*, 2001). Amino-acid replacement studies of one of the DDE transposases showed that the replacement of amino acid residues at different parts of the transposase (not directly involved in the formation of the DDE motif) significantly reduced the transposition efficacy of the IS element (up to $1x10^{-4}$), which is compatible with the decrease in transposition that was observed after the replacement of the residues in the DDE catalytic motif (Ohta *et al.*, 2002). CDSs of the transposases occupy the major region of the insertion sequence (except for the IS91, IS110 and IS200/065 families) and are flanked with terminal inverted repeats (IRs) that serve as sites for transposase binding. Upon transposition, many IS elements generate direct target repeats (DRs) in the site of insertion. The generation of DRs is characteristic for this family and reflects the mechanism of the transposition (for review, see Mahillon and Chandler (Mahillon and Chandler, 1998)). Depending on the transposition mechanism, the IS elements might have a greater or smaller impact on the host

genome and, thus, on the diversification of the bacterial population (Binnewies *et al.*, 2006; Griffiths *et al.*, 2000; Kleckner, 1981; Ton-Hoang *et al.*, 2012).

The initial report regarding the full genome sequence of *A. salmonicida* LFI1238, which was published in 2008, provided only a basic overview of the presence of IS elements in this microorganism (Hjerde *et al.*, 2008). The major families of IS elements in the genome of *A. salmonicida* LFI1238 are characterised in greater detail in the current study. Analysing different variances of the IS elements, we perform an assessment of their distribution, as well as of transposition success, in the genome of *A. salmonicida* LFI1238.

2. METHODS

2.1 Genomic data mining

The current study was performed using the full genome sequence of *A. salmonicida* LFI1238, with following the EMBL accession numbers: M178379, FM178380, FM178381, FM178382, FM178383, and FM178384. The refinement of the original prediction and the analysis of the data were performed using Blast-p, Blast-n, Pfam, and UniProt search algorithms with default parameters, as well as using manual curation. The prediction of chromosomal *ori*, *ter*, and *dif* sites was performed using a manual homology search with *Vibrio cholera*, *Escherichia coli* and *Bacillus subtilis* as references, as well using prediction functions and data of the DoriC 5.0 database of bacterial and archaeal *oriC* regions (Gao *et al.*, 2013).

2.2 Identification of potential integration sites

The identification of potential integration sites was performed using the MEME motif-based sequence analysis tool (Bailey *et al.*, 2009), which was combined with manual curation. To allow the prediction of putative integration sites, multiple alignments that were compiled of 300 bp up- and downstream regions of intact IS elements (independently for each family) were used as input data for the MEME sequence analysis tool. During the first prediction attempt, up- and downstream regions were concatenated into 600 bp sequences and uploaded to the MEME tool. During the second approach, each set of up- and downstream 300 bp fragments, which were accompanied by 100 bp 3' and 5' terminal regions of IS elements, were analysed individually.

2.3 Distribution of IS elements in the genome of *A. salmonicida* LFI1238

To provide easy tracking of a particular IS element, all insertion sequences were attributed with individual tags that contained data regarding the IS element number within each family (appear clockwise on circular chromosomes) followed by information regarding the chromosome that

contained this IS element, the IS element family name, the gene ID (according to initial annotation) and the distance to the IS element from the origin of replication (*ori*). For example, tag **1_Chr_I_Vsa_6_I0038_40023** indicates that the IS element belongs to the Vsa_6 family, is on Chr_1, has a gene ID of I0038, is of 40023 bp from the *ori* and, thus, is the closest Vsa_6 IS element to the origin of replication (IS element number 1).

The assessment of the distribution of IS elements was performed using multiple pairwise alignments of the sequences, followed by manual curation and phylogenetic reconstruction using the neighbour-joining algorithm with bootstrap analysis options that were provided in the MEGA5 software package (Tamura *et al.*, 2011). Trees with the highest observed bootstrap values, as well data regarding the location of the respective IS elements, were used to create a distribution pattern of the IS elements in the genome of the microorganism. The analysis of the transposition success was performed using the alignment and evaluation of the frequencies of the copies of IS elements within each IS family that are present in the genome of *A. salmonicida* LFI1238. First, we extracted and performed multiple pairwise alignments of the transposase amino acid (aa) sequences and, where relevant, placed IS elements into groups that shared 100% identity of their transposase CDSs. After allocation into different groups, DNA sequences of the IS elements were aligned and examined for identity. Identical IS elements (100% identity of whole IS element DNA sequences) were joined into clusters within each group. IS elements that contained unique amino acid mutations in the transposase CDSs, and which were thus not grouped with any other IS elements, were placed into a separate group within each family. After stratification, unique sequences that represented each cluster or group were used for the evaluation of the transposition success of particular variances within each individual family of IS elements.

2.4 Survey of Vsa transposases in other bacterial genomes

The identification of related IS elements in bacterial genomes that were available via GenBank was performed manually using the NCBI protein BLAST search for matches with parameters that were originally proposed by Mijenendonckx et al. for the examination of insertion sequences in the genome of *Cupriavidus metallidurans* CH34 (Mijnendonckx *et al.*, 2011). The bacterial genome sequence repository was searched for matches with *E*-value hits of at least $1e^{-20}$ and 35% amino acid identity over at least 50% of the transposase sequences for each family of IS elements in the genome of *A. salmonicida* LFI1238. The occurrence of each corresponding family was represented as a normalised value and calculated by dividing the number of hits with several bacterial species that contained similar IS elements within the family. The occurrence for each individual class was calculated as an average for all families that contained similar IS elements within the class and

mapped to a cladogram that represented relations of respective bacterial Classes (Phyla when designation to a class was not applicable). The cladogram was constructed using 16s RNA sequences that were selected to be representative for each Class/Phylum according to the All-Species Living Tree Project (Release LTPs106) (Munoz *et al.*, 2011). Sequences were aligned using the programme ClustalW (Larkin *et al.*, 2007) and were used for cladogram construction in the programme MEGA5.0 (neighbour-joining).

3. RESULTS AND DISCUSSION

3.1 Genomic data mining

A detailed survey revealed 291 intact or disrupted IS elements, which were placed into 20 groups. Although insertion sequences are found on both chromosomes, IS elements reside on only three of four LFI1238 plasmids. Among replicons that contain IS elements, their absolute numbers vary from one on the smallest pVS43 plasmid to 190 IS elements on the largest chromosome (Chr_I). The situation is different when seen as IS density per MB of DNA. This parameter is highest (250.0) for the smallest plasmid, pVS43, which consists of only 4327 bp, and is lowest (24.10) for the largest plasmid, pVS840, which contains 83540 bp. The IS densities per MB of DNA for the chromosomes are 57.14 (Chr_I) and 77.11 (Chr_II). For the pVS320 plasmid, the density per MB value is 133.33.

Surprisingly, only one of the 20 IS element families that were found in the genome of *A. salmonicida* is distributed over both chromosomal and plasmid DNA. This Vsa_9 IS element, which belongs to the IS91 family, can be found in almost equal amounts on both chromosomes (22 and 23 copies, respectively), in 3 copies on the pVS320 plasmid and as a single copy on the smallest plasmid, pVS43. Other IS elements demonstrate a certain degree of replicon specificity and can be found on either chromosomes or plasmids. In particular, Vsa_3, Vsa_4 and Vsa_19 reside on plasmids, whereas the other 16 remaining IS elements families have chromosomal locations. The copy number varies significantly (from 1 to 112 copies) for chromosomally located IS elements, whereas plasmid-located Vsa_3, Vsa_4 and Vsa_19 exist only in single copies.

The extreme density of the IS elements leads to many inactivated genes and reflects the adaptability of the host. The genome of *A. salmonicida* LFI1238 contains at least 156 genes that have been inactivated by the insertion of IS elements. In total, 106 elements have been inserted into functionally important genes, 25 IS elements have disrupted another IS element and 25 have affected cryptic CDSs with unknown functions. The majority of the inactivated CDSs belong to the chromosomes, and only 2 of the disrupted genes are on the pVS320 plasmid. In addition to 4 IS elements, the second largest *A. salmonicida* LFI1238 plasmid encodes 29 CDSs with primarily unknown functions. The only known protein (MobA) of the pVS320 plasmid has been inactivated by the simultaneous insertion of the upstream Vsa_19 (Tn3 superfamily) and downstream Vsa_9 IS elements (IS_91 superfamily). The original function of another pVS320 CDS, which was destroyed by the insertion of a Vsa_9 IS element, has yet to be determined [see Supplementary file 1 for the list of inactivated genes].

3.2 Identification of potential integration sites

The initial prediction of integration sites using concatenated 300 bp up- and downstream regions of IS elements from the original annotation have demonstrated relatively low target site specificity for most of the families of IS elements in the genome of *A. salmonicida* LFI1238. Therefore, no conserved sites have been identified for most of the studied families. Further manual curation of the data sets, as well as the implementation of the individual analysis of the up- and downstream regions of the insertion sequences, allowed us to refine the original annotation of the IS elements that was performed by Hjerde et al. (Hjerde *et al.*, 2008) and to identify potential integration sites for the Vsa_2, Vsa_6, Vsa_7, Vsa_8 and Vsa_9 families of insertion sequences. Although, Vsa_9 IS elements were found to integrate into a nearly perfect octanucleotide target, other families had lower specificity accepting imperfect integration sites. The insertion of the Vsa_6, Vsa_7, Vsa_8 and Vsa_9 sequences seems to split integration sites in the middle. Vsa_2 was found to integrate downstream of hexanucleotide motif. Vsa_7 IS elements have insertion specificity towards 5'-C..C-3' dimers within an imperfect hexanucleotide motif (Table 1). In addition to the detection of five target sites, the individual evaluation of up- and downstream areas of IS elements allowed us to identify several A/T reach regions near the IR of the Vsa_1 insertion sequences. Similar regions have previously been reported for other families of bacterial IS elements and are believed to be associated with the *rho*-independent termination of the transcription of these elements (Klaer *et al.*, 1981).

3.3 Distribution of IS elements in the genome of *A. salmonicida* LFI1238

Omitting the brief description of the minor families of Vsa insertion sequences will not allow us to correctly present the dynamics of the major families of IS elements in the genome of *A. salmonicida* LFI1238. The main characteristics and copy numbers of all 20 families of Vsa IS elements are summarised in Figure 1, where IS elements were mapped on both chromosomes of the microorganism and four plasmids. Although multi-copy insertion sequences are uniformly spread over the genome, low copy IS elements (1-3 copies) tend to be grouped near the *ori* or the *ter* regions. A similar phenomenon was reported for other bacterial species (Bickhart *et al.*, 2009; Craig, 1997; Garcillán-Barcia and Cruz, 2002; Gordon *et al.*, 1999). The concentration of low copy number IS elements near the *ori* and *ter* regions of the chromosomes suggests that these regions act as entry points for newly acquired insertion sequences into the genome of *A. salmonicida* (Figure 1). In support of such a hypothesis, we found that copies of 13 of 15 families of IS elements that are on Chr_I are locatred in the same area. However, it is necessary to indicate that although low copy insertion sequences located on Chr_I tend to place themselves at the beginning of the left replichore, members of the Vsa_7 family are located on the right as well as at the end of the left

replichore. In addition, the single-copy IS families Vsa_5 and Vsa_11 are distant from the origin of replication (Figure 1).

Chr_II has a smaller diversity and a smaller content of IS elements compared with Chr_I. In addition to a lower IS content, neither of the low copy number insertion sequences that are located on the Chr_II are integrated near the *ori*. Vsa_10 and Vsa_11 IS elements, which are present in single and double copies on chromosome II, are located in the proximity of its *ter* region (Figure 1). The Vsa_7 insertion sequence family present on Chr_I in 6 copies has 3 copies on Chr_II. Whereas one copy of Vsa_7 is near the *ori*, two other copies are near the *ter* region, which supports the integration manner of the families Vsa_10 and Vsa_11.

The detailed analysis of the major families of IS elements in the genome of *A. salmonicida* LFI1238 began with the pairwise alignment of the amino acid sequences of the transposases from each individual family. This analysis allowed us to systematise multi-copy Vsa IS elements and to place these elements into different groups within each family. The majority of multi-copy Vsa IS elements belong to DDE transposases, and only one, the Vsa_9 family, transposes by a RC mechanism.

The **Vsa_9** family is the only multi-copy family of *A. salmonicida* IS elements that is located on both chromosomes and on two plasmids of the microorganism. In contrast to DDE transposases, who primarily employ a cut and paste mechanism, Vsa_9 IS elements rely on RC transposition and, thus, are capable of the irreversible inactivation of genes that occasionally contain Vsa_9 target sites in their DNA sequences (Table_1). The transposase of the Vsa_9 IS element contains Y2-Tnp and Zn binding domains of the IS91 superfamily of bacterial insertion sequences but do not contain (De Palmenaer *et al.*, 2008) a terminal IR.

