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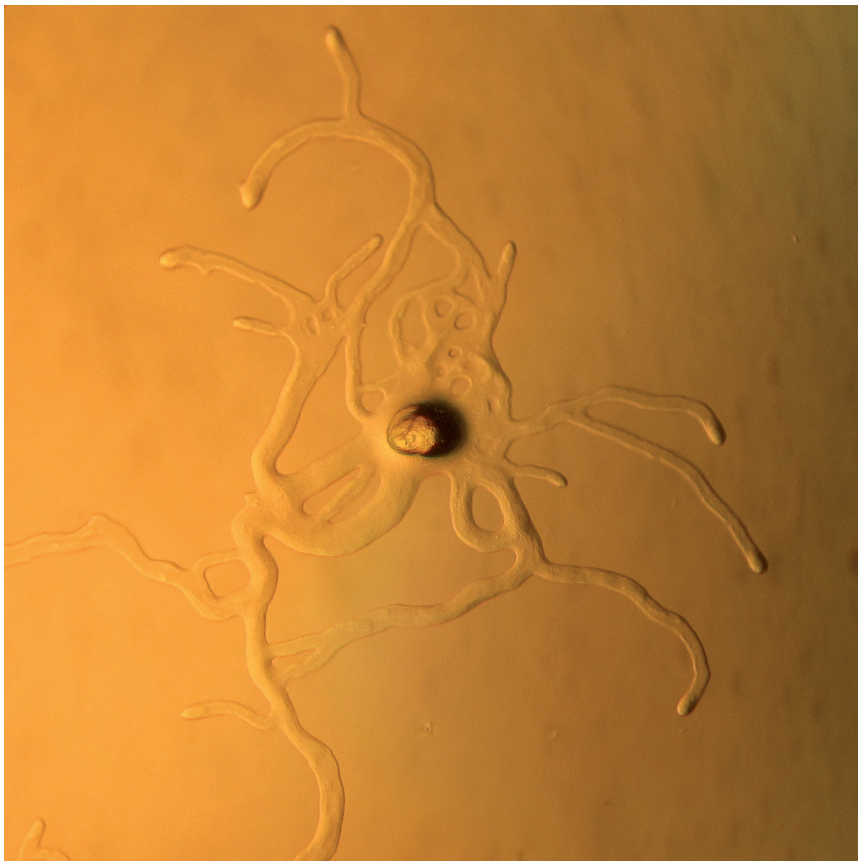
Reetta Penttinen

Genetic and Environmental Factors Associated with the Virulence of Fish Pathogen *Flavobacterium columnare*



JYVÄSKYLÄN YLIOPISTO

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Cover picture: The rhizoid colony type of *Flavobacterium columnare*.
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ABSTRACT

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Diss.

Flavobacterium columnare is an environmentally transmitted pathogen that annually causes fatal infections and economic harm in aquaculture on a global scale. However, its virulence mechanisms are largely unknown. In this thesis, I explored new virulence determinants of *F. columnare* by comparative studies on virulent and non-virulent colony types. First, the cell and colony surface structures were examined with high resolution scanning electron microscopy (HR-SEM). Second, a RT-qPCR protocol was developed to explore the gene expression of putative virulence factors and the effect of nutrient availability on virulence factor expression. Finally, the gene expression of gliding motility machinery and type IX secretion system (T9SS) components was studied in various nutrient conditions, as these systems putatively contribute to *F. columnare* virulence. HR-SEM revealed active production of outer membrane vesicles, which are used for virulence factor transport in several other bacterial species. The analysis of extracellularly secreted proteins revealed a ~13 kDa protein, which is specific for the virulent colony type. The gene expression studies showed significantly higher expression of tissue-degrading chondroitinase in the virulent colony type, confirming its role as a virulence factor. Furthermore, high nutrient availability increased the virulence factor expression and caused more rapid fish mortality. The environmental nutrients also regulated colony spreading in the motile colony types and part of the genes involved in gliding motility and T9SS. These findings demonstrate that nutrients are important regulators of *F. columnare* physiology with respect to virulence factor expression, motility and secretion – all of which are important virulence attributes. However, the molecular mechanisms and the regulatory pathways underlying these changes require further studies to fully understand the infection process of this pathogen.

Keywords: *Flavobacterium columnare*; colony type; gliding motility; nutrients; secretion; T9SS; virulence factor.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original paper and manuscripts that will be referred to in the text by their Roman numerals I–III.

I Elina Laanto, Reetta K. Penttinen, Jaana K. H. Bamford and Lotta-Riina Sundberg 2014. Comparing the different morphotypes of a fish pathogen - implications for key virulence factors in *Flavobacterium columnare*. *BMC Microbiology* 14: 170.

II Reetta Penttinen, Hanna Kinnula, Anssi Lipponen, Jaana K.H. Bamford and Lotta-Riina Sundberg 2016. High nutrient concentration can induce virulence factor expression and cause higher virulence in an environmentally transmitted pathogen. *Microbial Ecology*, in press.

III Reetta Penttinen, Ville Hoikkala, Jaana K.H. Bamford and Lotta-Riina Sundberg 2016. Gene expression analysis of gliding motility genes of spreading and non-spreading colony types of *Flavobacterium columnare*. Manuscript.

RESPONSIBILITIES OF REETTA PENTTINEN IN THE THESIS ARTICLES

I I participated in the preparation of the samples for the HR-SEM, performed the experimental infection of rainbow trout fry and did the sequence analysis of the identified OMV and ECP protein components. I participated in writing the article, although EL, L-RS and JB wrote most of it.

II I developed the RT-qPCR protocol, including the primer design, the reference gene validation and the determination of primer pair efficiency. The samples were prepared by me and HK. I performed the qPCR runs and handled the expression data. I performed the sequencing with the help of our research assistant and I did the sequence analysis. The infection experiment was conducted together with L-RS and our research assistants. L-RS performed all the statistical analyses. I and L-RS wrote the paper together with the co-authors.

III The samples used in this paper were prepared in manuscript II. I performed the qPCR runs and gene expression data analysis, excluding the statistical analyses, which were conducted by L-RS. The sequencing and sequence analysis were conducted by me and VH. I prepared the ECP profile and the proteolytic activity assays. I wrote most of the paper.

The author initials refer to: JB: Jaana Bamford; VH: Ville Hoikkala; HK: Hanna Kinnula; EL: Elina Laanto; RP: Reetta Penttinen; L-RS: Lotta-Riina Sundberg.

ABBREVIATIONS

aa	amino acid
cDNA	complementary DNA
Cq	quantification cycle
CTD	C-terminal domain
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
ECP	extracellular product
HR- SEM	high resolution scanning electron microscope
kDa	kilodalton
LPS	lipopolysaccharide
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
mRNA	messenger RNA
OMP	outer membrane protein
OMV	outer membrane vesicle
ORF	open reading frame
PCR	polymerase chain reaction
qPCR	quantitative PCR
QS	quorum sensing
R	rough colony type of *F. columnare*
RNA	ribonucleic acid
RT-qPCR	reverse transcription quantitative PCR
Rz	rhizoid colony type of *F. columnare*
S	soft colony type of *F. columnare*
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSH	suppressive subtractive hybridization
T9SS	type IX secretion system

1 INTRODUCTION

The biological world is full of various relationships within and between species. Biological interaction is something between at least two participants and is usually determined on the basis of how its existence affects them. The interaction can be beneficial, harmful or neutral, to either both or only one of the participants. Competition is a type of interaction in which the more efficient resource users can harm others indirectly by limiting their access to resources, or directly inhibiting their growth (Riley and Gordon 1999, Hibbing *et al.* 2010). Symbiotic relationships fall into a continuum of interactions from which one partner benefits, while the consequences to the other partner range from positive (mutualism) through neutral (commensalism) to negative (parasitism) (Barton and Northrup 2011). In parasitism, one participant takes advantage of the other, for example, by utilizing the host's macromolecules as resources for the parasite's own reproduction and growth, and harms it directly (Poulin 2008). In all ecosystems, these biological interactions occur across different trophic levels forming a multilateral network.

Different ways of biological interplay described above, including parasitism, are also adopted in the bacterial world (Barton and Northrup 2011). Parasitic micro-organisms causing disease are called pathogens. Harm caused to the host (i.e. virulence) is a result of pathogenic bacteria using its virulence weaponry (Casadevall and Pirofski 1999). Indeed, bacteria have developed various mechanisms for exploiting the host resources for surviving and reproducing (Barton and Northrup 2011). From the pathogen's point of view, the interaction with the host has to be established on the surface of the bacterial cell. Consequently, the virulence factors that are secreted outside the bacterial cell, as well as secretion systems operating in virulence factor translocation, are significant contributors in bacterial virulence (Costa *et al.* 2015). The pathogen's virulence, that is the ability to cause disease symptoms to the host organism, is affected by other biotic and abiotic factors, including environmental variables. Beyond the virulence mechanisms that are displayed to the exterior of the cell, there lies a complex network of metabolic pathways that are controlled by various regulatory mechanisms. In fact, the cell surface is, at the same time, the

site by which bacteria sense the environmental factors. Bacteria are exposed to variation in environmental factors, which are important regulators of bacterial metabolism; therefore, they can have a remarkable impact on host-pathogen interaction as well.

In my thesis, I explore the pathogen–environment interactions in a fish pathogen, *Flavobacterium columnare*. My aim is to find new possible factors contributing to the virulence of *F. columnare* by using molecular biology tools for comparison of virulent and non-virulent morphotypes of this bacterium. I also investigate how the environmental factors, specifically nutrients, contribute to bacterial virulence, the expression of putative virulence genes and gliding motility machinery, which is potentially used for virulence factor secretion.

1.1 Bacterial virulence is enabled by great genetic adaptiveness

Bacterial disease has been described as the outcome of the interaction between a pathogen and its host, mediated by either one or both of them (Casadevall and Pirofski 1999). However, not all bacteria are pathogenic. This raises a question: Why would any bacterium become pathogenic? According to the coincidental evolution of virulence hypothesis, bacterial virulence is seen as a by-product of an evolutionary event of a bacterium adapting to a new niche (Levin and Svanborg Eden 1990, Levin 1996).

Bacteria are everywhere, residing in a huge variety of diverse terrestrial and aquatic habitats, as well as within rock and in the atmosphere (Barton and Northrup 2011). Furthermore, while most of the organisms occupying extreme environments, such as highly saline or hot habitats, are found to be archaea, bacterial species also reside in these habitats (Oren 2002). These habitats are not uniform, but consist of various micro-environments, which may differ in their physico-chemical factors, such as pH, salinity, temperature, availability of oxygen, carbon, phosphorus, nitrogen, sulphur, and macro- or micronutrients. Bacteria also make a significant contribution to the earth's nutrient recycling processes in both soil and aquatic environments. In addition, bacteria are found living symbiotically with taxonomically very distinct organisms. These include, for example, the symbiotic interaction between cyanobacteria and fungi in lichen, nitrogen-fixing bacteria and legumes, and the mutualistic relationship of nutrient-digesting bacteria that colonize many animal intestines (Barton and Northrup 2011). The amazingly wide diversity in bacterial habitat repertoire comes with diversity in the metabolic pathways that allow them to survive in various environments.

Horizontal gene transfer through conjugation, transduction and transformation, together with mutations and relatively short generation times of bacteria, enable rapid genetic evolution that improves their chances of coping with a changing environment (Lawrence 2002). Taken together, bacteria have a great capacity for adaptation to varying environmental conditions, and consequently, colonization of new habitats. After all, the biological aim of life is

to survive and transfer genetic material to the next generations by reproducing. This is also the priority for pathogenic bacteria; they take advantage of the host cell components and utilize them as energy and nutrient sources in order to survive and replicate. In this sense, the damage caused to the host can be considered rather as a by-product than an intended consequence of a host–pathogen interaction (Levin and Svanborg Eden 1990).

