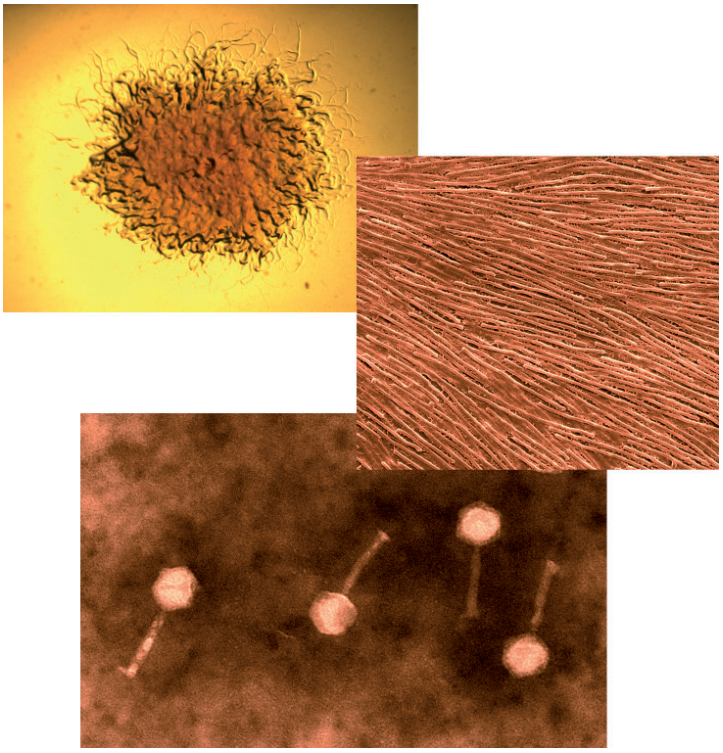
JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
290

Elina Laanto

Exploring Phage-Bacterium Interactions

New Ways to Combat a Fish Pathogen



JYVÄSKYLÄN YLIOPISTO

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2014

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Kaikilla vuorilla ja kivillä,
kaikilla puilla, pensailla ja pikku ruohoilla,
kaikilla metsän, ilman ja meren eläimillä
on omat kertomuksensa.
Mikään ei ole kuollutta,
kaikki elää, kaikki hengittää…

Zacharias Topelius
Mistä satuja saadaan

ABSTRACT

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Exploring phage-bacterium interactions – New ways to combat a fish pathogen
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Diss.

The risks associated with antibiotic use, including development of antibiotic resistant pathogenic strains and the spreading of resistance genes, have caused a search for novel therapeutics. After being forgotten because of the ease brought by antibiotics in disease control in the Western world, phage therapy has enjoyed reviving interest. Aquaculture is one of the major users of antibiotics and contributes greatly to the leakage of antibiotics into the surrounding environment. Increased severity of disease outbreaks caused by a common fish pathogen, *Flavobacterium columnare*, has led to concerns on its management by antibiotics and instigated a search for efficient alternatives. In this thesis, phages infecting *F. columnare* were isolated from fish farms, and their impact to the host bacterium as well as their efficiency as therapeutics was studied. Phages infecting *F. columnare* were shown to be host-specific compared to phages infecting *Flavobacterium* sp. isolated from fresh waters across Finland. *Flavobacterium* sp. phages with variable host-ranges were characterized, providing more information on the known diversity of fresh water bacteriophages. The first *F. columnare* phage genome was sequenced and annotated. The genome showed similarities to marine phages of *Cellulophaga*, a close relative of Flavobacteria. Resistance to phage had clear costs for the *F. columnare*, as the abilities of bacteria to cause disease and, in parallel, to move was lost. In a comparative study between virulent and phage induced non-virulent morphotypes new virulence factors for *F. columnare* were suggested. Finally, addition of phage in aquaria with experimentally infected fish had a significant influence on the longevity of fish in a flow-through system. These results suggest great potential for using phages in aquaculture against common fish disease. In the future new phages are needed and their properties well characterized but above all, strict legislation needs to be overcome before the actual production of therapeutics can begin.

Keywords: Bacteriophage; fish pathogen; *Flavobacterium columnare*; phage therapy; virulence.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV.

I Laanto E., Sundberg L.-R. & Bamford J.K.H. 2011. Phage specificity of the freshwater fish pathogen *Flavobacterium columnare*. *Applied and Environmental Microbiology* 77: 7868–7872.

II Laanto E., Bamford J.K.H., Laakso J. & Sundberg L.-R. 2012. Phage-driven loss of virulence in a fish pathogenic bacterium. *PLoS One* 7(12): e53157. doi:10.1371/journal.pone.0053157.

III Laanto E., Penttinen R.K., Bamford J.K.H. & Sundberg L.-R. 2014. Comparing the different morphotypes of a fish pathogen – implications for key virulence factors in *Flavobacterium columnare*. *BMC Microbiology* 14:170

IV Laanto E., Bamford J.K.H., Ravantti J. & Sundberg L.-R. 2014. Tackling a fish disease with a little help from a phage. Submitted manuscript.

RESPONSIBILITIES OF ELINA LAANTO IN THE ARTICLES OF THE THESIS:

I I isolated the material, did the experiments (except for RISA and TEM-imaging) and prepared the tables and figures. I analysed the data and wrote the paper together with the co-authors.

II I performed the experiments, except virulence tests, and participated in the preparation of figures and the writing of the article.

III I was involved in the original idea, I did most of the experiments and prepared the figures. Reetta Penttinen performed the virulence experiments and the sequence analysis. I analysed the data and wrote the article together with the co-authors.

IV I performed all the other experiments except the sequencing. Lotta-Riina Sundberg performed the statistical analysis. I analysed the data and wrote the manuscript with the co-authors.

ABBREVATIONS

bp	base pair
CFU	colony forming unit
ds	double-stranded
HR-SEM	high-resolution scanning electron microscopy
EPS	exopolysaccharide
TEM	transmission electron microscope
MDR	multi-drug resistant
OMTs	outer membrane-enclosed tubes
OMVs	outer membrane vesicles
ORF	open reading frame
PCR	polymerase chain reaction
PFU	plaque forming unit
RISA	ribosomal intergenic spacer analysis
rRNA	ribosomal RNA
RT	room temperature
ss	single-stranded
WHO	World Health Organization

CONTENTS

1 INTRODUCTION

A virus is an entity that consists of a genome and a capsid and requires a host to replicate itself. Thus it is not considered to be an organism. But as it enters the host it in a sense becomes biologically active and therefore could be considered as "alive". Indeed, a viriocell-concept has been used to describe the living form of a virus (Forterre 2011) as after viral infection the purpose of the cell changes only to produce new virions, instead of replicating. Viruses are found infecting all three currently classified domains of life, and even a virus infecting a virus exists (La Scola *et al.* 2008). Bacteriophage (phage) is a virus that infects a bacterial cell. Phage research has undoubtedly contributed greatly to the field of biology. Perhaps the greatest contributions that have been achieved with phages were the development of molecular biology and the concept of genes (Summers 2006). But maybe the long list of the importances of phages should start with the fact, that we all exist because of the interactions between bacteria (and archaea) and their viruses (Forterre and Prangishvili 2013). The continuous war between phages and their hosts developed the 'proving ground' for the biosphere and for the diversity of life.

Even though the existence of phages exceeds the lifespan of human kind by far, our first observation of their presence was done no earlier than in the beginning of the 20th century, after the establishment of bacteriology. Two microbiologists, Richard Twort and Felix d'Herelle, independently realized that something was destroying their bacterial cultures. d'Herelle quickly adopted the idea that these agents were invading bacteria and multiplying at their expense and called them bacteriophages. A few years later, it was d'Herelle who first thought about using phages as therapeutics after noticing that phage titres rapidly rose in a patient with an infectious disease at the point when recovery was taking place. Certainly, the concept of phage therapy is as old as the discovery of phages but it was neglected for a long time in the Western world because of the discovery of antibiotics. Now, after the global experiment with overuse of antibiotics, we are receiving the results: multidrug resistant (MDR) pathogens and the spreading of resistance genes. Development of MDR pathogenic bacteria is one of the greatest challenges of our time. One of the

ways that could be used to meet this challenge is phage therapy, and indeed, it has been drawing revived interest during the past decade.

Large amounts of data have already been collected from phages in different habitats, but we are only beginning to realise the impact of phage-bacterium interactions to the ecological processes. Moreover, even though there is very detailed information on model phages, the knowledge on phage interacting with its host bacterium in its real environment is poor, for example in the case of phage T4 and its interactions with *Escherichia coli* in the human intestine. Novel methods are being developed to overcome this barrier, e.g. the development of fluorescent T4 that can be visualized in the human tissue in real time (Kaźmierczak *et al.* 2014). But for comprehensive understanding of phage therapy, knowledge of the interactions *in vivo* is vital.

In this dissertation I aim to fill the gap in our knowledge concerning the phages in northern freshwaters infecting Flavobacteria and the fish pathogenic *Flavobacterium columnare*. I wish to lead the reader through the interesting aspects of phages and their role in bacterial life, and discuss how we could use these interactions for the benefit of our society. This thesis offers the first implications for the potential of phages in the fight against fish disease and is an opening in the development of phage therapy in fish farming and future sustainability in disease management.

1.1 Bacteriophages, everywhere

Bacteriophages were not considered to have any biochemical or ecological effect in natural systems until the discovery of their abundance in seawater (Bergh *et al.* 1989, Fuhrman 1999, Suttle 2005). Since then, estimates of the total number of phage particles, being 10^{31} or even more, have suggested that they exceed the number of their bacterial hosts by at least ten-fold. The turnover of the phage population in the oceans is possibly only few days (Suttle and Chen 1992) leading to the estimation of approximately 10^{23} infections per second is needed to meet this number (Suttle 2007). However, there might be some factors causing a bias to these enumerations. First, the abundance of bacterial vesicles found in marine environments (Biller *et al.* 2014) indicates that at least some of the phage-like particles counted by staining methods might have actually been membrane vesicles carrying DNA. Vesicles carrying DNA have been suggested to be a source of false detection of virus-like particles earlier by Soler *et al.* (2008). Second, the view on the number of different phage families is changing. For example the single-stranded DNA (ssDNA) phages seem to be more frequent than previously thought (Holmfeldt *et al.* 2013, Hopkins *et al.* 2014). Also, they are not visible by the DNA-staining methods (Holmfeldt *et al.* 2012), most probably decreasing the earlier estimates on phage numbers. But nevertheless, phages are everywhere where their hosts are, and there is evidence that at least 10 phage species can infect one species of bacteria at a time (Wichels *et al.* 1998), supporting the estimation of their total number and

diversity. The situation in freshwater environments follows that of marine (Wommack and Colwell 2000), but in more complex environments (soil and sugar beet phytosphere) the situation is not as clear (Ashelford *et al.* 1999b, 2003) with estimates ranging from lower to higher abundance than that of the host. Perhaps most importantly, it is coming increasingly clear that phages are the key players in nutrient cycling in the world's oceans. Marine phages are major drivers of global carbon cycling (Wilhelm and Suttle 1999) and also contribute to the reservoir and cycling of phosphorous (Jover *et al.* 2014). Phages also alter their host metabolism (Ankrah *et al.* 2014) which may have effect on microbial species composition and biogeochemistry and therefore influence their interactions with the environment.

1.1.1 Bacteriophage classification

The current view places the origin of phages to the time before the three contemporary domains of life separated (Ackermann 1999, Hendrix *et al.* 1999). Therefore, bacteriophages have been evolving for perhaps four billion years and the high amount of genetic exchange has led to a very diverse population. Detecting relationships by sequence comparisons has proven to be difficult because of the speed of genetic exchange; the classification of viruses cannot be based on a conserved gene marker. Therefore, other classification methods are needed and indeed, establishing a single phylogeny for viruses seems impossible (Abrescia *et al.* 2012). However, there is a way to trace back the evolutionary origins of viruses. Structural virology has revealed conserved structures, viral "selves", shared by viruses infecting hosts from all domains of life that are without any notable sequence similarity (Benson *et al.* 1999, Bamford *et al.* 2002).

Bacteriophages are numerically the largest virus group known with over 5500 species examined (Ackermann 2007). The international committee for taxonomy of viruses (ICTV, http://www.ictvonline.org/) uses 70 different properties for classifying phages, generally based on the nature of the genome, host range and replication system. From all the phages studied, the tailed phages form the majority, constituting approximately 96 % (Ackermann 2007). For some bacterial phyla no phages have yet been identified (Ackermann 2003). On the other hand, the phages examined so far are mostly ones infecting bacterial isolates that are somehow involved in human diseases or food production and can be easily grown in the laboratory. This leads to a very narrow perspective to the phage diversity in the biosphere. Table 1 lists the phage families known so far with their typical characteristics.

TABLE 1 Overview of phage families described so far, adapted from Ackermann (2007). Not to scale.

Order/Family	Genome	Type member and Shape
Caudovirales	dsDNA	
Myoviridae		T4 
Siphoviridae		λ 
Podoviridae		T7 
Microviridae	ssDNA	φX174 
Corticoviridae	dsDNA	PM2 
Tectiviridae	dsDNA	PRD1 
Leviviridae	ssRNA	MS2 
Cystoviridae	dsRNA	φ6 
Inoviridae	ssDNA	fd
Plasmaviridae	dsDNA	MVL2 

ds= double-stranded, ss= single-stranded

1.1.2 Tailed bacteriophages are oddballs in the viral world

Tailed phages have been found to infect only archaea and bacteria. Their head and tail structure is very unique amongst known viruses, although tectiviruses and some algal viruses possess tail-like structures that are unstable and thus not comparable. The genome comprises of dsDNA and it is packaged in the icosahedral capsid called the head. The tail is a special organelle functioning in host recognition, cell wall penetration and genome injection into the host. The tailed phages are the only phages that have been classified into an order – the *Caudovirales*. *Caudovirales* is further divided to three families according to the phage tail structures. *Myoviridae* possess a long and straight contractile tail, *Siphoviridae* have a characteristic long and flexible, non-contractile tail and the *Podoviridae* have a short and stubby, non-contractile tail. Capsid size of different tailed phages varies greatly, with the diameter ranging from 400 to 1700 Å (Ackermann 2007). The majority of known tailed phages fall under Siphoviridae 61 %, the second biggest is Myoviridae 25 % and the third Podoviridae 14 % (Ackermann 2003, 2007). Tailed phages have most probably a common evolutionary origin despite clear differences in their genomes and virion sizes.

The virions are built from similar building blocks through analogous pathways and their structural proteins have similar folds (Ackermann 1999, Fokine and Rossmann 2014).

It has been suggested that the tailed phages originate from a time point in early evolution when bacteria separated from the branch that later became archaea and eukarya (Hendrix 1999). So far, the largest phage genomes are of the tailed phages with their genome size ranging between 19 kbp all the way up to the largest phage genome known (Hendrix 2008), which at the time of the writing of this thesis according to the list of 100 giant viruses (http://www.giantvirus.org/top.html, read 13.6.2014) is *Bacillus megaterium* phage G with a genome of 497 512 bp, coding over 650 ORFs holding the place of 10th largest viral genome (largest *Pandoravirus salinus* 2 473 870). The genomes of tailed phages typically consist 90-95 % of protein-coding sequence and the genes are arranged to co-transcriptional groups that seem to be very tightly packed. In the genome, genes are transcribed either all in one direction or in both directions. The average gene size is approximately 200 codons, two-thirds of the average host gene (Hatfull *et al.* 2006). However, some of the largest proteins known are encoded by phages, for example the 7312 amino acid putative tail fiber protein of a cyanophage (Sullivan *et al.* 2005).

1.1.3 Life cycles

The life cycle of a phage can be divided into different stages. The first step is the search for susceptible host cells in the extracellular environment that is followed by phage adsorption into the cell. During the initial contact between a phage and a bacterial cell, the phage has the possibility to deciding against infection, as it has not yet become bound. But after the second step of adsorption an irreversible reaction occurs as the phage binds to the bacterium (e.g. tail fibres bind with the receptor). Adsorption is a highly specific process between the host cell surface receptors and phage attachment structures and determines the phage host range (Weinbauer 2004). After adsorption, the phage penetrates the host cell wall using specific enzymes, located for example in the tail, and its genome is injected into the cell while capsid remains outside. The genome stays in the cytoplasm or it is integrated in the host genome. If the genome is not integrated it is replicated, the proteins are translated, and new particles are assembled resulting in the packaging of new genomes into the capsids. In the tailed phages, endolysins attack the murein part of the cell wall and holins damage the plasma wall, allowing endolysins to break down the peptidoglycan (Young, 2005). As the host cell lyses, new phage particles are released to the extracellular environment and the search for a new susceptible host cell starts again (Young 1992, Young *et al*. 2000). The period from the adsorption to the cell lysis is called the latent period. The part of the latent period before the genomes are packaged to the capsids is described as the eclipse period. After the eclipse period, host cells contain numerous new phage particles ready for release via lysis. The release of new virions to the extracellular environment is described as the rise period.

In traditional textbooks, one often sees a schematic representation of phage infection types divided into two cycles – the lytic and the temperate, following the example of the well-studied phage Lambda. But phages can exhibit three different lifestyles that can be called productive, lysogenic and pseudolysogenic. Abedon (2008) divides infection types even further under the three higher-level categories to six lower-level categories and they are covered here shortly. In the lytic infection (i) the phage genome is replicated and new virions assembled leading to the release of virions and to the destruction of the host cell. A chronic infection (ii) is also productive as it results in phage survival, genome replication and virion release but doesn't affect bacterial survival as the virions are released chronically via extrusion or budding from the cell envelope of the infected bacterium (Ackermann and DuBow 1987). Chronic infection has been observed only in filamentous phages (Russel and Model 2006). Lysogenic (iii) lifestyle results from a temperate phage infecting a bacterium. It requires the down-regulation of phage gene expression for the establishment of a long-term relationship with the host (Echols 1972, Zeng *et al.* 2010). A phage residing in the genome is called a prophage. The genome is replicated along with the host genome and is usually integrated or can exist as an extra chromosomal plasmid as in case of phage N15 (Ravin 2011). Implications of the importance of lysogeny in bacterial evolution can be found from the bacterial genomes, which commonly possess multiple prophages or parts of them (Casjens 2003). Prophage DNA is responsible for interstrain genetic variability in many bacteria and prophage genes are many times the source of pathogenic traits such as toxins (Brüssow *et al.* 2004, Pallen and Wren 2007). In addition to toxins, it is becoming increasingly evident that prophages can also enhance the ecological fitness of their host (Schuch and Fischetti 2009). The host cell survives until/unless an induction for phage lytic cycle occurs, in many cases activation of the SOS response to host DNA damage provides the nessecary trigger to relieve the prophage repression (Quinones *et al.* 2005).

The other infection type categories include pseudolysogeny (iv), phage abortive infection (v) and restricted infection (vi). The molecular basis of pseudolysogeny is not understood but genetic and molecular data was gained from an investigation on phage P22 and its host, where the emergence of pseudolysogeny was visually observed for the fist time (Cenens *et al.* 2013). Characteristics of the life style is regulated by conditions that restrict host growth (Ripp and Miller 1997, Wommack and Colwell 2000). The phage genome enters the cell but establishes neither stable long-term relationship nor starts the lytic cycle, but rather resides in the cell and is carried on to the other daughter cell in division. When nutrients are again available, either a true lysogeny or lytic cycle is established (Ripp and Miller 1998). Abortive infection and restriction are the development of antiphage defenses that lead to phage decay (in abortive infection also to bacterial decay, as the cell performs an altruistic suicide to protect the rest of the population).

1.1.4 The impact of phage-bacterium interactions - phages are dictators of bacterial biology

The diversity of bacteria and archaea is theoretically estimated to be millions of species (Curtis *et al.* 2002). This has been greatly influenced by viruses as they play an important role in ecosystems as maintainers of microbial diversity. Phages have many effects on their hosts, they predate and lyse the cells, transfer genes between hosts and establish lysogeny, modifying host functions i.e. lysogenic conversion (Wommack and Colwell 2000, Weinbauer 2004, Abedon 2008). Several studies, empirical and theoretical, show evidence that the interaction between bacteria and phages results in host diversification (Rodriguez-Valera *et al.* 2009, Marston *et al.* 2012, Williams 2013). Many times the interaction between a phage and a bacterium leads to dynamic antagonistic coevolution as shown by microcosm experiments (Buckling and Rainey 2002). These interactions are of great importance in many natural and applied systems and therefore understanding them is crucial.

The role of phage-host interaction is also crucial in the evolution of pathogenic bacteria (Brüssow *et al.* 2004, Pallen and Wren 2007). Perhaps the best known example is phage-encoded cholera toxin, which is responsible for the virulence of *Vibrio cholerae* (Waldor and Mekalanos 1996). However, even though microbiological research is often focused on pathogenic bacteria, it should be kept in mind that the vast majority of bacteria populating our planet are living freely in different ecosystems or live as symbionts on or in animals and plants. In aquatic environments the bacterial growth is often slow, bacteria exist in low densities and typically bloom sporadically when nutrients are available (Bratbak *et al.* 1990). Many studies have found a connection between lysogen frequency and limited source of carbon and energy (Weinbauer and Suttle 1996, Paul *et al.* 1997, Jiang and Paul 1998, Weinbauer and Suttle 1999, McDaniel *et al.* 2002) suggesting that lysogeny is an important survival strategy for the virion. The same observation has also been done in terrestrial surroundings (Ashelford *et al.* 1999a). Lysogenic conversion is beneficial to the host as the prophage provides superinfection immunity i.e. the bacterium is not infected with similar phages. In addition there are many reported advantages for the host, such as the acquisition of an ability to produce bacteriocins and antibiotics (Miller and Day 2008). But in low-nutrient environments the direct contribution of a prophage to the host survival by suppressing metabolic activities (Paul 2008) might be the most important feature for maintaining lysogeny. In many species of bacteria the role of phages in the intra- and interspecies competition has become evident, as the lysogens can use the prophage to outcompete phage-sensitive strains when invading new niches (Brown *et al.* 2006, Selva *et al.* 2009), although the weapon-utility of a temperate phage might be a short-term benefit (Gama *et al.* 2013)

Phages play also a key role in shaping the microbiota of the eukaryote hosts (Koskella 2013), and may even be considered as a part of non-host immunity (Barr *et al.* 2013). Our gut microbiota is a very densely populated ecosystem. It comprises of up to 10^{13} bacterial cells per gram and is often

considered as an independent organ. Not surprisingly, phages are the key players in shaping this ecosystem too, driving the composition of gut microbiota and contributing to the well-being of the human host (Dalmasso *et al.* 2014).

In the continuous arms race between bacteria and phage, bacteria need to evolve to avoid extinction and as a response, phage evolve to outcompete the bacterial defence mechanisms (Samson *et al.* 2013). One example of such is the masking of the phage receptors under exopolysaccharide (EPS) –layer by *Pseudomonas putida* which the phage AF can overcome by possessing depolymerizing activity (Cornelissen *et al.* 2012). The clustered regularly interspaced short palindromic repeats/Cas (CRISPR/Cas) –system is considered as a bacterial adaptive immunity system against viral infections (Deveau and Garneau 2010, Bondy-Denomy and Davidson 2014). Quorum sensing, the ability to regulate gene expression according to cell-cell signaling (Waters and Bassler 2005), has also been found to play a role in bacteriophage defense mechanisms. *E. coli* was found to reduce the expression of receptors in response to quorum sensing signal, the N-acyl-L-homoserine lactone (AHL), leading to 2-fold decrease in phage adsorption rate (Høyland-Kroghsbo *et al.* 2013). The short generation times and thus rapid evolution makes the interactions between bacteria and their phages a feasible method for studying host-parasite systems over ecological and evolutionary timescales (Bohannan and Lenski 2000, Buckling *et al.* 2009). Indeed, the dynamics as well as the consequences of coevolution have been extensively studied under laboratory conditions. However, little is known of the role the bacterium-phage coevolution plays outside the laboratory but it is predicted to have a global impact for instance in nutrient cycling and climate (Fuhrman 1999, Suttle 2007).