All diversity of the Vsa_9 sequences falls into 7 groups, which are based on the identity of the transposase amino acid sequences (Figure 2). Group 1 consists of 24 IS elements that all share 100% identity of transposase amino acid sequences. Encoding identical transposases, Vsa_9 IS elements from group 1, however, contain several different synonymous mutations in their DNA sequences that allow their allocation to different clusters. Groups 2, 3, 4, 5 and 6 consist of 6, 3, 2, 2 and 2, insertion sequences with 100% identity of transposases within each group, respectively. The last group, group 7, contains 5 single-copy IS elements with different unique mutations. With several mutations in the transposase coding areas of the IS elements, Vsa_9 family demonstrates the remarkable consistency of the transposase non-coding areas of the IS elements. Only one of 45 IS

elements contains a point mutation (79:C→T) in the transposase non-coding area of the IS element (sequence **45_Chr_II_Vsa_9_II1097_1193529**). Figure 3 represents the evolutionary relations and distribution of the Vsa_9 IS elements in the genome of *A. salmonicida* LFI1238 [see Supplementary file 3 for an overview of the clustering].

As mentioned above, while the Vsa_9 family is the only family of RC transposases that occurs in the genome of *A. salmonicida* LFI1238, five other multi-copy families belong to DDE transposases. Sharing a known catalytic domain, Vsa_1, Vs_2, Vsa_6, Vsa7 and Vsa_8 IS elements resemble the main characteristic features of the respective families of bacterial transposases and, thus, known experimental evidence that has been obtained in other bacterial species can be projected onto these Vsa IS elements (Mahillon and Chandler, 1998).

The IS elements of the **Vsa_1** family are located on both chromosomes of *A. salmonicida* LFI1238 and, according to our Pfam search, belong to the IS4 family of prokaryotic IS elements. According to IS-finder, the reference centre for bacterial insertion sequences, the IS4 family is extremely heterogeneous and consists of IS elements that share relatively low sequence homology (Kichenaradja *et al.*, 2010). According to our findings, the Vsa_1 family has a typical distance of 103 bp between the D and E residues in the DDE motif, which correlates well with a general family description by Kichenaradja et al. (Kichenaradja *et al.*, 2010). The phylogenetic reconstruction of relations between different members of the IS4 family has demonstrated that *A. salmonicida* Vsa_1 insertion sequences reliably cluster with *E. coli* IS4 insertion sequences, which likely indicates the early acquisition of this type of IS element in the evolutionary history of both species (see Supplementary file 4). The first comprehensive study of the *E coli* IS4 insertion element was published by Klaer et al. (Klaer *et al.*, 1981). According to their initial annotation, the IS4 element encodes only one CDS, which is necessary for its transposition, and is generally present as a single copy in the genomes of *E. coli* isolates. The analysis of the regions that surround IS4 elements has demonstrated the presence of inverted AT-reach clusters at both termini of insertion sequences, which indicates the potential involvement of a *rho*-independent mechanism in the termination of the transcription of these IS elements. Studies of IS4-dependent transposase production, which were performed by the same authors, have demonstrated a low level of protein expression, thus proposing an explanation of the low abundance of this IS element in the genomes of *E. coli* (Klaer *et al.*, 1981).

In contrast to *E. coli*, the genome of *A. salmonicida* LFI1238 contains 11 intact copies of the Vsa_1 IS element. Multiple alignments and further manual curation allowed us to assign these copies into

2 groups. Whereas group 1 is multi-copy and contain 9 IS elements with 100% identity of the DNA sequences; group 2 contains various single-copy insertion sequences. One copy contains a deletion (772: C) and another one contains a transversion (50: G→T); both of these changes do not affect the transposase encoding sequence. In addition to the identification of point mutations in the transposase genes of the Vsa_1 family, the multiple alignment of the up- and downstream regions of the IS elements allowed us to identify inverted AT reach regions in the up- and downstream proximities of the Vsa_1 IS elements similar to those inversions that were previously reported for *E. coli* IS4 (see Supplementary file 2).

Transposases of the Vsa_1 family contain typical IS4 family N-terminal domains at positions 19-114, as well as DDE catalytic domains that span positions 128-352. The Blast-n search for intact copies of Vsa_1 IS elements in the genomes of other microorganisms provided several high probability hits with different members of the Vibrionacea family (*E* value ranging from 0.0 to e^{-119}), as well as 28 hits with *Rahnella aquatilis* (63% identity over 71% of the query sequence; e^{-17}), which supports the hypothesis of the early acquisition of the IS4 family in the evolutionary history of Gammaproteobacteria. Belonging to Enterobacteriaceae, *R. aquatilis* was originally described as a Gram-negative, rod-like, freshwater environmental bacteria (Izard *et al.*, 1979) and was recently recognised as pathogenic for humans (Harrell *et al.*, 1989). An extensive study of the IS4 family has demonstrated the predominant occurrence of IS4 insertion sequences in genomes of pathogens or extremophilic bacteria, which allowed Palmenaer and colleagues to propose the importance of this type of insertion sequence in bacterial adaptation [Palmenaer et al., 2008].

The **Vsa_2** family of *A. salmonicida* LFI1238 insertion sequences has much greater diversity than the Vsa_1 family. In addition to many copies, each Vsa_2 IS element encodes 3 CDSs that resemble common features of the IS66 family of bacterial insertion sequences. IS66 insertion sequences generally range between 2.5 - 2.8 kb and require all 3 CDSs (ORF 1 to 3) for their transposition (Kichenaradja *et al.*, 2010). The homology analysis that was performed by Han et al. demonstrated the presence of IS elements that are similar to IS679 in the genomes of *Agrobacterium*, *Escherichia*, *Rhizobium*, and *Pseudomonas*, as well as in *Vibrio* species (Han *et al.*, 2001), allowing us to directly apply their characteristics to the Vsa_2 insertion sequence from *A. salmonicida* LFI1238.

Based on the similarities of CDSs, all types of Vsa_2 IS elements of *A. salmonicida* LFI1238 can be divided into 6 large groups (see Supplementary file 5). The 1st and largest group includes 19 IS elements, the 2nd group consists of 15 IS elements, the 3rd group consists of 3 IS elements, the 4th group consists of 3 IS elements, the 5th group consists of 2 IS elements and the 6th group consists

of 50 IS elements, which contain many different mutations. In addition, group 6 contains single-copy sequences with unique mutations, and every other group can be further split into clusters based on the identity of the IS element nucleotide sequences. Entering Chr_I of the ancestral microorganism near the *ori*, Vsa_2 IS elements further spread over both chromosomes of *A. salmonicida* LFI1238 following the speciation process. The analysis of mutations that are carried by Vsa_2 IS elements, which was performed *via* phylogenetic reconstruction using whole IS element nucleotide sequences, has demonstrated the existence of specific microevolution and sub-microevolution lines within the Vsa_2 family. The spreading process of Vsa_2 IS elements was likely initiated by one of the sequences from group 1 cluster 1, which consists of 8 identical IS elements. Along with being ancestral for two major microevolution lines of Vsa_2 IS elements (Figure 4), group 1 cluster 1 has 6 additional sub-microevolution lines that are composed of several sequences that each exist as a single copy (see Supplementary file 5.1). These single-copy offspring do not reliably group with other Vsa_2 IS elements that are present in the genome of *A. salmonicida* LFI1238 and, thus, can be considered Vsa_2 IS element variants with a decreased level of transposition activity. As an alternative, the existence of the single-copy Vsa_2 variants can be explained by a recent divergence of the offspring that has not yet undergone numerous transpositions. Alignments of the nucleotide as well as the Vsa_2 amino acid sequences demonstrated the presence of numerous mutations at both CDSs and non-protein coding areas of IS elements, which likely indicates the decreased transposition ability of these variants. A final explanation of the phenomenon requires functional studies of each variant, which goes beyond the scope of the current study, which aims at prediction, rather functional testing, of the properties of Vsa IS elements.

The microevolution line 1 is the largest microevolutionary line of Vsa_2 IS elements and consists of 3 sub-microevolutionary lines (initiated by sequences **35_VSAL_I1745-1860051**, **5_VSAL_I0216-3067957** and **13_VSAL_I0781-866373**) and single-copy offspring **28_VSAL_I1415-1530171**, **48_VSAL_I2260-2433749**, **60_VSAL_I2730-2955955**. Similar to microevolution line 1, microevolution line 2 includes several sequences that do not further group with other IS elements from the Vsa_2 dataset (sequences **8_VSAL_I0289-337220** and **84_VSAL_II0391- 771987**) along with IS elements that group with several other Vsa_2 IS elements. Sequences **28_VSAL_I1415-1530171**, **48_VSAL_I2260-2433749**, **60_VSAL_I2730-2955955**, **8_VSAL_I0289-337220**, **84_VSAL_II0391- 771987** likely contain mutations that affect their further transposition or have recently been outgrouped.

The nucleotide BLAST search, which used a complete Vsa_2 IS element for the query, provided several matches with different members of Alteromonadaceae (59% identity over 64% of the query sequence; E=2e-^{42}) and Shewanellaceae (64% identity over 64% of the query sequence; E=e^{-45}), supporting the hypothesis regarding the vertical manner of acquisition of the Vsa_2 insertion sequences. The area corresponding to 64% of the query covers ORF 3 (transposase) and ORF 2 (accessory protein), which indicates that ORF 1 likely has a species-specific function. Such a conclusion is supported by a more specific Pfam search, which did not provide any significant matches for ORF 1, whereas ORF 2 and ORF 3 were reliably identified as IS66 ORF 2-like protein (E=2.8e^{-38}) and IS66 transposase (E=6.1e^{-95}).

Vsa_6 is the second largest family of IS elements in the genome of *A. salmonicida* LFI1238. Vsa_6 has 57 intact copies located on both chromosomes. The search against the UniProt database using the Vsa_6 transposase amino acid sequence provided a similarity match with a transposase from the IS982 family (identity 63.0% over 100% of the query sequence, E=7.0e^{-130}). According to the IS finder reference centre, until recently, members of this family were found only in Gram-positive bacteria and encoded only one ORF (Kichenaradja *et al.*, 2010). The Blast-n search over the collection of bacterial genomes, which was performed within the scope of the current study, has demonstrated that this information requires correction. In addition to several low score matches with Gram-positive microorganisms, the highest score (68% identity over 92% of the query sequence; E=6e^{-64}) was observed for the match with the *Glaciecola psychrophila* strain 170 (Yin *et al.*, 2013). Besides match with a *G. psychrophila,* search provided several hits with other Gammaproteobacteria species (E=2e^{-25}-9e^{-43}).

As typical members of the IS*982* family, Vsa_6, IS elements have only one CDS that covers most of the insertion sequences. The Vsa_6 IS elements in the genome of *A. salmonicida* LFI1238 can be divided into 3 groups that are closely related to each other [see Supplementary file 6]. The largest group (group 1) consists of 49 identical copies of the IS element. Thirty-six copies are located on chromosome I, and 13 copies are on chromosome II. Group 2 has four IS elements located on chromosome I. Group 3 consists of four single-copy offspring that are located on both chromosomes. IS elements belonging to Group 3 contain several mutations at different loci of their transposase CDSs as well as mutations in the non-coding area of the IS elements [see Supplementary file 6.1]. Transposases of the Vsa_6 family contain the typical DDE domain, which consists of three carboxylate residues that are responsible for coordinating metal ions that are required for catalysis.

The **Vsa_7** family is relatively small. In the genome of *A. salmonicida* LFI1238, this family is represented by nine copies that are spread over two groups. Group 1 consists of eight IS elements with identical transposase genes that are located on both chromosomes. The second group consists of only one IS element that is located on chromosome I. Two non-synonymous mutations place this IS element apart from the IS elements of group 1 (272: Gly/Arg; replacement of the 35 C-terminal amino acids on five amino acids due to a single nucleotide deletion that causes a frame-shift in the transposase gene). A UniProt database search for transposase aa sequences gave several high score hits with integrases (identity 40.0% over 94% of the query sequence, $E=8e^{-65}$), as well as with transposases of the IS30 superfamily (identity 40.0% over 97% of the query sequence, $E=2e^{-62}$). The Vsa_7 insertion sequences contain one single CDS that occupies almost the entire length of the IS elements. The insertion sequences have 27 bp terminal inverted repeats and an imperfect 6 bp integration site (Table 1). In addition to the transposase catalytic domain, the Vsa_7 transposase amino acid sequence provides a significant hit with a 43 amino acid HTH-38 helix-turn-helix domain at the N-terminus of the sequence. This domain can be found in DNA-binding proteins, which include transcriptional regulators and recombinases. Functional studies of the motif demonstrated that this motif is required for the insertional specificity of IS elements (Lysnyansky *et al.*, 2009). The presence of the motif likely determines the exceptional properties of this type of insertion element, which were discovered well before modern sequencing and protein function prediction tools became available. The initial identification and characterisation of the IS30 insertion sequence was reported by Caspers et al. (Caspers *et al.*, 1984). The authors indicated the presence of several copies of the IS30 insertion sequence in the genome of *E. coli* K12 and demonstrated their role in 3 different types of spontaneous inactivation of the prophage P1, which is present in strain K12. During extensive *in vivo* experiments, the authors demonstrated the ability of IS30 to mediate the integration of the pAW83 plasmid into the prophage P1, which contains target sites for the IS30 element (Caspers *et al.*, 1984). A subsequent study that was performed by Olasz et al. demonstrated that terminal inverted repeats of IS30 might serve as functional target sites for insertions of another copy of the IS30 element, which might lead to the formation of $(IS30)_2$ dimers. Moreover, left and right terminal inverted repeats of the IS element were found to have different attractivity for the insertion of the second copy of the IS30 insertion sequence (Olasz *et al.*, 1997).