1.1.1 To be or not to be pathogenic? – Opportunistic lifestyle

Generally, pathogens can be classified into obligate and opportunistic (i.e. facultative) and they are characterized by their pathogenic lifestyle in terms of an intra- or extracellular association with the host cell. Briefly, intracellular pathogens have the ability to replicate inside a host cell, while extracellular pathogens colonize sites such as host skin, mucosal surfaces or body cavity fluids of the host (Silva 2012). Obligate pathogens, such as *Mycobacterium tuberculosis*, have lost the ability to replicate in the outside-host environment and are, therefore, dependent on the host's existence (Casadevall 2008, Poulin 2008). Traditionally, medical literature has defined opportunists as organisms that are harmless in a healthy human host, but able to become pathogenic in a host that is weakened, for example, because of immuno-deficiency or a burn wound. This is the case in *Pseudomonas aeruginosa,* which is originally an environmental bacterium that colonizes human skin and the mouth, but is able to cause various infections in immunocompromised patients and those with cystic fibrosis (de Bentzmann and Plesiat 2011). Another example is *Staphylococcus aureus,* which is considered a commensal occupant of human skin and the nasal cavity but is capable of causing various infectious diseases ranging from skin infections to life-threatening diseases (Cogen *et al.* 2008). The up-to-date vision sees opportunistic pathogens capable of persisting and reproducing in the outside-host environment, but also causing disease when an appropriate host is encountered (Brown *et al.* 2012). This wider concept describes opportunists simply as non-obligate pathogens, extending the definition to environmentally transmitting pathogens also, such as *F. columnare* and *Vibrio vulnificus* both of which transmit to a host via water (Jones and Oliver 2009, Kunttu *et al.* 2012). Indeed, aquatic environments serve as habitats for a variety of pathogens, including opportunists from genera such as *Vibrio*, *Flavobacteria* and *Aeromonas* (see e.g. (Igbinosa *et al.* 2012, Loch and Faisal 2015, Le Roux *et al.* 2015)). For example, *Vibrio cholerae* also can be considered an opportunist. The bacteria are capable of surviving and replicating in the outside-host environment, which can significantly increase the transmission of the bacteria and influence disease outbreaks (Vezzulli *et al.* 2010, Vezzulli *et al.* 2013).

1.1.2 Antibiotic resistance: an emerging problem in treating infectious diseases

Bacterial infections commonly emerge in all parts of the world, yet until recently, most of them have been beatable with antibiotic treatment. However, the infectious diseases caused by bacterial pathogens have been in the public spotlight due to the increasing emergence of antibiotic-resistant bacterial strains (Anon. 2013, Anon. 2015).

The first antibiotics were found in the late 1930s and were followed by 'the golden era of antibiotics' in the 1950s and 1960s, when new antibiotic compounds were frequently discovered (Davies and Davies 2010). At that time, they were regarded as a panacea and were believed to be the end of infectious diseases. Thus, the focus of the research was directed on other emerging problems like cancer and heart disease. However, reports of antibiotic resistance kept on accumulating (Aminov 2010, Wilson *et al.* 2011). The acquisition of antibiotic resistance genes via horizontal gene transfer in combination with short generation times improve the survival and evolution of pathogenic bacteria. Now, increased and ever-spreading antibiotic resistance has become a severe global health threat as commonly occurring bacterial infections are more challenging to treat with conventional antimicrobial compounds, and new antimicrobial drugs are rarely discovered (Conly and Johnston 2005, Aminov 2010). Bacterial strains with resistance to multiple antibiotics have evolved, including MRSA (methicillin-resistant *Staphylococcus aureus*) and ESBL (extended-spectrum beta-lactamase) producing *Enterobacteriales* (Anon. 2013, Anon. 2015).

Disease-causing micro-organisms are virtually everywhere. Given that antibiotics are increasingly losing their power, there is an alarming need for new treatment methods for bacterial diseases. Mechanistic knowledge of how the disease is caused forms the basis for developing new methods for treating infectious diseases. Hence, the quote 'know your enemy' couldn't be more suitable; we can win only by thoroughly understanding what we are battling against.

1.2 Disease mechanisms are driven by virulence factors

Pathogenic bacteria exhibit their own characteristic ways to cause disease. Yet, all bacterial infections are characterized by certain common features. As the pathogen encounters its host, it needs mechanisms to adhere to host tissue in order to colonize the infection site. For infection, (i.e. acquisition of a microbe by the host), the host immune response has to be hindered, silenced or evaded (Casadevall and Pirofski 2000, Casadevall and Pirofski 2001). Bacteria have evolved a variety of ways to evade host defence systems. For example, bacteria that possess antigenic variation can produce different phenotypes with a wide repertoire of surface element variants that interfere with the functions of

antibodies (Deitsch *et al.* 2009). Moreover, pathogens may have mechanisms for manipulating the host signalling pathways and the immune system (Buttner 2012), such as secretion of effector proteins as exemplified in *Yersinia* and *Shigella* (Bliska *et al.* 2013, Ashida *et al.* 2015). Toxicity has been considered one of the virulence determinants as it damages the host through producing toxins, toxic metabolic end products, switching on allergic reactions and disrupting the host nutrition (Casadevall and Pirofski 2001, Lebrun *et al.* 2009). Finally, contagiousness (i.e. encompassing the ability to replicate and transmit between host organisms), is essential for the existence of the pathogen (Casadevall and Pirofski 2001).

For the successful completion of all infection stages (adherence, invasion, colonization, host-defence evasion, toxicity and transmission) (Casadevall and Pirofski 1999, Casadevall and Pirofski 2001), molecular virulence mechanisms are required. The components involved in these processes are referred to as virulence factors (Henderson *et al.* 1996, Casadevall and Pirofski 1999). The concept of 'a virulence factor' is broad, comprising not only the genes and products that interact directly with the host, but also those that regulate virulence gene expression, activate virulence factors at the translational level, enable colonization, function in immune evasion, enable intracellular survival or operate host-derived factors to promote the pathogen's own survival (Wassenaar and Gaastra 2001). In this thesis, virulence factors refer to the components that cause damage to the host as defined by Casadevall and Pirofski (Casadevall and Pirofski 1999). Bacterial infection and virulence are complicated phenomena, and even though the mediating virulence factors are unique to each host-pathogen interaction, these processes share common requisites.

1.2.1 Secretion of virulence factors in Gram-negative bacteria

The bacterial surface structures and products transported into the extracellular milieu act in the interface between the pathogen and its host; therefore they are important mediators of host–pathogen interactions (Henderson *et al.* 2004, Chagnot *et al.* 2013). In addition, these bacterial surface structures are often recognized by the host immune system (Mogensen 2009). Bacterial factors intended to interact with the extracellular environment have to be delivered out of the cytoplasm. Secretion refers to the active transport of substrates, such as proteins, DNA or smaller molecules, from the interior of the cell to the outside of the outer-most lipid layer. This definition covers the subcellularly located products that i) are released to the extracellular environment; ii) stay associated with the cell surface; and iii) are injected into the host cell (Economou *et al.* 2006, Desvaux *et al.* 2009, Costa *et al.* 2015). The bacterial secretome (i.e. secretion machineries and the products secreted through them) (Desvaux *et al.* 2009), have a significant role in bacterial virulence (Lantz 1997, Costa *et al.* 2015).

Bacteria have several mechanisms for transportation of the extracellularly secreted products out of the bacterial cytoplasm. Secretion systems are composed of a varying number of different protein components that form

complex nanomachineries on the cell membrane. Nine different secretion systems, T1SS–T9SS (type I–IX secretion systems), have been described in Gram-negative bacteria (Gram-negative secretion systems are reviewed in (Chagnot *et al.* 2013, Costa *et al.* 2015). Furthermore, secretion may occur via non-classical (NC) protein secretion, which refers to yet-to-be-characterized transportation mechanisms. The hypothesis of the existence of such systems has been provoked due to proteins that are predictably cytoplasmic and, lack defined N-terminal signal peptides, but are still found in the extracellular environment. NC also refers to transportation via mechanisms such as membrane vesicle production and the release of extracellular colicins via a lysis gene-dependent manner (Bendtsen *et al.* 2005, Lloubes *et al.* 2013).

In Gram-negative bacteria, secretion refers to the crossing of molecules through the inner (i.e. cytoplasmic) membrane to the periplasm and further through the outer membrane to the exterior of the cell (Desvaux *et al.* 2009). Generally, transportation occurs as a one- or two-step process (Rego *et al.* 2010). Secretion systems T1SS, T3SS, T4SS and T6SS extend through cytoplasmic and outer membranes, allowing the secretion to occur in a one-step process (Costa *et al.* 2015). In two-step secretion, the substrate crosses through cytoplasmic and outer membranes one at a time, which requires two separate secretion machineries. Transportation of substrates through the cytoplasmic membrane occurs via the general secretory pathway Sec, or Tat transport system (Lycklama A Nijeholt and Driessen 2012, Berks 2015). Once in the periplasm, the secreted molecules are targeted to a secretion system that mediates the delivery through the outer membrane (Rego *et al.* 2010). These systems include T2SS, T5SS, T7SS, T8SS and T9SS (Chagnot *et al.* 2013, Costa *et al.* 2015). While the well-characterized Sec and Tat systems are commonly found in Gram-negative and Gram-positive bacteria, the Gram-negative-specific T1SS–T9SS (which enable transportation through the outer membrane) are found only within certain species or taxonomic groups (McBride and Zhu 2013, Costa *et al.* 2015). However, a single bacterial species can possess several different systems for extracellular secretion. For example, *P. aeruginosa* uses 5 different systems (T1SS, T2SS, T3SS, T5SS and T6SS) for protein secretion. Diversity in secretion machineries, and consequently in exoproteomes, is thought to enable adaptation and survival in different kinds of habitats in which this bacterial species resides (Bleves *et al.* 2010).

The final subcellular location of a secreted protein is determined by the amino acid sequence of the nascent polypeptide chain. A secretion system-specific N-terminal signal peptide targets a protein to the cytoplasmic membrane where it is translocated via the Sec or Tat systems. A protein secreted through cytoplasmic membrane may stay attached to the cytoplasmic membrane, be delivered to periplasmic space, or be targeted to the outer membrane (Desvaux *et al.* 2009). Secreted proteins that are translocated through the outer membrane (via T2SS, T5SS, T7SS, T8SS and T9SS) have specific domains and signal peptides determining the secretion system by which the protein will be translocated. For example, the proteins that are secreted through T5SS, known as the autotransporter system, have multiple domains: N-terminal

signal peptide targeting the protein to the Sec system, a passenger domain and a transmembrane domain (van Ulsen *et al.* 2014). After the translocation of the secreted protein via Sec and the cleavage of the signal peptide, the transmembrane domain inserts itself into the outer membrane and forms a pore through which the passenger domain is translocated. The passenger domain is then cleaved and either non-covalently attached to the cell surface or released into the exterior of the cell (van Ulsen *et al.* 2014). Furthermore, proteins that are secreted through T9SS have a C-terminal domain (CTD) that targets them to the T9SS (Seers *et al.* 2006). Generally, after a protein has been targeted to the outer membrane, it can either be anchored to it or be released into the extracellular milieu (Desvaux *et al.* 2009, Costa *et al.* 2015).

Secretion systems have many essential functions of bacteria, such as biogenesis of the cell envelope (including filamentous structures e.g. pili, curli and flagella), adherence, nutrient acquisition, bacterial communication, biofilm production and motility (Gerlach and Hensel 2007, Chagnot *et al.* 2013, Costa *et al.* 2015). As many of these processes are also involved in pathogenicity, secretion systems have a special importance in bacterial virulence and can be considered as virulence factors or determinants. For example, T3SS, T4SS and T6SS are systems that can also extend through the membrane of the eukaryotic host cell; therefore they are able to deliver products directly into the host cytosol (Costa *et al.* 2015). These systems can inject effector proteins into the eukaryotic cytosol or plasma membrane in order to enable invasion by modifying the host physiology, including suppression of the host immune defence or elicitation of the host cell death (Alfano and Collmer 2004, Cornelis 2006, Alvarez-Martinez and Christie 2009, Cianfanelli *et al.* 2016). In addition to effector proteins, T4SS substrates include DNA, which can be transferred into another bacterial cell through conjugation via a contact-dependent manner. T4SS also functions in the secretion and intake of extracellular DNA (Alvarez-Martinez and Christie 2009). Overall, secretion systems have been harnessed for the delivery of virulence factors by a range of bacterial pathogens, emphasizing their significance in virulence (see e.g. (Chagnot *et al.* 2013, Costa *et al.* 2015).