1.2 Post-antibiotic era is in true need of alternatives

Antibiotics are natural antimicrobials produced by many bacteria (e.g. *Streptomyces* and *Bacillus*) and fungi (e.g. *Cephalosporium* and *Penicillium*). Most of the antibiotics in use are chemically modified versions of the natural substances and currently approximately 200 different compounds are available for clinical applications (Walsh 2003, Overbye and Barrett 2005). These compounds are divided into groups that possess different modes of actions and some have activity against a wide spectrum of bacteria. However, bacteria can develop resistance to antibiotics relatively quickly. The most common way for bacteria to acquire resistance is horizontally via gene transfer, by acquiring DNA directly from environment (transformation), with phage infection (transduction) or actively in direct cell-cell contact (conjugation) reviewed e.g. in Ochman *et al.* (2000).

The World Health Organization (WHO) 2014 report on antimicrobial resistance states that we have now entered a post-antibiotic era (Anon. 2014). The widespread use of antibiotics has led to the development of resistance in

fundamental human pathogens such as *Staphylococcus aureus*, *Enterobacteriaceae* (*Klebsiella pneumonia*, *E. coli)* and *P. aeruginosa*. These are causing increasing problems throughout the world due to their MDR properties (Anon. 2014). New antibiotics are sought but new launches are not frequent, only two new antibiotics (in human medicine) came on the market in the US during 2008-2012 (Spellberg 2012) most probably due to the cost of development compared to the income for pharmaceutical companies. Antibiotics used in human medicine are more or less the same as used in agriculture, which in fact, is the largest user of antibiotics and contributes greatly to the spread of resistance worldwide. The realization of the dangers related to the use of antibiotics has increasingly lowered the attractiveness of food products that have had antibiotic exposure, building pressure for the producers to find alternatives.

Bush and colleagues (2011) suggest ways to tackle antibiotic resistance i.e. using old discarded antibiotics, controlled usage of antibiotics, new antibiotic discovery and alternative therapeutics (e.g. phage therapy), and generally raising public awareness of antibiotic usage and maintaining public health and sanitation in parts of the world where it is needed. A recent study described the gate, which gram-negative bacteria use to transport lipopolysaccharides (LPS) to the outer surface of the cell and suggests that compounds could be developed to lock this gate, effectively killing the bacteria (Dong *et al.* 2014). These kinds of developments are highly important in the race against bacterial pathogens as well as new treatment methods. This thesis focuses on phages as an alternative.

1.2.1 Phage therapy – revival of an old idea

The concept of phage therapy is relatively simple – using bacteriophages, the natural parasites of bacteria, to specifically destroy pathogenic bacteria. The most beneficial side of using phages is that they exclusively infect and replicate in bacteria and are unable to infect eukaryotic cells. Felix d'Herelle can be considered to be the father of phage therapy. From his observations he concluded that phage multiplication and lysis of the pathogen were involved in the recovery from the diseases. He therefore named phages as "exogenous agents of immunity" (Summers 2005). This idea led him to employ phages in many different diseases, as therapeutics as well as prophylactics. Even though d'Herelle was the inventor of phage therapy, Bruynnoghe and Masin at Louvain performed the first reported trial (Summers 2005). They tested phages locally against cutaneous boils by injecting staphylococcal phage preparation and received reduction in swelling and pain. Overall, d'Herelle's and others experiments on phage therapy were speaking on the behalf of the potential of phages as therapeutics and many companies, such as L'Oréal in France, marketed commercial medicines including phages (Sulakvelidze *et al.* 2001). Soon, however, the invention of "user-friendly" antibiotics (after the discovery of penicillin by Alexander Fleming in 1928), and the World War II ended the development of phage therapy in the West. But trials continued in the Soviet Union and other Eastern European countries. The Eliava institute, founded by

Georgyi Eliava and d'Herelle in Tbilisi Georgia, was, and still is, one of the main centres of phage therapy (Sulakvelidze *et al.* 2001).

There is an increasing amount of data available on phage efficacy in clinical infections, including MDR bacteria (Golkar *et al.* 2013) but the expantion of phage therapy in real life systems is often limited by the lack of funding for large control studies. Evidence suggests that phage therapy is safe in treating clinically important pathogens (Bruttin and Brüssow 2005, Wright *et al.* 2009, Sarker *et al.* 2012, Brüssow 2012, Rhoads *et al.* 2013) and thus phage therapy is considered to be an attractive alternative for antibiotics. In addition to human associated pathogens, several efforts have been made to study the efficiency of phages in animals and plants that are used for food production (Callaway *et al.* 2008, Frampton and Pitman 2012, Jones *et al.* 2012,).

Using phages as antimicrobials has several advantages but also some limitations. Superior features when compared to antibiotics include specificity and the self-replicating feature. Specificity is a benefit when the infecting agent is well known and solely responsible of the infection but when there is not a precise knowledge of the cause of the disease a wider spectrum antibiotic can be a better solution. Phages affect only minimally normal flora whereas antibiotics tend to cause long lasting effects. Phages are also self-replicating and thus are able to increase in number specifically at the location of the host (Abedon and Thomas-Abedon 2010). Because of the specificity of phages the targeted bacteria will be the only ones that require resistance and this resistance is not spread. The disadvantages include the phage choice and host range. Actually, only a small proportion of phages possess therapeutic properties. The characteristics of the phage need to be thoroughly determined, most importantly the obligatorily lytic lifestyle of the phage must be ensured (Gill and Hyman 2010). A very narrow phage host range can be a disadvantage, if not all target strains are infected. The possibility of phage interacting with the host immune system and the capability to evolve during manufacture and use should also be examined. No data exists on a phage being able to infect a eukaryote, although it cannot be said for sure, this scenario can be considered unlikely (Merril 2008). Overall, the advantages of phage therapy can be considered to outnumber the disadvantages and phages be considered safe if precautions are taken care of.

1.3 Columnaris disease is one of the many diseases that plague aquaculture

Aquaculture, including all forms of aquatic animals, is the fastest growing food-production area (Bostock *et al.* 2010). Disease outbreaks are limiting the development and efficiency of aquaculture, and yearly economical losses due to pathogens have been estimated to be several billions of US dollars (Subasinghe *et al.* 2001). The aquatic environment in itself offers an easy transmission route for pathogens and when intensive farming with high host densities and

increased transmission opportunities are considered, aquaculture facilities are a pathogen paradise (Mennerat *et al.* 2010). Antibiotics have been widely and routinely used in aquaculture all over world as a treatment but in some parts also as a prophylaxis, even when there are no signs of disease (Cabello 2006). This has contributed to antibiotic leakage into the environment and to increased resistance not only in the pathogenic bacteria but also in the environmental bacteria in the aquaculture surroundings (Cabello *et al.* 2013).

Pathogenic flavobacteria are a major burden for the fish farming industry. In Finland, fish farms struggle with flavobacterial infections throughout the year: *F. psychrophilum* during the cold-water period (Nilsen *et al.* 2014) and *F. columnare* during the warm water period (Pulkkinen *et al.* 2010). *F. columnare* is an opportunistic fish pathogen that causes a columnaris disease in a wide variety of fish species including salmonids, channel catfish, common carp and ornamental fish (Snieszko 1974, Bernardet 1989, Decostere *et al.* 1998, Wagner *et al.* 2002). The cells of *F. columnare* are long and slender, gram-negative rods (Figure 1). Strains isolated from different parts of the world have been classified into three genomovars (I-III) based on restriction fragment length polymorphism (RFLP) of 16S rDNA (Triyanto and Wakabayashi 1999). The same typing method has been used later by others (Michel *et al.* 2002, Arias *et al.* 2004, Darwish and Ismaiel 2005). All the Finnish isolates have been designated as genomovar I. When ribosomal intergenic spacer analysis (RISA) was used, the Finnish isolates were further divided to eight genetically different groups A-H (Suomalainen *et al.* 2006a).

Typical symptoms of columnaris disease are gill nercosis, skin lesions and fin and jaw erosion, resulting in mortalities among young fish (Declercq *et al.* 2013). Mortalities caused by *F. columnare* cause substantial economic losses to the fish farming industry throughout the world (Wagner *et al.* 2002). Antibiotics and chemical bathing have been used for controlling disease outbreaks. Recently, biofilm properties (Cai *et al.* 2013) and the ability to survive in the environment (Arias *et al.* 2012, Sundberg *et al.* 2014) have been studied to elucidate how *F. columnare* persists and transmits in the fish farming conditions.

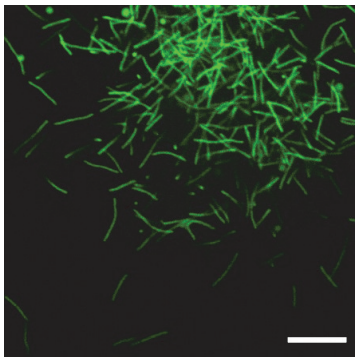


FIGURE 1 *F. columnare* cells with fluorescent expression. The long and slender shape of the cells is visible. Scale bar 10 µm.

Despite the severity and global distribution of *F. columnare*, its virulence factors are largely unknown. It is known that different strains vary in their virulence (Decostere *et al.* 1998, Thomas Jinu and Goodwin 2004, Suomalainen *et al.* 2006b). Associations of adhesion on gill tissue, chemotaxis to fish mucus and activity of the connective tissue degrading enzyme, chondroitin AC lyase, to its virulence have been made (Decostere 1999, Suomalainen *et al.* 2006b, Klesius and Shoemaker 2008). However, Olivares-Fuster *et al*. (2011) did not find adhesion itself to be a virulence determinant but rather additional factors were responsible for the disease. One tool to reveal virulence factors in *F. columnare* is the property of the bacterium to exhibit three different colony morphologies in the laboratory that differ in their virulence (Kunttu *et al.* 2009a, 2011). Only the Rhizoid morphotype is able to cause disease. Comparative studies on the virulent and non-virulent morphotypes are therefore important.

Genetic methods for flavobacteria are continuously being developed and in addition, the first genome sequence of *F. columnare* was published in 2012 (Tekedar *et al.* 2012). The research done on *F. columnare* by research groups in Asia, Europe and North- and South-America has multiplied during past years, indicating that virulence mechanisms could be elucidated in the near future.

The increasing trend in disease severity and the occurrence of virulent *F. columnare* strains (Pulkkinen *et al.* 2010), and the diminished effect of antibiotics have caused a demand for efficient alternatives. The interest in phage therapy against fish disease is rising, and successful reports have been published also in this field of animal production (Nakai and Park 2002, Khairnar *et al.* 2013), including phages against *F. columnare* (Prasad *et al.* 2011). Successful phage therapy against flavobacterial disease has been reported previously with phages of *F. psychrophilum* (Stenholm *et al.* 2008, Castillo *et al.* 2012). Therefore, it seems that phages are a promising tool for preventing major losses in aquaculture. Phages of *F. columnare* have been studied to some extent previously when the species was designated as *Chondrococcus columnaris* (Anacker and Ordal 1955, Kingsbury and Ordal 1966) and recently from the India (Prasad and Kumar 2010). The potential of phage therapy as a control and disease management method in the case of columnaris disease seems feasible, as the disease is mainly external.

2 AIMS OF THE STUDY

The current rise of antibiotic resistance among many important bacterial pathogens has led to investigations for alternative treatment methods during the past decades. Columnaris disease has shown increasing severity on a yearly bases, leading to increased antibiotic usage, but at the same time the efficiency of treatment has decreased. This thesis aimed to investigate the possibility of using phages to reduce the antibiotic load to the environment and to avoid the possibility of forming antibiotic resistant strains and also to efficiently fight against a lethal pathogen. Specific questions and aims set for the study were:

I. For developing phage therapy, phages are a necessity. The aim was to find whether *F. columnare* phages could be isolated directly from water samples and when and where these samples should be collected. The aim was also to collect a wide range of phages infecting Flavobacteria to study the host range of *F. columnare* phages in parallel to other *Flavobacterium* species and their phages and receive information on the naturally occurring phages of Flavobacteria in freshwaters.
II. One of the expected outcomes of phage therapy is the rise of phage resistant variants. Therefore it is of great importance to understand the characteristics of phage resistant population before applying phages. What are the causes of phage resistance in *F. columnare*?
III. Understanding the pathogen is crucial in developing treatment methods. The capability of forming various phenotypes of *F. columnare* under laboratory conditions exhibiting different virulence was considered as a tool. The aim was to examine factors contributing to the virulence of *F. columnare*.
IV. To examine if it is possible to control columnaris disease with phages and whether zebra fish (*Danio rerio*) could be used as a model species for screening efficient phages.

3 SUMMARY OF THE MATERIALS AND METHODS

The materials and methods not described in the original publication I are described here in detail. Other methods are listed in Table 2 with a reference to the Roman numerals of the original publication where the method has been described. All the bacterial strains and phages used were isolated and/or described during the study and they have been described in the original publications I and II, except the previously described *F. columnare*-strains A-H (Suomalainen *et al.* 2006a).

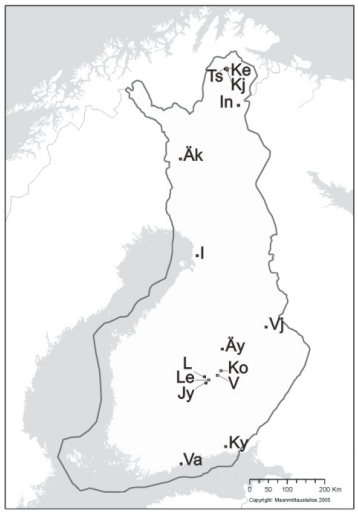
TABLE 2 Methods used in the original publications included in the thesis.

Method	Publication
Phage resistance	II
Gliding motility	II
Stability of colony morphotype	II
Virulence experiments	II, III
Electron microscopy of bacteria	III
Ectracellular and outer membrane protein extraction	III
Outer membrane vesicle isolation and purification	III
Phage therapy experiments	IV
Phage stability	IV
Phage whole genome sequencing	IV

3.1 Isolation of bacteria and phages

Water samples were collected from several locations in Finland (Figure 2) from freshwaters and fish farms during the warm water period (May to August) in the years 2008-2009 to isolate flavobacteria and their phages. One *F. columnare*

strain (B067), however was isolated from diseased fish during 2007 together with its phage FCL-1.



Location	Abbrevation	Longitude	Latitude
Lake Konnevesi	Ko	E 26° 35.644'	N 62° 31.446'
Lake Jyväsjärvi	Jy	E 25° 44.339'	N 62° 13.779'
River Vantaanjoki	Va	E 24° 58.072'	N 60° 15.948'
River Kevojoki	Ke	E 26° 59.429'	N 69° 45.337'
River Tsarsjoki	Ts	E 26° 59.447'	N 69° 45.430'
Lake Kevojärvi	Kj	E 26° 59.608'	N 69° 45.430'
Lake Valtimojärvi	Vj	E 28° 51.000'	N 63° 40.688'
River Kymijoki	Ky	E 26° 49.926'	N 60° 41.391'
Lake Leppävesi	Le	E 25° 54.633'	N 62° 16.064'
Lake Äkässaivo	Äk	E 24° 8.640'	N 67° 41.840'
River Äyskoski	Äy	E 26° 40.756'	N 63° 0.665'
Lake Inari	In	E 27° 53.097'	N 68° 47.783'
Fishery L [1)]	L		
Fishery V [1)]	V		
Fishery I [2)]	I		

[1)] Central Finland
[2)] Northern Finland

FIGURE 2 Map of the sampling sites in Finland.

An aliquot of water sample (100 µl) was spread on solid Shieh agar (Shieh 1980) or on diluted (1/5) Luria Bertani (Sambrook and David 1989) agar. Colonies, displaying orange to yellow pigment, and a spreading, flavobacterium-like growth, were pure-cultured on Shieh agar and in Shieh broth. Phages were grown with the enrichment method using isolated bacteria as hosts. Five-fold culture medium was diluted in a filtered (pore size 0.45 µm, Nalgene) freshwater sample. Cultures were grown at room temperature (RT), at 110 rpm on a benchtop shaker (New Brunswick Scientific) until they turned turbid. 300 µl of turbid sample was applied onto solid agar using the double agar overlay method (3 ml of 0.7 % top agar). After incubation at RT for 24 to 48 hours (depending on the bacterial growth) the plaques were picked. Three rounds of plaque purification were performed for each phage isolate. Two different methods were used to prepare phage stocks from incubated agarplates: (i) Top agar in which there was semi-confluent lysis was removed to an Erlenmeyer flask, 4 ml of pure culture media was added and the flasks were shaken for four hours. (ii) Alternatively 5 ml of culture media was added to a plate on which there was confluent lysis and shaken at 8 °C, 95 rpm on a benchtop shaker for 24 hours. In both methods the lysate was centrifuged (Sorvall RC-5, SS-34 rotor, 10 800 × *g*, 15 min, +4 °C) to remove the cell debris. The supernatant containing the phages was stored at 4 °C. Selected phages were grown by infecting the host bacterium (multiplicity of infection 5-10) at proper cell density (usually approximately 1×10^9 CFU/ml) and following the optic density of the bacterium. After the lysis of the cells, bacterial debris was removed by centrifugation (Sorvall RC-5, SS-34 rotor, 10 800 × *g*, 20 min, +4 °C). Phage particles were

precipitated with polyethylene glycol (PEG) and NaCl, and purified by rate zonal centrifugation either in a linear 5 to 30 % glycerol gradient (Beckman coulter L-90K, SW 41 rotor, 71 000 × *g*, 45 min, +4 °C), or in a CsCl (ρ=1,495, in 150 mM NaCl, 100 mM MgCl2, 50 mM Tris-HCl, pH 7.0) density gradient (Beckman coulter L-90K centrifuge, SW 41 rotor, 49 000 × *g*, 20 h, +18 °C). Visible bands (one or two) were collected and plaque-forming units/ml (PFU/ml) determined. Particles were collected by centrifugation (Beckmann coulter L-90K, 70 Ti rotor, 75 000 × *g*, 2 h, +4 °C) and resuspended in 20 mM Tris-HCl, pH 7.2 and PFU/ml was determined.

3.2 Genetic typing of the bacterial isolates

Bacterial DNA was exctracted from overnight liquid cultures with Genomic Wizard DNA isolation kit (Promega). *Flavobacterium columnare* –strains were studied in more detail using ribosomal intergenic spacer analysis (RISA) (Suomalainen *et al.* 2006a). In RISA, the intergenic spacer sequence between the ribosomal 16S and 23S rDNA was amplified with primers bRISA forward and bRISA reverse (Hartmann *et al.* 2005). The reaction mixture contained 0.3 µM of each primer, 0.2 mM dNTP mix, 1xDreamTaq PCR buffer (Fermentas) and 2U Dream Taq polymerase. The PCR-product was digested with HaeIII and HinfI and and run on a 12% polyacrylamid gel. The restriction patterns were compared to the ones described earlier (Suomalainen *et al.* 2006a).

For the other *Flavobacterium*-isolates a 900 bp -long fragment of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using DreamTaq™ Green DNA Polymerase (Fermentas) and primers Com2ph (Schwieger and Tebbe 1998) and fd1 (Weisburg *et al.* 1991). PCR-products were purified (Qiagen, PCR Purification Kit) and sequenced with BigDye® Terminator v3.1. Cycle Sequencing kit using 3130xl Genetic Analyzer (both from Applied Biosystems). Sequences were compared to the public database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3 Phage infection tests

In the initial screening for susceptibility of the bacteria to phages, each bacterium was infected with each phage isolate using the double layer method. Ten microliters of phage lysate was spotted on the surface of top agar pre-casted with the bacterium. If clear zones were detected after one or two days of incubation at RT the same host bacterium was infected again with serial tenfold dilutions of plaque suspension using the double layer method (300 µl of bacterium and 100 µl of phage suspension to the top agar) to measure the level of infection and to visualize single plaques. Titer (PFU/ml) of each phage on each bacterium was determined.

3.4 Analysis of phage genomes

A zinc chloride (40 mM final, Sigma) precipitation (Santos 1991) was used to concentrate phage for DNA extraction. Before the precipitation the phage lysate (approximately 10^8-10^{10} PFU/ml) was treated with RNase (1 µl/ml, Sigma) and DNase (10 µl/ml, Sigma) for 30 min at 37 °C. Precipitated phage were centrifuged (Thermo IEC MicroCL17 Centrifuge, 9 600 × g, 1 min). The pellet was resuspended in TES-buffer (0.1 M Tris-HCl pH 8; 0.1 M EDTA; 0.3 % SDS) and treated with Proteinase K (0.8 mg/ml). The DNA was purified using columns and protocol from Fermentas GeneJetTM Genomic DNA Purification Kit. Phage DNA was digested with EcoRI, HindIII, BamHI and PstI and 100-300 ng/µl was run on a 0.5 % agarose gel for 7 hours and genome sizes were calculated or estimated by comparing the genome band to the DNA size marker GeneRuler™ 1 kbp Plus DNA Ladder and GeneRuler™ High Range DNA Ladder (Fermentas). For four of the phages (FKj-2, FL-1, FCL-2 and FCV-1), which were purified by gradient centrifugation, particles were disrupted with SDS (2 %) and protease K (0.6 mg/ml) followed by two phenol and three ether extractions. The genomic DNA was precipitated with sodium acetate (0.3 M) and ethanol (70 %) (Bamford and Bamford 1991). Genomes were digested either with BamHI, EcoRI or HindIII restriction enzymes and the resulting fragments were cloned in pSU18 vector (Bartolomé *et al.* 1991). DNA fragments were sequenced using universal and reverse sequencing primers hybridizing to the vector sequence. Primer walking was performed in both directions with custom made 20- to 22-nucleotide long primers. Sequences were analysed using Vector NTI 11.0.0 (Invitrogen) and compared to database sequences in May 2011 using DNA and protein blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.5 Transmission electron microscopy (TEM) of phages

Transmission electron microscopy samples of the phage isolates were prepared from concentrated phage lysates or from purified phage particles. Ten microliters of concentrated phage suspension was spotted on carbon-stabilized formvar coated grids for 2 minutes. Excess suspension was drained with filter paper. Ten microliters of 1% phosphotungstate, pH 6.5 was applied on the grid for 1 minute for staining and the grid was dried with filter paper. Imaging was performed with Jeol JEM-1200EX operating at 60 kV and later with Jeol JEM-1400 at 80 kV.

4 RESULTS AND DISCUSSION

4.1 Description of *Flavobacterium* isolates and phages from Finnish freshwaters (I)

Major efforts have been made since the discovery of viral abundance of the aquatic phages in the 1990's (Bergh *et al.* 1989) to determine their diversity and role in the ecosystem (Weinbauer 2004, Breitbart 2011). The research on freshwater phages is following in footsteps of marine phages (Wilhelm and Matteson 2008) but data, for example on the diversity of phages inhabiting freshwaters, is still limited. Despite the ubiquitous appearance of the host bacteria (Eiler and Bertilsson 2004, 2007), phages infecting members of the genus *Flavobacterium* have not been previously described in freshwater environments. Phages infecting *F. psychrophilum* have been previously studied, but the isolation locations were strictly restricted to fish farms in Denmark (Stenholm *et al.* 2008). Our survey of the fresh waters and three fish farms in Finland resulted in total of 53 flavobacterial isolates and 49 phage isolates. The freshwater locations were not connected to fish farming. The three fish farms sampled were inland farms rearing mainly salmonid fingerlings. Yellow-pigmented bacteria with flavobacteria-like spreading growth characteristics were chosen for further analysis and were confirmed to belong to the genus *Flavobacterium* by sequencing of the 16S rRNA gene. From the isolates, 21 were identified as *F. columnare,* which were further typed to genetic groups according to RISA, and the remaining 32 isolates were grouped to the genus *Flavobacterium* without further species classification. *F. columnare* strains were isolated only from the fish farms. According to this data, occurrence of *F. columnare* seems to be connected to the fish farming environment; it was not isolated from natural waters However, the sample collecting in this study was not done systematically, and there is evidence that *F. columnare* is present also outside fish farms (Rickard *et al.* 2003, Revetta *et al.* 2005, Kunttu *et al.* 2012).