The **Vsa_8** IS family of *A. salmonicida* LFI1238 encodes 2 CDSs (130 and 178 aa) and is represented by 27 intact copies that are spread over both chromosomes of the microorganism. A single aa sequence of 321 aa in Vsa_7 carries two different functional domains, whereas ORFs of Vsa_8 insertion elements encode two functional domains that are on two individual protein molecules (308 aa). The smallest CDS of the Vsa_8 IS element encodes a HTH-29 winged helix-

turn-helix domain that is often found in transferases (HMM length 112, Pfam E=2.6e^{-7}) or transposases of the IS630 superfamily (41.0% identity over 69% of the query sequence, UniProt E=2e^{-19}). The largest CDS of the Vsa_8 IS elements contains a typical DDE motif, which however, does not possess significant matches with any experimentally characterised transposase. A blast-n search for the intact IS element in the collection of bacterial genomes provided a single significant match with *Photobacterium profundum* SS9 (83% identity over 100% of the query sequence, E=0.0), which likely indicates the vertical acquisition of the Vsa_8 IS element.

All copies of the Vsa_8 IS element that were analysed in the current study can be placed into 2 groups based on 100% identity of the transposase aa sequences. Group 1 consists of two clusters, which include 21 and 4 insertion sequences, respectively. Group 2 of the Vsa_8 IS elements includes only two sequences (**1_Chr_I_Vsa_8_I039_431696** and **4_Chr_I_Vsa_8_I1309_1419984**), which harbour a point mutation (260: M→I) and a large deletion (141-211: 71 AA) in the transposase gene [see Supplementary file 7].

The first attempts to identify the insertion specificity of the IS630 family were performed in 1990. In a series of *in vivo* functional studies that were performed on *Shigella sonnei* IS630 insertion sequences, the authors have demonstrated that, unlike other IS elements, IS630 does not mediate the co-integration of the replicons; however, IS630 can contribute to the formation of composite transposons that are capable of transferring several different genes into defined integration sites. Studies that were performed in *E. coli* have demonstrated the insertion of IS630 elements downstream of a 5'-TA-3' dinucleotide sequence, which was followed by its duplication during the insertion of IS630 (Tenzen *et al.*, 1990). The pairwise alignment of the *S. sonnei* IS630 and Vsa_8 complete insertion sequences that was performed in our study has demonstrated 35.3% identity between the 1129 bp and 1080 bp insertion sequences, respectively. Despite the low identity and dissimilar terminal inverted repeats, Vsa_8 insertion sequences were found to be integrated in the same manner as was previously described for *S. sonnei* IS630 (downstream of the 5'-TA-3' with its duplication).

3.4 Survey of the Vsa transposases in other bacterial genomes

At the time of our study, the analysis of taxonomy reports of the NCBI/ protein BLAST server for the presence of Vsa IS elements in other sequenced bacterial genomes demonstrated their predominant occurrence in Gammaproteobacteria (Table 2). Although most the transposase genes share only a certain degree of homology with Vsa IS elements, the Vsa_5 element has 100% homology with the *Salmonella enterica* subsp. *enterica* serovar Typhimurium protein NP_863365.

The Vsa_5 insertion sequence exists in a single copy, is on the largest *A(V.) salmonicida* chromosome, and destroys the ClpX subunit of the ATP-dependent Clp protease.

Despite great advances in high throughput sequencing, the number of sequenced bacterial genomes to date resembles only a tiny fraction of the total number of prokaryotic species that exist in the global ecosystem. As mentioned earlier, all 20 Vsa IS elements are abundant in Gammaproteobacteria, whereas other bacterial classes have significantly fewer insertion sequences that share similarity with Vsa IS elements. The search for homologs of Vsa IS elements was performed using a broad similarity range that allowed us to identify distantly related insertion sequences (lower border *E*-value = $1e^{-20}$ and 35% amino acid identity over at least 50% of the transposases sequence). Despite such a wide scope search, Vsa_9 and Vsa_20 IS elements have been found exclusively in Gammaproteobacteria, which indicates the high host specificity or the late emergence of these IS families. Although at the time of this analysis the average occurrence (AO) of Vsa_9 IS elements was 7.0 copies per Gammaproteobacteria genome, Vsa_20 was much less abundant, with the AO of 0.16 per genome. The Vsa_20 AO is not only significantly lower than the occurrence of the Vsa_9 IS elements but also is the lowest AO among all Vsa IS elements. The closest rarely occurring IS elements on average occur as 1.0 copy per bacterial genome, which is nearly 6 times higher than the AO of Vsa_20 IS elements (the highest AO was observed for Vsa_6 IS elements in Alphaproteobacteria, with 44.0 copies per genome). In the genome of *A. salmonicida* LFI1238, the Vsa_20 IS element exists as a single copy located on the Chr_I. In contrast to other low copy number IS elements, the only copy of *A. salmonicida* Vsa_20 is integrated near the Chr_I *ter* region, which place Vsa_20 apart from other low copy number Vsa IS elements located near the *ori* of Chr_I. Such IS elements demonstrate a significantly broader host range and can be found in the genomes of microorganisms from at least 14 other different classes. A similar tendency can be observed for two other Vsa IS families (Vsa_11 and Vsa_12) located near the Chr_II *ter* region. In addition to Gammaproteobacteria, matches for both families were found only in Betaproteobacteria, with AOs of 2.0 and 3.4 IS elements per genome for Vsa_11 and Vsa_12, respectively. Another Vsa family with rare occurrence in other bacterial classes is the Vsa_8 family. In contrast to the other 4 above-mentioned families, Vsa_8 is a multi-copy family that consists of 27 copies that are spread over both chromosomes of *A. salmonicida* LFI1238. Along with persistence in other Gammaproteobacteria species, with an AO of 13.2 copies per genome, similar IS elements were found in Cyanobacteria, which likely indicates the recent horizontal transfer of these IS elements between marine members of two bacterial classes (AO 1.0 copy per Cyanobacteria genome). Similarly to low copy number IS elements that are located on chromosomes of *A. salmonicida* LFI1238, single-copy Vsa IS elements that are located only on

plasmids of the microorganism demonstrate high (Vsa_4, Vsa_19) as well as low (Vsa_3) occurrence in other bacterial classes (Table 2).

The purely *in silico* approach that was applied to a single bacterial genome in the current study does not allow the prediction of integration sites for low copy (2-3 copies) or single-copy IS elements due to the lack of input information for the reliable performance of prediction algorithms. Limited data regarding the integration specificity of certain IS elements may explain their localisation; however, this explanation is largely speculative. Simultaneously, the localisation of these IS elements at distinctive areas of the chromosomes of *A. salmonicida* LFI1238 requires discussion. As for a typical member of the Vibrionaceae, genome *A. salmonicida* LFI1238 is spread over two chromosomes, which play distinct roles in the lifecycle of the microorganism. The examination of the replication of *V. cholerae* chromosomes demonstrated that Chr_II begins to replicate later in the C period of the cell cycle, when most of chromosome I has already been replicated. Despite the lag in the initiation of Chr_II replication, both chromosomes simultaneously complete replication (Rasmussen *et al.*, 2007; Stokke *et al.*, 2011). As well as initiation of replication of chromosomes in *V. cholerae,* initiation of replication of both chromosomes of *A. salmonicida* is controlled by DnaA and RctB proteins. In *V. cholera*, the termination of replication occurs at *ter* regions that are directly opposite the *ori* of the chromosomes, whereas chromosome dimer resolution sites (*dif*) are located near the *ter* regions (Mcleod and Waldor, 2004). As well as the *ori*, *ter* sites of the two chromosomes are independent of each other (Kono *et al.*, 2011). To date, three distinctive mechanisms have been described for the termination of the replication of bacterial chromosomes: fork-trap, fork collision and a *dif*-stop (Kono *et al.*, 2012). The fork-trap mechanism has been described in detail for *E. coli* and is based on the interaction of the 36 kDa Tus protein with ten 23 bp *ter* sites (TerA-J) that are located opposite of the *ori* (Mulcair *et al.*, 2006). Our search did not reveal the presence of the *Ter-Tus* system in *A. salmonicida* LFI1238 and, thus, likely indicates the importance of the other two mechanisms for the termination of replication in *A. salmonicida*. The search for characteristic *dif* sites that was performed in the scope of the current study has demonstrated their presence on both *A. salmonicida* LFI1238 chromosomes. The *dif* site of the Chr_I is located in the area opposite the *ori* and, thus, is near the *ter* region (Figure 1), whereas on Chr_II, the *dif* site is shifted clockwise by approximately 85 kbp from the putative *ter* region of the chromosome (area directly opposite *ori*). Despite the minor GC skew inconsistency, which is likely associated with recombination events (Hjerde *et al.*, 2008), major GC skew shift points on Chr_I of *A. salmonicida* LFI1238 are linked with *ori* and *ter* regions of the chromosome. The situation is different for Chr_II, in which GC skew shift points are associated with *ori* and *dif* sites. The experimental determination of replication dynamics, which was performed by Dryselius et al. on

exponentially growing *Vibrio parahaemolyticus*, demonstrated even replication progress of left and right replichores within and between the two chromosomes leading to a replication fork collision in the area that is diametrically opposite the *ori* (Dryselius *et al.*, 2008). The combination of our findings with the experimental data of Dryselius *et al.* led us to conclude that, in contrast to *E. coli*, which relies on a dedicated replication termination system, the termination of replication in *A. salmonicida* is likely occur by a conserved mechanism of fork collision at the area that is located opposite to the *ori*. Although the final explanation of the manner of localisation of the chromosomally located Vsa IS elements requires experimental verification (or at least an analysis of multiple genomes), the obvious discrepancy in the organisation and replication mechanisms might underlie the manner of localisation of Vsa_IS elements on *A. salmonicida* LFI1238 chromosomes. A similar explanation is likely applicable to the plasmids of the microorganism. Significantly differing in size, plasmids of *A. salmonicida* LFI1238 seem to replicate using Rolling-cycle (pVS43 and pVS54) and Theta mechanisms (pVS320 and pVS840) and thus, in addition to the differential presence of target sites for IS elements, might have varied susceptibility to the insertion of IS elements.

4. Summary

Despite being known for nearly three decades, as well as having a fully sequenced and annotated genome, *A. salmonicida* LFI1238 resembles a highly interesting model organism for both experimental and theoretical studies. The versatile lifecycle of this bacterium is reflected in its complex genome. Both chromosomes and plasmids of *A. salmonicida* LFI1238 are extremely enriched with IS elements that are capable of autonomous replication and pandemic-like spread over the host genome. While origination of the IS element is a major subject for debates, the vertical as well as horizontal manner of their transfer between different host species is well verified experimentally. According to the hypothesis that was tested in this study, after entering the host, IS elements initially transpose into several loci, then further evolve independently and create their own microevolution lines within the particular host. While actively transposing IS elements accumulate mutations with higher rates, IS elements with low transposition activity resemble a significant degree of conservation and appear as 100% identical sequences. The accumulation of mutations generally has negative effects on the fitness of IS elements, eliminating these elements from further transposition processes and creating dead-end IS element offsprings. Dependent on host transcription machinery and predominantly acting in *cis*, transposases of IS elements create excellent prerequisites for the diversification of bacterial populations responding to environmental factors. The differential transposition of IS elements from various chromosomal loci under variety of environmental conditions contributes to the creation of bacterial subpopulations with different arrays of inactivated genes and, thus, different fitness. Increased rates of bacterial evolution that is mediated by the differential transposition of IS elements correlate well with epidemiological observations of cold-water vibriosis outbreaks caused by *A. salmonicida*. Previously associated with many strains of *A. salmonicida*, modern cases of CWV outbreaks are caused exclusively by the *A. salmonicida* LFI1238 strain, which likely has not yet undergone the inactivation of functionally important virulence genes by the insertion of IS elements.