The investigation of secretion systems is of high importance not only to understand the biology of bacteria, but also to find novel ways by which they may be exploited in treating bacterial infections. As alternative therapies for antibiotics are needed urgently, targeting the secretion systems with antivirulence components would provide a potential approach to combat bacterial pathogens (Steadman *et al.* 2014). Hence, learning how these systems work in bacterial pathogenesis can give us insights into how to disrupt them. These machineries are the ones that also interact with the host, and thus it can be possible to interrupt the host–pathogen interaction by inhibiting their biological function. Already there are some promising results of inhibiting the activity of at least T3SS, T4SS and the biogenesis of curli (by T8SS) and pili (by T7SS) (Cegelski *et al.* 2009, Paschos *et al.* 2011, Duncan *et al.* 2012, Steadman *et al.* 2014).

1.2.2 T9SS and *Flavobacterium* gliding motility

The pathogenicity of several bacterial species is characterized by their ability to move. The most extensively studied bacterial motility apparatus are proteinaceous filaments of the bacterial cell surface, called flagella. Swimming motility occurs in liquid environments and is enabled by the rotation of flagella by individual cells (Jarrell and McBride 2008). Flagella also contribute to bacterial virulence by enabling chemotaxis (e.g. moving towards the host), but also they have important roles in biofilm formation, adherence and colonization (Haiko and Westerlund-Wikstrom 2013). In addition to swimming motility, bacteria have various strategies for migrating within or over solid surfaces, such as swarming, twitching, sliding and gliding. Swarming motility refers to multicellular bacterial movement on surfaces mediated by rotating flagella; however, swarming bacteria are distinguished from swimming bacteria by the increased number of flagella (Kearns 2010). Twitching motility is slow, jerky cell movement, independent of flagella, resulting from the extension and retraction of T4P (type IV pili) (Mattick 2002). Furthermore, a passive way of movement has been reported in *Mycobacteria* which do not need active motors for moving by sliding. However, in order to spread, they utilize the expansive forces resulting from cell replication (Martinez *et al.* 1999). Gliding motility is a process by which bacteria can crawl over surfaces. It is enabled by machinery that is located on the cell surface. Sequence analysis has revealed that components of this machinery are restricted within phylum Bacteroidetes, that includes bacterial species such as *Flavobacterium johnsoniae, F. psychrophilum* and *Cytophaga hutchinsonii* (McBride 2001, McBride and Zhu 2013). Gliding as a way of movement which has been described also in other bacterial species outside Bacteroidetes, for example in genus *Mycoplasma*, *Myxococcus xanthus* and in a cyanobacterium *Nostoc punctiforme* (Miyata 2010, Nan *et al.* 2011, Risser and Meeks 2013). However, those motility mechanisms are not related to the gliding motility apparatus unique to Bacteroidetes (McBride and Zhu 2013) to which I refer in this thesis.

In the phylum Bacteroidetes, gliding motility machinery is studied most closely in *F. johnsoniae*, which can be considered as a model of this system (Fig. 1). Cells that are capable of gliding motility form spreading colony morphology (McBride 2001), which is an important phenotypic indicator of the intact and active gliding motility system. Gliding motility genes have been identified mainly with the help of gliding motility mutants which form non-spreading colonies indicating a malfunction of the gliding motility machinery. Genetic techniques have revealed several *gld* and *spr* genes that are involved in gliding or spreading (*Flavobacterium* gliding motility and T9SS are reviewed in (McBride and Nakane 2015)). Mutations in *gld* genes result in defects in gliding, whereas *spr* mutants fail to form spreading colonies while some individual cells may remain motile (Braun *et al.* 2005, Nelson *et al.* 2007, Nelson *et al.* 2008, Rhodes *et al.* 2011a, Rhodes *et al.* 2011b). In addition, certain genes that possess only a redundant function in motility have been designated *rem* genes (Shrivastava *et al.* 2012).

The gene products of *gldA*, *gldF* and *gldG* form an ATP-binding cassette, but its role in gliding has not been determined (Agarwal *et al.* 1997, Hunnicutt *et al.* 2002). *gldI* is a lipoprotein located in the periplasm, and based on sequence analysis, it contributes to the protein folding of other gliding motility components (McBride and Braun 2004). The products of genes *gldB*, *gldD*, *gldH* and *gldJ* are also lipoproteins but their exact function is not known (Hunnicutt and McBride 2000, Hunnicutt and McBride 2001, McBride *et al.* 2003, Braun and McBride 2005).

gldK, *gldL*, *gldM*, *gldN*, *sprA*, *sprE* and *sprT* encode proteins that form a secretion system, designated as the type IX secretion system (T9SS) (Braun *et al.* 2005, Nelson *et al.* 2007, Sato *et al.* 2010, Rhodes *et al.* 2011b, McBride and Zhu 2013). Gliding motility machinery and T9SS are considered as intertwined systems as they share common protein constituents (Fig. 1) (Shrivastava *et al.* 2013). T9SS genes are commonly found in Bacteroidetes, though not all species harbouring T9SS genes have the genes needed for gliding motility, as exemplified in nonmotile *Porphyromonas gingivalis* (McBride and Zhu 2013). Gliding motility is dependent of functional T9SS probably because it is used for secretion of the gliding adhesins SprB and RemA (Shrivastava *et al.* 2012). Several additional factors are responsible for the secretion of specific products; SprF is required for successful secretion of SprB, whereas SprC and SprD assist SprB functionality. However, their exact roles are unknown (Rhodes *et al.* 2011b). PorV is a component needed for the secretion of *F. johnsoniae* RemA and several other proteins (Kharade and McBride 2015). In the model of the gliding mechanism, the cell movement is enabled by a helical movement of adhesins along the cell surface (Nakane *et al.* 2013). In *F. johnsoniae*, the surface adhesins SprB, RemA – and presumably other adhesins – mediate the adhesion to the surface, which is a prerequisite for gliding (Nelson *et al.* 2008, Shrivastava *et al.* 2012, Nakane *et al.* 2013). The adhesins are propelled rapidly along a helical track on the cell surface by a motor that has not been characterized; however it is thought to be composed of Gld proteins (Nakane *et al.* 2013, Shrivastava and Berg 2015).

Besides gliding motility, T9SS has various other roles. It acts in the biofilm formation of *Capnocytophaga ochracea* (Kita *et al.* 2016). T9SS also plays a crucial role in periodontal pathogen *P. gingivalis*, which uses it for virulence factor secretion and pigmentation (Sato *et al.* 2005, Sato *et al.* 2010, Nakayama 2015). *F. johnsoniae* chitinase is secreted through T9SS, which is also involved in starch utilization (Kharade and McBride 2015). T9SS is needed for cellulose utilization in *Cytophaga hutchinsonii* and plant colonization in *Flavobacteria* residing in rhizospheres (Zhu and McBride 2014, Kolton *et al.* 2014).

The secretion via T9SS occurs in a two-step process. Predictably the secreted proteins are first targeted to the Sec system, but more detailed studies of common secretory systems, such as Sec, are not available in *Flavobacteria*. However, it has been demonstrated in *F. johnsoniae* that a mutation in *secDF*, which is involved in the secretion process via the Sec secretion system, leads to disrupted secretion of *gldJ* (Nelson and McBride 2006). In *Escherichia coli*, the Sec system has been characterized in more detail and SecDF are crucial components

in the secretion of substances across the cytoplasmic membrane (Pogliano and Beckwith 1994). The products secreted through Sec carry N-terminal signals (Natale *et al.* 2008) which can also be found in the corresponding homologs of *F. johnsoniae* (Nelson and McBride 2006). The conserved CTD targets the secreted products further to be translocated across the outer membrane through T9SS (Seers *et al.* 2006). However, the comprehensive molecular mechanisms of protein translocation via T9SS are still to be characterized in *Flavobacteria*.

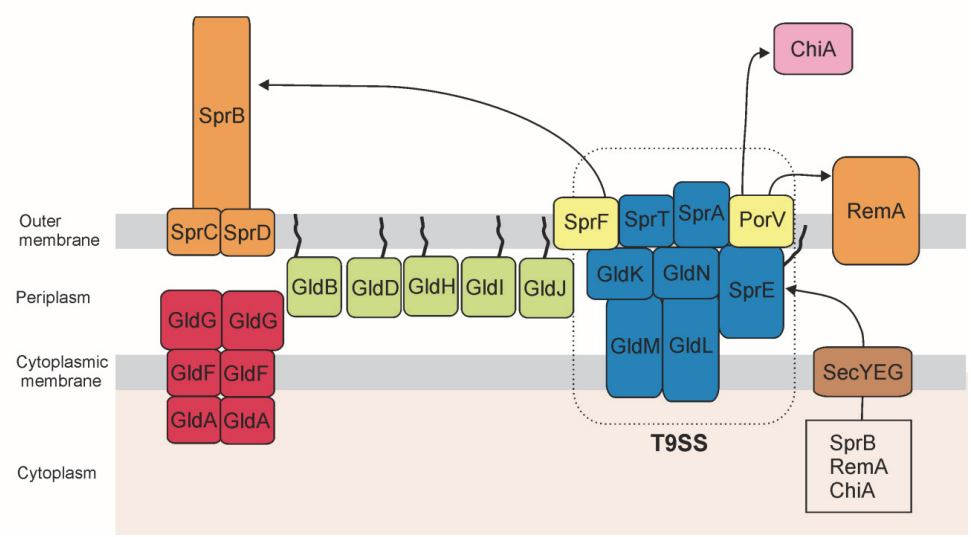

FIGURE 1 *F. johnsoniae* gliding motility machinery. GldA, GldF and GldG form an ABC transporter (red). GldB, GldD, GldH, GldI and GldJ are lipoproteins (green). GldK, GldL, GldM, GldN, SprA, SprE and SprT compose T9SS (blue) and are required for secretion of adhesins SprB and RemA (orange) and chitinase ChiA (pink), which is not related to motility. SprC and SprD assist the function of SprB. SprF is required for the secretion of SprB and PorV for the secretion of RemA and ChiA. Proteins secreted through T9SS have N-terminal sequences that target them first to the Sec system (brown) and C-terminal domains, which direct them to T9SS (modified from McBride and Nakane 2015).

1.2.3 Proteases are potential virulence factors

Proteolytic enzymes, often referred to as proteases, proteinases or peptidases, are enzymes that hydrolyse peptide bonds (Rawlings and Salvesen 2013). Proteases possess a variety of physiological functions in the normal cell metabolism of animal, plant and microbial cells. Intracellular proteases are usually involved in the regulation of metabolic functions, whereas extracellularly secreted proteases act in the hydrolysis of large protein components into smaller molecules that are absorbable into the cell (Rao *et al.* 1998). Extracellular proteases have various roles in bacterial pathogenesis (Lantz 1997). Proteases acting in immediate contact with the host tissue can

cause direct damage because they often exhibit collagenolytic or elastinolytic activity for the degradation of connective tissue components. Extracellular proteases are needed not only for the processing and maturation of the pathogen's own virulence factors but also for inactivating the host defence systems and interfering with the host cell signalling and membrane permeability. Proteases also have roles in activating the tightly regulated host protease systems, resulting in host tissue degradation by its own enzymes. Furthermore, extracellular proteases can target small antimicrobial peptides secreted by the host, thus enabling evasion from the host defence systems. Proteases are involved in the timely coordination of biofilm formation which is an important determinant of virulence in many bacterial species (Lebrun *et al.* 2009, Frees *et al.* 2013).

1.3 Environment as a regulator of bacterial virulence

Opportunistic pathogens shuttle between the within-host and outside-host environment (Brown *et al.* 2012). As these two environments differ from each other *per se*, the bacteria are required to adapt accurately to the changing environmental factors, both biotic and abiotic. In addition to the host organism, pathogens are involved in other biotic interactions with predators (e.g. bacteriophages, protozoans) and other bacteria. Interaction with other bacteria may be an interspecific or an intraspecific competition for resources (Hibbing *et al.* 2010, Foster and Bell 2012). On the other hand, pathogenic bacteria can cooperate, in which case the pathogenesis is coordinated by communication between bacteria (Antunes and Ferreira 2009). A well-established example of such a communication system is quorum sensing (QS). Bacteria capable of QS produce and respond to small signal molecules to detect the surrounding cell density and to control the virulence factor production accordingly (Antunes *et al.* 2010).