Phages were enriched using flavobacterial isolates from freshwaters, fish farms and previously described *F. columnare* strains. In the case of the

previously described *F. columnare* strains, phages were isolated only against bacteria of the genetic groups C and G. The phages were named according to their host species and their isolation site. First letters in the name indicate the host, F for *Flavobacterium* and FC for *F. columnare*, following letters refering to the sampling site (e.g. FCL-1 is a *F. columnare* phage from fish farm L).

Many of the phages isolated in this study produced low titer lysates, and were not subjected to further characterization but all of the phages that were characterized in more detail under TEM were found to be tailed phages belonging to the families of *Myoviridae*, *Podoviridae* or *Siphoviridae*. Most of the studied phages fell to the range between 50 and 70 nm in their head size but five of the isolates belonging to *Myoviridae* (FJy-3, FKo-2, FKj-2, FKy-1 and FKy-3) had capsid sizes around and larger than 100 nm. Indeed, amongst isolates there were phages (e.g. FJy-3) that had very small plaque morphology (unpublished data), a feature that is associated with large genomes and correspondingly to large heads (Serwer *et al.* 2007). The smallest isolate that was characterized, FV-3, had a head diameter of approximately 40 nm. Two of the further analyzed *F. columnare* phages, FCV-1 and FCL-2, belonged to the *Myoviridae*-family with head diameters of approximately 50 nm. The third analyzed *F. columnare* phage FCL-1 was sensitive to the purification treatments and no full particles were observed under the EM, but detached contractile tails were seen designating the phage to *Myoviridae*.

Data of the diversity of the *Flavobacterium* phages is somewhat biased because of the isolation technique used. Enrichment of phages favours the phages that are able to reproduce most efficiently under specific conditions (Dunbar *et al.* 1997). But as it has been previously stated, the vast majority of phages studied according their morphology are tailed phages (Ackermann 2007) and our data supports this view. Although, it must be kept in mind that tailed phages are most certainly overrepresented because they make more visible plaques and therefore cause bias to estimations of phage diversity. In freshwater environments only a few tailless isolates have yet been observed, such as a membrane-containing cystovirus phiN (Mäntynen S., Laanto E., Kohvakka A., Poranen M., Bamford J.K. & Ravantti J. submitted). But there is also evidence of ssDNA-phages in freshwaters, as a novel type of ssDNA-phage infecting *Flavobacterium* sp. was recently isolated from lake water (Laanto E., Mäntynen S., Marjakangas J., Bamford J.K., Ravantti J. & Sundberg L.-R. unpublished). The application of new molecular approaches to the study of freshwaters will certainly reveal much more of the diversity of phages also in these environments in future. As flavobacteria are major players in the biochemical cycles and contribute to water quality in fresh waters (Eiler and Bertilsson 2007), it is important to study the bacteriophage community relevant to these bacteria as the evidence of phages key role in the oceans nutrition cycles is indisputable (Suttle 2007, Jover *et al.* 2014).

4.1.1 Phage genomes (I, IV)

The phage genomes were initially digested with restriction enzymes BamHI, EcoRI, HindIII and PstI. Many of the genomes were left uncut and their size was roughly estimated against DNA size markers. For the genomes that were cut the size was calculated from the resulting bands in the restriction profile. From four phages, FKj-2, FL-1, FCL-2 and FCV-1, digested genomes were cloned to pSU18-cloning vector and inserts form resulting clones were partly sequenced. Sequencing resulted to approximately 3000 bp from three genomes and from one phage genome, FCV-1, to 1600 bp. No significant DNA sequence similarities were found in the database in May 2011. This is expected because of the enormous genetic diversity of phages and the lack of conserved genes; generally there is no sequence similarity between phages that infect different hosts (Hatfull 2008). When the predicted ORFs were compared to the database using BLAST, best matches were: for FCL-2 to a hypothetical protein of the Vibrio phage VP16T (score 57.8) and to a hypothetical protein of the Vibrio phage VP16C (score 55.1). Comparison of the FCV-1 resulted to a hypothetical protein B40-8030 of a Bacteroides phage B40-8. According to the list of 1613 complete phage genomes in the database of the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI) in 7.6.2014 (http://www.ebi.ac.uk/genomes/phage.html) there are two complete genome sequences of phages infecting *Flavobacterium*. A prophage 6H, that has been detected from *F. psychrophilum* isolates globally (Castillo *et al.* 2013) and phage 11b which infects a psychrophilic *Flavobacterium* sp from Arctic ice (Borriss *et al.* 2007). This makes the whole genome sequence of FCL-2 to be the first complete genome of a phage infecting *F. columnare* (see below).

The genome of the phage FCL-2 was further characterized in more detail to evaluate its potential in phage therapy against *F. columnare*. Sequencing of the whole genome resulted in a 47 129 bp long genome with a G + C content of 30.2 %. There were 71 predicted ORFs of which 46 were predicted as hypothetical proteins leaving 25 ORFs (35.2 %) without any result (Figure 3). Predicted function was assigned to 15 of the ORFs, including proteins involved in packaging (terminase and phage portal protein) and structural proteins (e.g. tail and tail tape measure proteins). The structural proteins were found to match the *Cellulophaga* phage phiSM. Also matches to seven other *Cellulophaga* phages were received. Although the matching *Cellulophaga* phages were isolated from marine environments (Danish costal waters) (Holmfeldt *et al.* 2007) the genus *Cellulophaga*, a member of the Bacteroidetes group, is a close relative to genus *Flavobacterium*. In the genome of phage FCL-2 only a few ORFs were related to those in *Flavobacterium* phages but this is most probably due to the low number of these phage genomes in the database. There was one match to the *Flavobacterium* phage 11b and two hits to the genome of prophage 6H. The two closely related proteins in 6H, IbrA and IbrB, are also found in *F. psychrophilum* (the host of 6H) and in *F. columnare*. In the genome analysis of 6H (Castillo *et al.* 2013), a hypothesis was made that these two proteins could be possible virulence factors because they were related to the IbrAB complex in *E.*

coli. This complex is possibly involved in host-pathogen interactions as they show resistance to serum and Ig-binding (Sandt and Hill 2000). If these two proteins play a role in the virulence of *F. columnare* is an interesting question to be investigated in the future. Interestingly, the two ORFs before IbrA and IbrB are located in the same order in the sequenced genome of *F. columnare* ATTC 49512, implying a common origin for these regions. Whether this applies also to the host of FCL-2, *F. columnare* strain B185, will be evaluated when its genome is available.

The FCL-2 genome was also run in the online version of PHACTS (www.phantome.org) that predicts the lifestyle from the phage proteome against phages of known lifestyles (McNair *et al.* 2012). The result obtained from PHACTS was slightly (0.518 %) in favor of a temperate lifestyle. Although no clear signs of temperate lifestyle such as integrases or recombinases were found in the FCL-2 genome, the finding of the IbrA and IbrB and their possible role in bacterial adaptive functions might prevent the usage of this phage as pharmaceutic (Gill and Hyman 2010). Nevertheless, these traits do not prevent the use of FCL-2 in the phage therapy model system under laboratory conditions.

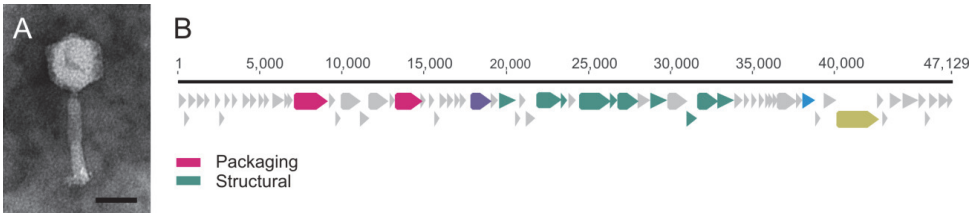


FIGURE 3 In panel A the phage FCL-2 viewed under TEM (scale bar 40 nm) and in panel B the graphic presentation of the genome of FCL-2 with colours indicating the putative functions for ORFs as shown in the figure. In addition, light purple indicates putative ORF for protease, blue indicates putative ORF for hydrolase and yellow the putative ORF for DNA-methylase.

4.2 Host range studies suggest that *F. columnare* phages are host specialists (I)

When isolated phages infecting *Flavobacterium* sp. and *F. columnare* were tested against 31 *Flavobacterium* sp strains and new and previously described *F. columnare* strains belonging to genetic groups A-H (Suomalainen *et al.* 2006a), versatile infection profiles were observed. Few of the single host enrichment phages were virulent to nearly all bacterial strains tested, whereas some phages (especially *F. columnare* phages) were strictly host-specific according to the collection of *Flavobacterium* strains tested. The finding of diverse host ranges is in accordance to previous findings that phages of marine flavobacteria may have a high variation in host range (Holmfeldt *et al.* 2007). Generally, phages were able to infect a range of isolated *Flavobacterium*-species, although some

studies suggest that single host enrichments might select for narrow host range phages (Jensen *et al.* 1998, Wichels *et al.* 2002). The broad host range phages infected bacteria were isolated from both freshwater and fish farm samples. Two phages with the broadest host ranges (infecting 24 bacteria) were isolated from freshwater samples; river Tsarsjoki (FTs-1) in Northern Finland and lake Jyväsjärvi (FJy-3) in Central Finland. Sensitivity of bacteria to these phages differed; FTs-1 had relatively high titers on nearly every bacterium it infected whereas FJy-3 had relatively low titers. Five phages (FTs-1, FV-10, FV-8, FJy-2 and FKj-1) produced higher titers on other bacteria than the one that was used for the isolation. According to the studies on marine tailed phages, members of the *Myoviridae* and *Siphoviridae* often have the broadest host ranges while the *Podoviridae* phages show a narrow host range (Suttle 2005). Our study is in agreement with this view. The only confirmed *Podoviridae* phage FV-4 had a narrow host range infecting only two of the bacterial isolates. However, phage FJy-1 (*Myoviridae*) was also host-specific infecting only one bacterial species. It should however be noted that host ranges of *Flavobacterium* sp. phages were not related to geographical location; the phages had equally versatile infection patterns independent of where they were isolated.

Interestingly, of the characterized phages four of the ones with large genomes were among the most broad host range phages. Phages with large genomes (>100 kbp) infecting for example *Bacillus cereus* and *E. coli* have been detected also to possess broad host ranges (Schwarzer *et al.* 2012, El-Arabi *et al.* 2013). The large genome offers flexibility in coding for functions to replicate in different hosts. Also, in bacteria the genome size has been shown to correlate with lifestyle and host adaptations (Toft and Andersson 2010). It is possible that reduction in genome size leads to specialization also in viruses by deletion of genes related to host range (Drillien *et al.* 1981).

No infection was observed in *F. columnare* strains by *Flavobacterium* sp. phages and vice versa. *F. columnare* strains isolated from one location were only susceptible to the phage isolated from the same location. *F. columnare* phages didn't infect any of the previously studied *F. columnare* strains in cross infection tests unless the bacterium belonged to the same genotype (analysed by RISA) as the isolation host. Therefore *F. columnare* phages were concluded to show a narrow host range, limited to specific host genotype. What is the benefit of such limited host range remains unclear. Indeed, the understanding of the complex phage-bacterium networks in nature is still narrow (Koskella and Meaden 2013). Columnaris disease occurs only during the summer when the water temperature is high enough (approximately above 18 ^{o}C). During the rest of the year host density is probably too low for the phage to initiate replication (Wiggins and Alexander 1985). This could promote for higher phage virulence during the host bloom in order to replicate efficiently and ensure over-wintering survival of at least some of the virions and also limit the host range. When considering the use of *F. columnare* phages in phage therapy, such specificity is an advantage, as other bacterial species are left undisturbed. On the other hand, different genotypes of *F. columnare* co-occur during columnaris outbreaks (Suomalainen *et al.* 2006a, Kunttu *et al.* 2012). Consequently, this

means that treatment should include several phages, i.e. phage cocktails, which would infect several genotypes of *F. columnare* -strains. Phage cocktails are also needed to avoid possible resistance problems. However, it seems that at fish farms, some phage types can be recurrently isolated. Phages FCL-2 and FCL-3 were isolated from the same fish farm during different years and surprisingly, they were discovered to have the same infection profiles. In addition, I have since isolated phages with similar restriction profiles and host ranges from another fish farm during three different years (unpublished data). These findings together suggest that the same phage type is persisting at the fish farm, which could indicate that also its bacterial hosts may reside there. This is an interesting finding as it can provide information on the co-evolutionary dynamics of phage-host interactions, which is very important considering the possible application of phage therapy in these environments.

When *Flavobacterium* sp. phages were tested against different *F. columnare* strains no individual plaques were observed but clear spots were detected. Phage lysates of 11 *Flavobacterium* sp. phages (FTs-1, FKo-2, FL-1, FV-1, FKy-1, FKy-2, FKy-3, FV-3, FV-4, FV-5, FV-6 and FV-8) inhibited the growth or lysed the bacterial culture on the double layer plate assay on all *F. columnare* strains tested. This inhibition occurred most strongly in undiluted phage lysate but in some cases also in the 1/10 and/or 1/100 dilutions (unpublished data). There are possible explanations for this phenomenon. Either the phages were able to bind to the bacteria and cause death but were not able to produce progeny, or the clear spots in the underlying bacterial lawn could be an indication of bacteriocin activity. Bacteriocins are antimicrobial peptides produced by bacteria that are active against other bacteria of the same strain or closely related species (Riley and Wertz 2003). *F. columnare*, as well as other Flavobacteria, have been reported to produce bacteriocins (Anacker and Ordal 1959, Dalsgaard 1993), but these antimicrobial substances have not been characterized. Phage lytic enzymes (lysins) present in the phage lysate are also a possible cause for this phenomenon (Fischetti 2008).

4.3 Cost of phage resistance in *F. columnare* (II)

Bacteria and their parasitoid phage are in a continuous arms race – bacteria have several mechanisms to resist phages as well as phages are constantly evolving to overcome these defenses, reviewed e.g. in Labrie *et al.* (2010) and Koskella and Brockhurst (2014). In *F. columnare,* the development of phage resistance was detected visually as phage resistant colonies differed clearly in their morphotype from the ancestral ones (Figures 4 A and C). Phenotypic change in phage-resistant variants is a known phenomena also in another opportunistic pathogen, *P. aeruginosa* (Hosseinidoust *et al.* 2013a). It has been previously shown that *F. columnare* can exhibit different colony morphotypes that are induced in the laboratory during cultivation and starvation from the Rhizoid morphotype (Kunttu *et al.* 2009a). When four *F. columnare* strains (B067,

B185, B245 and Os06) exhibiting the original Rhizoid morphotype were conferred with 10^6 - 10^9 PFU/ml of phage, all of the 100 to 400 CFU of bacteria changed their colony morphotype to Rough, except in B185, for which 50 % fewer colonies appeared compared to the phage free control. The phage-resistant Rough morphotype differed from the previously described spontaneous Rough type by having small colonies with solid edges, whereas the spontaneous type has irregular edges with sporadic rhizoid protrusions (Kunttu *et al.* 2009a). The ability to form spreading colonies, indicating ability for gliding motility, was also lost according to the measurement of colony size on different nutrition concentrations. The diameter of the phage-resistant Rough colonies was consistent but the Rhizoid colonies varied greatly accordingly. Phage-induced reduction of motility has previously been observed at least in *E. coli*, *Bacillus*-species and *P. aeruginosa* (Joys 1965, Schade *et al.* 1967, Heierson *et al.* 1986, Darzins 1993) usually, because the phages use the motility apparatus as a receptor.

Virulence of the two morphotypes, the ancestral Rhizoid and the phage-resistant Rough morphotype, was tested using zebra fish as host. In all four bacterial strains the Rhizoid morphotype caused columnaris disease and mortality in fish, and in two strains the mortalities were 100%. In one strain, B185, the phage-resistant Rough was able to cause minor fish mortality (12.5 %) while Rough types of the other strains were non-virulent. Together, the results from this and previous studies (Kunttu *et al.* 2009a) strongly suggest, that gliding motility has a connection with virulence in *F. columnare*. Indeed, motility has been found to be connected to virulence in other bacterial species (Ottemann and Miller 1997, Josenhans and Suerbaum 2002). The sites where the *F. columnare* phages attach have not yet been studied, but because of the direct loss of gliding motility in the phage-resistant morphotypes it is possible that these phages use the motility apparatus as their receptor. Interestingly, the flavobacterial gliding motility apparatus may have a dual function, serving also as a secretion system for virulence factors. Indeed, a Por secretion system (PorSS), named type IX secretion system, was recently described for secretion of virulence factors in *Porphyromonas gingivalis,* a member of Bacteroidetes (Sato *et al.* 2010). This secretion system differs from other previously described secretion machineries and it appears to be widespread among the phylum (McBride and Zhu 2013). In *F. johnsoniae* the genes involved in the PorSS are a subset of the gliding motility machinery (Sato *et al.* 2010). These genes are found also in *F. columnare* (Dumpala *et al.* 2010) and the connection of virulence and motility suggests that virulence factors are secreted via this machinery also in *F. columnare*.

The stability of the Rhizoid and Rough morphotypes was measured in serial culture. Three strains (B67, B185 and B245) maintained their morphotypes with occasional spontaneous changes from Rhizoid to Rough. The less virulent strain Os06, however, was more unstable during the ten serial passages. It is hypothesized, that the rapid switch between phenotypes could serve as a survival strategy for the less virulent strain, as it can less efficiently use the fish host, but should be able to maintain itself alive under variable environmental

conditions. So far isolations of Finnish *F. columnare* strains have been of the Rhizoid morphotype in spite of the source (fish or water) (Kunttu *et al.* 2012, my observations) even when the phages have been isolated simultaneously. Recently it was shown, that the phage-resistant and starved Rough types have a lower fitness inside and outside host environments compared to the virulent Rhizoid morphotype, including lower resistance to protozoan grazing (Zhang *et al.* 2014). This observation could explain the absence of Rough morphotypes in the initial isolations even though they would exist at the farm.

Overall, the results in study II show an example of how *F. columnare* responds to strong selective pressure caused by a phage. Such negative outcomes from phage resistance in outside host environment have been observed before (Santander and Robeson 2007, Capparelli *et al.* 2010, Friman *et al.* 2011, Zhang *et al.* 2014), but also opposite results have been reported. In *P. aeruginosa* increased virulence was detected when phage resistance was evolved in exposure to heterogeneous phage population (Hosseinidoust *et al.* 2013b). However, considering the possibility to use phage therapy against columnaris disease, the direct loss of motility and virulence in the bacteria is encouraging. The results suggest that development of phage resistance might not be a problem in the case of *F. columnare*, at least in shorter time frames.

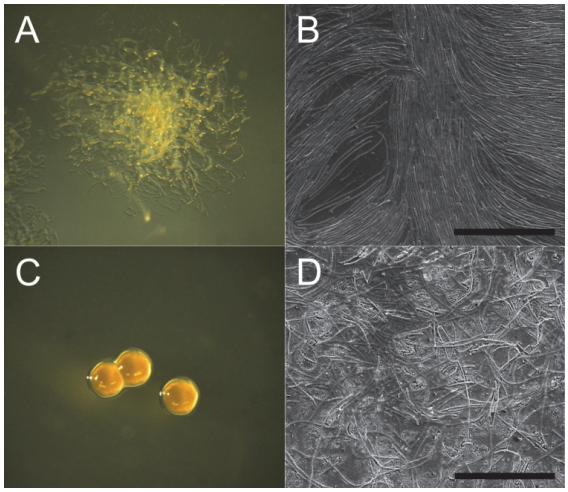


FIGURE 4 Rhizoid (panel A) and Rough (panel C) colonies on Shieh agar (II) and a closer view of the cells in a colony of Rhizoid in panel B and of Rough in panel D under SEM (III) (scale bars in panels B and D are 20 μm).

4.4 What changes after phage exposure? (III)

The virulence of different colony morphologies of *F. columnare* strains has been previously studied (Kunttu *et al.* 2009a, II) suggesting strongly that only the cells of the Rhizoid morphotype are able to cause disease. This implies, that by

comparing the differences of these morphotypes, insights could be gained to the largely unknown virulence factors of *F. columnare*, as functional genetic manipulation methods are not yet available (but see Staroscik *et al.* (2008)). Here, phage FCL-1 was used to induce the non-virulent Rough morphotype from the ancestral Rhizoid morphotype of strain B067. The Soft morphotype was spontaneously formed among the culture of the Rhizoid type. Virulence of the three morphotypes was confirmed by infecting rainbow trout (*Oncorhynchus mykiss*) fingerlings. Rhizoid morphotype was indeed virulent (30 % end survival) compared to the Rough (90 % end survival) and Soft (70 % end survival) that were comparable to the control group (70 % end survival).

The cell organization in a colony as well as the planktonic cells of the three morphotypes was visualized under high resolution scanning electron microscopy (HR-SEM). The surface of the colonies varied greatly, which is consistent with the appearance of the colonies on agar; Rhizoid and Rough colonies have a hard surface while a Soft colony can be easily picked by a loop. On the colony surface of the Rough morphotype only a thick layer of extracellular filamentous material was seen and no cells were observed underneath. The same filamentous structure was also detected on the surface of the Rhizoid colony but the surface was missing in some areas, and the bacterial cells were visible there. This extracellular layer was completely missing from the Soft morphotype. It is likely, that this extracellular matrix contributes to the biofilm formation and offers shelter to the cells e.g. from predators (Matz *et al.* 2005). In addition, filamentous layer could be involved with the adherence of bacteria on surfaces, suggested by the absence of both the layer and adherence in the Soft type. Also the internal structure of the colonies was visualized. The organized arrangement of the cells within the Rhizoid colony was evident (Figure 4B), while the cells of the Rough type were scattered without any pattern (Figure 4D). Also, no clear organization was observed from the internal colony of the Soft type, but the absence of adherence during sampling most likely affected this result, because the organized structure of the colony could be detected from the colony surface mentioned above. In previous studies it has been confirmed, that during infection, *F. columnare* forms a biofilm on the fish's fins, skin and gills (Olivares-Fuster *et al.* 2011, Bullard *et al.* 2013) and there degrades the host tissue. It is not yet known how well the external structures and internal arrangement of the cells within the colony observed here correspond to the behaviour of *F. columnare* during infection. However, as only the Rhizoid type with both adherence capacity and organized cell arrangements is virulent, it can be assumed that these capacities are important for infectivity.

Generally, bacterial cells in the biofilm have been shown to display different gene expression profiles compared to planktonic cells (Whiteley *et al.* 2001, Schembri *et al.* 2003). Planktonic cells are the ones that attach to the surface and initiate infection when transmitting from one host to another, and understanding this step is therefore crucial in the lifestyle of *F. columnare*. No differences were found between the planktonic cells of the Rhizoid and phage-resistant Rough morphotype when visualized under HR-SEM. In addition, the extracellular protein (ECP) profiles of the Rhizoid and phage resistant Rough

type were compared. In Rhizoid, a small (13 kDa) protein was observed in relatively high quantities and was absent or in very small quantities in the profile of the Rough type. This same protein was also observed in the Rhizoid morphotypes of two other strains (B185 and B245, data not shown) also lacking from the Rough type, and therefore it is suggested that the protein is possibly connected to virulence in *F. columnare.* The identification of the protein did not result to any known function, but the sequence was found to be absent from the genomes of two other closely related bacteria, *F. johnsoniae* and *F. psychrophilum.*

4.5 Outer membrane vesicles (OMVs) may be potential mediators of virulence (III)

Gram-negative bacteria produce OMVs ubiquitously. Proposed roles for OMVs include virulence mediators and functioning as the response to envelope stress (Ellis and Kuehn 2010, Baumgarten *et al.* 2012). OMVs have been even shown to protect cells from phage infection (Manning and Kuehn 2011). The noticeable abundance of OMVs on the cells of the Rhizoid and Rough morphotype led to a question of their importance in *F. columnare*. Small vesicles in *F. columnare* have been previously reported (Arias *et al.* 2012, Cai *et al.* 2013) but no further analyses have been executed. In the SEM analysis the Rhizoid and Rough morphotype planktonic cells were observed to possess large vesicles (100-500 nm). These structures were not observed in the cells of the Soft morphotype. It could be that the lack of vesicles in the Soft type has a connection with the lack of adherence; the S type is non-adherent on the surface whereas the Rhizoid and Rough are highly adherent (Kunttu *et al.* 2009a, 2011). Vesicles were seen on the surfaces of bacteria, usually at the end of a chain and when attached to surface, they seemed erupted anchoring the cell to the surface. However, whether the sample processing causes the vesicle eruption is unsure. In the TEM analysis, only small size (approximately 50 nm) vesicles with a lipid bilayer in the Rhizoid and Rough types were observed. The absence of the large vesicles in the TEM samples might have been caused by sample processing in thin-sectioning.