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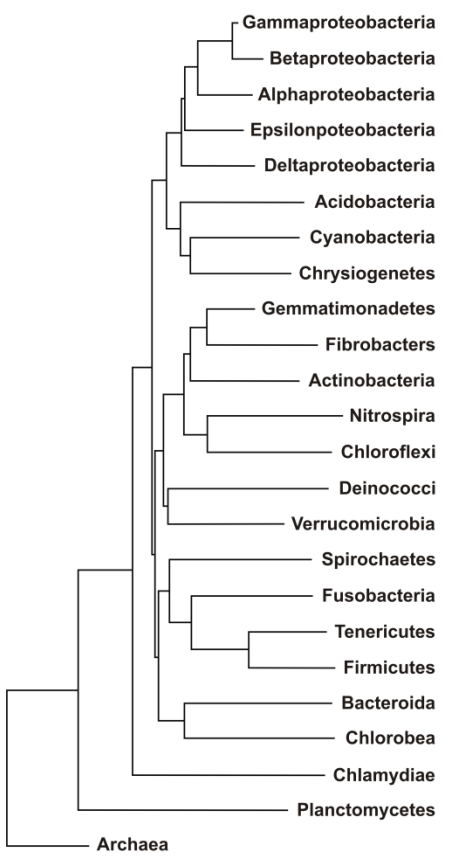
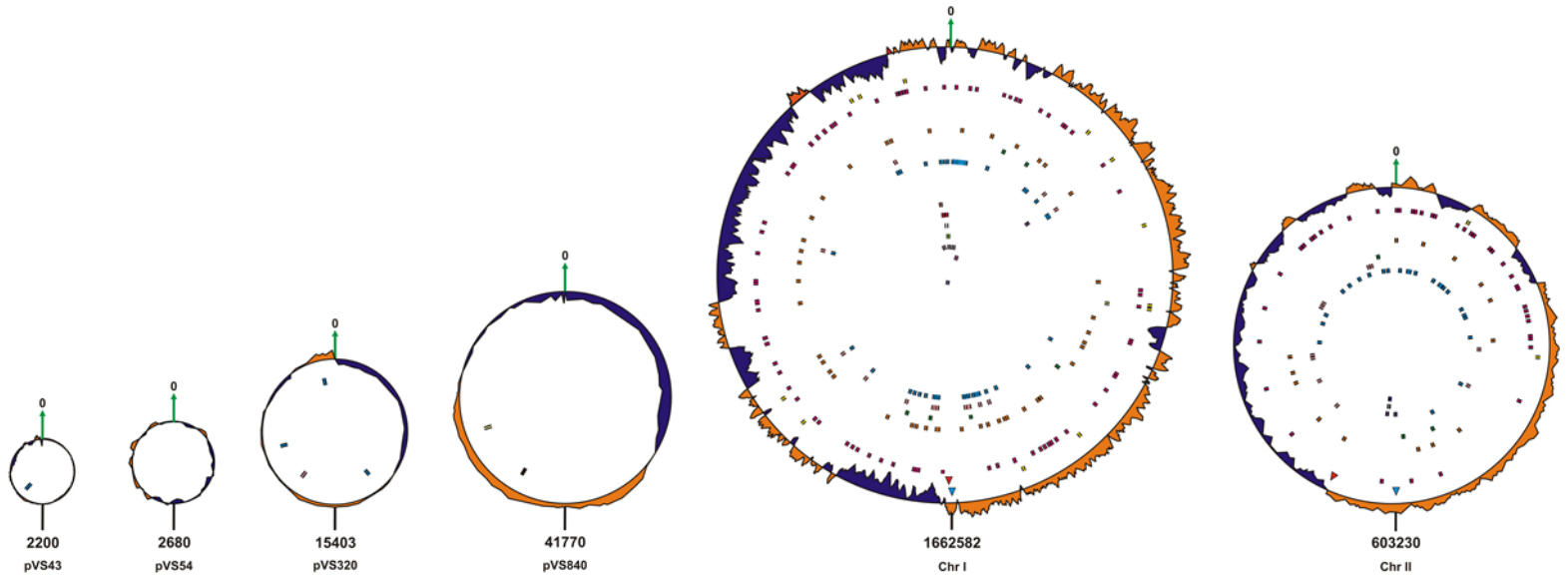
Table 1. Insertion characteristics of the major families of IS elements in *Aliivibrio salmonicida* LFI1238.

IS family	number of copies	integration site	cis- A/T reach region	terminal inverted repeat
Vsa_1	11	-	-17, -5	5'-TAATGCCGA-3'
Vsa_2	89	Downstream of [AT][TCG][ATC]A[AG][CA]	-	5'-GTAAGCGTGCGGGGAACTACACCT-3'
Vsa_6	57	TA[AG][TC]..[TAGC][TCAG]TA	-	5'-TAACCTGAATTCTGGATAA-3'
Vsa_7	9	[AT][TAGC]C...CA[TA]	-	5'-CTAGGTTCCAAGAATAACCGACACACT-3'
Vsa_8	27	[TCA][GT][TA]...[TCAG][CT][AT]	-	5'- TAAGTGTTATTGAAAACGCACTGATGAAT -3'
Vsa_9	45	GAAC...CCT[Tg]	-	-

Table 2. Distribution of IS elements in sequenced bacterial genomes. The numbers represent the average numbers of IS elements in a certain bacterial Class. Values are calculated as the average for all families of the Class/Phylum (when designation to a Class was not applicable) that contained a particular type of IS element. The occurrence within families is calculated as the number of IS elements divided by the number of sequenced species in this family. The cladogram on the left side of the table represents relations between respective Classes/Phyla based on 16s RNA sequences that are representative for each of the groups.



	Vsa_1	Vsa_2	Vsa_3	Vsa_4	Vsa_5	Vsa_6	Vsa_7	Vsa_8	Vsa_9	Vsa_10	Vsa_11	Vsa_12	Vsa_13	Vsa_14	Vsa_15	Vsa_16	Vsa_17	Vsa_18	Vsa_19	Vsa_20
Gammaproteobacteria	2,6	6,6	2,3	3,2	4,2	9,4	6,3	13,2	7,0	2,6	4,6	5,1	6,9	7,8	4,1	9,0	4,1	6,3	2,7	0,16
Betaproteobacteria	4,0	7,8		5,1		16,7	7,0			2,7	2,0	3,4	8,0	7,0	3,6		8,0	7,0	1,8	
Alphaproteobacteria	2,5	5,2		4,9	2,0	44,0	9,0			3,7			6,8	7,5	3,4			10,9	3,0	
Epsilonpoteobacteria	8,8	4,3	5,6	5,9			4,0												2,0	
Deltaproteobacteria	8,2	13,0	14,3	6,5			2,0			4,2			7,4	5,5						
Acidobacteria				4,3						18,0			10,0	6,1	6,0					
Cyanobacteria						6,6		1,0												
Chrysiogenetes																1,7				
Gemmatimonadetes		3,4					2,0													
Fibrobacters		2,7		7,7			4,0													
Actinobacteria	1,7			4,7	1,0		3,0								2,2					
Nitrospira		3,3		3,9	1,0		2,0													
Chloroflexi						2,0														
Deinococci		2,0		6,5																
Verrucomicrobia														5,4						
Spirochaetes						16,0	30,0											19,8		
Fusobacteria							2,0													
Tenericutes					2,3															
Firmicutes		4,1		2,5	2,0		6,7			5,3			1,0	3,6	5,3			5,6	2,2	
Bacteroida		1,8		5,4	1,0	7,3	5,7			2,5			10,0	10,0	1,0			1,8	1,8	
Chlorobea													4,8	5,2						
Chlamydiae						6,2	4,3						6,0	4,3		6,8				
Planctomycetes		3,7		1,6			6,5						9,0	10,9						



	Vsa_1	Vsa_2	Vsa_3	Vsa_4	Vsa_5	Vsa_6	Vsa_7	Vsa_8	Vsa_9	Vsa_10	Vsa_11	Vsa_12	Vsa_13	Vsa_14	Vsa_15	Vsa_16	Vsa_17	Vsa_18	Vsa_19	Vsa_20
Chr_I	12	75	-	-	1	45	6	17	22	-	1	-	1	2	1	1	3	2	-	1
Chr_II	2	37	-	-	-	14	3	10	23	2	1	1	-	-	-	-	-	-	-	-
pVS43	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
pVS54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pVS320	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	1	-
pVS840	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IS family	IS4	IS66	IS91	IS6	IS4	IS982	IS30	IS630	IS91	IS91	IS3	IS3	ISAs1	ISAs1	IS5	IS630	ISNCY	IS5	Tn3	IS5

Figure 1. Distribution of intact insertion sequence elements over replicons of *A.(V.) salmonicida* LFI1238. On chromosomes, IS element families are mapped from the outside (Vsa_1) to the inside (Vsa_20) of the circular diagrams. On plasmids, all IS families are mapped at the same circle. Predicted origins of replication are on top of the diagrams and are indicated by green arrows. Chromosomal *ter* and *dif* sites are indicated by blue and red triangles. Orange and violet represent GC skew scores above and below average, respectively. Colour bar on the top of the table represent the colour coding for each individual IS family.

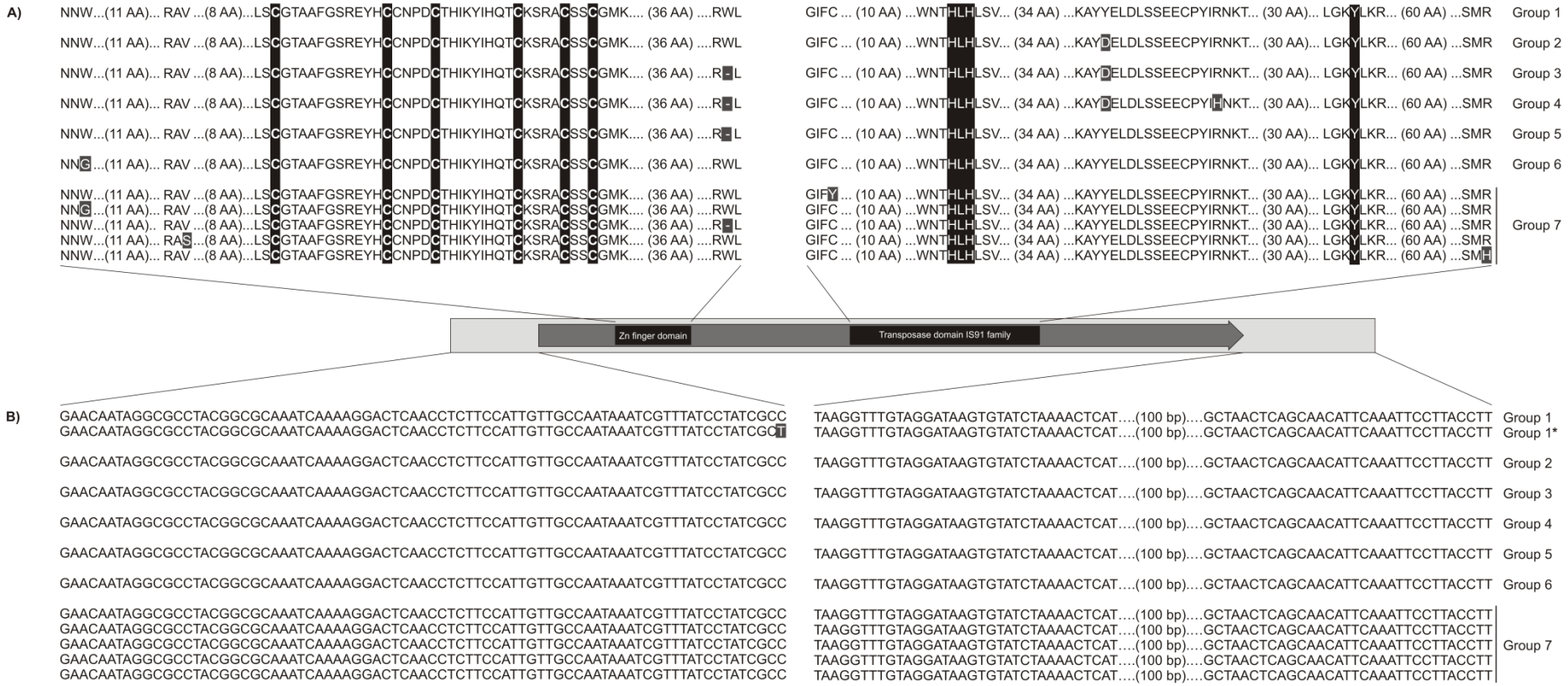


Figure 2. Distribution of the mutations in the catalytic (A) and terminal (B) domains of Vsa 9 IS elements of *A. salmonicida* LFI1238. Black shading indicates identical residues, and grey shading highlights point mutations. Although groups 1-6 contain multiple identical sequences, only single respective sequences are shown on figure 2A, and all 5 unique sequences of group 7 are demonstrated on both figure 2A and 2B. Being relatively variable in the catalytic domain (A) Vsa_9 IS elements demonstrate remarkable conservation of the terminal domains of the elements (B). The only point mutation (79:C→T) that was found at the terminal domain of the Vsa_9 IS elements belongs to the sequence 45_Chr_II_Vsa_9_II1097_1193529 of group 1 (indicated by asterisks on figure 2B).