Bacteria constantly sense the physico-chemical properties of their environment. In a changing environment bacteria can only survive by having a broad capacity to change their metabolism according to the environmental conditions. Abiotic factors such as pH, temperature and the availability of nutrients (especially carbon and nitrogen), oxygen and metal ions (Fe, Mn, Zn) are important mediators of bacterial metabolism. These factors can be signals of host presence, thus they are also important regulators of virulence (Mekalanos 1992, Somerville and Proctor 2009, Rohmer *et al.* 2011). In addition to the simple physical and chemical factors, the virulence gene expression can be regulated by specific host-derived nutrients (Commichau and Stulke 2015). Furthermore, several virulence mechanisms are triggered by physical contact with the host or specific host products detected by the pathogen (Guiney 1997).

For a pathogen, the animal host represents a nutrient-rich habitat, but at the same time exploitation of the host requires different metabolic pathways than those needed in the outside-host environment (Fuchs *et al.* 2012). This is

why the host–pathogen interaction is characterized by the most fundamental aim of the pathogen: to transfer energy and nutrients from the host for its own survival and replication (Rohmer *et al.* 2011). Therefore, the role of environmental factors, especially nutrients, is central in controlling the metabolism of opportunistic pathogens, and thereby in their virulence (Somerville and Proctor 2009). Entering the within-host environment requires global changes in gene expression. Bacteria have specialized mechanisms for transmitting the external and internal signals of nutrient availability into changes in gene expression (Chubukov *et al.* 2014). Moreover, it has been elucidated that bacterial virulence and its regulation are often integrated in the global regulatory pathways that mediate normal cell metabolism. These include systems such as carbon catabolite repression, the phosphotransferase system of nitrogen, stringent response and envelope stress response (Gorke and Stulke 2008, Pfluger-Grau and Gorke 2010, Dalebroux *et al.* 2010, Flores-Kim and Darwin 2014).

1.4 The opportunistic fish pathogen *Flavobacterium columnare*

Flavobacterium columnare is a Gram-negative, 3−5 µm long, rod-shaped bacterium that belongs to the *Flavobacteriaceae* family in the phylum Bacteroidetes (Bernardet *et al.* 1996, Declercq *et al.* 2013a). *F. columnare* is the causative agent of columnaris disease in various wild, ornamental and cultured freshwater fish species around the world (Decostere *et al.* 1998, Declercq *et al.* 2013a). The bacterium has been isolated in wild fish species such as threadfin shad (*Dorosoma pretenense*), channel catfish, (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and freshwater drum (*Aplodintous grunniens*) (Olivares-Fuster *et al.* 2007). In the fish farming industry, it is a severe problem. *F. columnare* is the second most relevant pathogen in the channel catfish industry of the United States and causes extensive economic loss annually (Wagner *et al.* 2002). In addition, *F. columnare* infects cultured salmonids such as salmon (*Salmo salar*), trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*), but also Nile tilapia (*Oreochromis niloticus*) and Red tilapia (*Oreochromis sp.)* (Figueiredo *et al.* 2005, Shoemaker *et al.* 2008, Pulkkinen *et al.* 2010, Dong *et al.* 2015). The external symptoms of the disease are seen as tissue lesions and erosions, typically in gills, fins and skin (Pulkkinen *et al.* 2010). However, internal symptoms are rarely reported or are completely absent (Koski *et al.* 1993). Given the ability to degrade tissue of vital organs such as fish gills, the *F. columnare* infection may be fatal. Furthermore, the bacterium profits from the capability of a saprophytic lifestyle, which enhances the transmission between hosts via water (Kunttu *et al.* 2009b), potentially leading up to 100 % mortality within a fish tank (Suomalainen *et al.* 2005b). Despite the prevalence of *F. columnare* to the aquaculture, the detailed molecular mechanisms by which it causes infection are largely unknown. For destruction of the fish tissues, several degradative enzymes are needed. *F. columnare* is known to produce chondroitinase that

degrades components of the host extracellular matrix and has been shown to be connected to virulence (Suomalainen *et al.* 2006). Presumedly also other tissue-degrading proteases are involved, but even though there are some studies on proteases and proteolytic activities of *F. columnare* available, these studies do not clearly connect them to virulence (Bertolini and Rohovec 1992, Newton *et al.* 1997). Gliding motility of *F. columnare* may be essential for a successful infection (Kunttu *et al.* 2009a, Kunttu *et al.* 2011, Laanto *et al.* 2012, Zhang *et al.* 2014), as this phenomenon couples the adherence to a surface and the colonization process of the host. However, studies on the use of gliding motility apparatus of *F. columnare* are limited. Efforts on finding new virulence-related factors has been made using proteomics and suppressive subtractive hybridization (SSH) but the role of the resulted virulence factor candidates have not been characterized further (Olivares-Fuster and Arias 2008, Dumpala *et al.* 2010).

Due to its opportunistic lifestyle, *F. columnare* can survive long periods in the outside-host environment and still retain its pathogenicity (Arias *et al.* 2012, Sundberg *et al.* 2014). The bacterium enters the fish farms probably from the environmental waters (Olivares-Fuster *et al.* 2007, Kunttu *et al.* 2012, Sundberg *et al.* 2016) but it also resides in natural biofilms (Kunttu *et al.* 2012, Cai *et al.* 2013). There are several approaches involved in disease prevention which concern controlling the rearing conditions and water quality. The high fish density used in the rearing units increases the transmission of the pathogen, but also the resultant organic matter in the fish tanks (Suomalainen *et al.* 2005c). Consequently, low fish density is suggested to be a cost-effective way to control the columnaris disease (Suomalainen *et al.* 2005c). Indeed, organic matter and nitrite have been shown to increase the adherence capacity of *F. columnare* (Decostere *et al.* 1999a). Other methods for disease prevention include bathing of the fish in acidic or saline water or in certain concentrations of $CuSO_4$ or $KMnO_4$ (Davis 1922, Suomalainen *et al.* 2005a, Darwish *et al.* 2009). Several trials to develop a vaccine against *F. columnare* have also been reported (Declercq *et al.* 2013a). Currently there is no vaccine available in Europe, but in the USA a modified live *F. columnare* vaccine for channel catfish and largemouth bass fry has been licenced (Shoemaker *et al.* 2011). However, the most effective way to treat columnaris disease is with antibiotics (see e.g. (Declercq *et al.* 2013a)). Furthermore, antibiotics have also been shown to be effective when used prophylactically (Thomas-Jinu and Goodwin 2004). However, antibiotic use also affects the water systems surrounding the fish farms (Tamminen *et al.* 2011, Cabello *et al.* 2013). Nevertheless the growing concern towards the emergence of antibiotic resistance (Declercq *et al.* 2013b, Mohammed and Arias 2014) demands new ways to treat the disease.

F. columnare displays different colony morphologies (Kunttu *et al.* 2009a). Bacteria that are isolated from fish suffering from columnaris disease have a characteristic appearance of spreading colony morphology exhibiting tentacle-like protrusions, designated as the rhizoid (Rz) colony type (Fig. 2). However, in laboratory conditions, *F. columnare* may express also rough (R) and soft (S) colony types (Bernardet 1989, Kunttu *et al.* 2009a). R and S colonies are typically more or less round and lack the protrusions characteristic of a Rz colony (Fig.

2). A R colony has, in accordance with its name, a rough and hard appearance, with a darker yellow colour, whereas S colonies are soft and moist, and usually a lighter yellow in colour (Kunttu *et al.* 2009a) (Fig. 2).

Changes in colony morphology appear in the presence of phage, spontaneously during serial passage and, starvation (Kunttu *et al.* 2009a, Kunttu *et al.* 2012, Laanto *et al.* 2012, Sundberg *et al.* 2014). Also, a β-lactam selection with ampicillin leads to changes in colony morphology (Bader *et al.* 2005). Furthermore, although the change in colony type was first believed to be irreversible (Kunttu *et al.* 2009a), further observations have proven that it can actually be switched back and forth between the Rz, R and S types (Laanto *et al.* 2012). In addition to the colony appearance, *F. columnare* morphotypes differ with respect to adherence and, motility, but most importantly, to virulence. Rz has been shown to be virulent, whereas R and S show no or only intermediate capability of causing the disease in fish (Kunttu *et al.* 2009a, Kunttu *et al.* 2011, Laanto *et al.* 2012). Both the Rz and R are adherent, whereas S has only a limited adherence capacity (Kunttu *et al.* 2011). When comparing the Rz and the R type of a same *F. columnare* strain, Rz exhibits higher growth rate and is more resistant to protozoan predation (Zhang *et al.* 2014). This may explain why the other colony types have not been isolated from the farming environment. Even though an effort has been put to understand the role of these colony morphologies, their relevance in the lifecycle of *F. columnare* and in the bacterial community has remained unclear

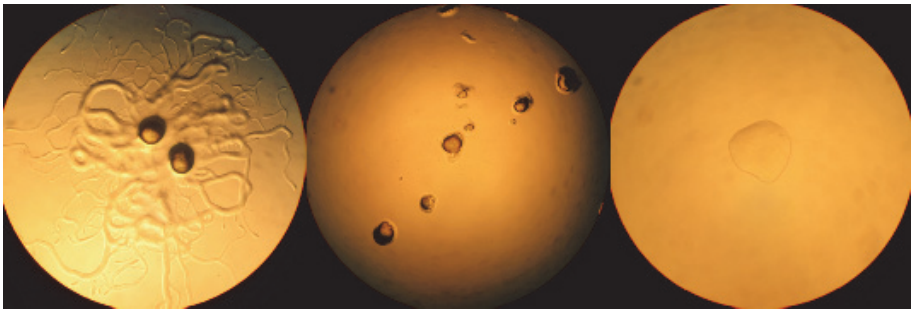

FIGURE 2 Different colony morphologies of the fish pathogen *F. columnare*. From left: rhizoid, virulent (Rz); rough, non-virulent (R); soft, non-virulent (S).

2 AIMS OF THE STUDY

The virulence mechanisms of the fish pathogen *F. columnare* are poorly known. The spreading colony appearance results from gliding motility and is connected to the virulence. *F. columnare* forms colony types that differ in their virulence and gliding motility. Therefore, colony types are useful tools for studying virulence determinants by comparative studies. More specifically, the aims of this PhD work were:

i. To explore the cellular and colony surface structures of different colony types with a high-resolution scanning electron microscope (HR-SEM) and to determine the proteinaceous composition and secretion patterns of the colony types in order to find factors associated with virulence.

ii. To develop a RT-qPCR protocol for studying the gene expression of *F. columnare.*

iii. To explore the impact of the environmental nutrient level on the virulence and on the virulence factor production in virulent and non-virulent colony morphologies by using RT-qPCR.

iv. To study the gene expression of gliding motility and T9SS genes in the spreading and non-spreading colony types grown in conditions that are expected to induce or reduce motility.

3 SUMMARY OF THE MATERIALS AND METHODS

The methods used in this thesis are listed in Table 1 and the bacterial strains in Table 2. For further details, see the original papers I–III.

TABLE 1 Methods used in this thesis.

Method	Publication
Experimental infection	I, II
High-resolution scanning electron microscopy (HR-SEM)	I
Isolation of outer membrane vesicles (OMV)	I
Outer membrane protein (OMP) extraction	I, III
Extracellular product (ECP) profiling	I, III
SDS-PAGE	I, III
Protein identification by mass spectrophotometry	I
Reference gene validation for RT-qPCR	II
RNA sample preparation, RNA quality assessment and cDNA synthesis	II, III
RT-qPCR	II, III
Protease activity	III
DNA sequencing (by Sanger method) and sequence assembly	II, III

TABLE 2 Bacterial strains used in this thesis.

Bacterial species	Strain	Colony type	Reference
Flavobacterium columnare	B067	Rz	(Laanto *et al.* 2011)
F. columnare	B067	R	(Laanto *et al.* 2012)
F. columnare	B067	S	(Laanto *et al.* 2012)
F. columnare	B185	Rz	(Laanto *et al.* 2011)

4 RESULTS AND DISCUSSION

4.1 Colony types are potential tools in the study of *F. columnare* virulence

During recent years, although the pathogenesis of *F. columnare* has been studied by genome sequencing (Tekedar *et al.* 2012), proteomics (Dumpala *et al.* 2010), gene deletion methods (Li *et al.* 2015), and different experimental approaches (Kinnula *et al.* 2015, Declercq *et al.* 2015), the molecular mechanisms of how it causes the disease are still poorly known. The different colony types possessing differences in virulence (Kunttu *et al.* 2009a, Laanto *et al.* 2012, Dong *et al.* 2015) offer a considerable possibility to study *F. columnare* virulence via comparative analyses. The phenotypic difference between the colony morphologies is dramatic and is hypothesized to reflect changes in the cell surface structures and the molecular composition. In this study, the colony types were compared with respect to the surface structure of the colonies and individual cells, as well as protein composition and extracellular secretion. Environmental resources are important mediators of several metabolic pathways, including those related to virulence. Here, the effect of nutrient availability on *F. columnare* virulence and virulence factor expression was explored. Furthermore, the expression of T9SS and *Flavobacterium* gliding motility genes was measured in motility-inducing and reducing environments.