To analyse the OMVs of *F. columnare* in more detail, they were extracted and purified by the protocol described by Kulp and Kuehn (2010). The size range of these purified vesicles was approximately from 60 nm to 350 nm supporting the suggestion that the lack of large vesicles in thin-sectioned cells was caused by sampling bias. Five protein bands extracted from vesicles were analysed in more detail by nanoLC-ESI-MS/MS. One protein band (approx. 55 kDa) was identified as the OmpA-family outer membrane protein P60 of the *F. columnare* strain ATCC 49512. OmpA is often associated with adhesion to host tissues and is known to be a virulence factor in several pathogens (Smith *et al.* 2007, Confer and Ayalew 2012). The possibility of the connection of OmpA and adhesion also in *F. columnare* is supported by the above-mentioned lack of vesicles in the non-adherent Soft type. The other four analysed proteins were

not assigned with any function so their sequences were compared to database sequences. The 35 kDa protein gave a hit on flavobacterial gliding motility protein SprF of *F. psychrophilum* and *F. johnsoniae,* and based on amino acid similarity, was thus designated as SprF. SprF is one of the proteins needed in the formation of spreading colonies and for secretion of SprB, another important protein involved in gliding in *F. johnsoniae* (Rhodes *et al.* 2011). In *F. coumnare* the role of SprF remains still unknown. The spreading e.g rhizoid morphotype is needed for virulence (Kunttu *et al.* 2009a, 2011, II), perhaps for secretion of virulence factors and SprF could be part of this also in *F. columnare*. However, it cannot be excluded that the production of OMVs is a stress response for Rhizoid and Rough morphotypes. But in that case, the reason why Soft morphotype lacks OMVs would be unclear, unless it could tolerate better laboratory culture conditions or absence of OMVs would be caused by systematic bias in sample preparation.

To conclude, virulence in *F. columnare* may be a sum of several co-occurring characteristics. It seems that the OMVs play an important role in the life of *F. columnare*. Although OMVs were found also in the non-virulent Rough type, it is suggested that they may have a role in virulence, as they were absent from the cells of the Soft type. In addition, gliding motility and coordinated cell organization are also needed for virulence, as their absence correlate with loss of virulence in the Rough morpotype. Interestingly, the studies of OMVs in gram-negative bacteria have been increasing during the past few years. Hopefully, the cumulating information of OMVs will reveal new functions that contribute to bacterial behavior in the future.

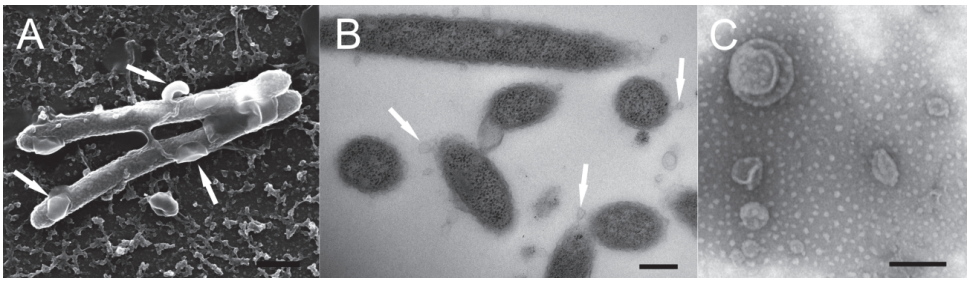


FIGURE 5 OMVs observed on the cells of *F. columnare* strain B067 Rhizoid morphotype (III). In panel A are the planktonic cells under HR-SEM. In panel B are thin-sectioned cells and in panel C purified OMVs, both under TEM. Arrows in panels A and B indicate some of the vesicles observed. Scale bars in A 500 nm, B and C 200 nm.

4.6 The impact of phage FCL-2 on survival of fish infected with *F. columnare* (IV)

The true efficiency of a phage as a potential therapeutic agent should be measured as its capacity to destroy bacterial cells on the site of bacterial

infection (Levin and Bull 2004). Therefore, the capability of phage FCL-2 in controlling columnaris disease was studied with two fish species in laboratory conditions. Rainbow trout is the common natural host of *F. columnare,* and one of the species mostly affected by the pathogen in Finnish fish farming. Because of the general difficulties associated with working with rainbow trout (e.g. poor availability of disease-free fingerlings and sensitivity to laboratory conditions), zebra fish were also used to confirm their suitability as a model system for screening therapeutic phages.

The most important results of phage therapy experiments were obtained from a population experiment with rainbow trout fingerlings. Here, fish (n=20) were held in aquaria where the water flow was constant, similarly to the actual conditions in the rearing tanks at fish farms. After a seven-day period the survival of fish infected with *F. columnare* was only 8 %. This was significantly lower compared to the survival of fish receiving phage treatment after bacterial exposure (45 % survival with a phage to bacterium ratio 10:1 and 50 % at ratio 1:1). It indeed seems, that phage FCL-2 works efficiently at the site of the infection, although the phage was given only as a single treatment.

The results of the population experiment are relevant also when considering the transmission of columnaris disease in the rearing units. The shedding rate of *F. columnare* cells can be 10 times greater from dead than from diseased living fish (Kunttu *et al.* 2009b). Despite their regular removal, dead fish are common in the rearing tanks during columnaris outbreaks, contributing greatly to the spreading of the disease. In the population experiment, the dead fish were removed at 12-hour intervals allowing time for the bacterial cells to shed into the water. According to our results phage can work efficiently under these circumstances, and protect the fish, as the mortalities remained low in the phage treated aquaria even when the bacteria shedding from the fish maintain a constant infection pressure.

Secondly, the zebra fish experiment (individually tested) showed a good potential as an infection model for future phage therapeutic evaluations, as the end survival of the fish treated with phage was 60 % compared to the infection control fish with end survival of 0 %. The 100 % survival of the fish that were not exposed to bacteria supports the reliability of using zebra fish in screening, indicating that the zebra fish tolerate experimental conditions very well. Rainbow trout were also tested individually and significant differences were also detected in these experiments, although the sensitivity of the fish to the experimental conditions caused high mortalities in the control treatments. Altogether, the results from phage therapy trials in animal production (e.g. in poultry, swine and cattle) in general have given encouraging results (Smith and Huggins 1983, Berchieri *et al.* 1991, Barrow *et al.* 1998, Jamalludeen *et al.* 2009, Santos *et al.* 2010).

The stability of FCL-2 in a six-week period showed only slight decay and even better results could be obtained by optimization. It remained also lytic during the experiment showing potential as a therapeutic (Gill and Hyman 2010). But the results gained from the genome sequence might prevent its usage as mentioned previously.

As a conclusion, the survival of phage treated fish was significantly improved in all of the experiments, even though phage was administrated only once. Therefore development of phage therapy applications has great potential for control of columnaris disease in aquaculture. However, the timing and dosage of phages during columnaris infection should be studied further to improve the efficacy of the treatments. The main obstacle for future experiments, however, is the strict legislation, which at the moment prevents testing of phage therapy at fish farms. European directive 2001/83/EG considers a product to be medicinal if it contains active compounds that kill the external human or animal parasites. At the moment phage is classified as such and is therefore subjected to specific requirements. Therefore, there is a need for changes in the legislation to be able to establish phage therapy as a genuine treatment or biocide method. This needs to be overcome by clear definition of phage in the legislation (Huys *et al.* 2013).

4.7 Future aspects

During the studies of this thesis interesting questions arose that are, in my opinion, worth investigating in the future. The strong inhibition observed by some of the *Flavobcaterium* sp phage lysates against all tested *F. columnare* strains (I) is an interesting finding that could possibly offer new antimicrobials against *F. columnare*. First, the origin of the causative agent (e.g. phage lytic enzyme or bacteriocins) should be studied, the nature of the agent solved, and finally if the agent is convenient for production, to test if there's a possibility for it be used as an antimicrobial. Many of the substances produced by, for example Cyanobacteria, are showing potential as antimicrobials (Silva-Stenico *et al*. 2014) and this property should be studied in Flavobacteria as well. Indeed, the demand of new antimicrobials is tremendous.

OMVs were found to be abundant in the *F. columanre* strain B067 (morphotypes Rhizoid and Rough) (III). The growing evidence for the importance of OMVs in different mechanisms of bacteria intrigues questions for future studies also for the OMVs of *F. columnare.* The social role of the OMV's is interesting as has been suggested for the social soil bacterium *Myxococcus xhanthus* that OMVs transport memebrane proteins that stimulate social activity (Kahnt *et al.* 2010, Remis *et al.* 2014). The work on OMVs of *F. columnare* could be continued by analyzing the cargo in the vesicles in more detail as the study here composed of total OMVs (III). The data of the proteins packed in the vesicles of the virulent strains compared to the vesicles of non-virulent strains could bring new insights to the virulence mechanisms of *F. columnare*.

During the studies efforts were made to produce *F. columnare* phages in liquid i.e. using one step growth experiment. For efficient phage therapeutics also the phage production should be efficient. In the case of *F. columnare* phages this area needs more developing. It might be that the phages use the gliding motility machinery as a receptor, which is not functioning properly in the liquid

medium. Phages do adsorb but some part of the infection cycle might be hampered. Or it might as well be that the conditions in the liquid culture contribute to the problem and more screening is needed for optimal conditions.

One of the most interesting obstacles conferred was the immediate aggregation of cells in liquid culture when high titre of phage was added. Under the SEM, many extremely long filaments attaching cells were observed in the planktonic cell samples suggesting direct contact of the cells. Recently, the outer membrane-enclosed tubes (OMTs) were described for social bacterium *M. xhanthus* (Ducret *et al.* 2013, Wei *et al.* 2014) that exceeded multiple cell lengths and I suggest that similar structures could exist also in *F. columnare*. The aggregation indicates fast signalling in the homogenous cell population in the liquid media and could be mediated through these structures connecting cells.

More testing is also needed to assess whether phages are efficient and powerful method for aquaculture in real life situation, it certainly shows great potential. Overall, all the possibilities phages (or parts of them) offer in the continuous race against bacterial pathogens are yet to be clarified but options vary from early detection of pathogens to bacterial manipulation and to treatment and disinfection of the pathogenic agent. The fish farmers have shown strong interest towards testing the efficiency of phages at the actual site (personal communication) but the major obstacle in the way is the strict legislation, which prevents experiments at the fish farms if fish are included in the rearing tank where the phage is added.

5 CONCLUSIONS

The main conclusions of this thesis are:

I. Bacteriophages and their ubiquitous Flavobacterial hosts are widespread also in the northern fresh waters. The collection of phages included broad host range phages and more host-specific ones. Phages infecting *F. columnare* can be isolated from the fish farms during warm water period when columnaris outbreaks occur and they are very host specific. This quality makes them good candidates for phage therapy but could also cause limitations when designing phage cocktails.

II. Phage resistance has a cost for *F. columnare*: it looses its virulence when developing resistance. Virulence of the bacterium seems to associate with gliding motility.

III. Both the virulent Rhizoid and the phage-resistant Rough morphotypes possess OMVs that might be connected to virulence. The loss of coordinated cell organization is suggested to prevent the virulence capacity of the OMVs in the Rough morphotype.

IV. Phage is efficient in rescuing fish from disease caused by *F. columnare* in two fish species in a flow-through system. Zebra fish can be used as a screening system for potential phage therapeutics. This is a promising start for developing phage therapy against *F. columnare*

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Faagi-bakteeri -vuorovaikutusten tutkiminen – Uusia keinoja kalapatogeenin torjuntaan

Virukset ovat solujen loisia, jotka koostuvat yksinkertaisimmillaan perimästä eli genomista ja sitä ympäröivästä proteiinikuoresta. Niiden alkuperästä on useita teorioita, mutta yleisesti hyväksytty arvio on, että virukset ovat peräisin samalta muinaiselta kaudelta kuin ensimmäiset solut. Joka tapauksessa virukset ovat olleet luomassa elämän suurta kirjoa lisätessään geneettistä monimuotoisuutta. Bakteerit ja niiden virukset, bakteriofagit (lyhyemmin faagit), ovat hyviä mallisesimerkkejä virusten ja isäntäsolun vuorovaikutusten tutkimisessa. Faagit ovat erittäin monipuolinen ryhmä viruksia, joiden merkitys planeettamme elämälle on alkanut selvitä vasta viime vuosikymmeninä. Niillä on suuri rooli esimerkiksi ravinteiden kierrossa meriympäristössä. Kun faagit löydettiin 1910-luvulla, ne valjastettiin heti taudinaiheuttajabakteerien torjumiseen. Länsimaissa faagit kuitenkin unohdettiin kun käyttäjäystävälliset antibiootit saapuivat markkinoille. Antibioottien laaja käyttö on kuitenkin tuonut mukanaan antibioottiresistentit bakteerikannat, jotka ovat muodostuneet yhdeksi suurimmista ongelmista terveydenhuollossa.

Tämän väitöskirjan lähtökohtana oli kaloilla tautia aiheuttavan *Flavobacterium columnare*n vuosittain pahentuneet tautitapaukset ja tappiot kalanviljelyssä. Uusien hoitomenetelmien etsiminen antibioottien tilalle on eduksi niin yhteiskunnalle antibioottiresistenssin leviämisen estämiseksi, kuin kalanviljelijän näkökulmasta tautien tehokkaaksi torjumiseksi. Väitöskirjan ensimmäisessä osatyössä tutkittiin flavobakteereita infektoivien faagien esiintymistä Suomen vesistöissä ja kalanviljelylaitoksilla. Erityispaino tutkimuksessa oli tautia aiheuttavan *F. columnaren* faageissa. Selvisi, että *F. columnare*n faagit olivat erittäin isäntäspesifisiä, kun taas yleisesti Flavobakteereita infektoivien faagien joukosta löytyi niin spesialisteja kuin laajan isäntäkirjon tyyppejä. Lisäksi tutkimuksessa *F. columnare*a infektoivia faageja eristettiin ainoastaan kalanviljely-ympäristöistä.

Toisessa osatyössä tutkittiin tarkemmin faagivastustuskyvyn kehittymisen vaikutusta bakteeriin ja huomattiin, että faagille vastustuskykyiset bakteerit eivät kyenneet aiheuttamaan tautia seeprakalalla. Lisäksi selvisi, että vastustuskykyiset solut olivat menettäneet flavobakteereille ominaisen liikkumiskykynsä. Liikkumiskyvyn puuttuminen oli havaittavissa pesäkemuodosta: liikkuvat solut muodostivat bakteerimaljalla leviäviä pesäkkeitä, kun taas liikkumiskyvyttömät muodostivat pienen pesäkkeen, jonka läpimitta ei muuttunut ravinteiden vähetessä. Tutkimuksen tuloksista voidaan arvioida, että mahdollisten faagille vastustuskykyisten solujen ilmentyminen ei välttämättä ole *F. columnare*n tapauksessa ongelmallinen. Lisäksi huomattiin, että solujen liikkuminen on oleellinen taudinaiheuttamiskykyyn vaikuttava tekijä *F. columnare*lla

F. columnare on maailmanlaajuinen taudinaiheuttaja, joka aiheuttaa vakavia oireita usealla kalalajilla. Vaikka *F. columnare* on aiheuttanut taloudellisia

tappioita jo pitkään, sen taudinaiheuttamiskyvyn syytä ei vielä tarkkaan tiedetä. Jotta taudinaiheuttajaa vastaan voidaan tehokkaasti kehittää hoitomenetelmiä, on tärkeää ymmärtää sen biologiaa. Väitöskirjan kolmannessa osatyössä käytettiin hyväksi *F. columnare*n ominaisuutta muodostaa eri pesäkemuotoja laboratorio-olosuhteissa. Pesäkemuodoista vain yksi on taudinaiheuttaja, joten tätä ja kahta muuta pesäkemuotoa vertailtiin eri tavoin mahdollisten taudinaiheuttamistekijöiden tunnistamiseksi. Tutkimuksessa käytettiin hyväksi pyyhkäisy- ja läpäisyelektronimikroskopiaa, joilla tutkittiin solujen pintoja ja rakenteita biofilmissä sekä planktonisessa muodossa. Näistä huomattiin runsas membraanivesikkeleiden esiintyminen kahdella pesäkemuodolla. Lisäksi havaittiin, että taudinaiheuttamiskykyisellä muodolla erittyy kasvatusliuokseen huomattava määrä pienikokoista proteiinia. Tutkimuksen tulosten perusteella ehdotettiin kolmea uutta taudinaiheuttamiskyvyn tekijää *F. columnare*lla: solujen koordinoitu rakenne, membraanivesikkelit sekä eritettävä proteiini.

Neljännessä osatyössä testattiin faagin kykyä torjua *F. columnare*n aiheuttamaa columnaris-tautia kokeellisesti infektoiduilla kirjolohen poikasilla ja seeprakaloilla. Faagikäsitellyillä kaloilla kuolleisuus oli merkittävästi alhaisempi kuin faagikäsittelemättömillä. Merkittävin tulos saatiin populaatiokokeessa, jossa käytettiin läpivirtausallasta; faagi pystyi säilymään altaassa ja torjumaan tautia tehokkaasti. Lisäksi selvisi, että laboratorio-olosuhteissa paremmin toimiva seeprakala sopii seulontamalliksi tehokkaiden faagien selvittämiseksi.

Väitöskirjan tulokset ovat lupaavia faagiterapian kehittämiseksi columnaris-tautia vastaan; faagille vastustuskykyiset solut eivät aiheuta tautia ja faagi pystyy torjumaan merkittävästi kalakuolemia. Tulevaisuuden haasteita ovat faagiannoksen optimointi ja testaaminen taudille oleellisissa olosuhteissa, eli kalanviljelylaitoksilla. Testaaminen ei tällä hetkellä onnistu tiukan lainsäädännön takia, mutta faagiterapiatutkimuksen yleistyessä ja ennenkaikkea sen positiivisten tulosten kautta testaaminen saattaa tulla mahdolliseksi. Väitöskirjan tutkimus ja sen tulokset ovat merkittävä avaus faagiterapian kehittämiselle niin suomalaisessa kalanviljelyssä kuin laajemminkin uusien taudintorjuntamenetelmien kehittämisessä.

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ORIGINAL PAPERS

I

PHAGE SPECIFICITY OF THE FRESHWATER FISH PATHOGEN *FLAVOBACTERIUM COLUMNARE*

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Phage Specificity of the Freshwater Fish Pathogen *Flavobacterium columnare*▽

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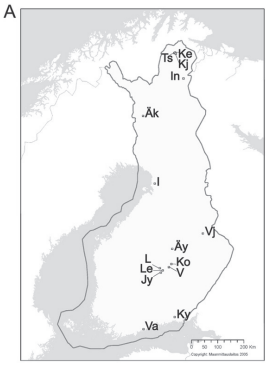
Flavobacteria and their phages were isolated from Finnish freshwaters and fish farms. Emphasis was placed on finding phages infecting the fish pathogen *Flavobacterium columnare* for use as phage therapy agents. The host ranges of the flavobacterial phages varied, phages infecting *F. columnare* being more host specific than the other phages.

Species of the genus *Flavobacterium* are widely distributed in nature, and they have been found in diverse habitats (3, 19, 30, 32, 36, 37, 38, 39). In general, flavobacteria are nonpathogenic, but some species are opportunistic pathogens (2). Columnaris, a disease caused by the fish pathogen *Flavobacterium columnare*, can cause up to 100% mortality among salmonid fingerlings (28). Antibiotic treatment must be applied to prevent mass mortalities at fish farms. Despite effective treatment, columnaris occurs repeatedly during the summer (18). Therefore, the number of antibiotic treatments and the amount of antibiotics used can be extremely high. Increased resistance of environmental bacteria to antibiotics in fish farms and their surroundings has been reported (10, 24, 26, 31), and antibiotic-resistant fish-pathogenic flavobacteria have also emerged (7). To avoid risks related to antibiotic use, enrichment of bacteriophages could be used as an ecological method of decreasing the number of *F. columnare* infections. To our knowledge, this is the first study on European *F. columnare* phages.

In this study, a total of 53 flavobacterial isolates were received during the warm-water period (May to August) in 2008 and 2009 from water samples that included both open freshwater environments (rivers and lakes not connected to fish farming) and three inland land-based fish farms rearing mainly salmonid fingerlings in Finland (Fig. 1; Table 1). One bacterium (B67) was isolated from diseased fish in 2007. Water samples were cultured on Shieh agar (25) and 1/5× Luria



Location	Abbrevation	Longitude	Latitude
Lake Konnevesi	Ko	E 26° 35.644'	N 62° 31.446'
Lake Jyväsjärvi	Jy	E 25° 44.339'	N 62° 13.779'
River Vantaanjoki	Va	E 24° 58.072'	N 60° 15.948'
River Kevojoki	Ke	E 26° 59.429'	N 69° 45.337'
River Tsarsjoki	Ts	E 26° 59.447'	N 69° 45.430'
Lake Kevojärvi	Kj	E 26° 59.608'	N 69° 45.430'
Lake Valtimojärvi	Vj	E 28° 51.000'	N 63° 40.688'
River Kymijoki	Ky	E 26° 49.926'	N 60° 41.391'
Lake Leppävesi	Le	E 25° 54.633'	N 62° 16.064'
Lake Äkässaivo	Äk	E 24° 8.640'	N 67° 41.840'
River Äyskoski	Äy	E 26° 40.756'	N 63° 0.665'
Lake Inari	In	E 27° 53.097'	N 68° 47.783'
Fishery L [1)]	L		
Fishery V [1)]	V		
Fishery I [2)]	I		

[1)] Central Finland
[2)] Northern Finland

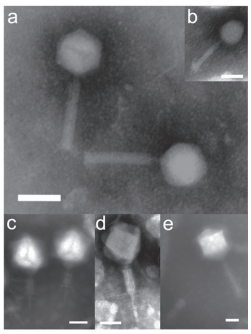


FIG. 1. (A) Sites in Finland where flavobacteria and their phages were isolated. Sampling sites are marked on the free map obtained from the National Land Survey of Finland (Maanmittauslaitos, 2005). The sites and their abbreviations and coordinates are listed on the right. (B) Electron micrographs of purified and negatively stained tailed *Flavobacterium* phages. (a) FCV-1; (b) FCL-2; (c) FJy-3; (d) FKo-2; (e) FKy-1. Bar, 50 nm.