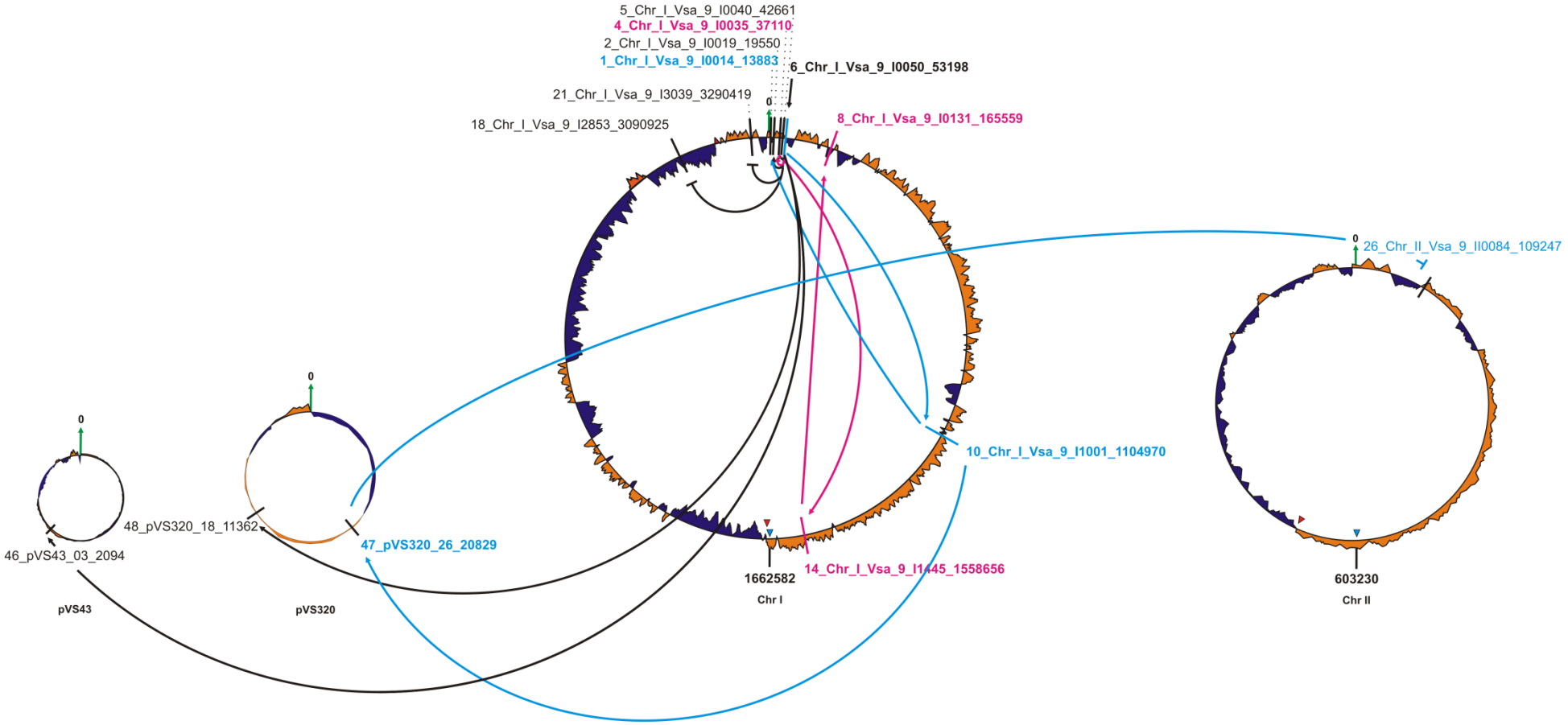


Figure 3. Reconstruction of the transposition history of the Vsa_9 IS elements of *A. salmonicida* LFI1238. Two larger circles on the right side of the figure represent chromosomes, whereas two plasmids, pVS43 and pVS320, are shown on the left side of the figure. Pink, blue and black represent the major microevolution lines of the Vsa_9 IS elements. For simplicity, each cluster is represented by single IS elements, whereas all unique single-copy sequences are shown. Arrowheads indicate the direction of transposition, whereas blunt end lines represent IS elements that are likely incapable of further transposition. Predicted origins of replication are on top of the diagrams and are indicated by green arrows. Chromosomal *ter* and *dif* sites are indicated by blue and red triangles. Orange and violet represent GC skew scores above and below average, respectively (see additional file 3 for a complete overview of the clusters).

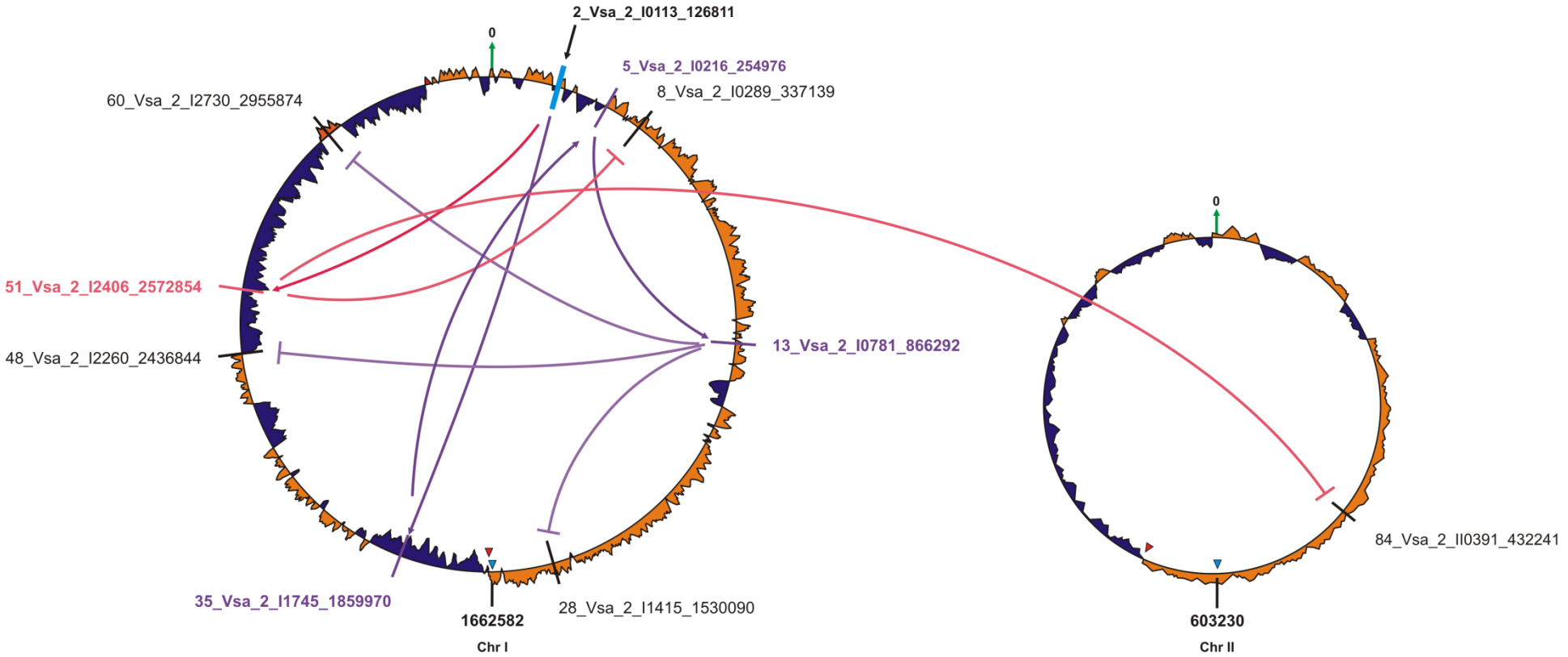


Figure 4. Reconstruction of the transposition history of the Vsa_2 IS elements of *A. salmonicida* LFI1238. Two circles represent chromosomes of the microorganism, where orange (above average) and violet (below average) outline the GC skew scores. Predicted origins of replication are on top of the diagrams and are indicated by green arrows. Chromosomal *ter* and *dif* sites are indicated by blue and red triangles. Purple and pink represent two major microevolution lines of the Vsa_2 IS elements, which were initiated by the sequences 51_VSAL_I2406 - 750079 and 35_VSAL_I1745 - 1860051. Clusters of different sequences are represented by single IS elements, whereas all unique single-copy sequences that belong to two major microevolutionary lines and that are likely incapable of further transposition are represented in black. Arrowheads indicate the direction of transposition, whereas blunt end lines lead to IS elements that are incapable of further transposition. For simplicity, sub-microevolution lines that are directly initiated by sequence 2 are not shown (see Additional file 5 for an overview of the clusters and Additional file 5.1 for a reconstruction of transposition for the remaining Vsa_2 IS elements that are not shown on figure 5).

SUPPLEMENTARY FILES

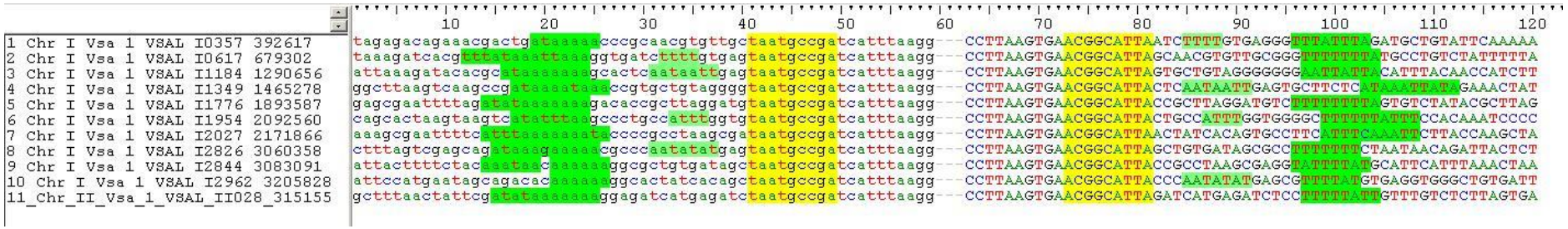
Supplementary file 1.

IS element	Chromosome	Locus	Location bp	Inactivated gene
Vsa_2	I	VSAL_I0070	71139..73455	Trk System Potassium Uptake Protein Trkh
	I	VSAL_I0516	569549..571809	Isopentenyl-Diphosphate Delta-Isomerase
	I	VSAL_I0766	853348..855660	Chitinise A
	I	VSAL_I0903	1003833..1006144	Chitinase A
	I		1103023.. 1106776	1-Acyl-Sn-Glycerol-3-Phosphate Acyltransferase
	I		1154076..1155792	Aldo/Keto Reductase
	I		1201769..1204080	1-Acyl-Sn-Glycerol-3-Phosphate Acyltransferase
	I	VSAL_I1105	1201769..1204080	Similar to Vibrio fischeri Macrolide-efflux protein
	I	VSAL_I1239	1349200..1351515	Extracellular serine Protease
	I	VSAL_I1266	1373905..1376219	Metallo-Beta-Lactamase Family Protein
	I	VSAL_I1278	1387349..1389663	Mlta-Interacting Mipa Family Protein
	I	VSAL_I1415	1530090..1532404	Chitinase
	I	VSAL_I1745	1859970..1862284	Vgrg Protein
	I	VSAL_I0155	192815..195130	Phosphopantetheine Adenylyltransferase
	I	VSAL_I1809	1932875..1935188	Diheme-Containing Soxa-Like Protein (chemotaxis protein)
	I	VSAL_I1943	2077283..2079597	Chitinise A
	I	VSAL_I2011	2150701..2153014	Long-Chain Fatty Acid Transport Protein
	I	VSAL_I2044	2188541..2190855	Long-Chain Fatty Acid Transport Protein
	I	VSAL_I2161	2323172..2325486	Protease III
	I	VSAL_I2088	2237128..2239433	3-Oxoacyl-ACP Synthase
	I	VSAL_I2180	2339251..2341565	Uncnown/ Ompa Domain-Containing Protein
	I	VSAL_I2116	2264933..2267247	hlyB hemolysisn protein
	I	VSAL_I2460	2631935..2634251	Sodium-Solute Symporter Putative
	I	VSAL_I2484	2660733..2663047	Type I Restriction-Modification System Restriction Subunit R
	I	VSAL_I2951	3197054..3199368	Sensory Transduction Protein Kinase
	I	VSAL_I3055	3307151..3309466	Na(+) Driven Multidrug Efflux Pump
	II		7297..10618	B-Type Cytochrome
	II		57609..59831	Glycogen Branching Enzyme
	II	VSAL_II0048	60897..63211	High-Affinity Iron Permease
	II	VSAL_II0071	94804..97020	Hypothetical Nitrate Reductase Large Subunit
	II		118419.. 121740	Threonine Efflux Protein
	II		152301..156053	Transporter, Nadc Family
	II	VSAL_II0234	257692..260006	Maltose/Maltodextrin Transporter ATP-Binding Protein