4.1.1 Virulence of the colony types (I)

The ability of the Rz colony type to form spreading colonies was earlier connected to *F. columnare* virulence (Kunttu *et al.* 2009a, Laanto *et al.* 2014). In these previous studies, R and S morphotypes were either not virulent or caused only a low mortality when used for challenging fish hosts. The virulence of *F. columnare* strain B067 colony types Rz, R and S (used in I–III) was determined in an experimental infection. Small rainbow trout fingerlings were bathed in water containing an equal amount of bacteria (1×10^5 colony forming unit ml^{-1}) for 1 h and were then transferred in fresh water. After monitoring for 97 hours, the

survival of 30 % was observed in the fish group exposed to Rz, whereas in the fish exposed to R, S and in the control fish there was a minimum survival of 70 %. The survival of fish treated with Rz differed statistically from other treatments ($p = 0.007$), whereas the R, S and control groups did not differ from each other. These results support the previous proposals connecting colony type to virulence, and indicate that morphologies are sufficient for studying *F. columnare* virulence.

4.1.2 Colony and cell surface structures of the colony morphologies (I)

The phenotypic distinction between the colony types can be expected to result from the different internal organization and structure of bacterial cells that form the colony. Rz, R and S colony types of *F. columnare* grown on a filter paper and between a glass slide and Shieh agar (Song *et al.* 1988) were visualized under HR-SEM to explore their ultrastructural differences (I). Cells in Rz and S colonies were organized in rows and lines forming whirl- and wave-like shapes within a colony. By contrast, in R colonies, the cells showed no organization and were located randomly. As R is not virulent, the internal organization of cells within a colony might be one of the requirements for *F. columnare* virulence. However, organized behaviour was also seen in the S type, and thus, deeper studies are needed to confirm this hypothesis. Inter-cellular communication is crucial for bacteria living in communities. Many bacteria communicate via QS molecules in order to express coordinate behaviour (Henke and Bassler 2004). For example, in *P. aeruginosa,* QS molecules are essential for biofilm maturation (de Kievit 2009). Currently, the use of QS in *F. columnare* has not been published. However, according to the analyses it seems that *F. columnare* does not produce any of the known acetylated homoserine lactones (Sundberg & Diggle, personal communication), which are molecules commonly used in QS by Gram-negative bacteria (Williams *et al.* 2007).

A thick, filament-rich layer was observed on top of the Rz and R bacterial growth, whereas this layer was not detected on the S type colonies. Individual cells of the Rz and R type had rope-like elements that connected them to the adjacent cells and to the surface. Bacteria use elongated and filamentous structures for cell adhesion and biofilm formation, for example (Niemann *et al.* 2004, Gerlach and Hensel 2007). Indeed, on the agar plates, Rz and R showed a great adherence capacity, whereas S was only loosely attached to the growth surface (Kunttu *et al.* 2011). In HR-SEM micrographs, the S type cells did not have the filaments but did possess a moister and smoother appearance and seemed to attach to neighbouring cells along the length of the whole cell. Compositional analysis of these filaments, as well as other cell surface structures, such as capsule and lipopolysaccharide (LPS), would provide insights on how they contribute to the adherence of *F. columnare* or if they are important in pathogenesis.

Each colony type was able to produce outer membrane vesicles (OMV) in some circumstances. All the colony types produced vesicles when grown on a solid surface; however, the planktonic cells of S had no OMVs but had only

small blebs on the cell surface. OMVs are spherical pieces of outer membrane that are generated through membrane budding. They are produced by pathogenic as well as non-pathogenic bacteria and they possess a variety of functions related to cell-to-cell communication and modification of cell surface, for example. Most importantly, OMVs have been studied because of their relevance in bacterial pathogenesis as they cargo virulence factors (Schwechheimer and Kuehn 2015). Previously, *F. columnare* was shown to produce OMVs (Arias *et al.* 2012, Cai *et al.* 2013), but in these studies the constitution of vesicles was not studied. To explore the protein composition of OMVs, 5 protein components were identified with nanoLC-ESI-MS/MS (liquid chromatography/electrospray ionization tandem mass spectrometry) from OMVs produced by the Rz type. According to BLAST search (Altschul *et al.* 1990), each protein was encoded by genes in the *F. columnare* genome, and protein function was predicted for 2 proteins, whereas 3 of the proteins remained hypothetical. A protein band of approximately 55 kDa in size was identified as OmpA, which is one of the most abundant components in the outer membranes of Gram-negative bacteria (Koebnik *et al.* 2000) and is commonly found in OMVs (Bonnington and Kuehn 2014). OmpA is an outer membrane porin that enhances the membrane stability by binding to periplasmic peptidoglycan (Park *et al.* 2012) and it has several other roles related to OMV production, adhesion and pathogenesis, for example (Smith *et al.* 2007, Krishnan and Prasadarao 2012). The second identified protein was SprF, which is an outer membrane protein related to the secretion and motility of *F. johnsoniae* (Rhodes *et al.* 2011a). Bacteria with SprF deletion fail to secrete SprB, which is an adhesin required for gliding motility (Nelson *et al.* 2008, Rhodes *et al.* 2011a). Consequently, absence of SprF leads to the formation of non-spreading colonies. The presence of SprF in OMVs supports the previous suggestions that flavobacterial gliding motility is active in *F. columnare* (Olivares-Fuster and Arias 2008, Klesius *et al.* 2010) (I). However, the predicted identity of SprF in *F. columnare* is based on sequence similarity; therefore its role as an OMV component requires further studies.

The protein profiles of OMVs produced by the Rz and R types were similar to each other. When Rz OMV was compared to whole outer membrane protein (OMP) profile, it was not found to contain any extra components. By contrast, it seemed that constituents of OMVs represent only a minority of the whole OMP composition, which could indicate for selective packaging. Selective packaging of OMVs has been reported, for example, in *P. gingivalis*, *Bacteroides fragilis* and *B. thetaiotaomicron* (Haurat *et al.* 2011, Elhenawy *et al.* 2014). In these bacteria, the OMVs are enriched with proteolytic enzymes, and in the periodontal pathogen, *P. gingivalis*, they are enriched with proteolytic virulence factors (Elhenawy *et al.* 2014). Many pathogenic bacteria pack OMVs with virulence factors. Transportation via OMVs has several advantages compared to secretion system-dependent translocation; it offers a suitable environment for membrane-bound proteins and protects the cargo from the extracellular environment (e.g. degrading enzymes). Furthermore, OMVs allow the host tissue to be attacked from a distance with a more concentrated load of

substances (Bonnington and Kuehn 2014). In this study, the vesicles were analysed further only from liquid cultures, but OMVs were also present in the biofilms. The OMV protein constituents are expected to differ between the planktonic and biofilm lifestyles (Schooling and Beveridge 2006). The comparison of OMVs from different growth styles and more detailed analysis of their contents – specifically with regards to proteolytic activity – would give a wider understanding of *F. columnare* membrane vesicles and their putative role in pathogenesis.

4.2 Development of a RT-qPCR protocol for studying gene expression in *F. columnare* (II)

RT-qPCR is a technique that can be utilized for measuring the gene expression level of any specific gene of interest. RT-qPCR is a sensitive method, but with a careful experimental design it offers a reliable approach for studying gene expression of any gene with a pre-determined DNA sequence. In 2009, MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines were published in order to increase the quality of published qPCR results. It provided general guidelines for publishing gene expression results generated with the RT-qPCR method (Bustin *et al.* 2009).

Following the MIQE guidelines, a RT-qPCR protocol was developed for *F. columnare* to study gene expression in different colony types. Generally, RT-qPCR determines the amount of mRNA of a target gene in a sample at a given time point (Wong and Medrano 2005). However, mRNAs are unstable and sensitive to degradation. Therefore the MIQE guidelines address the importance of validating the RNA sample quality, as low-quality (highly degraded) RNA produces unreliable gene expression results. In the *F. columnare* qPCR protocol, DNAse-treated RNA samples were analysed with the Agilent Bioanalyzer on RNA Nano Chips to determine the level of sample degradation, and only high-quality samples were used in the further stages. After the RNA had been reverse-transcribed into complementary DNA (cDNA), they were used as templates in qPCR reactions. SYBR® green chemistry was used in the qPCR. SYBR® green is a DNA binding dye that binds only to double-stranded DNA. This emphasizes the importance of an accurate primer design as the primers determine the specificity of qPCR. The specificity of each primer pair binding was tested with both a standard curve and a melting curve analysis to exclude any unspecific binding.

SYBR® green binding to DNA results in a fluorescence signal that can be detected with a specific qPCR instrument. The signal is detected after each cycle in each qPCR reaction, resulting in an amplification curve. The fluorescence intensity correlates with the double-stranded DNA (dsDNA) concentration; therefore, the amplification curve represents the amount of present PCR amplicon during the reaction. The time-point when fluorescence first reaches

the threshold line, (i.e. the intensity required for a detectable signal), is called the quantification cycle (C_q). The C_q value correlates with the initial concentration of the target cDNA (mRNA) and this value can be used for the quantification of gene expression (Heid *et al.* 1996).

Data normalization plays a key role in generating reliable gene expression results with qPCR. The gene expression of target genes has to be normalized with the expression of stably expressed genes, which are referred to as reference genes. Housekeeping genes are often used as reference genes because they tend to be stably expressed regardless of the environmental conditions. However, the expression stability of each candidate reference gene has to be evaluated. It is recommended that gene expression stability is measured in a sample set that covers all the conditions of the upcoming qPCR experiment. There are several available software tools for measuring gene expression stability; for example, geNorm (Vandesompele *et al.* 2002), NormFinder (Andersen *et al.* 2004) and Bestkeeper (Pfaffl *et al.* 2004). Many of these have been integrated into commonly used qPCR data analysis software.

In this study, the gene expression stability of five reference gene candidates, 16S rRNA, *gapdh*, *glyA*, *rplQ* and *rpoD*, was measured. The expression stability was measured in a sample set which included samples from Rz, R and S colony types grown on 1x Shieh agar plates and in liquid 1x Shieh medium. Using the geNorm and NormFinder methods (Vandesompele *et al.* 2002, Andersen *et al.* 2004), the most stably expressed genes were *gapdh* and *glyA*. These two genes were validated as suitable reference genes to be used in the RT-qPCR studies of *F. columnare* colony types (II). However, as the expression stability needs to be assessed in all the treatment groups, the stability values were calculated for the entire dataset whenever this protocol was used (II, III).

4.3 Secretion in different colony morphologies (I, II, III)

Proteases are suggested to play a significant role in the virulence of a range of pathogens belonging to phylum Bacteroidetes, including *F. columnare* (Thomas *et al.* 2011). Considering the external symptoms of the columnaris disease, which are seen as lesions, erosions and necrosis of fish skin, gills and fins (Pulkkinen *et al.* 2010), the involvement of secreted proteases seems logical. For the degradation of fish tissues, bacteria need enzymes that specifically target the structure components of vertebrate tissues. Therefore, proteases are important for the infectivity of many fish pathogens, including *Yersinia ruckeri* and *Aeromonas hydrophila* (Leung and Stevenson 1988, Secades and Guijarro 1999). *F. columnare* produces several of proteases that degrade at least gelatine, casein, hemoglobin, fibrinogen and elastin (Bertolini and Rohovec 1992, Newton *et al.* 1997). However, the responsible enzymes resulting in these lytic activities have not been identified and it has remained unclear whether they are connected to virulence. In the present study, the secretion of colony types Rz, R

and S was studied in order to find factors that could explain the differences in their morphology and virulence (I, II, III). RT-qPCR was used to measure the gene expression of putative secreted proteolytic virulence factors (II). In addition, the extracellular product (ECP), the OMP profiles and general proteolytic activity were explored in each of the colony types (I, III).