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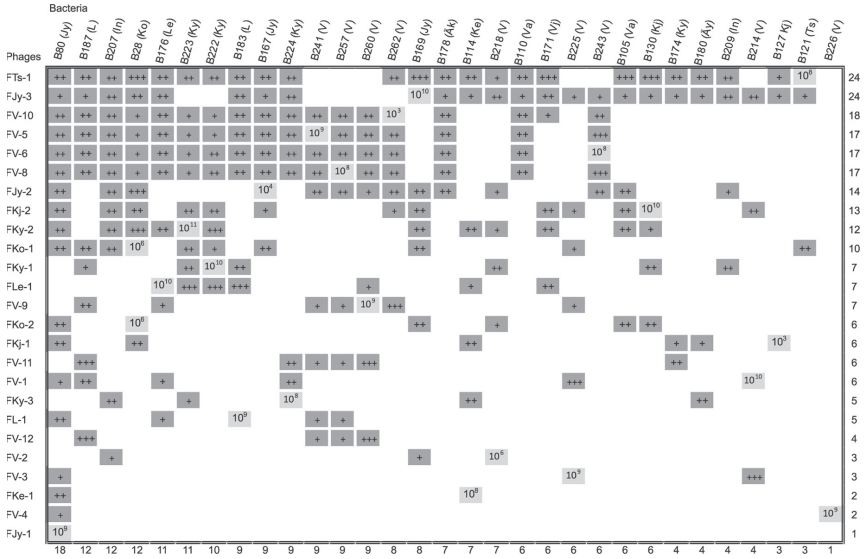

FIG. 2. Host ranges of the phages infecting different *Flavobacterium* sp. strains. Dark gray squares indicate infection; white indicates no infection, and light gray squares mark the strain which was originally used for isolation of the phage. The numbers at right and bottom are the total number of different host ranges of the phage and susceptibility of bacteria to phages, respectively. The approximate titer of each phage on the bacteria is marked with plus signs (+, $\leq 10^5$; ++, 10^6 to 10^8; and +++, $\geq 10^9$ PFU/ml). The titer (PFU/ml) of the phage on the isolation strain is marked on the light gray squares. In parentheses after the name of the bacterial strain is the abbreviation of the place of isolation, which is also included in the phage nomenclature (Fig. 1). *F. columnare* phages infected only one specific *F. columnare* RISA group, and they are listed in Table 2.

Bertani agar (22), and yellow- and orange-pigmented colonies were selected for analyses. The flavobacterial isolates were subjected to PCR with universal primers (UP-PCR) (for methods, see references 5, 6,, and 13), and the 16S rRNA genes of different groups were sequenced (Table 1). All *F. columnare* isolates fell into the same UP-PCR group, and thus they were further analyzed with ribosomal intergenic spacer analysis (RISA) (8, 29). According to RISA, the isolates were assigned to five groups (Table 1). Based on our data, the occurrence of *F. columnare* seems to be connected to the fish farming environment; this organism was not isolated from natural waters. However, it is likely that the initial source of *F. columnare* at the farms is nature, because there is evidence that it is also present outside fish farms (20, 21; H. Kunttu, L.-R. Sundberg, and E. T. Valtonen, unpublished data).

Previous reports describe phages infecting the genus *Flavobacterium* and their interaction with the bacterial host mostly in marine environments (4, 9, 12, 14), but the phage-host relationship of the fish pathogen *Flavobacterium psychrophilum* has also been studied (27). A total of 49 bacteriophages were isolated from water samples (Table 2). Phages were enriched using flavobacterial isolates from freshwaters, fish farms, and previously described *F. columnare* strains. Phage stocks were prepared, and selected phages were grown by infecting the host bacterium (multiplicity of infection, 5 to 10) at the proper cell density, concentrated, and purified (22). Many of the isolated phages produced low-titer lysates, and they were used only for infection tests (see below for host range studies). Phage genomic DNA was extracted (for methods, see references 1 and 23) and digested with BamHI, EcoRI, HindIII, and PstI. For the genomes that were cut, the genome size was calculated from the resulting restriction profile (Table 2). For the phages that were sequenced (FKj-2, FL-1, FCL-2, and FCV-1), approximately 3,000 bp of each genome (except FCV-1, for which 1,600 bp was used) was subjected to BLAST searching (http://blast.ncbi.nlm.nih.gov/Blast.cgi; May 2011), and no significant DNA sequence similarity was found in the database. Putative protein-coding genes were analyzed using Vector NTI 11.0.0 (Invitrogen). Best matches for FCL-2 were to a hypothetical protein of the *Vibrio* phage VP16T (score, 57.8) and to a hypothetical protein of the *Vibrio* phage VP16C (score, 55.1). For FCV-1, the best match was to a hypothetical protein, B40-8030, of the *Bacteroides* phage B40-8 (score, 53.9).

Transmission electron microscopy (TEM) was used to study phage morphology. All of the flavobacterial phages that were characterized were tailed phages of the families *Myoviridae*, *Podoviridae*, and *Siphoviridae* (Fig. 1 and Table 2). Most of these phages had an average head size of 50 to 70 nm, but some of the myovirus isolates had capsid sizes of about 100 nm or more (FJy-3, FKo-2, FKj-2, FKy-1, and FKy-3).

Phages infecting *F. columnare* were isolated only from fish

TABLE 1. Bacterial strains isolated and used in the study

Bacterial strain[a]	UP-PCR or RISA group[b]	EMBL accession no.[c]	Sampling site	Source or reference	Bacterial strain[a]	UP-PCR or RISA group[b]	EMBL accession no.[c]	Sampling site	Source or reference
B67	A		Fishery L	This study	B244	ND		Fishery V	This study
B28	1	FR696328	Lake Konnevesi	This study	B245	C		Fishery V	This study
B80	2	FR696329	Lake Jyväsjärvi	This study	B247	C		Fishery V	This study
B105	3	FR696330	River Vantaanjoki	This study	B257	26	FR696355	Fishery V	This study
B110	4	FR696331	River Vantaanjoki	This study	B259	C		Fishery V	This study
B114	5	FR696332	River Kevojoki	This study	B260	ND	FR696356	Fishery V	This study
B121	6	FR696333	River Tsarsjoki	This study	B261	C		Fishery V	This study
B127	7	FR696334	Lake Kevojärvi	This study	B262	ND	FR696357	Fishery V	This study
B130	8	FR696335	Lake Kevojärvi	This study	B263	ND	FR696358	Fishery V	This study
B167	9	FR696336	Lake Jyväsjärvi	This study	B267	C		Fishery V	This study
B169	10	FR696337	Lake Jyväsjärvi	This study	B268	C		Fishery V	This study
B171	11	FR696338	Lake Valtimojärvi	This study	B269	ND		Fishery V	This study
B174	12	FR696339	River Kymijoki	This study	B270	C		Fishery V	This study
B176	13	FR696340	Lake Leppävesi	This study	B271	C		Fishery I	This study
B178	14	FR696341	Lake Äkässaivo	This study	B272	J		Fishery I	This study
B180	15	FR696342	River Äyskoski	This study	B273	C		Fishery I	This study
B183	16	FR696343	Fishery L	This study	B274	C		Fishery I	This study
B185	G	FR696344	Fishery L	This study	B275	C		Fishery I	This study
B187	17	FR696345	Fishery L	This study	Rz-A	A			29
B207	18	FR696346	Lake Inari	This study	R-B	B			29
B209	19	FR696347	Lake Inari	This study	Rz-C	C			29
B214	20	FR696348	Fishery V	This study	S-C	C			29
B218	21		Fishery V	This study	R-D	D			29
B222	22	FR696349	River Kymijoki	This study	S-D	D			29
B223	23	FR696350	River Kymijoki	This study	Rz-E	E			29
B224	24		River Kymijoki	This study	R-E	E			29
B225	24	FR696351	Fishery V	This study	S-E	E			29
B226	25	FR696352	Fishery V	This study	S-F	F			*F. columnare* type strain NCIMB 2248
B230	I		Fishery V	This study					
B234	C		Fishery V	This study					
B235	C		Fishery V	This study	Rz-G	G			29
B236	ND		Fishery V	This study	R-G	G			29
B237	C		Fishery V	This study	S-G	G			29
B241	26	FR696353	Fishery V	This study	R-H	H			29
B243	27	FR696354	Fishery V	This study					

[a] The previously studied *F. columnare* strains (genomic groups A to H and colony morphologies 1 to 4) are referred to in this study by placing the colony morphology after the genomic group: Rz, rhizoid (previously 1); R, rough (previously 2 and 3); and S, smooth (previously 4). For example Rz-C corresponds to the previous designation C1.

[b] UP-PCR group (numbers) for *Flavobacterium* sp. or RISA group (letters) for *Flavobacterium columnare*. ND, not determined.

[c] For the partial 16S rRNA sequence.

farms during disease outbreaks. These phages might survive inside the host cell during the cold-water period and start a lytic cycle when nutrients become available for the host cell and enough energy is available.

Studies on aquatic phage-host interplay have been conducted extensively in marine environments (15), but less is known about this interplay in freshwaters (15, 34, 35). In our study, the host ranges differed greatly between the phage isolates (Fig. 2). In the initial screening for susceptibility of the bacteria for phages, each bacterium was infected with each phage isolate by spotting the phage lysate on top agar containing the host bacterium. Each bacterium was then infected with each phage using a plaque assay. Some of the isolated phages infecting *Flavobacterium* species were identified as having a broad host range. These phages infected bacteria isolated from both freshwater and fish farm samples, although it has been suggested that single-host enrichments select for phages with narrow host ranges from sewage and marine environments (11, 33). Some of the phages (especially all *F. columnare* phages) were more host specific, as determined with our collection of *Flavobacterium* strains. *F. columnare* strains isolated from the same location as the phage were the only ones susceptible to that specific phage.

No infection of *F. columnare* strains by *Flavobacterium* sp. phages was observed, and vice versa. However, 11 *Flavobacterium* sp. phage lysates (FTs-1, FKo-2, FL-1, FV-1, FKy-1, FKy-2, FKy-3, FV-3, FV-4, FV-5, FV-6, and FV-8) inhibited the growth or lysed the underlying bacterial culture on all *F. columnare* strains tested but produced no individual plaques (data not shown). It could be that the phages were able to bind to the bacteria and cause death but were not able to produce progeny, or the clear spots could be an indication of bacteriocin activity. The causative agent of this strong inhibition or lysis should be studied further for the possibility of developing antimicrobial agents. One of the phages (FCL-1) was isolated from a fish suffering from columnaris. The presence of the *F. columnare* phages in the fish indicates the possibility of controlling a fish disease by enrichment of these phages. A number of successful reports on phage

TABLE 2. Bacteriophages isolated and characterized in this study

Phage[b]	Sampling site	Isolation strain	Phage family	Approximate genome size (kbp)[c]	RISA group[d]
FJy-1	Lake Jyväsjärvi	B80	*Myoviridae*	30	
FJy-2	Lake Jyväsjärvi	B167	ND[a]	>48	
FJy-3	Lake Jyväsjärvi	B169	*Myoviridae*	L	
FKo-1	Lake Konnevesi	B28	*Myoviridae*	25–48	
FKo-2	Lake Konnevesi	B28	*Myoviridae*	20–30	
FKe-1	River Kevojoki	B114	*Myoviridae*	ND	
FTs-1	River Tsarsjoki	B121	ND	25–48	
FKj-1	Lake Kevojärvi	B127	ND	>48	
FKj-2	Lake Kevojärvi	B130	*Myoviridae*	25–48	
FKy-1	River Kymijoki	B222	*Myoviridae*	20–48	
FKy-2	River Kymijoki	B223	*Podoviridae*?	25–48	
FKy-3	River Kymijoki	B224	*Myoviridae*?	L	
FLe-1	Lake Leppävesi	B176	*Siphoviridae*?	20–30	
FL-1	Fishery L	B183	*Myoviridae*	55	
FV-1	Fishery V	B214	*Myoviridae*	28	
FV-2	Fishery V	B218	ND	25–48	
FV-3	Fishery V	B225	*Myoviridae*	ND	
FV-4	Fishery V	B226	*Podoviridae*	25–48	
FV-5	Fishery V	B241	ND	L	
FV-6	Fishery V	B243	ND	30–40	
FV-8	Fishery V	B257	ND	L	
FV-9	Fishery V	B260	*Podoviridae*?	L	
FV-10	Fishery V	B262	ND	L	
FV-11	Fishery V	B263	ND	>48	
FV-12	Fishery V	B278	*Podoviridae*?	60	
FCL-1	Fishery L	B67	*Myoviridae*/*Podoviridae*	50	A
FCL-2	Fishery L	B185	*Myoviridae*	30	G
FCL-3	Fishery L	R-G	ND	30	G
FCL-4	Fishery L	R-G	ND	ND	G
FCV-1	Fishery V	Rz-C	*Myoviridae*	50	C
FCV-2	Fishery V	B235	ND	ND	C
FCV-3	Fishery V	B236	ND	ND	C
FCV-4	Fishery V	Rz-C	ND	ND	C
FCV-5	Fishery V	Rz-C	ND	ND	C
FCV-6	Fishery V	Rz-C	ND	ND	C
FCV-7	Fishery V	Rz-C	ND	ND	C
FCV-8	Fishery V	Rz-C	ND	ND	C
FCV-9	Fishery V	B245	ND	ND	C
FCV-10	Fishery V	B247	ND	ND	C
FCV-11	Fishery V	Rz-C	ND	ND	C
FCV-12	Fishery V	Rz-C	ND	ND	C
FCV-13	Fishery V	B261	ND	ND	C
FCV-14	Fishery V	Rz-C	ND	ND	C
FCV-15	Fishery V	Rz-C	ND	ND	C
FCV-16	Fishery V	Rz-C	ND	ND	C
FCV-17	Fishery V	Rz-C	ND	ND	C
FCV-18	Fishery V	Rz-C	ND	ND	C
FCV-19	Fishery V	Rz-C	ND	ND	C
FCV-20	Fishery V	Rz-C	ND	ND	C

[a] ND, not determined.
[b] The first letter(s) of the phage name indicates the isolation host (F, *Flavobacterium* sp.; FC, *F. columnare*); subsequent letters refer to the sampling site.
[c] L, much larger than the typical tailed phage genome (50 kb).
[d] The *F. columnare* RISA group that the phage is specific to.

therapy against fish diseases have been published; in these studies, the phages were applied directly to the water (16, 17). Another possible benefit related to phages is that they could be developed for use as diagnostic tools. In the present study, we show that bacteriophages and flavobacteria are widespread in northern freshwaters. We found phages of *F. columnare* to be host specific, making them good candidates for phage therapy.

Nucleotide sequence accession numbers. All sequences have been submitted to the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) under the accession numbers given in Table 1 and FR714876 (FCL-2), FR714877 (FL-1), FR714878 (FKj-2), and FR865436 (FCV-1).

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II

PHAGE-DRIVEN LOSS OF VIRULENCE IN A FISH PATHOGENIC BACTERIUM

by

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Phage-Driven Loss of Virulence in a Fish Pathogenic Bacterium

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Abstract

Parasites provide a selective pressure during the evolution of their hosts, and mediate a range of effects on ecological communities. Due to their short generation time, host-parasite interactions may also drive the virulence of opportunistic bacteria. This is especially relevant in systems where high densities of hosts and parasites on different trophic levels (e.g. vertebrate hosts, their bacterial pathogens, and virus parasitizing bacteria) co-exist. In farmed salmonid fingerlings, *Flavobacterium columnare* is an emerging pathogen, and phage that infect *F. columnare* have been isolated. However, the impact of these phage on their host bacterium is not well understood. To study this, four strains of *F. columnare* were exposed to three isolates of lytic phage and the development of phage resistance and changes in colony morphology were monitored. Using zebrafish (*Danio rerio*) as a model system, the ancestral rhizoid morphotypes were associated with a 25–100% mortality rate, whereas phage-resistant rough morphotypes that lost their virulence and gliding motility (which are key characteristics of the ancestral types), did not affect zebrafish survival. Both morphotypes maintained their colony morphologies over ten serial passages in liquid culture, except for the low-virulence strain, Os06, which changed morphology with each passage. To our knowledge, this is the first report of the effects of phage-host interactions in a commercially important fish pathogen where phage resistance directly correlates with a decline in bacterial virulence. These results suggest that phage can cause phenotypic changes in *F. columnare* outside the fish host, and antagonistic interactions between bacterial pathogens and their parasitic phage can favor low bacterial virulence under natural conditions. Furthermore, these results suggest that phage-based therapies can provide a disease management strategy for columnaris disease in aquaculture.

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Introduction

Over the last few years, there has been a growing concern regarding the emergence of disease outbreaks in livestock. It has become clear that environmental changes (e.g., climate warming, human intervention, enhanced transmission by transportation, use of antibiotics) have resulted in the development of new pathogens and diseases, and in addition, diseases that were previously under control have re-emerged [1–3]. In particular, intensive farming environments have been found to be evolutionary hot spots for pathogens. For example, the ecological and epidemiological features of intensive farming, including high host densities, effective transmission, and potential for serial passage, can select for high virulence of pathogens [4], [5]. Indeed, over the past 20 years, several new viral, bacterial, and eukaryotic/parasitic diseases have emerged in salmonid (*Salmo salar*, *S. trutta*, and *Oncorhynchus mykiss*) farming [2]. In particular, the occurrence of columnaris disease (caused by the opportunistic pathogen, *Flavobacterium columnare*, Bacteroidetes) has increased dramatically [3].

Traditionally, the evolution of pathogens is viewed as a reciprocal arms race competition between a pathogen and its host. Theories of virulence evolution predict that within-host growth of a pathogen, which is associated with virulence, is restricted by the availability of hosts [4]. This is referred to as the transmission-virulence trade-off. While obligate pathogens are dependent on their hosts and suffer from the transmission-virulence trade-off, opportunistic pathogens are able to survive and reproduce in environment, outside their hosts. In the latter case, the outside-host environment provides fluctuating selection pressures for opportunistic pathogens (e.g., predation, parasitism, and ecological changes), which may have correlated effects on pathogen virulence. Antagonistic species interactions between bacteria and their parasitic viruses (i.e. phage) are profoundly important regulators of bacterial abundance and traits [6]. Phage are the most abundant entities in the biosphere. They exceed the number of their host by ten-fold and thus have the capacity to control bacterial populations [7]. They also have a major impact on ecosystems and carbon cycling, especially in aqueous environments [8–10]. In the continuous arms race between phage and their hosts, bacteria need to evolve rapidly to avoid extinction [11].

Indeed, lytic phage have been shown to drive evolution in bacterial communities and to lead to strain diversification [12–15]. However, overlapping host-parasite interactions (e.g., bacterium-host and phage-bacterium) remain poorly understood. It is evident that theories regarding host-parasite interactions need to be expanded to involve three or more players in order to more accurately represent the evolution of virulence that is observed in complex environmental settings.

The ecological and evolutionary pressures that limit or increase the virulence of opportunistic bacterial pathogens are poorly known. In some cases, bacteria that gain resistance against lytic phage have a lower virulence, as has been demonstrated for *Serratia marcescens* [16], *Salmonella enterica* [17], and *Staphylococcus aureus* [18]. On the other hand, phage can invade the host as a prophage, and then modify and induce virulence of opportunistic bacteria by encoding virulence factors like the cholera toxin in *Vibrio cholera* [19]. This increases the pathogenicity of the host bacterium, and makes it a better competitor in the bacterial population [20], [21]. However, there is still very little information available regarding phage-host interactions that affect bacterial virulence under natural or intensive farming conditions. Moreover, the key question is, how do phage drive the evolution of opportunistic bacteria in the environment outside of a host. This aspect is especially important for understanding the emergence of opportunists, including opportunist saprotrophic pathogens which are able to replicate in, and transmit from, dead hosts. In these cases, the bacterial virulence is not necessarily limited by the virulence-transmission tradeoff.

F. columnare inhabits environmental microbial communities, but is also an opportunistic fish pathogen [22]. Disease outbreaks of columnaris disease rarely occur in nature, and the strains isolated from nature are less virulent than those isolated from fish farms [22]. Since the 1990s, columnaris infections have become increasingly more frequent and problematic in freshwater aquaculture, resulting in severe infections, increased mortality, and economic losses for fish farms producing salmonid fingerlings and fry [3]. This is mainly caused by the ability of *F. columnare* to survive in water, and outside of fish hosts for up to several months, thereby eluding antibiotic treatments [23]. *F. columnare* is also able to exploit dead fish material present in fish ponds for saprotrophic growth and as a means of transmission [23].

In nature and at fish farms, *F. columnare* encounters commensals and enemies from different trophic levels (e.g., protozoa, bacteria, viruses). However, studies of the interactions between *F. columnare* and other aquatic organisms present in the water body are scarce [24], [25]. Moreover, the impact of these interactions on bacterial virulence, and the mechanisms mediating bacterial virulence, are largely unknown. Under laboratory conditions, *F. columnare* exhibits three different colony morphologies, rhizoid (Rz), rough (R), and soft (S). The rhizoid morphotype is isolated in primary cultures from diseased fish, tank water, and biofilms. However, when the bacterium is cultured in the laboratory, or maintained under starvation, it loses its rhizoid morphology and manifests a parallel change in colony morphotype and decline in virulence and in susceptibility to phage infection [22], [26], [27], (Laanto et al., unpubl).

We have recently isolated and characterized phage capable of infecting the fish pathogen, *F. columnare* [28]. The results of previous studies, as described above, have demonstrated that lytic phage can act as a selective pressure against bacterial virulence, or can invade bacterium as a prophage and promote virulence. Based on the phage sensitivity of *F. columnare* [28], and the correlation between colony morphotype and virulence [26], [27], it is hypothesized that exposure of *F. columnare* to phage will cause a decline in bacterial virulence as a trade-off for acquiring phage resistance. Characterization of this phage-host relationship will provide novel insight into the virulence mechanisms of *F. columnare* in a fish host, as well as understanding on the infection dynamics of opportunistic pathogens outside their hosts. Moreover, studies of the complex host-parasite interactions that occur in intensive farming has the potential to facilitate our understanding of emerging new diseases, and to improve disease management in aquaculture.

Materials and Methods

Bacteria and Phage

Four previously isolated *F. columnare* strains from three different fish farms located in Central and Northern Finland were used in this study (Table 1) [28]. The bacteria were originally isolated from diseased fish and tank water obtained from fish farms as part of a disease surveillance project. To obtain isolates from fish, B67 and Os06, fish were euthanized by cutting the spinal cord prior to sampling. Isolates B185 and B245 were isolated from tank water. Standard culture methods were used to isolate bacteria, and included Shieh medium [29] for B185 and B245, Shieh medium supplemented with tobramycin [30] for B67, and AO-agar [31] for Os06. Pure cultures were stored at -80°C with 10% glycerol and 10% fetal calf serum. For the analyses performed, bacterial strains were grown in Shieh medium at room temperature (RT, approximately 24°C) with constant shaking (110 rpm). The genetic groups and phage susceptibility of B67, B185, and B245 were previously determined [28], while isolate Os06 was characterized in the present study (Table 1). Genetic grouping was based on the ribosomal intergenic spacer copy and length profile, and was performed as previously described [32]. In this study, the ancestral rhizoid morphotype is abbreviated, Rz, and the phage-induced morphotype rough is abbreviated, R.

Three lytic phage used in the present study were previously isolated from tank water from two different fish farms in Central Finland (Table 1) [28]. These *F. columnare* phage are genotype-specific [28], and therefore, phage FCL-2 were used for two bacterial strains in the genetic group G (Table 1). Phage used were enriched with bacteria using the standard double layer method. Briefly, 300 µl fresh bacterial culture was mixed with 3 ml soft agar (0.7%) and 100 µl phage suspension and poured on Shieh agar plates. After 48 h incubation at RT, phage were isolated by adding 5 ml Shieh-medium on top of the agar plates that exhibited confluent lysis. The plates were shaken at 95 rpm for 24 h at 8°C, after which the phage lysates were filtered through a 0.45 µm Supor® Membrane (PALL Corporation) and stored at 4°C.

Selection for Bacterial Colony Morphotypes with Phage

Phage that were used to select for phage-resistant morphotypes are listed in the Table 1 along with the corresponding bacterial strain. Phage lysates (50 µl of approximately 10^9 to 10^{11} plaque forming units (PFU) ml^{-1}) were spread on one half of a Shieh agar plate. Fresh bacterial culture (diluted to 10^{-1} and 10^{-2}) was then spotted on both halves of the plate (in the presence and absence of phage). After 48 h, rough (R) *F. columnare* colonies that grew in the presence of phage were selected, cultured to assure the loss of the parental Rz growth, and then stored at -80°C for further analysis.