	II	VSAL_II0241	264526..266840	Nitrate Reductase Large Subunit
	II		280688..283002	Phosphopantetheine Adenylyltransferase
	II	VSAL_II0272	300370..302586	Aerobactin Siderophore Biosynthesis Protein Iuca
	II	VSAL_II0333	376163..378477	Multidrug ABC Transporter Atpase And Permease
	II		376163..378477	Toxin (Type I Secretion Target Repeat Protein)
	II	VSAL_II0391	432241..434555	Penicillin Hydrolase (Choloylglycine Hydrolase)
	II	VSAL_II0517	573434..575748	Dihydropteridine Reductase, NAD(P)H-Dependent, Oxygen-Insensitive
	II	VSAL_II0766	841127..843441	Methyl-Accepting Chemotaxis Protein
	II		1015816..1018130	Maltose Transporter Membrane Protein
	II	VSAL_II1005	1102140..1104356	Mshq Protein
	II		1102140..1104356	HTH-Type Transcriptional Regulator Galr
	II		1144794..1146742	Transporter, Nadc Family Protein
	II		1185562..1187778	Cytocrome C
Vsa_6	I		240800..241804	Competence Protein F
	I		495222..496226	Aspartate Kinase III
	I		830359..831363	Serine Transporter/Inner Membrane Transport Protein Yhao
	I		960274..961278	Inner Membrane Lipoprotein
	I		1015633..1016637	5-Methyltetrahydropteroyltriglutamate/Homocysteine S-Methyl Transferase
	I		1072318..1073322	Alanine Racemase
	I		1325363..1326367	Threonine Aldolase
	I		1454948..1455952	Tyrosine-Specific Transport Protein
	I		1526468..1527472	Glycine Betaine Transporter
	I		1622806..1623810	Arginase/Agmatinase/Formimionoglutamate Hydrolase
	I		1711500..1712504	Acyl-Coa Synthetase
	I		1736675..1737796	Na+/Solute Symporter
	I		1787876..1788880	Trna-(Ms[2]Io[6]A)-Hydroxylase
	I		2135010..2136014	ATP-Dependent Helicase Hrpa
	I		2156965..2157969	ABC Transporter: Transmembrane And ATP-Binding Protein
	I		2156965..2157969	Multidrug Resistance Efflux Pump
	I		2222473..2223477	ABC Transporter: Transmembrane And ATP-Binding Protein
	I		2801590..2802594	Serine Protease
	I		2946747..2947751	Fimbrial Assembly Protein Pilq
	II		184729..185733	Multidrug ATP-Binding/Permease Fusionabc Transporter
	II		217387..218391	Trimethylamine-N-Oxide Reductase 2
	II		553043..554047	Signal Transduction Protein
	II		694168..695172	GGDEF Domain-Containing Protein
	II		835813..836817	PAS/PAC Sensor-Containing Diguanylate

				Cyclase/Phosphodiesterase
Vsa_7	I		1832986..1834039	Diguanylate Cyclase
	II		514950..516003	ATP-Dependent Transcriptional Regulator
Vsa_8	I		512434..513555	Capsular Polysaccharide Synthesis Enzyme Cpsd
	I		1581613..1582734	Methyl-Accepting Chemotaxis Protein
	I		1589920..1591033	Acyltransferase Family Protein
	I		2045266..2046387	Penicillin-Binding Protein 2
	I		2183802..2184923	Regulator Of Competence-Specific Genes
	I		3096142..3097263	Minor Curlin Subunit Csgb
	II		306734..307855	Aerobactin Siderophore Biosynthesis Protein Iucc
	II		878929..880050	Peptidase C39, Bacteriocin Processing
	II		1003822..1004951	Maltose Transporter Membrane Protein
	II		1153865..1154986	Lactoylglutathione Lyase
Vsa_9	I		59768..61206	Acetolactate Synthase 2 Catalytic Subunit
	I		1270158..1271596	Chitinase A
	I		1450223..1451661	Na+ Driven Multidrug Efflux Pump
	I		1541621..1543059	U32 Family Peptidase
	I		1558656..1560094	Methyl-Accepting Chemotaxis Protein
	I		1777502..1778940	Lrga-Associated Membrane Protein Lrgb
	I		1805026..1806461	Methyl-Accepting Chemotaxis Protein
	I		3090925..3092363	NADH Pyrophosphatase
	I		3285768..3287206	Methyl-Accepting Chemotaxis Protein
	II		18442..19877	Multidrug Efflux System Protein
	II		40074..43835	Acriflavin Resistance Plasma Membrane Protein
	II		130799..132237	Trimethylamine-N-Oxide Reductase 2
	II		230847..232285	Reticulocyte-Binding Protein 2d
	II		284304..285743	Agmatinase
	II		908950..910388	Multidrug Resistance Protein A
	II		959345..960783	Dehydrogenase
	II		983378..984816	Short Chain Dehydrogenase
	II		1060457..1061892	Cytotoxic Necrotizing Factor 1
	II		1193529..1194967	Acriflavin Resistance Plasma Membrane Protein
	pVS320		20829..22388	MobA

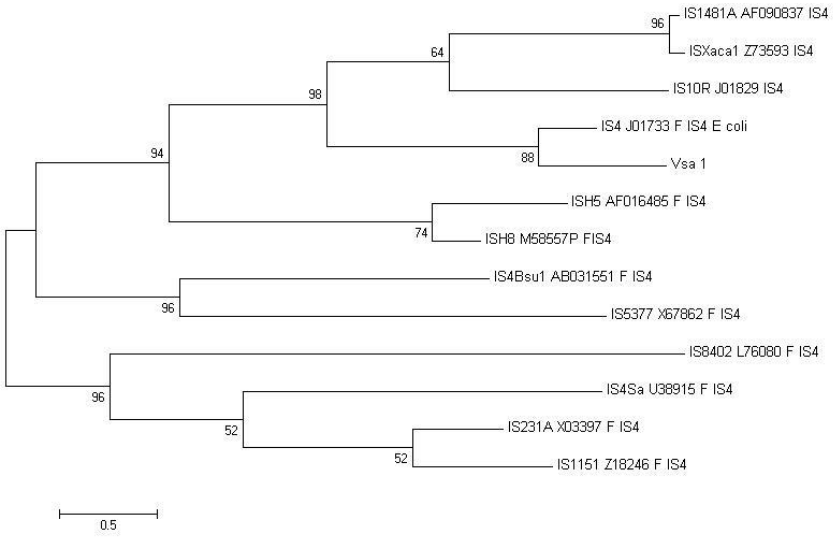
Supplementary file 2.



Supplementary file 3.

		Transposases	
Group 1	Cluster 1	6_Chr_I_Vsa_9_I0050_53198	
		11_Chr_I_Vsa_9_I1166_1270158	
		15_Chr_I_Vsa_9_I1683_1777502	
		28_Chr_II_Vsa_9_II0107_130799	
		29_Chr_II_Vsa_9_II0117_142202	
		31_Chr_II_Vsa_9_II0188_214356	
		32_Chr_II_Vsa_9_II0202_225761	
		33_Chr_II_Vsa_9_II0208_230847	
		43_Chr_II_Vsa_9_1143355	
		44_Chr_II_Vsa_9_II1086_1181771	
	Cluster 2	10_Chr_I_Vsa_9_I1001_1104970	
		12_Chr_I_Vsa_9_I1335_1450223	
		13_Chr_I_Vsa_9_I1430_1541621	
		30_Chr_II_Vsa_9_II0130_152946	
		35_Chr_II_Vsa_9_II0359_402119	
		36_Chr_II_Vsa_9_II0442_500931	
		37_Chr_II_Vsa_9_II0829_908950	
		38_Chr_II_Vsa_9_II0875_959345	
		39_Chr_II_Vsa_9_II0900_983378	
		41_Chr_II_Vsa_9_II0990_1083739	
	Cluster 3	7_Chr_I_Vsa_9_I0050_59768	
		45_Chr_II_Vsa_9_II1097_1193529	
		46_pVSAL43_03_2094	
		48_pVSAL320_18_11362	
G2		14_Chr_I_Vsa_9_I1445_1558656	
		19_Chr_I_Vsa_9_I2969_3214581	
		20_Chr_I_Vsa_9_I3035_3285768	
		22_Chr_I_Vsa_9_I3056_3310617	
		34_Chr_II_Vsa_9_II0256_284304	
		3_Chr_I_Vsa_9_I0022_23773	
G3		4_Chr_I_Vsa_9_I0035_37110	
		16_Chr_I_Vsa_9_I1708_1805026	
		40_Chr_II_Vsa_9_II0970_1060457	
G4		8_Chr_I_Vsa_9_I0131_165559	
		17_Chr_I_Vsa_9_I1720_1828835	
G5		1_Chr_I_Vsa_9_I0014_13883	
		27_Chr_II_Vsa_9_II0106_129363	
G6		2_Chr_I_Vsa_9_I0019_19550	
		23_Chr_II_Vsa_9_II0017_18442	
G7		5_Chr_I_Vsa_9_I0040_42661	
		21_Chr_I_Vsa_9_I3039_3290419	
		26_Chr_II_Vsa_9_II0084_109247	
		18_Chr_I_Vsa_9_I2853_3090925	
		47_pVSAL320_26_20829	

Supplementary file 4.

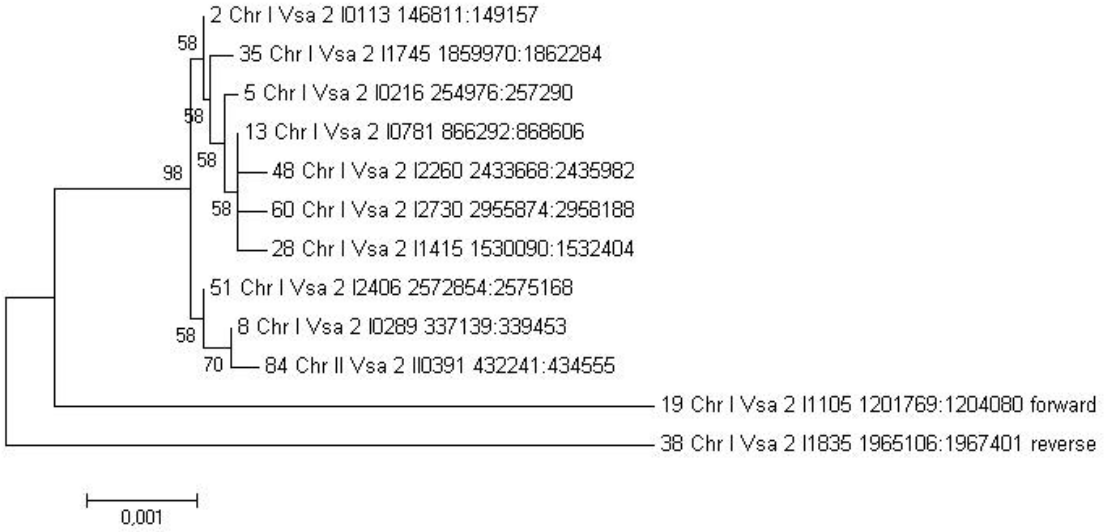


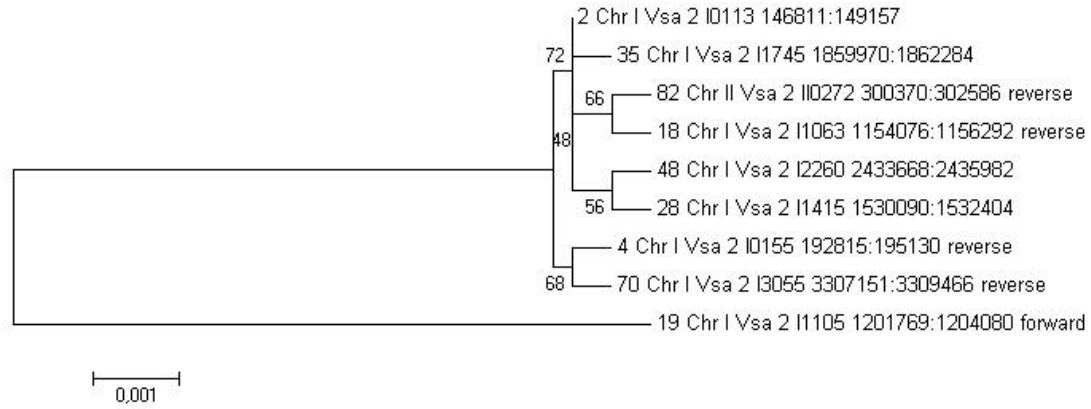
Supplementary file 5.