The capacity to degrade chondroitin sulphates has been connected to the virulence of *F. columnare* (Suomalainen *et al.* 2006). This activity is due to chondroitinase, an enzyme that degrades chondroitin sulphates of the extracellular matrix in vertebrate tissues. In study II, the expression of chondroitinase (encoded by *cslA*) in different colony types (Rz, R and S) was analysed using RT-qPCR. The virulent Rz type showed significantly higher (over 5-fold change) chondroitinase expression in liquid Shieh medium and on agar plate cultures when compared to other colony types (main effects of colony type on chondroitinase [$p = 0.01$] and collagenase expressions [$p = 0.002$]). This result is consistent with the previous studies; recently, the role of chondroitinase was demonstrated with a gene deletion experiment (Li *et al.* 2015). That study showed that mutants lacking this gene, and consequently, the ability to degrade chondroitin sulphate were less virulent, confirming the importance of chondroitinase in *F. columnare* virulence. The same study reported another *F. columnare* gene with lytic activity of chondroitin, *cslB*. However, the total lytic activity of chondroitin was shown to result mainly from *cslA*.

Collagenases are enzymes that degrade collagen fibres of vertebrate tissues. Due to their function, they are important virulence factors in several bacterial pathogens (Harrington 1996); also, they have been proposed to be involved in the virulence of *F. columnare*. This suggestion is based on a SSH study in which *F. columnare* was compared to its non-virulent relative, *F. johnsoniae*, to reveal genes unique to *F. columnare* (Olivares-Fuster and Arias 2008). The collagenase gene was found by SSH only in *F. columnare,* but its role in virulence has not been further analysed. The current study performed with RT-qPCR shows that collagenase is expressed in each of the *F. columnare* colony types; however, its expression was not associated with the virulent Rz type (II). By contrast, the highest collagenase expression was observed in the R type, which is not able to cause disease symptoms in fish.

RT-qPCR can be used for measuring the expression of a target gene at the transcriptional level (Wong and Medrano 2005). However, secreted proteins undergo various modification steps prior to external localization. Even though bacteria regulate their gene expression mainly at the transcriptional level (Commichau and Stulke 2015), regulation also occurs after transcription and translation. Collagenolytic activity is undoubtedly necessary only in the extracellular environment where it can degrade collagen fibres of the host tissue. The successful secretion of proteases requires active processing that enables, for example, the correct folding of the polypeptide chain into a mature protein and an intact secretion system through which the product can be translocated (Natale *et al.* 2008, Costa *et al.* 2015). The processes needed for maturation of *F. columnare* collagenase and chondroitinase are yet to be

characterized. Therefore, it remains uncertain whether all of the colony types are able to successfully secrete chondroitinase and collagenase to the exterior of the cell. However, the overall ECP and OMP profiles of Rz, R and S grown in a liquid Shieh medium (Song *et al.* 1988) did not differ remarkably, except for the overrepresented ~13 kDa protein band, found in the ECP profile of Rz, which was barely detectable in R or S (I; discussed in more detail below). Furthermore, all the colony types possessed extracellular proteolytic activity with no perceptible differences on skim milk agar plates (III). These results indicate that each colony type has functional systems for the secretion of proteolytically active compounds.

When the ECP profiles of different colony types were compared, a ~13 kDa protein band, unique to virulent Rz type was detected. The intensity of this protein band was only moderate in the non-virulent R and S types (I). Using mass spectrophotometry analysis, the protein was identified as an open reading frame (ORF) FCOL_04265 of *F. columnare* ATCC 49512 (NCBI: NC_016510). A BLAST search of this gene did not provide any additional information for predicting the gene function, and the ORF was found to be specific to *F. columnare*. The association between the virulent Rz type and the high expression level of this protein could indicate that it has a role in *F. columnare* virulence, but to confirm this suggestion, a more detailed investigation is required. For example, a mutation in ORF FCOL_04265 and its impact on virulence could be tested in an infection experiment. This approach could give us insight into the importance of this protein in pathogenesis of *F. columnare*.

The gene sequences of chondroitinase (*cslA*), collagenase and the 13 kDa protein with an unknown function were determined with the traditional Sanger sequencing method and were compared between Rz, R and S types (II, III). The sequences of these genes did not differ between the colony types, even though their expression in gene (chondroitinase, collagenase) or protein (13 kDa) levels was different. This result indicates that the colony types have differing cascades for regulating these genes. The regulatory pathways of *F. columnare* have not been studied further, but their investigation would help to understand the physiology of the different colony types.

4.4 Effects of nutrient availability on *F. columnare* (II, III)

Environmental factors are important regulators of bacterial metabolism (Somerville and Proctor 2009, Chubukov *et al.* 2014). Pathogenic bacteria also use these signals for detecting the host presence and regulating the virulence mechanisms. In *F. columnare*, water quality, including nutrient composition, temperature, salt concentration and hardness, have direct influences on bacterial adhesion and pathogenicity (Wakabayashi 1991, Decostere *et al.* 1999b, Straus *et al.* 2015). High water hardness also has been shown to increase biofilm formation in *F. columnare*, whereas increased salinity and water temperature have the opposite effects (Cai *et al.* 2013). Environmental nutrient availability

has a wide impact on bacterial metabolism and virulence, but its role in opportunistic pathogens has not gained too much attention. The changing nutrient level is predicted to contribute to the regulation of virulence mechanisms as the fish host represents a considerably different habitat compared to the outside-host niche in which different metabolic processes need to be activated. In the present work, general nutrient availability as an environmental regulator of *F. columnare* virulence and related attributes was explored. Nutrient availability of the outside-host environment not only increased *F. columnare* virulence, but also induced the virulence factor expression (II). Furthermore, the decreased nutrient level on the agar plates enhanced spreading behaviour and changed the expression of the genes needed for motility (III).

4.4.1 Nutrients have a significant impact on *F. columnare* virulence (II)

Proteolytic enzymes contribute to the bacterial virulence by degrading the host tissue components (Lantz 1997). *F. columnare* produces chondroitinase and collagenase, which exhibit proteolytic activities on the extracellular matrix components, chondroitin sulphate and collagen, respectively. The gene expression of these enzymes in Rz, R and S types was measured with RT-qPCR on agar plates on which the medium composition was either halved (0.5x), normal (1x) or doubled (2x). Both chondroitinase and collagenase were upregulated at higher nutrient levels in the Rz type. The same pattern was observed in the R and S types with regard to chondroitinase, but not with collagenase.

To determine whether the increased virulence factor production was also reflected on virulence, rainbow trout fry were exposed to two bacterial strains; B067 and B185, which represent two different genotypes, A and G, respectively. Prior to the infection, the bacteria had been cultivated in nutrient-modified media (0.5xN, 1x and 2xN Shieh), in which the concentration of yeast extract and peptone were either halved or doubled. The concentrations of the rest of the Shieh components were not modified, but in both of the media, they were equal to those of 1x Shieh. In addition to the peptone and yeast extact, the Shieh medium contains salts such as iron sulphate. Because iron is an important regulator of bacterial virulence gene expression (Litwin and Calderwood 1993), the modifications were restricted only to the peptone and yeast extract to exclude the possible influences of the other medium substances. It is also worth mentioning that the bacteria originated from same culture before dividing into different growth conditions. This ruled out the possibility that the observed differences in the virulence experiment were due to the differences between bacterial cultures. Both bacterial strains with a history in high-nutrient environment caused significantly more rapid fish mortality compared to bacteria grown in low-nutrient concentrations and control treatments ($p \leq 0.041$ for B067; $p \leq 0.006$ for B185). The results of this work connect a high nutrient level with an increased virulence factor production and virulence. Nutrient availability was also predicted to turn microbes from mutualistic into virulent

for their fish host (Wedekind *et al.* 2010). Furthermore, even a slight increase in nutrient resources promoted disease symptoms in coral diseases (Bruno *et al.* 2003). However, a recent study demonstrated that the effects of the nutrient level can also be opposite, and the nutrient quality may have remarkable impact on the outcome of host–pathogen interaction (Ketola *et al.* 2016). Nutrient quality was not assessed in this study, and more detailed studies are needed to specifically determine which nutrient components contribute to the regulation of *F. columnare* virulence activity. In the infection experiment of the study II, a fish mortality of 93.75–100 % was observed among the infected fish. The mortality in the control fish group reached approximately 45 %. Control fish mortality is usually detected in experimental infections of rainbow trout fry, which is adapted to cold water. However, in the laboratory conditions, a successful infection of *F. columnare* requires temperatures above 23 °C (Kunttu et al., personal communication). Therefore the infection conditions, especially the higher temperature, may contribute to the control fish mortality.

A nutrient-rich environment may affect bacterial virulence through increased growth rate, which in turn may increase the bacterial dose (Wedekind *et al.* 2010). However, this study shows that nutrients of the outside-host environment may trigger on those genes that are putatively relevant only in the within-host environment. The expression of chondroitinase and collagenase in the absence of the host may be a response to the environment nutrients other than those derived from the host. Even though these enzymes may not be needed in the outside-host environment, their increased expression may influence the bacterial virulence. Consequently, these bacteria are able to cause the disease more rapidly when they encounter the host. This result is an important finding considering the disease epidemics at fish farms. Once the bacterium has entered the fish farm and the first infections appear, the fish farming conditions may, in fact, support the process of infection outbreak. The fish carcasses, together with fish faeces and uneaten food, can maintain the virulence machinery by serving as additional nutrient resources. Due to the saprophytic lifestyle and the ability of *F. columnare* to survive even under starvation, the farming conditions offer various niches for its outside-host survival (Kunttu *et al.* 2009b, Sundberg *et al.* 2014). The intensive farming conditions appear to select for more virulent *F. columnare* strains that can reach high population densities (Sundberg *et al.* 2016). Taken together, elevated nutrient resources may drive this process by favouring those bacteria that have a strong metabolic response to the external nutrient resources.

The protease activity may not be 'on' or it is expressed only at basal level when substrates are not available. For example in *Leptospira interrogans*, the collagenase expression is induced only in cell cultures (Kassegne *et al.* 2014). As the collagenase expression analysis in the current study was conducted in the absence of a host, it cannot be excluded that the expression of this enzyme is controlled by appropriate substrate concentration. Furthermore, bacterial proteases that are subject to post-transcriptional and post-translational processing and regulation on these levels can also influence mature protein

expression. This, in turn, is undetectable with RT-qPCR, which measures the expression at a transcriptional level.

The environmental nutrient variables used in the study II were either all components of the Shieh medium (in the virulence gene expression experiment) or only the peptone and yeast extract (in the infection experiment). Even though these nutrients are not absolutely comparable to the organic matter found in the fish tanks, this result opens new directions for studying the impact of nutrients on *F. columnare* virulence. The enzymes studied in this work, chondroitinase and collagenase, are predicted to function in the host tissue degradation. However, the bacterial infection process usually includes several initial steps, such as chemotaxis towards the host, attachment to the infection site and colonization of the host tissue. The factors involved in these steps of *F. columnare* infection are not characterized; therefore it would be useful to explore the impact of environmental nutrients on the adherence capacity or chemotaxis. Chemotaxis towards fish mucus has been recognized in *F. columnare* (Klesius *et al.* 2008).

4.4.2 Gliding motility in different nutrient conditions (III)

Bacteria use environmental cues for controlling the activity of their motility apparatus. Generally, nutrient availability may have opposite responses: some bacteria invest in motility when nutrients are abundant, while others are more motile in low-nutrient conditions in order to search for resources elsewhere.