The number of phage needed to achieve growth of only rough colonies of *F. columnare*, was analysed by taking 100 colony forming units (CFU) of bacteria (400 CFU in the case of B185) and plating the bacteria on Shieh agar with and without phage (i.e. 100 µl phage suspension with known PFU ml^{-1} at three different dilutions). The phage suspension was first spread on a plate,

Table 1. The *Flavobacterium columnare* strains and phage used in this study.

Bacterial strain*	Genetic group	Location	Source	Year Isolated	Phage used
B67	A	Farm L	Fish (*Salmo trutta*)	2007	FCL-1
B245	C	Farm V	Tank water	2009	FCV-1
B185	G	Farm L	Tank water	2008	FCL-2
Os06	G	Farm O	Fish (*Salmo salar*)	2006	FCL-2

*Bacteria and phage were previously characterized (28), except for Os06 which was genetically characterized in this study.
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dried, and then inoculated with a fresh culture of *F. columnare*. Bacterial CFU was estimated from the optical density of the liquid culture.

Phage Resistance and the Presence of Phage in Rough (R) Morphotypes

Phage resistance of the rough (R) colonies that grew in the presence of phage was tested using a standard double layer method with slight modifications. Briefly, 300 µl fresh R morphotype bacterial culture was mixed with 3 ml soft agar and poured on Shieh agar plates. Five microliter aliquots from each of three dilutions of phage lysate were then spotted on the surface of the top agar. After 48 h at RT, the presence of plaques was recorded.

To determine if a phenotype change from ancestral rhizoid (Rz) to R was caused by lysogenic conversion (e.g., incorporation of phage into bacterium), the R morphotypes were tested for the release of phage into the culture medium. The lysogenic release of phage from R morphotypes into the supernatant was expected produce visible plaques on the Rz lawn. Three hundred µl of overnight grown Rz bacteria were plated with 3 ml soft agar (0.7%) on Shieh agar plates. R bacteria were centrifuged for 3 min at 13 000×g, diluted 10- and 100-fold, then 10 µl aliquots were spotted on the surface of the top agar. After 48 h at RT, the presence of plaques was recorded.

Stability of Colony Morphotypes in Serial Culture

To evaluate the stability of the colony morphotype of Rz and R bacteria, ten serial culture transfers were performed for all strains and their morphotypes. For these experiments, cultures and samplings were not replicated, since the initial bacterial strains were considered to be replicates of each other. Briefly, bacteria were cultured in Shieh broth for 22–24 h, after which 1 ml of the grown bacteria was transferred into 5 ml of fresh Shieh medium. Ten serial transfers were performed for each bacterium. From each transfer, a sample was plated onto agar plates, and the proportion of colony morphotypes present were estimated. The frequencies of the colony types observed were further analyzed using factorial analysis of variance (ANOVA), with the day of measurement, strain identity, colony class, and day*strain and strain*colony class interactions evaluated as factors. In addition, the phage resistance of a single colony of Os06 that changed colony morphotype from R to Rz was tested as described above.

Gliding Motility in Different Nutrient Conditions

Rz and R morphotypes of all bacterial strains were cultured on 2× concentrated Shieh, 1× (normal) Shieh, and 0.5× diluted Shieh agar plates. After 48 h, the colony diameter of 10 individual colonies were measured. Because the data did not fulfill the assumptions of normality, data were analysed using ANOVA on ranked data values. The sum of squares (SS) and mean squares (MS) were used to calculate test value H which was tested against chi square distribution with dfs corresponding to dfs of original treatment effects [33]. The analyses were performed using SPSS 20 (IBM).

Virulence Experiments

Fish experiments were conducted according to the Finnish Act on the Use of Animals for Experimental Purposes, under permission ESAVI-2010-05569/Ym-23 granted for L-RS by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland.

Unsexed, adult, disease-free zebra fish (*Danio rerio*) were obtained from Core Facilities (COFA) and research services of Tampere (Tampere University, Finland). These zebra fish were infected with both colony morphotypes of the four *F. columnare* strains by bathing. The infection method and bacterial dose used were developed and optimized in preliminary experiments (Kinnula et al. unpublished). The fish were individually challenged in 100 ml water with 1×10^7 CFU ml^{-1} freshly grown bacteria for 30 min at 25–26.5°C. Each infection included eight replicates and four replicates of a negative control (i.e. fish exposed to sterile Shieh medium). After each challenge, fish were transferred into separate 3 liter aquaria, with one fish per aquarium with 1 liter bore hole water. Fish were monitored for 3 d for disease symptoms and mortality. Mortality data were analyzed using Cox regression conducted by SPSS 20 (IBM).

Results

Effect of Phage on Bacterial Colony Morphotype

When cultured in the presence of phage, all bacterial strains studied here lost their rhizoid (Rz) colony morphotype. The phage resistant bacteria growing with the phage lysate had a rough (R) colony morphotype, with solid edges and small size (Figure 1). In contrast, the spontaneously formed rough morphotypes reported in previous papers [26], [27] have irregular edges with sporadic rhizoid protrusions. Moreover, these spontaneously formed rough morphotypes also exhibit gliding motility when cultured in low nutrient media (our unpublished data). For all bacterial strains evaluated, the R colonies were confirmed to be phage-resistant, and showed no signs of lysogenic release of phage into culture supernatant.

For each bacterium-phage combination assayed, approximately 100 (B67, B245, Os06) or 400 (B185) Rz colonies were observed to convert to phage-resistant R colonies over two days of culturing on agar plates at 22°C when the number of phages on each plate was 10^6 or more. To change all the 100 ancestral Rz colonies to R colonies, 7.4×10^6 PFU ml^{-1} phage FCL-1 and 6.5×10^6 PFU ml^{-1} phage FCV-1 were needed for B67 and B245, respectively. Phage FCL-2 was not as efficient in causing a

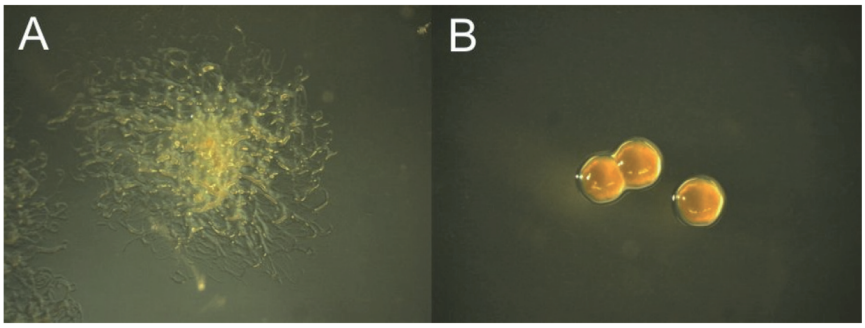

Figure 1. ***Flavobacterium columnare*** **colony morphotypes.** A) Ancestral rhizoid (Rz) colony and B) phage-resistant rough (R) colony on Shieh agar.
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morphology change, with 2×10^9 PFU ml^{-1} needed for Os06, and 3×10^9 PFU ml^{-1} was needed for B185, to obtain phage-resistant colonies. For bacterial strains, B67, B245, and Os06, colony counts on plates with and without phage were similar. However, for B185, approximately 50% fewer total colonies were observed to grow in the presence of phage compared with a parental phage-free control. This may be due to the high cost of developing phage resistance in this strain, possibly by mutation.

Gliding Motility in Different Nutrient Conditions

The colony diameter of Rz and R colonies grown on agar plates with different nutrient concentrations were measured to characterize the ability of the morphotypes to move by gliding. Overall, colony diameter was found to be significantly affected by colony morphology ($H = 129.6$, $p<0.001$) and nutrient concentration ($H = 10.6$, $p<0.001$), and in addition to main effects, also significant interaction between colony morphology and nutrient concentration was found ($H = 20.1$, $p<0.001$) (Figure 2).

Stability of Colony Morphotypes in Serial Culture

The stability of the two colony types during serial culture was found to depend on the strain identity and the strain colony morphotype (strain identity: Wald $\chi2 = 364.5$, $df = 3$, $p<0.001$; strain×colony morphotype: Wald $\chi2 = 17.5$, $df = 4$, $p = 0.002$) (Figure 3a). For example, the phage-resistant strains, B67, B185, and B245, almost completely maintained their R colony morphology during the ten serial transfers. However, among the ancestral Rz morphotype spontaneous R colonies did appear. These spontaneously formed R colonies had irregular edges with some rhizoid protrusions, thereby differentiating them from the phage-resistant R colonies. The ancestral Rz and phage-resistant R morphotypes of strain Os06 were the most unstable, as they changed morphotypes in each culture. The soft (S) morphotype in particular was observed frequently (Figure 3b). Furthermore, the Os06 R morphotype did change back to the Rz morphotype and maintained its phage resistance.

Virulence Experiments

Virulence of the ancestral Rz morphotype was compared to the phage-resistant R type using a zebrafish infection model. The Rz morphotype was associated with a significantly higher mortality rate than the phage-resistant R morphotype (Cox regression, Wald = 27.4, df = 1, $p<0.001$, Table 2). Moreover, in most cases, the R morphotype bacteria were completely non-virulent, but experiments involving B185 R did result in the death of one fish. The virulence of the bacterial strains also significantly differed from each other (Cox regression, Wald = 13.1, df = 1, $p<0.001$). For example, the Rz morphotype of B185 and B67 were the most virulent, and resulted in the death of all eight fish tested within 24 h. In contrast, experiments with B245 resulted in the death of 4/8 fish, and for Os06 2/8 fish died (Table 2). The fish that died showed no obvious external signs of columnaris disease, indicating that the rapid progression of the disease can cause a symptomless death, possibly by blocking the gill surfaces. The remaining fish did not develop any disease symptoms during the three day post-infection monitoring.

Discussion

Antagonistic co-evolution between a bacterial host and its parasitic phage can represent a selective pressure for bacterial virulence. In this study, the ability of specialized parasitic phage to modulate the virulence of opportunistic pathogen outside its vertebrate host was investigated by exposing four strains of the opportunistic fish pathogen, *Flavobacterium columnare*, to corresponding lytic phage. When bacteria manifesting an ancestral rhizoid (Rz) morphotype were cultured in the presence of lytic phage, the colony morphotype changed to rough (R), and was accompanied by a loss of virulence and gliding motility. These results demonstrate that phage can cause phenotypic changes in *F. columnare* outside of the fish host, and thus, modulate its virulence.

While there are various mechanisms that can select for high virulence of pathogens, including within-host strain competition [34] and formation of persistent spores [35], opportunistic pathogens diverge fundamentally from the assumptions of epidemiological models. Opportunistic pathogenic bacteria are exposed to different environments during their life cycle, and they can have different strategies for living inside and outside a host [36]. Outside of a host, bacteria can be exposed to predators and parasitic phage, and they also need to compete for resources. On the other hand, within host growth requires traits for overcoming the immune defense of a multicellular host while growing otherwise in an enemy-free, high-resource host tissue. These conditions can either decrease or increase infectivity. Furthermore, opportunistic saprotrophic bacteria can reproduce and transmit from dead hosts, and can actively grow outside of a host as part of

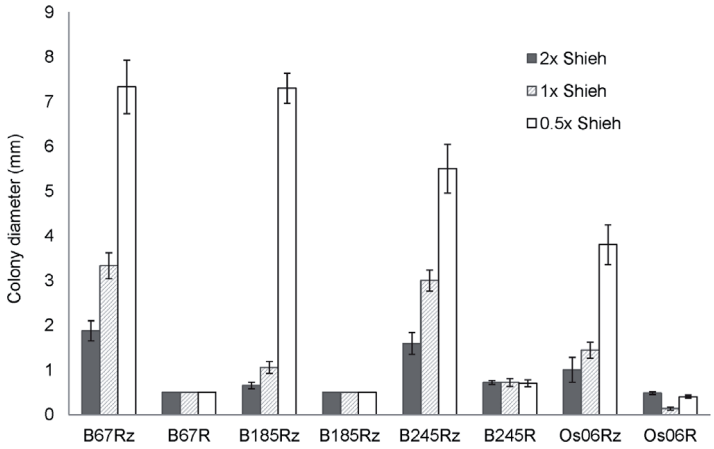

Figure 2. Gliding motility of colony morphotypes with different nutrient concentrations. Colony diameter (mm) was used to indicate the capacity for gliding motility of the *Flavobacterium columnare* rhizoid (Rz) and rough (R) colony morphotypes under different nutrient conditions. Motility is presented as the mean colony diameter (mm±SE) obtained from ten colonies grown for 48 h on 2× concentrated, normal (1×), and diluted (0.5×) Shieh agar. Colony diameter was found to be significantly affected by colony morphology ($H = 129.6$, $p<0.001$), and by nutrient concentration ($H = 10.6$, $p<0.001$, colony*nutrient interaction $H = 20.1$, $p<0.001$).
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the normal aquatic food web. Indeed, *F. columnare* transmits most efficiently from dead hosts (making high virulence traits beneficial), and can survive for long periods (at least five months) in water outside of a host [22], [23]. These traits may explain why columnaris disease has become problematic in fish farms [3]. However, the impact of the selective pressures generated by the environment outside of a host on the virulence of *F. columnare* has not yet been elucidated.

Based on the dramatic loss of virulence associated with phage-resistant *F. columnare* exhibiting a rough colony morphotype, we hypothesize that virulence is related to gliding motility in this bacterium. Accordingly, motility and virulence have been found to be connected in many bacteria [37], [38]. However, unlike many other bacteria, *F. columnare* does not have flagella or pili. Rather, it moves on surfaces by gliding, a motility characteristic associated with flavobacteria. Moreover, the flavobacterial gliding motility system has been shown to be orthologous to the Por secretion system (PorSS) of virulence factors in *Porphyromonas gingivalis* [39]. The corresponding genes needed for gliding/PorSS also exist in *F. columnare* [40], thereby suggesting that the motility apparatus may play a role in the secretion of virulence factors also in this species. This hypothesis would also be consistent with the motility-dependent virulence of this bacterium [26], [27]. Phage may reduce bacterial motility by using the bacterial motility apparatus as a receptor [41]–[43]. Therefore, to resist phage infection, bacteria may mutate, or down-regulate, the expression of the motility apparatus to impair motility (and virulence as well if it is coupled with motility). In other bacterial species, the negative effect of phage resistance on bacterial virulence outside a host has been observed [16]–[18]. In contrast, traditionally in the phage-host literature, more emphasis has been given to the capacity for phage to provide new genetic material and virulence factors for a host bacterium, thus increasing the fitness of the host bacterium [44], [21]. Phage resistance may, however, have trade-offs with respect to bacterial growth [18], and this may be true for *F. columnare* as well (Zhang et al., unpublished). Therefore, it is essential to recognize that phage can have both negative and positive effects on the virulence traits of their hosts, with the former highlighted in this study.

In addition to changes in colony morphotype selected by the presence of phage, spontaneous morphology changes from Rz to R in serial cultures of *F. columnare* were also detected, a phenomenon that has been previously reported [26], [27], [45], [46]. However, the appearance and biological significance of spontaneous R morphotypes are different than those of phage-resistant R types. For example, spontaneously formed R colonies appear during serial culture in the laboratory, they often exhibit weakly rhizoid or irregular edges, and they retain their gliding motility in low nutrient conditions. In contrast, phage-resistant R colonies are small with solid edges. The number of Rz morphotype colonies has a tendency to decrease in the bacterial population during culture (see Figure 3), indicating that colony morphotype is probably regulated by gene expression and phenotypic changes in response to environmental conditions. However, in the three virulent bacterial strains studied (e.g., B67, B185, and B245), the Rz morphotype was maintained at higher numbers during serial culture, suggesting that selection for the virulent Rz phenotype in these strains is strong, even under laboratory conditions. Also, the phage resistant R morphotypes of the same strains were stable, indicating that phage resistance may involve genetic effects (e.g., mutation of the phage receptor), which are not rapidly reversed under laboratory conditions, especially if phage cause a strong selective pressure as in case of B185 and FCV-2. Altogether, the general instability of the bacterial colony morphotype seems to be limited to certain strains (e.g., Os06), possibly to allow these bacteria to rapidly switch between phenotypes as necessary [47], [48]. This would increase the chances of survival in an unpredictable environment, rather than having changes in

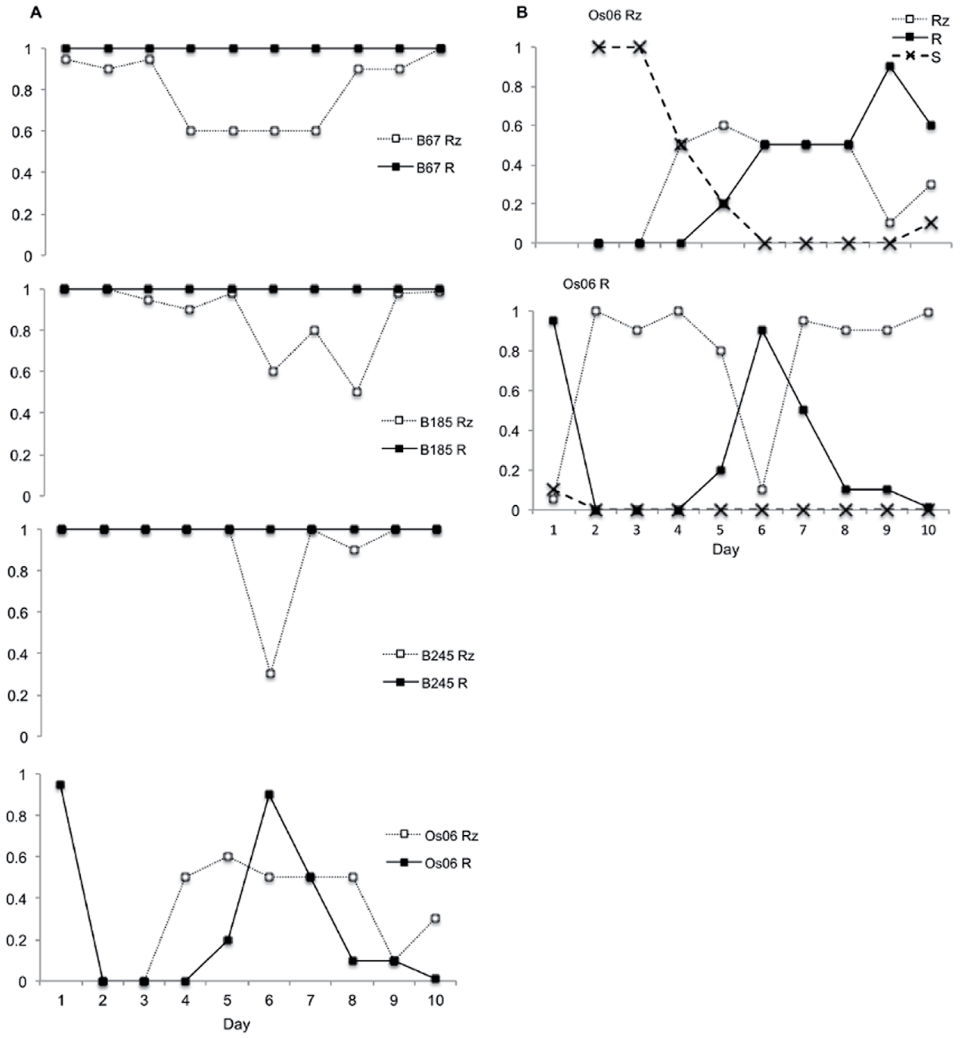

Figure 3. Proportion of *Flavobacterium columnare* colony morphotypes in serial culture. The proportion of colony morphologies (Rz = rhizoid, R = rough, and S = soft) in serial cultures of ancestral rhizoid and phage-resistant rough morphotypes of four *F. columnare* strains were estimated from pure cultures taken from each passage in Shieh broth. ND = not determined, but the proportion of Rz colonies was >50%. A) The proportion of ancestral Rz and phage-resistant R morphotypes that maintained their morphotype in serial culture, B) The fluctuation in the proportion of different morphotypes detected in Rz and R cultures of strain Os06.
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phenotype/expression induced by environmental cues. Moreover, rapid shuffling between morphotypes in a low-virulence strain may be less costly than shuffling in more virulent strains, as less virulent bacteria (e.g., Os06) may not suffer from a loss of motility since they may not always be dependent on fish hosts for their survival. Furthermore, it can be assumed that less virulent strains living outside of a host are more frequently exposed to trophic interactions, and therefore the ability to change morphotype rapidly is beneficial for avoiding enemies and adapting to changing

Table 2. Zebrafish mortality caused by *Flavobacterium columnare* strains with ancestral rhizoid (Rz) versus phage-induced rough (R) morphotypes.

	Fish mortality[a] caused by morphotypes	
Strain	**Ancestral Rz**	**Phage-resistant R**
B67	100%	0%
B185	100%	12.5%
B245	50%	0%
Os06	25%	0%

[a]Zebrafish (n = 8) were individually tested for each morphotype.
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environmental conditions [22]. The history of *F. columnare* as an environmental bacterium is consistent with these hypotheses.

The observation that phage resistance correlates with loss of virulence is especially important when designing novel treatment methods for intensive farming. Columnaris disease is an economically significant fish disease in freshwater aquaculture around the world. In addition, columnaris disease is an epidermal disease and transmits via water. Therefore, based on previous studies that have shown phage therapy to be successful for other fish diseases [49]–[51], and the potential for phage to be applied directly to tank water, we hypothesize that phage represent a valuable disease targeting strategy. Moreover, if phage use the gliding motility apparatus of *F. columnare* as a receptor, as suggested by the results of the present study, phage-host interactions would be mediated at the surface of the skin and gills of infected fish (or in the biofilms of the fish tank). Therefore, as fingerling fish are reared in relatively small water volumes, the amount of phage needed for treatments would be at a manageable level. In addition, the development of phage resistance would not hamper the success of the therapy, if the bacteria maintain their R morphotype as efficiently in the fish farming environments as they did in the laboratory. Additional studies will be needed to confirm these hypotheses, and to further elucidate the details of phage-host interactions.

In this study, zebrafish was used as the model organism rather than rainbow trout or other salmonids, which are common hosts of columnaris disease at fish farms. Therefore, it can be argued that the use of a model organism that is not the natural host of the studied disease could produce different outcomes [52]. However, *F. columnare* has a wide host range, and correspondingly, has been isolated from European graylings (*Thymallus thymallus*), whitefish (*Coregonus lavaretus*), bream (*Ambramis brama*), pikeperch (*Zander lucioperca*), and perch (*Perca fluviatilis*) in Fennoscandia [22], [32]. Furthermore, the use of zebrafish for *F. columnare* infections has previously been described [53], and our unpublished studies suggest that zebrafish can be used as a reliable model for studying columnaris disease instead of rainbow trout (Kinnula et al., unpublished). There are other advantages to using zebrafish as well. Zebrafish is established as model organisms for a wide range of experimental studies, disease-free fish are available year round, and they tolerate laboratory conditions better than rainbow trout. However, it will be important to confirm the phage-host interactions identified in the present study in salmonids, especially for the development of therapeutic applications.

To conclude, parasitic phage of *F. columnare* can select against bacterial virulence and motility, as indicated by colony morphology, in an environment outside a fish host. By studying phage-driven selection pressures and their impact on bacterial virulence, we have the opportunity to gain insight into the virulence factors of *F. columnare* and understand how its virulence evolves on both ecological and molecular scales. To our knowledge, this study represents the first report of the effects of phage-host interactions on a commercially important fish pathogen where phage resistance directly correlates with a decline in bacterial virulence. The phage-driven loss of virulence observed also supports the hypothesis that antagonistic co-evolution can reduce the virulence of opportunistic pathogens outside of a host due to the associated costs of defending against parasitic or predatory enemies [16]. Our results also indicate that further characterization of phage-host interactions is needed, particularly in regard to impact of phage on the evolution of virulence in opportunistic pathogens that affect intensive farming, and to develop applications for disease control.

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Author Contributions

Conceived and designed the experiments: EL JKHB LRS. Performed the experiments: EL LRS. Analyzed the data: EL JKHB JL LRS. Wrote the paper: EL JKHB JL LRS.