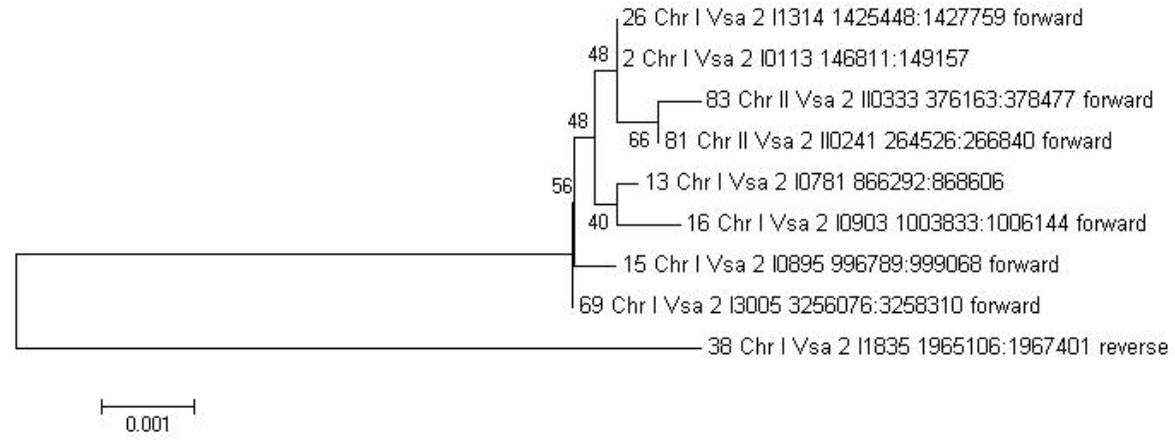
				100	200	300	400	500	600	700	
Group 1	Cluster 1	2_VSAL_I0113 - 146904	1								
		3_VSAL_I0142 - 181578	2								
		30_VSAL_I1574 - 1685410	3								
		66_VSAL_I2941 - 3188568	9								
		72_VSAL_II0033 - 116309	10								
		73_VSAL_II0048 - 114333	11								
		88_VSAL_II0749 - 823511	16								
		92_VSAL_II0975 - 139057	18								
	Cluster 2	35_VSAL_I1745 - 1860051	4								
		42_VSAL_I2044 - 1134392	5								
		45_VSAL_I2161 - 2323253	6								
		75_VSAL_II0082 - 1098362	12								
		76_VSAL_II0173 - 198406	13								
		85_VSAL_II0472 - 673559	15								
		86_VSAL_II0517 - 630794	17								
		94_VSAL_II1016 - 111373	19								
	Clr 3	51_VSAL_I2406 - 750079	7								
		53_VSAL_I2484 - 2660814	8								
		78_VSAL_II0227 - 250199	14								
Group 2	Clr 1	5_VSAL_I0216 - 3067957									
		6_VSAL_I0234 - 280769									
	Clr 2	13_VSAL_I0781 - 866373									
		20_VSAL_I1207 - 2008555									
		24_VSAL_I1278 - 1387430									
		32_VSAL_I1662 - 1758749									
		62_VSAL_I2761 - 332405									
	Clr 3	10_VSAL_I0435 - 484735									
		46_VSAL_I2180 - 983682									
	Clr 4	28_VSAL_I1415 - 1530171									
		48_VSAL_I2260 - 2433749									
		60_VSAL_I2730 - 2955955									
	Clr 5	8_VSAL_I0289 - 337220									
		67_VSAL_I2951 - 3197135									
		84_VSAL_II0391 - 771987									
Group 3		14_VSAL_I0809 - 898508									
		31_VSAL_I1654 - 1753065									
		33_VSAL_I1670 - 1765750									
Group 4		55_VSAL_I2600 - 518033									
		61_VSAL_I2751 - 2980853									
		91_VSAL_II0930 - 188411									
Gr6		1_VSAL_I0070 - 3251792									
		27_VSAL_I1363 - 1842268									

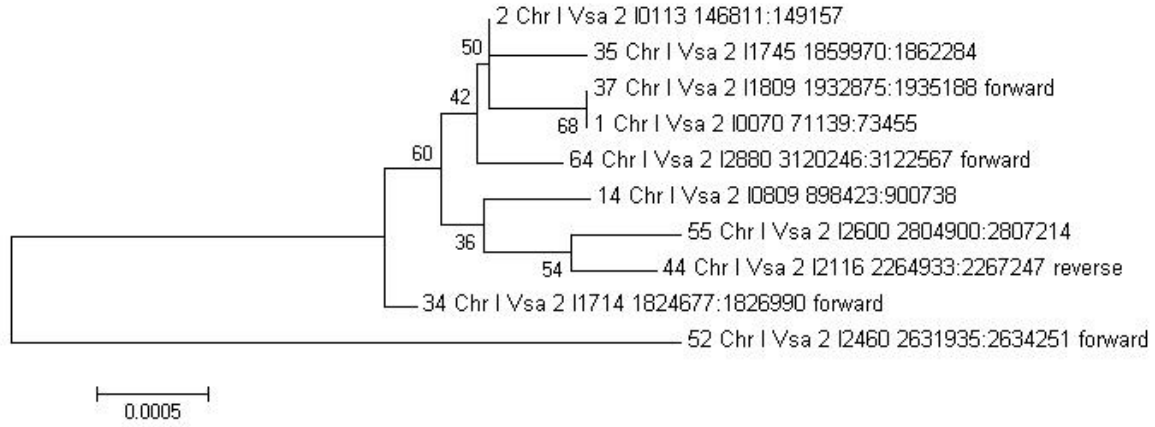
				1	2	3	4	5	6	7
Group 7		4_VSAL_I0155 - 3130117								
		7_VSAL_I0279 - 328784								
		11_VSAL_I0516 - 2753438								
		12_VSAL_I0766 - 2469587								
		15_VSAL_I0895 - 996870								
		16_VSAL_I0903 - 1003914								
		18_VSAL_I1063 - 1155995								
		19_VSAL_I1105 - 1201850								
		21_VSAL_I1239 - 1349281								
		22_VSAL_I1256 - 1365834								
		23_VSAL_I1266 - 1373986								
		25_VSAL_I1291 - 1400496								
		26_VSAL_I1314 - 1425529								
		34_VSAL_I1714 - 1824758								
		37_VSAL_I1809 - 1932956								
		38_VSAL_I1835 - 1967007								
		39_VSAL_I1928 - 2063208								
		40_VSAL_I1943 - 2077364								
		41_VSAL_I2011 - 1172233								
		43_VSAL_I2088 - 1085814								
		44_VSAL_I2116 - 1058000								
		47_VSAL_I2248 - 2418149								
		49_VSAL_I2266 - 886090								
		52_VSAL_I2460 - 2632016								
		54_VSAL_I2489 - 658801								
		56_VSAL_I2613 - 496218								
		57_VSAL_I2622 - 489161								
		58_VSAL_I2686 - 416265								
		59_VSAL_I2709 - 391161								
		63_VSAL_I2807 - 3039055								
		64_VSAL_I2880 - 3120327								
		65_VSAL_I2935 - 144232								
		68_VSAL_I2960 - 120112								
		69_VSAL_I3005 - 3256157								
		70_VSAL_I3055 - 15781								
		71_VSAL II0025 - 1172220								
		74_VSAL_II0071 - 94804								
		77_VSAL_II0221 - 959858								
		79_VSAL_II0234 - 257774								
		81_VSAL_II0241 - 264608								
		82_VSAL_II0272 - 300370								
		83_VSAL_II0333 - 376245								
		87_VSAL II0698 - 766582								
		89_VSAL_II0766 - 841210								
		90_VSAL II0831 - 912048								
		93_VSAL_II1005 - 1102140								
		95_VSAL - 18763								

Supplementary file 5.1.





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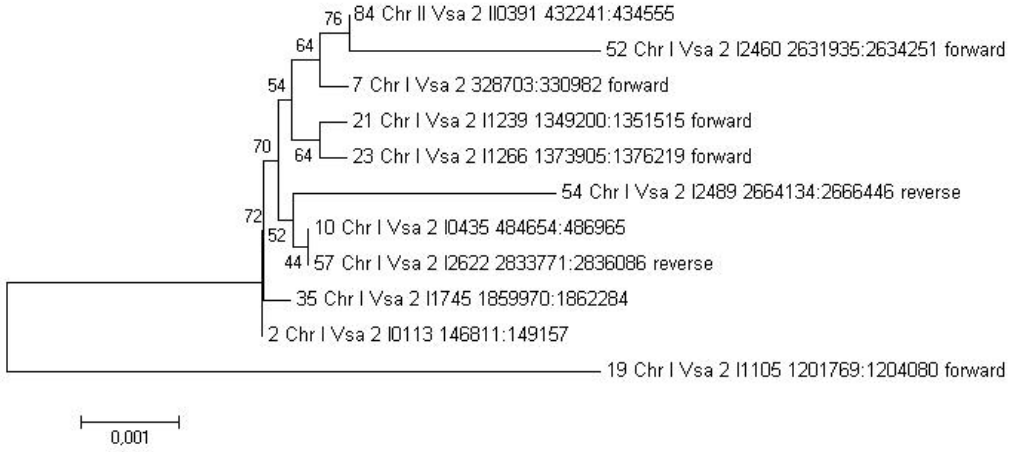
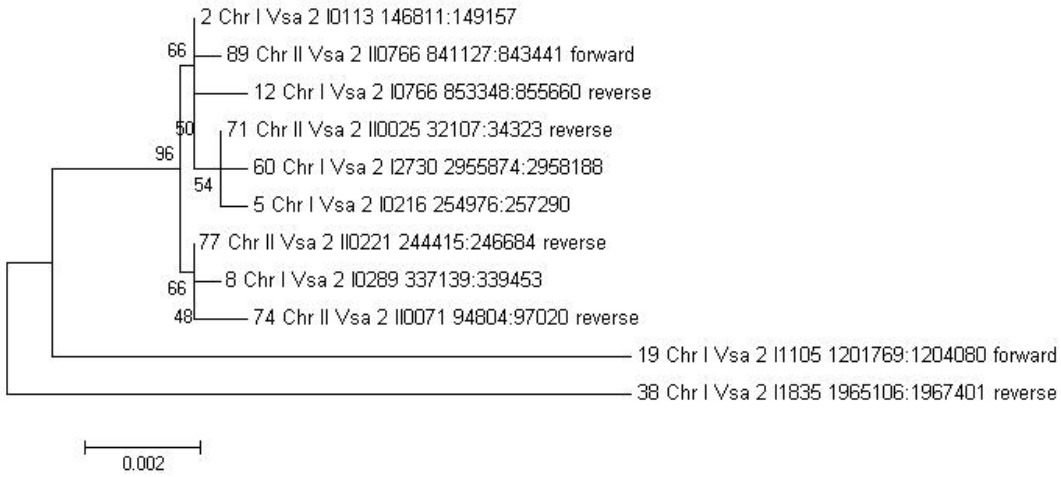
50
35 Chr I Vsa 2 I1745 1859970:1862284
42
37 Chr I Vsa 2 I1809 1932875:1935188 forward
68
1 Chr I Vsa 2 I0070 71139:73455
60
64 Chr I Vsa 2 I2880 3120246:3122567 forward
14 Chr I Vsa 2 I0809 898423:900738
36
55 Chr I Vsa 2 I2600 2804900:2807214
54
44 Chr I Vsa 2 I2116 2264933:2267247 reverse
34 Chr I Vsa 2 I1714 1824677:1826990 forward
52 Chr I Vsa 2 I2460 2631935:2634251 forward
0.0005