Virulent *F. columnare* colonies show great phenotypic plasticity when exposed to different nutrient conditions (Laanto *et al.* 2012). Adaptation to a changed nutrient availability is seen as changes in spreading behaviour, which can be quantified by an increase in colony diameter. Here, the effect of the nutrient level on the spreading behaviour was determined on agar plates with low (0.5x Shieh) and high (2x Shieh) nutrient concentrations. An increased colony diameter change in response to a decreased environmental nutrient level was observed in Rz and S types, whereas R type did not have this ability (III). Consistent results were observed earlier (Laanto *et al.* 2012), but the current study also includes the S type in the comparison. The spreading colony morphology of *F. columnare* can be considered also as an indicator of functional gliding motility machinery. Given that the *F. columnare* genome (Tekedar *et al.* 2012) carries the majority of the genes needed for *Flavobacterium* gliding motility and T9SS, it is assumed that these systems are required for the formation of spreading colony morphology. The function of *F. johnsoniae* gliding motility machinery is disrupted by even a small mutation in one of its proteinaceous components (see e.g. (Braun and McBride 2005)). In this study, the DNA sequences of gliding motility and T9SS genes *sprA*, *sprE*, *sprT*, *porV* and operons *gldKLMN* and *sprCDBF* were determined in spreading (Rz and S) and non-spreading (R) colony types. However, no genetic differences were found. Given the ability of *F. columnare* to switch colony type in a reversible manner, it can be hypothesized that the phenotypic changes are due to gene regulation rather than a random mutation in these gliding motility genes. Finally, the expression

of genes involved in these machineries, *gldG, gldH, gldL, sprA, sprB, sprE, sprF, sprT* and *porV,* was determined in Rz, R and S types grown in 0.5x Shieh, which induced the spreading behaviour, and in 2x Shieh, which reduced it. For measuring the gene expression, the previously developed RT-qPCR protocol was used (II).

The gene expression of *gldG, gldH, gldL* and *sprE* differed significantly between the colony types. Nutrient concentration had significant impact on *gldL*, *sprA*, *sprB* and *sprF* expression. In the gene expressions of *gldH*, *gldL*, *sprA* and *sprE,* a significant interaction between the nutrient level and the colony type was observed. Pairwise comparisons revealed that *gldG* and *gldH* expressions were slightly higher in R compared to Rz and S, and *gldH* expression of R type also differed between the nutrient levels. *gldL* expression of Rz differed significantly from that of R and S. Expression of *sprE* differed between all the colony types. Rz type expressed *sprE* at the lowest level in both nutrient conditions, while R showed high *sprE* expression in 0.5x Shieh. Nutrient level had a significant effect on *sprA* expression in Rz and R. In both colony types, *sprA* expression was higher in low-nutrient conditions. A similar effect was detected in the *sprF* expression of R type. Taken together, some of the gliding motility genes were expressed differently between the colony types, and some were observed to be associated with nutrient availability. However the expression differences did not correlate directly with virulence (Rz type) or spreading behaviour (Rz and S).

Most of the differences were detected between Rz and R type, and R type seemed to respond more dramatically to the environmental nutrients by altered gene expression (*gldH, sprA, sprF*). In contrast, in Rz, the nutrients affected only *sprA* expression, but they did not have a significant impact on any of the studied genes in the S type. To conclude, the gene expression differences between Rz (spreading, virulent), R (non-spreading, nonvirulent) and S (spreading, non-virulent) colony types and nutrient levels are an indicator of metabolic cascades that are either i) different or ii) subject to different regulatory pathways. This might be a result of intentional switching between alternative metabolic pathways, or a random genetic change leading to different phenotypes. Yet, because the colony type can be switched back, it is not likely that colony type changes appear coincidentally; rather they are a consequence of coordinated regulative processes.

The gene expression data showed high variability among the samples even within the same sample group. This variance may be due to the fact that all cells on the same agar plate do not have a similar gene expression profile. In this study, an RNA sample was prepared from the whole bacterial lawn on one Shieh agar plate. In the case that cells have intracolonial division of labour; that is, cells have different tasks depending on where they are located within a colony, global gene expression patterns would be expected to differ even within a single colony. Therefore, the expression pattern of gliding motility genes may not be similar in different parts of the colony, for example, on the edges versus in the middle of a colony. Thus, the variance in gene expression within a same treatment observed in this experiment may be due to the fact that gliding

motility gene expression is not 'on' in every cell, but, for example, is only in cells located on the edges of the colony. An example of this kind of differentiation within a bacterial colony has been reported in *Pseudomonas fluorescens* (Kim *et al.* 2016).

The differences in colony type appearance, between colony types and nutrient conditions, are expected to result from changes in the bacterial cell envelope. T9SS and gliding motility proteins are located on the cell surface (McBride and Nakane 2015), but even though they are assumed to be responsible for *F. columnare* gliding motility, their gene expression does not relate to the spreading phenotype. For assembling functional gliding motility and secretion machineries, the involved components have to be successfully processed and secreted to their final location. The mechanisms of how these protein complexes are built are not known, and only limited data of the processing of T9SS-secreted products are available. However, these studies have been conducted on model species of these systems, *F. johnsoniae* and *P. gingivalis*, but there are no T9SS studies of *F. columnare*. As the mechanisms of gliding motility machinery and T9SS are not fully characterized, there may be additional genes or regulatory mechanisms involved in gliding and secretion that are still to be discovered.

Secretion through T9SS has been studied more closely in *P. gingivalis*. Proteins that are secreted through T9SS have typically N-terminal peptide signals that target them first through the cytoplasmic membrane via the Sec system (Natale *et al.* 2008) and a conserved CTD, which serves as signal for secretion via T9SS through outer membrane (Seers *et al.* 2006). After translocation into the periplasm, the N-terminal signal of the CTD protein is cleaved. As the Sec transports only unfolded polypeptides, the maturation of these proteins requires further processing (Stathopoulos *et al.* 2000, Natale *et al.* 2008). CTD is cleaved off after translocation across the outer membrane (Glew *et al.* 2012), after which the secreted proteins are subjected to post-translational modifications, and finally, are anchored onto the cell surface via A-LPS (anionic lipopolysaccharide) (Gorasia *et al.* 2015). It is not known how *F. columnare* modifies the secreted products translocated via T9SS, but obviously the role of LPS in this process needs to be investigated. In *P. gingivalis*, *porX* and *porY* putatively form a two-component signal transduction system that has been shown to regulate a subset of *gld* and *spr* genes (Sato *et al.* 2010). The homologs of genes *porX* and *porY* are found in the genome of *F. columnare*, but their relevance in the regulation of gliding motility requires deeper investigation. Furthermore, the mechanism of how the signal of the external nutrient availability is transferred into metabolic changes that lead to a visible response in spreading behaviour also may be a cascade that is crucial for pathogenesis.

Tissue-degrading enzymes chondroitinase and collagenase are predictably secreted to the cell surface, but their secretion route is not known. The CTD that is typical for the proteins translocated through T9SS (Seers *et al.* 2006) was detected in chondroitinase (*cslA*) but not in the collagenase amino acid sequence. The ~13 kDa protein that was found to be secreted abundantly in the Rz type also lacked this domain. However, it has been stated that the typical

70–100 amino acid (aa) long C-terminal signal sequence targeting T9SS is highly variable and cryptic (Seers *et al.* 2006, Veith *et al.* 2013, Kharade and McBride 2014), and it has not been characterized in more detail in *F. columnare*. Hence, it remains unclear whether chondroitinase, collagenase and the 13 kDa protein are secreted through the outer membrane via T9SS or by some other route.

4.4.3 Secretion in different nutrient conditions

The nutrient level of the growth medium was shown to regulate the transcription of genes encoding tissue-degrading enzymes chondroitinase and collagenase (II). The whole extracellular secretion was studied by ECP profiling of Rz, R and S cultured under high (2xN Shieh) and low (0.5xN Shieh) nutrient conditions (III). Nutrient availability did not have remarkable effects on ECP profile. OMP profile of the colony types did not differ between the nutrient concentrations (0.5x, 1x, 2x Shieh). The impact of nutrient level on general proteolytic activity was tested on 0.5xN, 1x and 2xN Shieh agar plates supplied with 1.5 % skim milk agar. The nutrient level did not influence protease activity and it was comparable between the colony types. Clear differences in cell surface composition were not found between Rz and R types. However, a phage-induced colony type change from Rz into R is coupled with phage-resistance (Laanto *et al.* 2012) and with the loss of spreading. This result suggests that colony types can be expected to differ in their surface structures as viruses are known to use the cell surface elements for host recognition (Samson *et al.* 2013). Also, components of gliding motility machinery are putatively used by phages infecting *F. johnsoniae* as mutants lacking the lipoproteins GldH, GldI, GldJ are resistant to phages (McBride *et al.* 2003, McBride and Braun 2004, Braun and McBride 2005). Hence, it remains to be studied whether the gliding motility or T9SS components are used as receptors also by *F. columnare* phages.

4.5 Concluding remarks and future prospects

Flavobacteria are important habitants in the freshwater and soil microbial communities (Bernardet and Bowman 2006, Eiler and Bertilsson 2007). In addition to the normal flora of aquatic and soil environments, the genus *Flavobacterium* includes animal pathogens that are relevant in aquaculture (Bernardet and Bowman 2006). However, bacterial pathogenesis has been studied most extensively in the human pathogens and the underlying molecular virulence mechanisms have been well characterized amongst others in model species such as *E. coli* and *P. aeruginosa*. These findings have implicated that the bacterial cell surface structures such as the LPS layer, secretion systems and extracellularly secreted toxins are prominent virulence factors (Wu *et al.* 2008). The knowledge derived from studies using the model species is ideally used to study the virulence of non-model bacterial pathogens. Therefore, the research of non-model organisms, such as *F. columnare,* is

important for understanding its molecular virulence mechanisms, developing sustainable treatment methods and securing our food production.

F. columnare colony types have radically different appearances when cultivated on agar plates. Even though the bacteria isolated from disease outbreaks represent the virulent Rz type, the other non-virulent (R and S) types are systematically observed in laboratory cultivations. The roles of these morphologies are not known, and the mechanisms by which the morphology is switched are yet to be determined. In general, the ability to express morphological diversity is important because it can protect the bacteria not only from predators, such as protozoans and phages (Jurgens and Matz 2002, Laanto *et al.* 2012), but also from host immune systems and antimicrobial treatments (Justice *et al.* 2008). Furthermore, cooperative bacteria may have different types of cells contributing to the common goal, for example, biofilm formation or pathogenesis (Kuemmerli *et al.* 2009, Verplaetse *et al.* 2015). Morphological changes are expected to result from altering the gene expression profiles. It has been proposed that the *F. columnare* colony type diversity could be due to phase variation (Kunttu *et al.* 2009a), which is switching on/off the expression of certain genes (van der Woude 2006), but no mechanistic evidence of such process has been reported. Hence, the molecular level differences resulting in such dramatic phenotypic changes remain to be characterized. For exploring these differences, whole genome sequencing and transcriptomic analysis of the colony types could potentially open new insights into their metabolism and on the virulence mechanisms. These approaches would potentially help to identify new genetic factors that are only specific for virulent Rz and potentially related to virulence. To be able to study the proposed virulence factors further, a genetic toolbox for generating gene deletions and targeted mutagenesis is needed. However, the study of *F. columnare* virulence has been substantially hindered by a lack of tools for genome manipulation. The attempts to develop genetic manipulation techniques for this bacterium have not been completely successful (Staroscik *et al.* 2008, Zhang Jin *et al.* 2012). Plasmids have not been found in *F. columnare* isolates (Sundberg *et al.* 2016) and foreign DNA introduced by transformation is not accepted or is subjected to processing that makes the plasmids deficient (Heidi Kunttu, personal communication). Recently, Li *et al*. developed a successful gene deletion strategy for *F. columnare* (Li *et al.* 2015). This method is a promising tool for future studies and is inevitably important in investigating the virulence mechanisms of this pathogen. For example, the role of collagenase or any other virulence factor candidate could be studied by generating a null mutant of the gene of interest. Finally, the relevance of the gene could be tested in an experimental fish infection using a mutated bacterial strain. The study with deletion mutants requires also a complementation test, in which a wildtype gene is introduced into the cell in order to restore the gene function. In case the gene deletion leads to a loss of virulence, and the complementation in turn restores it, the gene can be considered a virulence factor. However, one future challenge in the development of new genetic tools for manipulating the *F. columnare* genome is the unstability of the plasmids when introduced to the cells.

5 CONCLUSIONS

The main findings of this thesis were:

I Colonies of the virulent Rz type exhibited advanced organization of individual cells, whereas a lack of order was seen in the non-virulent R colony, as revealed by SEM. *F. columnare* cells produced OMVs and part of the OMV composition was identified, but their role in virulence remains unknown. The ECP profile revealed a ~13 kDa protein with an unknown function. Its uniqueness to virulent Rz suggests that it contributes to *F. columnare* virulence. The role of this protein remains to be determined in the future; for example, with the help of a deletion mutant.