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III

COMPARING THE DIFFERENT MORPHOTYPES OF A FISH PATHOGEN – IMPLICATIONS FOR KEY VIRULENCE FACTORS IN *FLAVOBACTERIUM COLUMNARE*

by

Elina Laanto, Reetta K. Penttinen, Jaana K.H. Bamford & Lotta-Riina Sundberg
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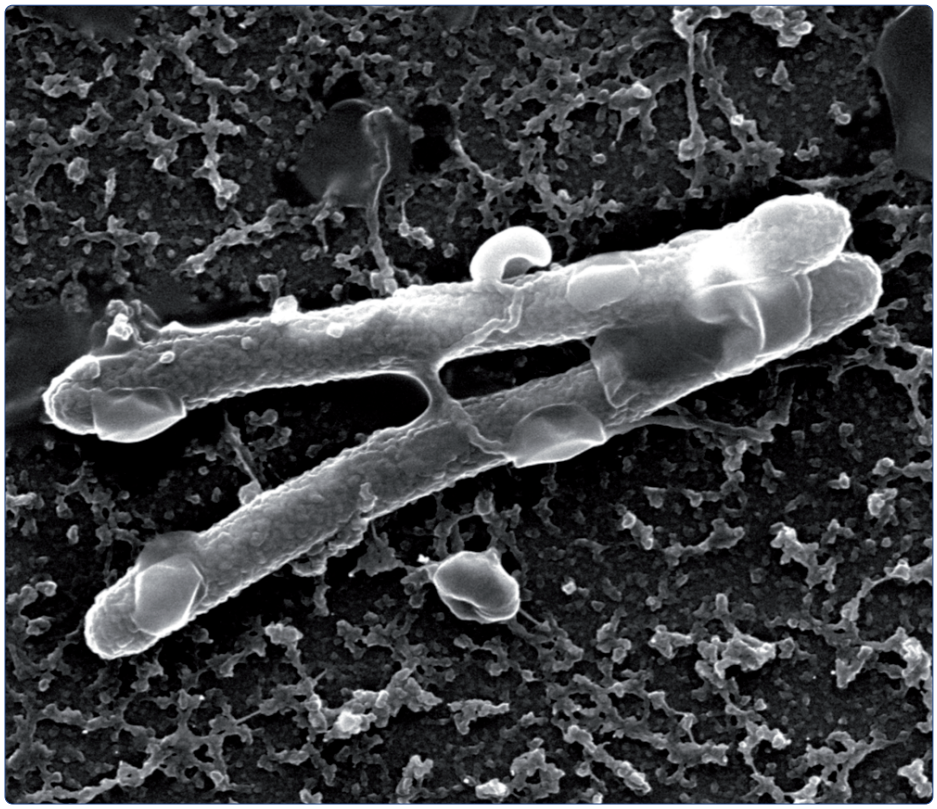

Comparing the different morphotypes of a fish pathogen - implications for key virulence factors in *Flavobacterium columnare*

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Comparing the different morphotypes of a fish pathogen - implications for key virulence factors in *Flavobacterium columnare*

Elina Laanto*, Reetta K Penttinen, Jaana KH Bamford and Lotta-Riina Sundberg

Abstract

Background: *Flavobacterium columnare* (Bacteroidetes) is the causative agent of columnaris disease in farmed freshwater fish around the world. The bacterium forms three colony morphotypes (Rhizoid, Rough and Soft), but the differences of the morphotypes are poorly known. We studied the virulence of the morphotypes produced by *F. columnare* strain B067 in rainbow trout (*Onconrhynchus mykiss*) and used high-resolution scanning electron microscopy to identify the fine structures of the cells grown in liquid and on agar. We also analysed the proteins secreted extracellularly and in membrane vesicles to identify possible virulence factors.

Results: Only the Rhizoid morphotype was virulent in rainbow trout. Under electron microscopy, the cells of Rhizoid and Soft morphotypes were observed to display an organised structure within the colony, whereas in the Rough type this internal organisation was absent. Planktonic cells of the Rhizoid and Rough morphotypes produced large membrane vesicles that were not seen on the cells of the Soft morphotype. The vesicles were purified and analysed. Two proteins with predicted functions were identified, OmpA and SprF. Furthermore, the Rhizoid morphotype secreted a notable amount of a small, unidentified 13 kDa protein absent in the Rough and Soft morphotypes, indicating an association with bacterial virulence.

Conclusions: Our results suggest three factors that are associated with the virulence of *F. columnare*: the coordinated organisation of cells, a secreted protein and outer membrane vesicles. The internal organisation of the cells within a colony may be associated with bacterial gliding motility, which has been suggested to be connected with virulence in *F. columnare*. The function of the secreted 13 kDa protein by the cells of the virulent morphotype cells remains unknown. The membrane vesicles might be connected with the adhesion of cells to the surfaces and could also carry potential virulence factors. Indeed, OmpA is a virulence factor in several bacterial pathogens, often linked with adhesion and invasion, and SprF is a protein connected with gliding motility and the protein secretion of flavobacteria.

Background

Understanding the behaviour of pathogenic bacteria is a key component in elucidating host-pathogen interactions. The visualisation of the physical characteristics of bacteria, detailing the organisation and interactions between cells in different culture conditions, can provide new insights into the ecology of diseases and reveal why some bacteria are more difficult to eradicate than others. Indeed, bacterial cells often have structures that facilitate surface adhesion, biofilm formation and cell-cell interactions [1-3].

The ubiquitous ability of bacteria to form biofilms can influence virulence and promote persistent infections [4-6]. Bacteria in the biofilm are covered by an extracellular polymeric substance (EPS) layer that protects the cells from hostile environmental factors [7]. The EPS layer is comprised of a complex mixture of proteins, DNA and other materials, like outer membrane vesicles (OMVs). OMVs are abundant in the extracellular material of many gram-negative bacteria, including *Helicobacter pylori*, *Myxococcus xanthus* and *Pseudomonas aeruginosa* [8-10], and they are often associated with virulence. OMVs have also been detected in the fish pathogens *Flavobacterium*

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psychrophilum and *Flavobacterium columnare* [11,12]. A proteome analysis from the extracellular matrix proteins of *P. aeruginosa* PAO1 revealed that the OMVs constituted a large amount of the proteins in the biofilm [13]. The same study compared the proteomes of the OMVs from planktonic cells and cells in biofilm, which were observed to differ substantially. The planktonic OMVs of *P. aeruginosa* contained virulence factors such as LasA protease precursor, elastase LasB and alkaline protease whereas these were missing from the biofilm OMVs, indicating that planktonic cells may be important mediators of disease [13]. The role of OMVs has also been studied extensively in many other pathogenic bacteria, and there is no doubt of their significant role in the virulence of bacterial pathogens [14].

F. columnare, a member of Bacteroidetes, is a major bacterial pathogen of farmed freshwater fish around the world [15,16]. During the warm water period, the bacterium can be isolated from nature and fish tanks, both from biofilms and free water [17]. It is known that *F. columnare* can survive outside the fish host for long periods [18] and may respond to stressful conditions by entering into a viable but non-dividing state [12]. However, the infection mechanisms in this fish pathogen are still largely unknown.

We have previously observed that in the laboratory *F. columnare* can be induced to form different colony morphotypes by exposure to phage infection, starvation and serial culture [18,19]. Only the ancestral Rhizoid type has been shown to be virulent in fish, in which the derivative Rough and Soft types are non-virulent [18-20]. Therefore, identification of the structures and cell organisation of these virulent and non-virulent types can provide valuable information on how bacteria behave outside the host and offer clues about the possible virulence mechanisms. In this study we used high-resolution scanning electron microscopy (HR-SEM) to observe the cell organisation architecture and identify cell surface structures in both virulent (Rhizoid) and its derivative non-virulent (Rough and Soft) morphotypes of *F. columnare* strain B067 under different culture conditions. The parental Rhizoid type was originally isolated from a diseased trout (*Salmo trutta*), the Rough type was obtained by phage selection [19] and the Soft type appeared spontaneously during culture. Of the morphotypes, Rhizoid and Soft are able to form spreading colonies on agar [19, Sundberg et al., unpublished observations], which often indicates the ability for gliding motility, but may not always be in direct association [21]. As previous electron microscopy studies on *F. columnare* are scarce [12,22-25], information is needed on how bacterial cells with different levels of virulence interact with each other and with surfaces. Our aim is to discover the connections between bacterial cell characteristics and virulence.

Results

Virulence of the different colony morphotypes

Rainbow trout fry were exposed to Rhizoid, Rough and Soft morphotypes of *F. columnare* in a bath challenge, and the signs of disease and morbidity were recorded. Bacteria of the different colony types caused significant differences in fish survival (Kaplan-Meier survival analysis, $\chi^2 = 12.007$, df = 3, p = 0.007). The survival of the fish infected with the Rhizoid type was significantly lower than those infected with the Rough or Soft types (p-values, compared to Rhizoid, 0.008 and 0.036, respectively). The outcomes of the infections with Rough and Soft types were comparable to those of the negative control (p-value 0.032 for Rhizoid vs. negative control) (Figure 1).

Surface structure of the colonies

To observe the surface structures of the bacterial colonies formed by the three different morphotypes, the colonies were grown on a filter paper and visualised under SEM. Biofilms of Rhizoid and Rough morphotypes were covered by a thick layer of extracellular filamentous material that was absent in the biofilm of the Soft morphotype (Figure 2 and Additional file 1). However, the layer covering the biofilm of the Rhizoid morphotype was not as complete as in the Rough morphotype, as cells were seen underneath (Figure 2A). In the Rhizoid and Soft morphotypes, the bacterial cells were accompanied by large vesicles with widely ranging sizes (up to 1.5 μm in diameter) (Figures 2C and D). Neither vesicles nor cells were seen underneath the thick extracellular material layer of the biofilm of the Rough morphotype (Additional file 1). Typical for the colony of the Soft morphotype were the wave-like arrangements formed by the cells with deep pores in regular intervals (Figure 2B).

Internal structure of the colony types

The cell organisation and internal structure of the colonies of the different morphotypes grown between a glass slide and a Shieh agar plate were visualised under SEM (Figure 3 and Figures 4D-F). Cells of the virulent Rhizoid morphotype formed organised structures on the glass slide, with characteristics of coordinated social movement (Figure 3A). The bacteria were attached to the surface and to each other by numerous thin fimbriae-like strings (Figures 3a and 4D). Cells in the colony of the non-virulent Rough morphotype were randomly scattered on the glass surface without any organised population structure, in contrast to that observed in the virulent Rhizoid type (Figure 3B). Cells of the Rough morphotype also exhibited slightly thicker fimbriae than the Rhizoid type that did not appear regularly on the cell surfaces (Figures 3a and b). Membrane vesicles were observed on the surface of both Rhizoid and Rough morphotypes (Figures 3a and b). Numerous vesicles of different sizes were detached from

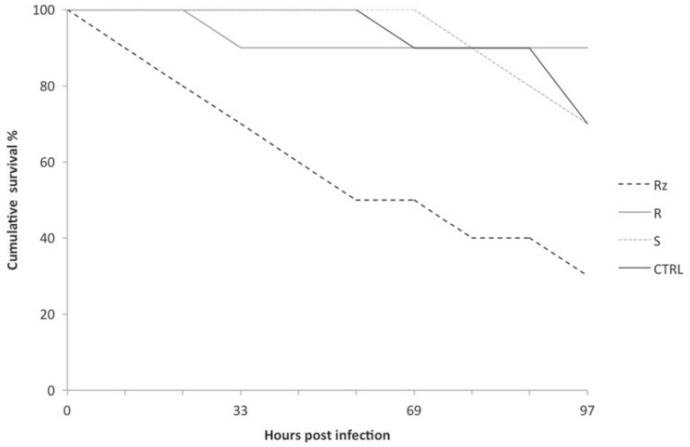

Figure 1 Virulence of different morphotypes of *F. columnare*. Rainbow trout (*Oncorhynchus mykiss*) survival after challenge with Rhizoid, Rough and Soft morphotypes of *F. columnare*.

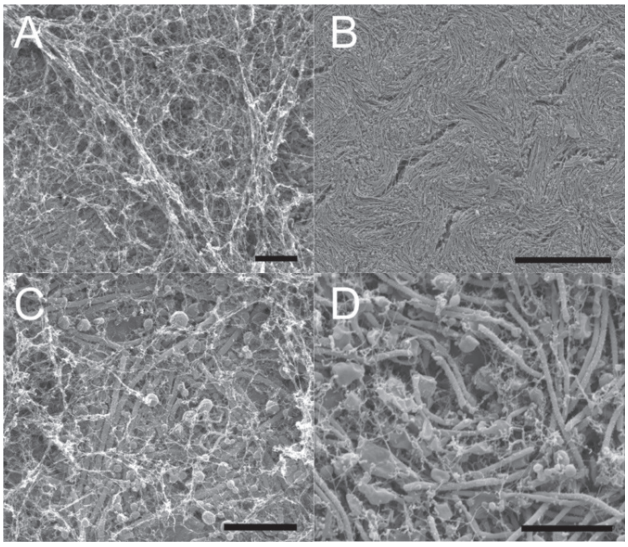

Figure 2 Colony surfaces of the Rhizoid and Soft morphotypes of *F. columnare* B067. The cells were grown on a filter paper on top of a Shieh agar plate and were visualised under HR-SEM. **Panel A**: Biofilm of the Rhizoid morphotype covered with an extracellular layer. **Panel C**: A closer view of a typical area where the surface layer is thin or missing, and large vesicles are visible (size approximately 1 μm). **Panels B and D**: Views of the colony/biofilm surface of the Soft morphotype where the fibrous layer found in other morphotypes was not detected. For the surface of the Rough morphotype, see Additional file 1. Notice the abundance of large vesicles in the Soft morphotype. Scale bar in A is 10 μm, in B, 40 μm, and in C and D, 4 μm.

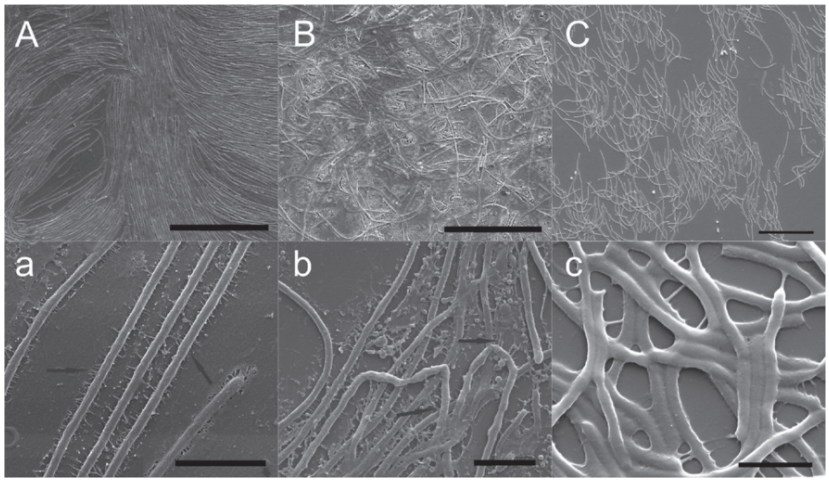

Figure 3 Internal structure of colonies formed by Rhizoid, Rough and Soft morphotypes of *F. columnare* B067. *F. columnare* cells were grown between a glass slide and Shieh agar and visualised under HR-SEM. **Panel A**: Rhizoid morphotype, **Panel B**: Rough morphotype and **Panel C**: Soft morphotype. A closer view of the cells can be seen underneath each panel marked with a matching lower case letter. Short filaments attaching cells to the surface and connecting cells are indicated by arrows in **3a** and **3b**. Scale bars in panels **A** to **C** 20 μm and in **panels a to c** 2 μm.

and scattered around the cells. Smaller vesicles (approximately 200 nm) formed vesicle chains, middle size vesicles (approximately 500 nm to 1 μm in size) were also abundant and a few larger vesicles (approximately 1,5 μm) were seen (Figures 4E and F). Furthermore, the shape of the Rough type cells was uneven compared to Rhizoid type cells. The non-virulent Soft morphotype cells formed wave-like 'dunes' on the glass (Figure 3C). Fimbriae connecting the bacteria to the glass were observed, and the cells appeared to be attached to the surface more along their length, but the vesicles were absent from the cells of the Soft morphotype (Figure 3c).

Planktonic cells of the colony types

Liquid bacterial cultures were visualised on Concanavalin A (ConA) plates under SEM. Large surface-associated vesicles were seen on cells of the Rhizoid and Rough morphotypes, but not on those of the Soft type (Figures 5, 4A and C). Individual cells had several vesicles that were spread evenly across the cell length. The surface of the vesicles was smoother than the surface of the bacterial cell, indicating that the membrane of the vesicles may be lipid, which was confirmed by transmission electron microscopy (TEM) analysis (see later). Also, in the Rhizoid and Rough morphotypes the bacteria produced thick rope or pearl chain -like structures to attach to each other and to the surface (Figures 4A and B). The liquid cultures were observed to contain aggregates (Figure 5 and Additional file 2) that were designated to originate from the growth medium according to the control sample containing only Shieh medium (Additional file 3C). A wider view of the typical samples of planktonic cells visualised under SEM is provided in Additional file 2. TEM analysis did not reveal a difference between the OMVs in the Rhizoid and Rough morphotypes in liquid culture, which is consistent with the results received by SEM. Vesicles with a bilayer and clustered electron-dense material were seen on the surface of both morphotypes, but their size was less than in the SEM analysis (average 50 nm under TEM vs. 100–500 nm under SEM) (Figure 6A).

Differences in the extracellularly secreted protein profiles by the colony types

The proteins concentrated from the supernatant of 18-hour cultures of the three morphotypes were analysed on Tricine-SDS-PAGE (Figure 7A). A notable amount of a small protein was present in the profile of the Rhizoid morphotype that was found missing or in very low amounts in the Rough and Soft morphotypes (MW approximately 13 kDa) (data for the Soft morphotype not shown). The protein was identified using nanoLC-ESI-MS/MS as a hypothetical protein FCOL_04265 in *F. columnare* ATCC

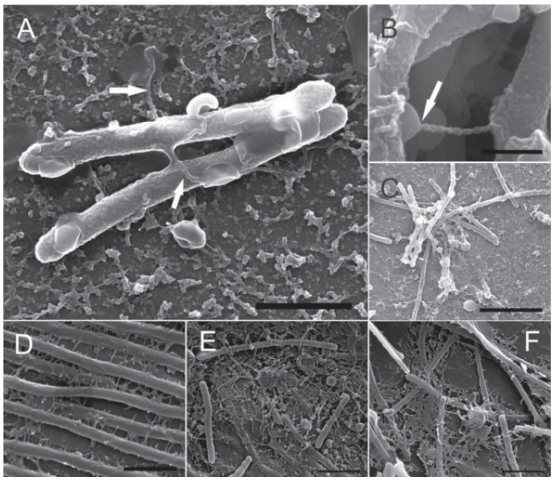

Figure 4 Surface structures and vesicles observed in *F. columnare* B067 cultured in liquid and on agar. Panels A to C represent the planktonic cells, and **panels D to F** show cells grown on agar. **Panel A**: A typical view of the planktonic cells of the Rhizoid morphotype. Vesicles and rope-like structures attaching vesicles to the cells (arrows) were abundant. **Panel B**: A closer view of the rope-like structure connecting a vesicle (arrow) and a cell from the Rough morphotype. **Panel C**: Typical clusters of cells with vesicles and long filaments in Rough morphotype. **Panel D**: An example of the short filaments on the cells of the Rhizoid morphotype. **Panels E and F**: cells and vesicles in a colony of the Rough morphotype. The scale bar in **Panel A** was 1 μm, in **B**, 500 nm, in **C**, 4 μm, in **D**, 1 μm, and in **Panels E and F**, 2 μm.

49512 (Table 1), but the function of the protein is unknown. Furthermore, this protein is specific for *F. columnare* and is not present in its close relatives, *F. psychrophilum* and *F. johnsoniae*.

Characterisation of OMVs

The OMVs of the Rhizoid morphotype were isolated and purified. The purification was done by density gradient centrifugation and resulted in three light-scattering bands. The bands were pelleted and visualised under TEM, which revealed the different sizes of the purified vesicles, ranging from approximately 60 nm to 350 nm (Figure 6B). These vesicles were run on a 14% Tricine SDS-PAGE (Figure 7B) and compared to the outer membrane protein profile of the Rhizoid morphotype without any notable additional bands (Figure 7C). Five protein bands from vesicle profile were commercially analysed in more detail by nanoLC-ESI-MS/MS. One protein band was identified as the OmpA outer membrane P60 of the *F. columnare* strain ATCC 49512 (Table 1). Others were identified as hypothetical proteins of the same bacterium. The resulting proteins were compared to database sequences using the BLAST algorithm and according to their match to the *F. columnare* ATCC 49512 genome. One of the identified proteins with an unknown function recorded a hit for flavobacterial gliding motility protein SprF of *F. psychrophilum* and *F. johnsoniae,* and based on amino acid similarity, it was designated as SprF (Table 1).

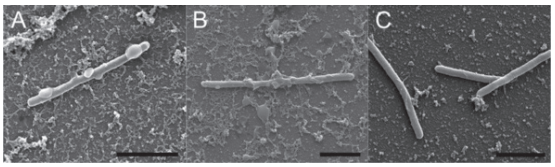

Figure 5 Planktonic cells of Rhizoid, Rough and Soft morphotypes of *F. columnare* B067. A typical view of the cells grown in liquid medium visualised under HR-SEM of the three morphotypes. Large vesicles were typical on the surface and surroundings of the **A)** Rhizoid and **B)** Rough morphotypes. **Panel C**: On the surface of the cells of the Soft morphotype, only small blebs were seen. The scale bar was 2 μm.

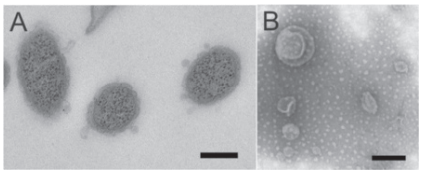

Figure 6 OMVs of Rhizoid morphotype *of F. columnare* B067. **Panel A**: Outer membrane vesicles visualised under TEM from the thin sections of the cells of the *F. columnare*; and **Panel B**: Purified vesicles under TEM. In the thin sections, the size of the observed vesicles was under 100 nm, whereas the purified vesicles ranged from 60 to 350 nm. The scale bar was 200 nm.

Discussion

Genetic properties and whole cell protein profiles of the different colony morphologies of *F. columnare* have been studied previously, but no differences have been detected [18]. We used HR-SEM to study parental virulent (Rhizoid) and its derivative non-virulent (Rough and Soft) morphotypes of the same bacterial strain in different culture conditions, and also compared their extracellular protein profiles. We found clear differences in the cell organisation, cell surface structures and extracellular protein profiles between the virulent and non-virulent morphotypes and suggested new factors that are potentially connected to the virulence of *F. columnare*. Virulence of the Rhizoid type was clearly high in rainbow trout fry, whereas the Rough and Soft types produced mortality rates comparable to the control treatment (Figure 1). The virulent Rhizoid type secreted a high amount of a small (approximately 13 kDa) protein, whose function is unclear, but which is not found in other bacterial species. Furthermore, our experiments revealed OMVs with variable sizes in the Rhizoid and Rough morphotypes. The vesicles were found to contain proteins with unknown functions and a OmpA-family protein, which is associated with virulence in other bacterial pathogens (see later).