76
84 Chr II Vsa 2 II0391 432241:434555
64
52 Chr I Vsa 2 I2460 2631935:2634251 forward
54
7 Chr I Vsa 2 328703:330982 forward
21 Chr I Vsa 2 I1239 1349200:1351515 forward
70
64
23 Chr I Vsa 2 I1266 1373905:1376219 forward
54 Chr I Vsa 2 I2489 2664134:2666446 reverse
72
52
10 Chr I Vsa 2 I0435 484654:486965
44
57 Chr I Vsa 2 I2622 2833771:2836086 reverse
35 Chr I Vsa 2 I1745 1859970:1862284
2 Chr I Vsa 2 I0113 146811:149157
19 Chr I Vsa 2 I1105 1201769:1204080 forward
0,001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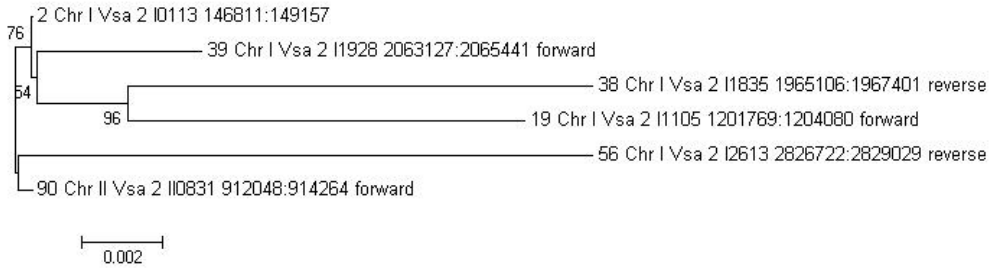
66
89 Chr II Vsa 2 II0766 841127:843441 forward
12 Chr I Vsa 2 I0766 853348:855660 reverse
50
71 Chr II Vsa 2 II0025 32107:34323 reverse
96
60 Chr I Vsa 2 I2730 2955874:2958188
54
5 Chr I Vsa 2 I0216 254976:257290
77 Chr II Vsa 2 II0221 244415:246684 reverse
66
8 Chr I Vsa 2 I0289 337139:339453
48
74 Chr II Vsa 2 II0071 94804:97020 reverse
19 Chr I Vsa 2 I1105 1201769:1204080 forward
38 Chr I Vsa 2 I1835 1965106:1967401 reverse
0.002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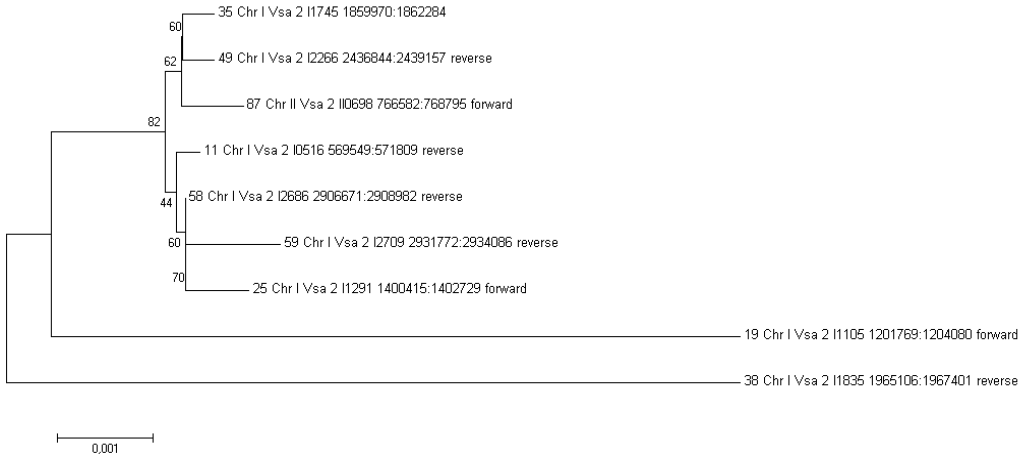
76
39 Chr I Vsa 2 I1928 2063127:2065441 forward
54
38 Chr I Vsa 2 I1835 1965106:1967401 reverse
96
19 Chr I Vsa 2 I1105 1201769:1204080 forward
56 Chr I Vsa 2 I2613 2826722:2829029 reverse
90 Chr II Vsa 2 II0831 912048:914264 forward
0.002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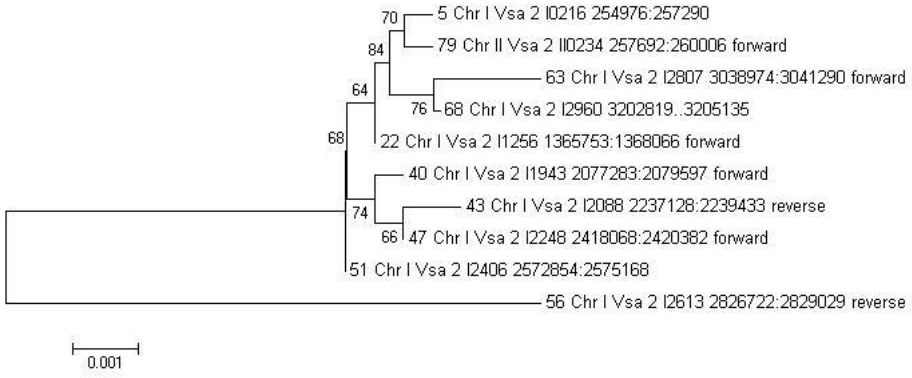
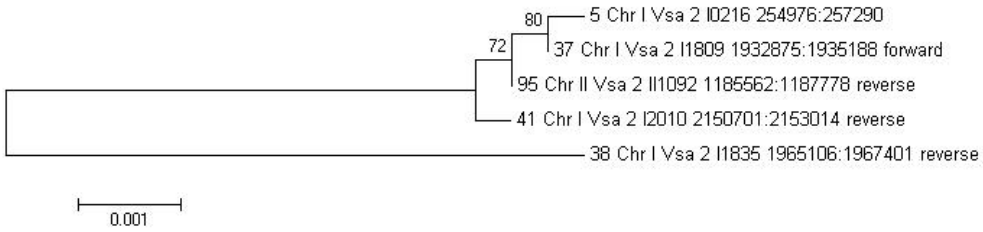
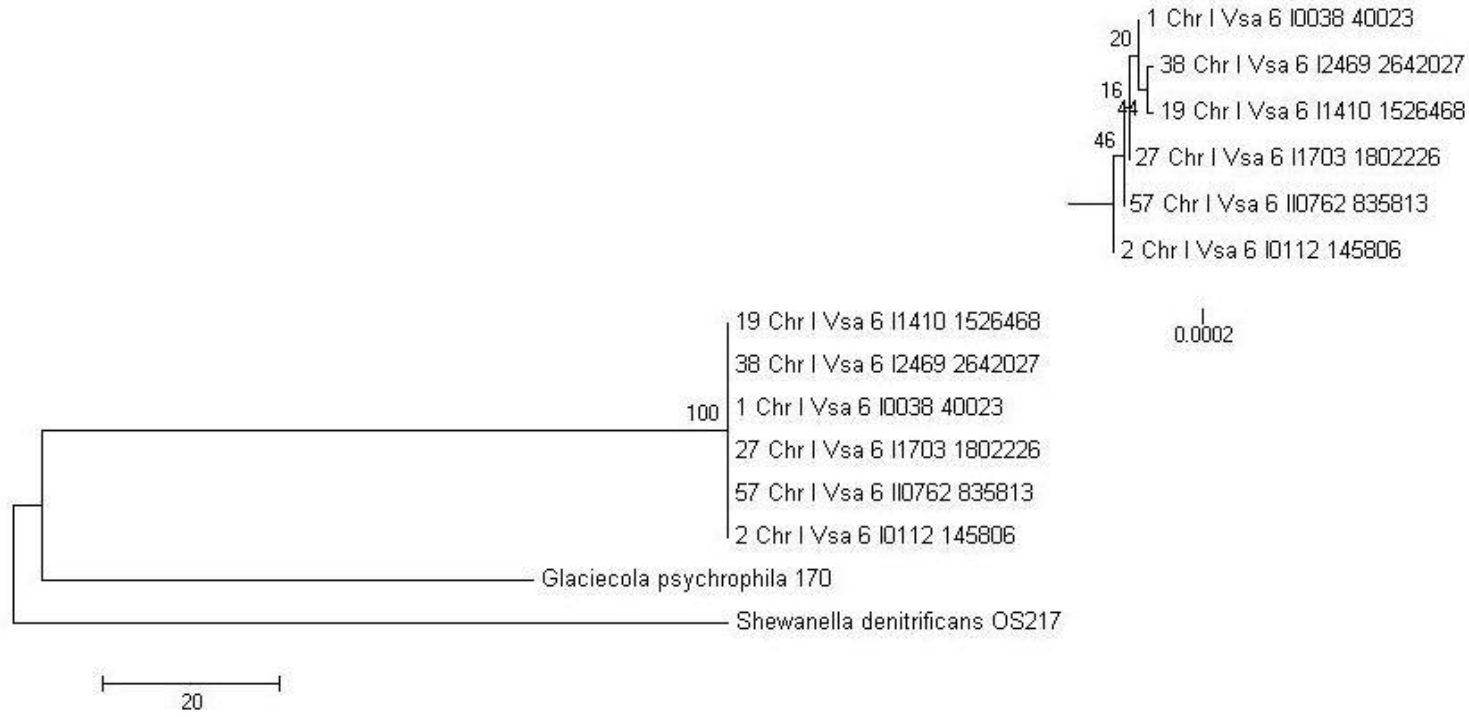
60
49 Chr I Vsa 2 I2266 2436844:2439157 reverse
62
87 Chr II Vsa 2 II0698 766582:768795 forward
82
11 Chr I Vsa 2 I0516 569549:571809 reverse
58 Chr I Vsa 2 I2686 2906671:2908982 reverse
44
59 Chr I Vsa 2 I2709 2931772:2934086 reverse
60
25 Chr I Vsa 2 I1291 1400415:1402729 forward
70
19 Chr I Vsa 2 I1105 1201769:1204080 forward
38 Chr I Vsa 2 I1835 1965106:1967401 reverse
0,001


70
5 Chr I Vsa 2 I0216 254976:257290
79 Chr II Vsa 2 II0234 257692:260006 forward
84
63 Chr I Vsa 2 I2807 3038974:3041290 forward
64
76
68 Chr I Vsa 2 I2960 3202819..3205135
68
22 Chr I Vsa 2 I1256 1365753:1368066 forward
40 Chr I Vsa 2 I1943 2077283:2079597 forward
43 Chr I Vsa 2 I2088 2237128:2239433 reverse
74
66
47 Chr I Vsa 2 I2248 2418068:2420382 forward
51 Chr I Vsa 2 I2406 2572854:2575168
56 Chr I Vsa 2 I2613 2826722:2829029 reverse
0.001


80
5 Chr I Vsa 2 I0216 254976:257290
72
37 Chr I Vsa 2 I1809 1932875:1935188 forward
95 Chr II Vsa 2 II1092 1185562:1187778 reverse
41 Chr I Vsa 2 I2010 2150701:2153014 reverse
38 Chr I Vsa 2 I1835 1965106:1967401 reverse
0.001

Supplementary file 6.



Supplementary file 6.1.

				1-50	50-100	100-150	150-200	200-250	
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		3_Chr_I_Vsa_6_I0201_ 240800							
		4_Chr_I_Vsa_6_I0286_ 333593							
		5_Chr_I_Vsa_6_I0317_ 360103							
		6_Chr_I_Vsa_6_I0444_ 495222							
		7_Chr_I_Vsa_6_I0744_ 830359							
		8_Chr_I_Vsa_6_I0862_ 960274							
		9_Chr_I_Vsa_6_I0917_ 1015633							
		10_Chr_I_Vsa_6_I0959_ 1055516							
		11_Chr_I_Vsa_6_I0972_ 1072318							
		12_Chr_I_Vsa_6_I1051_ 1143005							
		14_Chr_I_Vsa_6_I1218_ 1325363							
		15_Chr_I_Vsa_6_I1276_ 1385568							
		16_Chr_I_Vsa_6_I1339_ 1454948							
		17_Chr_I_Vsa_6_I1348_ 1464233							
		18_Chr_I_Vsa_6_I1365_ 1485887							
		20_Chr_I_Vsa_6_I1441_ 1553923							
		21_Chr_I_Vsa_6_I1459_ 1578008							
		22_Chr_I_Vsa_6_I1522_ 1622806							
		23_Chr_I_Vsa_6_I1601_ 1708691							
		24_Chr_I_Vsa_6_I1605_ 1711500							
		25_Chr_I_Vsa_6_I1652_ 1751400							
		26_Chr_I_Vsa_6_I1692_ 1787876							
		28_Chr_I_Vsa_6_I1777_ 1895284							
		29_Chr_I_Vsa_6_I1993_ 2135010							
		30_Chr_I_Vsa_6_I2015_ 2156965							
		31_Chr_I_Vsa_6_I2051_ 2197970							
		32_Chr_I_Vsa_6_I2070_ 2222473							
		33_Chr_I_Vsa_6_I2251_ 2420710							
		35_Chr_I_Vsa_6_I2323_ 2496560							

		36_Chr_I_Vsa_6_I2394_ 2562751							
		37_Chr_I_Vsa_6_I2430_ 2602097							
		39_Chr_I_Vsa_6_I2543_ 2723385							
		43_Chr_I_Vsa_6_I2863_ 3097930							
		44_Chr_I_Vsa_6_I2874_ 3114201							
		45_Chr_I_Vsa_6_I3002_ 3251062							
		46_Chr_II_Vsa_6_II0008_ 7700							
		47_Chr_II_Vsa_6_II0041_ 57689							
		48_Chr_II_Vsa_6_II0097_ 119219							
		49_Chr_II_Vsa_6_II0158_ 184729							
		50_Chr_II_Vsa_6_II0192_ 217387							
		51_Chr_II_Vsa_6_II0260_ 288541							
		52_Chr_II_Vsa_6_II0419_ 471284							
		53_Chr_II_Vsa_6_II0465_ 526398							
		54_Chr_II_Vsa_6_II0495_ 553043							
		55_Chr_II_Vsa_6_II0643_ 694168							
		58_Chr_II_Vsa_6_II0778_ 855062							
		56_Chr_II_Vsa_6_II0689_ 754301							
		59_Chr_II_Vsa_6_II0809_ 886328							

				1-50	50-100	100-150	150-200	200-250	
Group 2		2_Chr_I_Vsa_6_I0112_ 145806							
		13_Chr_I_Vsa_6_I1209_ 1317680							
		40_Chr_I_Vsa_6_I2597_ 2801590							
		41_Chr_I_Vsa_6_I2721_ 2946747							
Group 3		19_Chr_I_Vsa_6_I1410_ 1526468							
		27_Chr_I_Vsa_6_I1703_ 1802226							
		38_Chr_I_Vsa_6_I2469_ 2642027							
		57_Chr_II_Vsa_6_II0762_ 835813							

Supplementary file 7.