Biofilms are important reservoirs of bacteria in nature [26]. Therefore, it is important to understand how bacteria form and interact within biofilms. We visualised both the surface and internal structures of the bacterial biofilm of different colony types of *F. columnare* grown on agar. We found that the virulent Rhizoid morphotype produced an organised biofilm within the colony with indications of social movement, whereas in the phage-resistant Rough morphotype this behaviour was absent, and the cells were randomly scattered (Figure 3). Also, according to the surface view, the Soft type colony had an organised structure (Figure 2). This was not clear when the internal structure of the colony was studied, which is probably due to the fact that cells of the Soft type are not adherent and are therefore unable to stay fully attached to the visualised glass slide. As both

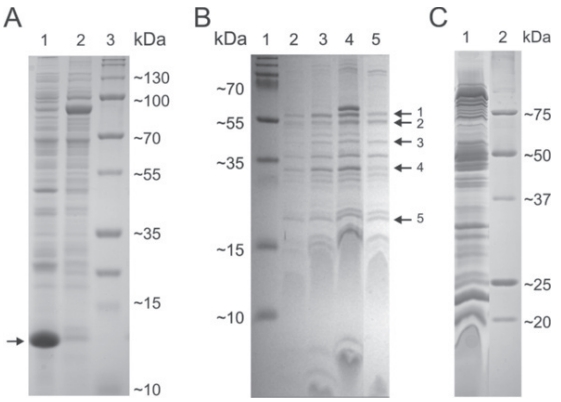

Figure 7 Protein profiles of *F. columnare* B067 morphotypes. Panel A: Extracellular protein profiles of the Rhizoid (Lane 1) and Rough (Lane 2) morphotype of the *F. columnare* strain B067 run in a 14% Tricine SDS-PAGE -gel. The protein band that was analysed further by nanoLC-ESI-MS/MS is indicated by an arrow. PageRuler Plus Prestained Protein Ladder (Thermo Scientific) MW marker was used for Lane 3. **Panel B**: OMV fractions from the 20–45% OptiPrep gradient run in a 14% Tricine SDS-PAGE gel. OMVs were purified from both Rhizoid and Rough morphotypes. Lane 1: PageRuler Plus Prestained Protein Ladder (Thermo Scientific) MW marker. Lanes 2 to 4: Rhizoid morphotype, three light-scattering fractions. Lane 5: Rough morphotype, middle fraction. Arrows indicate the protein bands that were further analysed from the middle fraction of the Rhizoid morphotype by nanoLC-ESI-MS/MS. **Panel C**: Outer membrane protein profile of the Rhizoid morphotype (Lane 1) and Precision Plus Protein™ Standard (BioRad) MW marker (Lane 2) run in a 16% SDS-PAGE.

Table 1 Identified proteins

Band name	Size (kDa) on gel	Protein identification by nanoLC-ESI-MS/MS	Match to ORF [Accession number in NCBI]	Best hit in BLAST: identity % (query cover %)	Predicted size (kDa)
1	≈ 55	Hypothetical protein	FCOL_07765 [YP_004942163.1]	FCOL_11410 47% (100%)	53.5
2	≈ 55	OmpA family outer membrane protein P60	FCOL_09105 [YP_004942423.1]	FP0156 Outer membrane protein precursor; OmpA family P60 [*F. psychrophilum* JIP02/86] 70% (100%)	50.2
3	45	Hypothetical protein	FCOL_02860 [YP_004941210.1]	FP1486 Protein of unknown function [*F. psychrophilum* JIP02/86] 55% (98%)	44.5
4	35	Hypothetical protein	FCOL_08865 [YP_004942378.1]	FP0017 Putative cell surface protein precursor SprF [*F. psychrophilum* JIP02/86] 68% (94%)	37.9
5	20	Hypothetical protein	FCOL_11765 [YP_004942947.1]	FP2260 Protein of unknown function precursor [*F. psychrophilum* JIP02/86] 48% (100%)	24.4
ECP	13	Hypothetical protein	FCOL_04265 [YP_004941480.1]	-	18,1

Protein identification of the membrane vesicle contents and the extracellular protein of *Flavobacterium columnare* B067 by nanoLC-ESI-MS/MS, and the subsequent identification by BLAST search. ORF annotation refers to *F. columnare* ATCC 49512 complete genome [NCBI: NC_016510].

Rhizoid and Soft types can form spreading colonies on agar, it is possible that the organisation of cells within the colony is associated with gliding motility. When the surface of the colonies was studied, the Rhizoid and Rough morphotype cells were observed to be covered by a fibrous extracellular layer that was missing in the non-virulent Soft morphotype (Figure 2). The fibrous surface layer may protect the bacteria from environmental stressors, such as protozoan grazing [4], but it may also be connected to the strong adherence of the colonies on agar. The Soft type, missing this fibrous layer, is indeed non-adherent, compared to the Rhizoid and Rough types [20].

Extracellularly secreted proteins have been suggested to be important for virulence in *F. columnare* [27,28]. In the current study, the comparison of extracellular protein profiles revealed a major difference between the virulent and non-virulent morphotypes. A notable amount of a small protein (MW ~ 13 kDa) was present in the protein profile of the Rhizoid type that was absent or present only in small quantities in the Rough and Soft types. The protein was designated as a hypothetical protein of *F. columnare*, but no function for the protein was identified. We have also observed this protein in the Rhizoid morphotypes of two other virulent *F. columnare* strains (unpublished), and in minor quantities in the non-virulent Rough morphotypes of these strains. Due to its association with the Rhizoid colony type, we suggest that it could have a role in the virulence of *F. columnare*. However, the exact function of this protein requires future elucidation.

In previous studies on *F. columnare*, evidence has been found for narrow extensions and slender projections from the outer membranes of the cells [22-24]. Furthermore, small membrane vesicles and extracellular polysaccharide substances were observed in recent studies [12,25], but their role has not been confirmed, although it has been observed that *F. columnare* can rapidly adhere to and colonise surfaces and initiate biofilm formation [25]. OMVs are described in a majority of gram-negative bacteria, and they play a significant role in the virulence of bacteria [14]. Vesicles can contain toxins or adhesins that are delivered directly into the host cells [14,29-31]. Moreover, OMVs are a functional part of natural biofilms, having proteolytic activity and binding antibiotics, such as gentamycin [10]. Generally, the size of an OMV ranges between 50 and 250 nm [29]. We observed two kinds of membrane vesicles in *F. columnare* grown in liquid. Under SEM, large (100–500 nm) vesicles were abundant on the surface of the Rhizoid and Rough (but not Soft) bacteria, as well as smaller (approximately less than 100 nm) surface vesicles, which also formed chain-like structures between individual bacterial cells. When thin-sectioned cells were visualised under TEM, the vesicles were observed to have a lipid bilayer, but the size was approximately 50 nm. TEM analysis of the purified vesicles revealed vesicles ranging in size from 60 to 350 nm. The reason for the absence of the large vesicles in the thin-sectioned samples is unclear, but it could be due to the sampling process. SEM analysis suggests that the large vesicles may be connected to the surface adhesion of the bacteria. The bacteria have several vesicles on their surface, which seem to erupt by contact, anchoring the bacteria to the surrounding surface. This result was supported by an analysis of vesicle contents, where the OmpA family outer membrane protein was identified. OmpA is often associated with adhesion to host tissues [32]. Indeed, the Rhizoid and Rough morphotypes are highly adherent, whereas the Soft morphotype (lacking the vesicles in liquid culture) is not [18]. However, the Soft type also produced large vesicles when grown on agar, though it is not clear whether these vesicles are the same

as those found in liquid cultures or in the Rhizoid and Rough types.

Although the function of the small vesicles and pearl-like vesicle chains observed in *F. columnare* was not analysed in depth in the current study, in TEM analysis they were shown to contain electron-dense material. The vesicle chains in the liquid cultures typically connected the cells to each other and to their surroundings. Usually there was a larger vesicle at the end of the chain, which in some cases appeared to have erupted by contact, possibly serving as an adhesin. Similar to *F. columnare*, small vesicles and their chain-like formations have been found in *F. psychrophilum*. *F. psychrophilum* produces small vesicles that bleb from the surface in pearl-like chain structures and exhibit proteolytic activity [11,33]. Although observed under both TEM and SEM, the nature of these pearl-like structures or ropes produced by all morphotypes of *F. columnare*—and whether they are ultrastructural artefacts caused by sample preparation—remains unclear. Recently, however, vesicle chains were also reported in *M. xanthus*, and were suggested to connect the cells in biofilms at the level of the periplasmic space, enabling the transfer of membrane proteins and other molecules between cells [34]. In contrast to *M. xanthus*, which had an increased abundance of vesicle chains in the biofilms, the vesicle chain-like structures observed in *F. columnare* were more common in the liquid cultures, though they were also observed in colonies (Figures 3 and 4).

In the initial protein identification, the proteins extracted from the vesicles remained hypothetical, except for one band, which was identified as the OmpA-family outer membrane protein P60 (see Table 1), but they all matched the *F. columnare* ATCC 49512 genome. After a basic local alignment search tool (BLAST) analysis, one protein was further identified as SprF. OmpA-family proteins are known to be virulence factors in several bacterial pathogens. The way in which OmpA-family proteins associate with *F. columnare* virulence is unclear, but our data implies that OmpA is involved with adhesion, and therefore might be a candidate virulence factor. Although the same protein band was present in the vesicles isolated from the non-virulent Rough type (Figure 7B), the virulence of the Rough type is probably affected by the loss of gliding motility. In addition, vesicles were not detected from the cells of the Soft morphotype that possess gliding motility, according to the spreading of colonies. Indeed, OmpA has been demonstrated to act as an adhesin and invasin, for example in *Pasteurella multocida* [35], several *E. coli* strains [36], *Neisseria gonorrhea* [32], *Leptospira interrogans* (causative agent of leptospirosis) [37], *Riemerella anatipestifer* (pathogen of domestic ducks) [38] and many other pathogens [39]. The protein has a strong immunogenic capacity [36,40]. In *F. psychrophilum*, OmpA has been identified as a promising candidate for the immunisation of rainbow trout against bacterial cold-water disease [41]. The role of the *F. columnare* OmpA-family protein for adhesion and invasion, and, on the other hand, as an immunogenic protein requires further study to reveal the mechanisms of how it interacts with the host tissue. However, the absence of functional genetic techniques hampers the genetic manipulation and verification of the role of OmpA as a virulence factor of *F. columnare*.

The protein identified as SprF is involved with flavobacterial gliding motility. In *F. johnsoniae*, Spr proteins (SprB together with SprC, SprD and SprF) are needed for the formation of spreading colonies on agar [21]. In *F. columnare*, the Rhizoid colony morphology (and corresponding gliding motility) is needed for virulence [18-20], possibly because of the role of flavobacterial gliding motility machinery as a type IX secretion system of virulence factors [42]. Indeed, SprF is needed for the secretion of SprB on the cell surface [21], but so far the specific role of SprF in *F. columnare* remains cryptic. Moreover, in the Rhizoid type, we observed numerous cell surface filaments that seemed to be situated at regular intervals along the cell, and appeared to attach bacterial cells to the glass surface and to neighbouring bacterial cells (Figure 3A). As these regularly appearing filaments were detected in lower numbers and in a less organised manner in the non-motile rough type, it is possible that these filaments are connected with gliding motility. It should be noted that the non-spreading Rough type colonies might not directly correlate to a loss of gliding motility. In *F. johnsoniae*, it has been observed that non-spreading colonies may not directly indicate loss of gliding motility, as this loss depends on whether mutations occur in *gld* or *spr* genes [21]. The surface adhesin SprB needed for flavobacterial gliding motility is a filament, approximately 150 nm long, on the cell surface [43,44]. As the structure of individual SprB proteins is fragile, and as the platinum sputter used in coating the samples can cover the finest structures, it is likely that the filaments visible in the Rhizoid type are adhesive structures other than SprB.

Conclusions

Our results suggest candidate virulence factors for *F. columnare*, factors that are still poorly understood, despite the problems caused by columnaris disease in the aquacultural industry. Additional questions are raised, especially on the role of OmpA and other unidentified proteins carried within the vesicles and secreted outside the cell, on adhesion to surfaces and invasion into the fish host. Also, the loss of an organised internal structure within the colony in the phage-resistant Rough type bacteria suggests that connections between neighbouring cells and social behaviour might be important for virulence in *F. columnare*.

Methods

Bacterial cultures

Flavobacterium columnare strain B067 was originally isolated from diseased trout (*Salmo trutta*) in 2007, and was stored frozen at −80°C in Shieh medium [45] with glycerol (10%) and foetal calf serum (10%). The derivative Rough phenotype of the strain was obtained by exposure to phage FCL-1 (see [19] for details). The Soft morphotype was isolated as a spontaneous transformant from the Rhizoid type. Bacteria were grown in a Shieh medium at 24°C under a constant agitation of 110 rpm in an orbital shaker.

Virulence experiments

The virulence of the bacteria producing the Rhizoid, Rough and Soft colony types of strain B067 was studied in an infection experiment using rainbow trout fry (*Onconrhynchus mykiss*, mean weight 0.57 g). Ten fish per colony type were individually exposed to 1×10^5 colony-forming units of bacteria ml^{-1} in 100 ml of ground water for 1 hour (T = 25°C). As a control treatment, 10 fish were individually exposed to sterile growth medium. After exposure, the fish were transferred to a 1-litre aquaria with 500 ml of fresh ground water (T = 25°C), and disease signs and fish morbidity were monitored in two-hour intervals for 97 hours. Morbid fish that had lost their natural swimming buoyancy, and which did not respond to external stimuli, were considered dead, removed from the experiment and euthanatised by cutting the spinal cord to avoid the suffering of the fish. The experiment was conducted according to the Finnish Act on the Use of Animals for Experimental Purposes, under permission granted by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland for L-RS. The virulence of the different colony types on rainbow trout infection was analysed by Kaplan-Meier survival analysis using IBM SPSS Statistics 20.

Treatment of SEM samples

F. columnare cell and biofilm structures were studied in three replicates in three different culture conditions: in liquid, in biofilm grown on a filter and in biofilm formed between a glass slide and an agar plate. To visualise the structure of the cells in liquid culture, *F. columnare* culture was placed on a ConA plate, incubated for 30 minutes and fixed (see later). To study the organisation of cells in a colony, the bacteria were cultured on Shieh plates with a glass slide (18×18 mm) placed on top of the culture. After 48 hours, the slide was detached, fixed and processed for SEM. To study the colony structure on an agar plate, a sterile 0.45 μm cellulose nitrate filter was placed on the Shieh agar, and 50 μl of the bacteria was spread on top of it. After 24 hours of growth, the filter was detached, fixed and processed. As control samples for SEM visualisation, sterile Shieh medium, supernatant from the cultures and *Escherichia coli* and *Salmonella enterica* cells (both grown in Shieh medium) were used. Samples were fixed (2% glutaraldehyde in 0.1 M NaCacodylate buffer, pH7.4) and washed twice with 0.1 M NaCac buffer, osmicated at RT, and washed twice with 0.1 M NaCac buffer. The cells were then dehydrated by exposure to a graded series of ethanol washes [50%, 70%, 96% and 100% (2×); each 3 min]. Filter samples were dried using the critical point method. The samples were then coated with platinum using platinum splutter and observed with an FEI Quanta™ 250 FEG-SEM.

Treatment of cells for TEM

Thin sections from the liquid cultures of Rhizoid and Rough morphotypes were also visualised using TEM. Samples were prepared from 5 ml of cultures grown for 16 hours, mixed with a final concentration of 3% glutaraldehyde and kept on ice for 45 minutes. The cells were washed three times with 0.1 M sodium phosphate buffer (pH 7.2). The pellet was overlaid with 1 ml of 1% osmium tetroxide in a phosphate buffer, washed once with the same buffer and then dehydrated in a rinsing ethanol series. The cells were embedded with Epon (Fisher) and sectioned.

Extraction of proteins from the ECPs

Rhizoid, Rough and Soft morphotype cells of *F. columnare* were grown in 50 ml of Shieh medium for 18 hours and pelleted (Megafuge 1.0R, 2500 × *g*, 15 min). Supernatant was filtered (0.8/0.2 μm pore size, Supor® membrane, PALL Life Sciences). Proteins were concentrated from 50 ml of the filtered supernatant with Amicon® Ultra Centrifugal filters (Ultracel®, 10 K, Millipore) to 500 μl in final volume. The protein concentration was determined with the Bradford protein assay [46]. Samples were run in a 14% Tricine SDS-PAGE [47] at 80 V, 30 mA for 20 hours. One protein band was excised and further analysed.

Isolation and analysis of membrane vesicles

The OMVs of the cells of the Rhizoid and Rough morphotypes of *F. columnare* strain B067 were isolated following the general outline for purification of natural OMVs in [48]. Bacteria were grown in 125 ml Shieh medium for 22–24 hours at RT with 110 rpm agitation. The cells were then removed by centrifugation (Sorvall RC-5, GSA- rotor, 10 400 × g, 30 min, RT) and the supernatant was filtered through a bottle-top filter unit (0.45 μm pore size, PES membrane, Nalgene), passing only vesicles less than 450 nm in size to the filtrate. The filtered supernatant was pelleted (Beckman coulter L-90 K, 45 Ti rotor, 60 000 × g, 2 h 30 min, 4°C) and resuspended in 1XPBS (phosphate buffer saline). The pellet was loaded on top of a 20–45% OptiPrep gradient and centrifuged (Beckman Coulter

L-90 K, SW 41 rotor, 49 000 × g, 17 h, 15°C). The light scattering fractions were collected, pelleted (Beckman coulter L-90 K, 70.1 Ti rotor, 54 000 × g, 3 h, 4°C) and resuspended in 1XPBS. The fractions were analysed under TEM. Samples were spotted on carbon-stabilised formvar-coated grids and fixed with 2% glutaraldehyde/0.1 M NaPOH for 1 minute and were washed three times with distilled H_2O and stained with 1% phosphotungstate, pH 6.5 for 1 minute. Imaging was performed with a Jeol JEM-1400. Fractions were also run in a 14% Tricine SDS-PAGE [47] at 80 V, 30 mA for 22 hours. Five protein bands were excised and further analysed.

Extraction of outer membrane proteins

The cell pellet from the ECP extraction of the Rhizoid morphotype was subjected to OMP extraction. The cells were disrupted by freeze-thawing three times, and cell debris was removed by centrifugation (5000 × g, 15 min, 4°C). Supernatant was then centrifuged (50 000 × g, 60 min, 4°C) and the pellet was suspended in 30 ml of 0.5% N-lauroylsarcosine/20 mM Tris–HCl, pH 7.2, and incubated for 20 minutes on ice in a cold room. Centrifugation was repeated, and the pellet was suspended in 4 ml of 0.5% N-lauroylsarcosine/20 mM Tris–HCl, pH 7.2, and centrifuged. The pellet was washed twice with 4 ml and suspended in 100 μl of 20 mM Tris–HCl. The sample was run in a 16% SDS-PAGE [49] at 100 V, 30 mA for 22 hours.

Protein identification

Protein identification using nanoLC-ESI-MS/MS was performed by ProteomeFactory (Proteome Factory AG, Berlin, Germany). The MS system consisted of an Agilent 1100 nanoLCsystem (Agilent, Waldbronn, Germany), a PicoTip electrospray emitter (New Objective, Woburn, MA) and an Orbitrap XL or a LTQ-FT Ultra mass spectrometer (ThermoFisher, Bremen, Germany). Protein spots were in-gel digested by trypsin (Promega, Mannheim, Germany) and applied to nanoLC-ESI-MS/MS. Peptides were trapped and desalted on the enrichment column (Zorbax SB C18, 0.3 × 5 mm, Agilent) for five minutes using 2.5% acetonitrile/0.5% formic acid as an eluent, then they were separated on a Zorbax 300 SB C18, 75 μm × 150 mm column (Agilent) using an acetonitrile/0.1% formic acid gradient from 5% to 35% acetonitril within 40 minutes. MS/MS spectra were recorded data-dependently by the mass spectrometer according to the manufacturer's recommendations. Proteins were identified using an MS/MS ion search with the Mascot search engine (Matrix Science, London, England) and the nr protein database (National Centre for Biotechnology Information, Bethesda, USA). The ion charge in the search parameters for ions from ESI-MS/MS data acquisition were set to '1+, 2+ or 3+', according to the instrument's and method's common charge state distribution. The resulting proteins were compared to database sequences using the BLAST algorithm [50].

Additional files

Additional file 1: A view of the colony surface of the Rough morphotype of *F. columnare*. Only the extracellular material was seen on the colony surface of the Rough morphotype, and cells were not observed. The scale bar was 4 μm.

Additional file 2: A wider view of a typical sample of the planktonic cells from the three morphotypes visualised under HR-SEM. Panel A: Rhizoid morphotype cells. Panel B: Rough morphotype cells. Panel C: Soft morphotype cells. The scale bar in A was 30 μm and in B and C, 40 μm.

Additional file 3: Controls used in the HR-SEM studies. Panel A: *E. coli* cells grown in Shieh medium. Panel B: *E. coli* cells grown on a filter paper (on Shieh agar). Panel C: Sterile Shieh medium. No vesicles, vesicle chains or filaments were seen in the controls. The scale bar in Panel A was 5 μm and was 10 μm in Panels B and C.

Competing interests

The authors declare that no competing interests exist.

Authors' contributions

EL performed the experiments, participated in the sample preparation and design of the study and drafted the manuscript. RKP participated in the sample preparation and performed the virulence experiments and sequence comparisons. JKHB participated in the design of the study and helped to draft the manuscript. LRS conceived of the study, participated in its design and coordination and drafted the manuscript. All authors read and approved the final manuscript.

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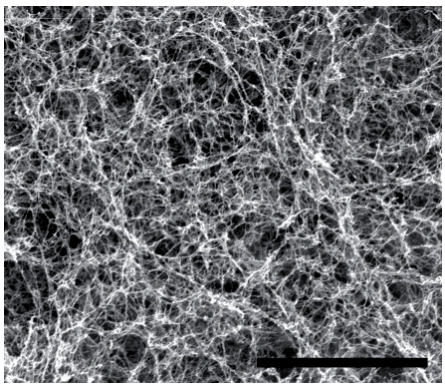
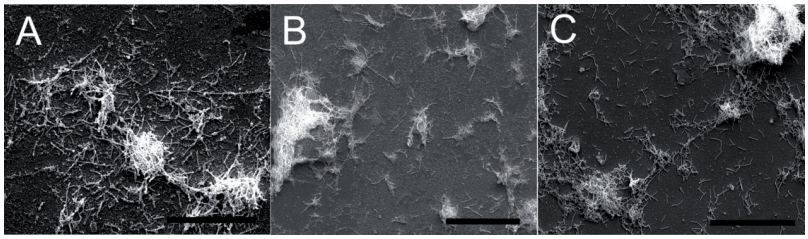
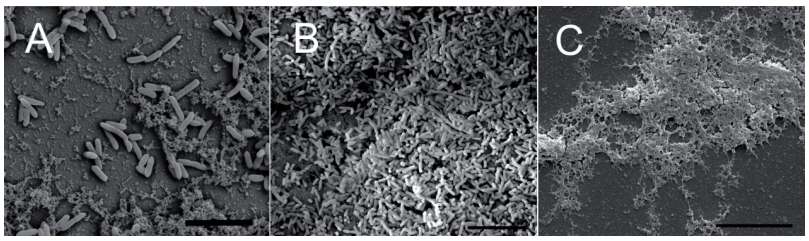


Additional file 1 (.pdf)
A view of the colony surface of the Rough morphotype of *F. columnare*
Only the extracellular material was seen on the colony surface of the Rough morphotype, and cells were not observed. The scale bar was 4 μm.



Additional file 2 (.pdf)
A wider view of a typical sample of the planktonic cells from the three morphotypes visualised under HR-SEM
Panel A: Rhizoid morphotype cells. Panel B: Rough morphotype cells. Panel C: Soft morphotype cells. The scale bar in A was 30 μm and in B and C, 40 μm.



Additional file 3 (.pdf)
Controls used in the HR-SEM studies
Panel A: *E. coli* cells grown in Shieh medium. Panel B: *E. coli* cells grown on a filter paper (on Shieh agar). Panel C: Sterile Shieh medium. No vesicles, vesicle chains or filaments were seen in the controls. The scale bar in Panel A was 5 μm and was 10 μm in Panels B and C.

IV

TACKLING A FISH DISEASE WITH A LITTLE HELP FROM A PHAGE

by

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Submitted manuscript