II RT-qPCR protocol was developed for studying gene expression in *F. columnare* colony types. The expression of two, already published, tissue-degrading enzymes was induced by increased environmental nutrients. Furthermore, elevated nutrient levels caused more rapid mortality in fish. These results suggest that a high nutrient level increases the bacterial virulence by increasing virulence factor production.

III *F. columnare* colony types responded differently to altered nutrient concentration by i) spreading behaviour and ii) gene expression of gliding motility and T9SS genes. These observations demonstrate that in addition to the colony appearance, these colony types have distinct regulatory programs for controlling their metabolism. The mechanisms underlying these processes require deeper investigation.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Kalapatogeeni *Flavobacterium columnare*n virulenssiin vaikuttavat geneettiset ja ympäristölliset tekijät

Patogeeniset bakteerit ovat loisia, jotka hyödyntävät isäntänsä soluista ja kudoksista vapauttamiaan ravinteita omassa aineenvaihdunnassaan. Tällaista bakteerin ominaisuutta kutsutaan taudinaiheutuskyvyksi eli virulenssiksi. Kullakin patogeenilla on oma erityinen tapansa aiheuttaa tautia, mutta infektioon tarvitaan bakteerilajista riippumatta tiettyjä yhteisiä ominaisuuksia. Onnistuneen infektion aiheuttamiseksi bakteerin täytyy ensin kiinnittyä isäntäänsä (adherenssi), levittäytyä ja lisääntyä siinä (kolonisaatio), sekä selviytyä sen immuunipuolustusmekanismeista. Lisäksi patogeenilla täytyy olla kyky siirtyä uuteen isäntään (transmissio). Näitä tapahtumia varten bakteeri tarvitsee virulenssikoneistoja, joiden toimintaan osallistuvia tekijöitä kutsutaan virulenssitekijöiksi. Isännän kudosten hajottamiseen bakteeri tarvitsee erilaisia proteolyyttisiä entsyymejä, joiden avulla kudosten rakennuskomponentit voidaan pilkkoa pienempiin osiin ja käyttää edelleen bakteerin omassa metaboliassa. Bakteerisolun pintarakenteista sekreetiokoneistoilla on usein tärkeä rooli virulenssissa, sillä niiden avulla bakteeri erittää virulenssitekijöitä välittömään ympäristöönsä tai suoraan isäntäsolun sisälle.

Opportunistiset bakteerit kykenevät elämään isännän ulkopuolella, mutta kohdatessaan sopivan isännän, ne pystyvät aiheuttamaan infektion. Tällainen elämäntapa vaatii huolellista ympäristön ominaisuuksien havainnointia, jonka avulla bakteeri säätelee metaboliaansa vallitsevien olosuhteiden mukaisesti. Ympäristön ominaisuuksia aistimalla bakteeri havaitsee isäntäsolun läsnäolon, joka usein käynnistää virulenssikoneistojen toiminnan. *Flavobacterium columnare* on kaloja infektoiva opportunistinen patogeeni, joka on ongelmallinen kalanviljelyssä ympäri maailmaa. Onnistuneen infektion seurauksena tämä tehokkaasti leviävä bakteeri voi aiheuttaa jopa kokonaisen viljelyaltaan kalojen kuoleman. Virulenssimekanismien toiminnan tunteminen voi olla merkittävä avain uusien hoitomuotojen kehittämiseen. Molekulaarisella tasolla *F. columnare*n virulenssikoneisto on kuitenkin jäänyt vielä suurelta osin määrittelemättä. Laboratorio-olosuhteissa se muodostaa kolmenlaisia pesäketyyppejä; Rz (*engl.* rhizoid), R (*engl.* rough) ja S (*engl.* soft). Näistä ainoastaan Rz on taudinaiheutuskykyinen. Rz-tyypin pesäkkeen juurimainen ja leviävä muoto on yksi virulenssiin yhdistetyistä ominaisuuksista. Tehokkaaseen leviämiseen kykenevä pesäke on indikaatio bakteereista, jotka liikkuvat pintoja pitkin niin kutsutun 'gliding motility' -menetelmän avulla. Todennäköisesti tämän liikkumistavan mahdollistaa *F. columnare*lla Bacteroidetes-pääjaksolle ominainen, solun pinnalle sijoittunut proteiinikompleksi, jonka toiminta on nivoutunut yhteen tyypin IX sekreetiosysteemin (T9SS) kanssa.

Tämän väitöskirjan tavoitteena oli selvittää *F. columnare*n virulenssiin vaikuttavia geneettisiä tekijöitä sekä tutkia, miten isännän ulkopuolisen ympäristön ominaisuudet vaikuttavat bakteerin taudinaiheutuskykyyn. Väitöskirjan

kaikissa osatöissä hyödynnettiin bakteerin virulenttia ja ei-virulentteja pesäketyyppejä. Väitöskirjan ensimmäisessa osatyössä eri pesäketyyppien muodostamien biofilmien sekä yksittäisten solujen pintarakenteita tutkittiin korkean resoluution pyyhkäisyelektronimikroskoopilla. Tutkimuksessa havaittiin, että bakteerisolut tuottavat ulkomembraanivesikkeleitä, joita useiden patogeenien tiedetään käyttävän virulenssitekijöiden kuljetukseen. Vesikkeleiden tarkemmassa analyysissä pystyttiin tunnistamaan proteiinit OmpA, joka on yleinen membraaniproteiini, sekä SprF, joka on yksi 'gliding motility' -koneiston komponenteista. Kun tarkasteltiin solun ulkopuolelle eritettyjä proteiineja, havaittiin, että Rz tuottaa suuria määriä molekyylikooltaan noin 13 kDa:n kokoista proteiinia. Tutkimuksessa tämä proteiini pystyttiin identifioimaan geenitasolle, mutta sen tarkempi tehtävä ja mahdollinen yhteys virulenssiin jäävät selvitettäviksi tulevaisuudessa.

Ympäristön ominaisuuksien aistiminen on tärkeä bakteerien ominaisuus, sillä ne joutuvat sopeutumaan nopeastikin muuttuneisiin olosuhteisiin ja säätelemään metaboliaansa sen mukaisesti. Opportunististen patogeenien elinkierto sisältää vaiheita sekä isännässä, että sen ulkopuolisessa ympäristössä, jotka eroavat lähtökohtaisesti toisistaan. Väitöskirjan toisessa osatyössä kehitettiin RT-qPCR-menetelmä, jonka avulla tutkittiin isännän ulkopuolisen ravinnepitoisuuden vaikutusta virulenssitekijöiden geenien ekspressioon eli ilmentymiseen, sekä taudinaiheutuskykyyn. Työssä havaittiin, että korkeassa ravinnepitoisuudessa kasvaneet bakteerit ekspressoivat tutkittuja kudosta hajottavia entsyymejä, kondroitinaasia ja kollagenaasia, merkittävästi enemmän kuin matalassa ravinnepitoisuudessa kasvaneet bakteerit. Lisäksi huomattiin, että korkeassa ravinnepitoisuudessa kasvaneet bakteerit aiheuttivat nopeammin taudinpurkauksen kaloissa.

Väitöskirjan kolmannessa osatyössä tutkittiin 'gliding motility'-koneiston sekä T9SS:n proteiinikomponenttien ekspressiota aiemmin kehitetyn RT-qPCR-menetelmän avulla. Geenien ekspressioita vertailtiin sellaisten pesäketyyppien välillä, jotka pystyvät tai eivät pysty muodostamaan leviäviä pesäkkeitä. Lisäksi geenien ekspressioista tutkittiin leviämistä indusoivassa sekä vähentävässä ravinneympäristössä. Havaittiin, että liikkumiskoneiston geenejä ekspressoidaan eri tavoin eri pesäketyypeillä, ja että ravinneolot vaikuttavat ainakin osaan näiden geenien ekspressioista.

Bakteeritautien ehkäisemisessä ja uusien hoitomuotojen kehittämisessä on tärkeää tuntea taudinaiheutuskyvyn taustalla olevat molekyylitason tekijät, sekä millaiset ympäristön ominaisuudet näitä mekanismeja ohjaavat. Väitöskirjan tulosten pohjalta voidaan todeta, että *F. columnare*n pesäketyypit ovat oiva työkalu uusien virulenssitekijöiden löytämiseen sekä sen virulenssimekanismien tutkimiseen. Lisäksi ravinteiden saatavuudella on merkittävä vaikutus bakteerien virulenssiin. Viljelyaltaissa ravinneolosuhteet voivat olla tautien leviämisen kannalta erityisen hyvät, jos niihin kertyy syömätöntä rehua ja kalojen jätöksiä. Tuloksilla voi olla merkitystä kalanviljelyn käytäntöjä ajatellen, sillä viljelyolosuhteet voivat jopa edesauttaa infektioiden käynnistymistä. Tulevaisuuden haasteita tulevat olemaan geneettisten menetelmien soveltaminen *F. columnare*n

virulenssimekanismien tarkemmaksi tutkimiseksi, sekä virulenssin säätelymekanismien tutkimus molekyylitasolla.

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ORIGINAL PAPERS

I

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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RESEARCH ARTICLE Open Access

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

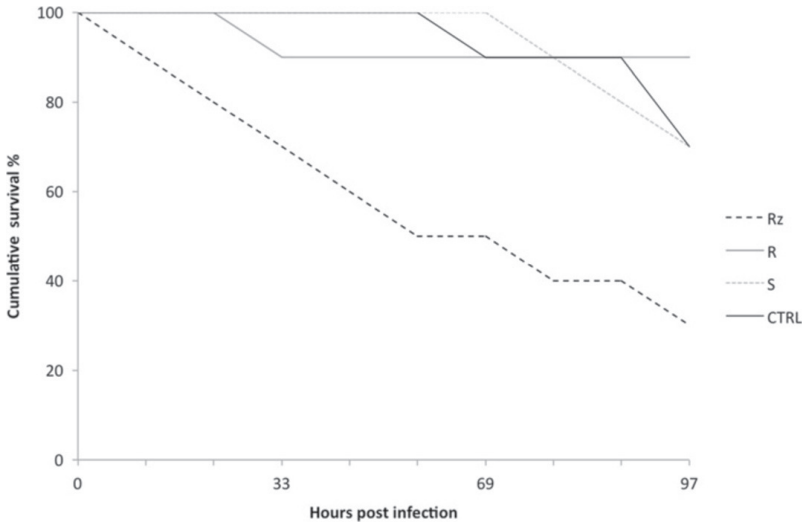

Figure 1 Virulence of different morphotypes of *F. columnare*. Rainbow trout (*Oncorhynchus mykiss*) survival after challenge with Rhizoid, Rough and Soft morphotypes of *F. columnare*.

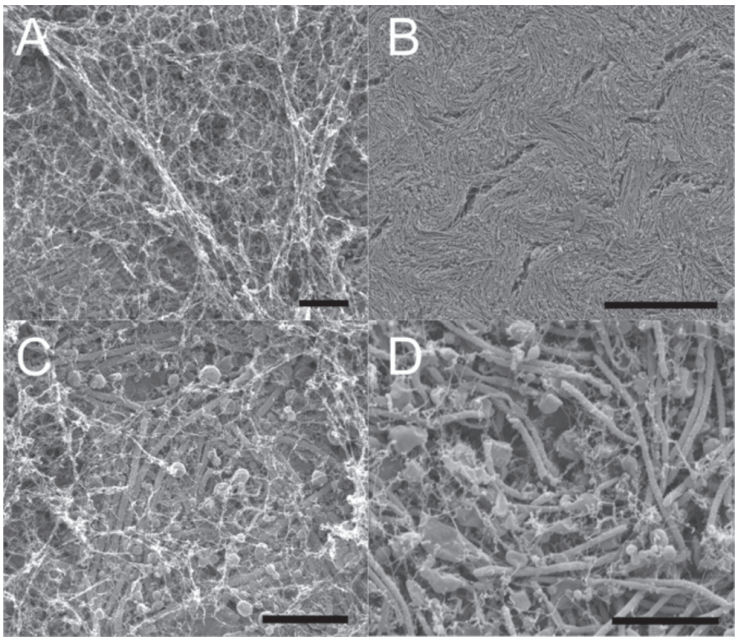

Figure 2 Colony surfaces of the Rhizoid and Soft morphotypes of *F. columnare* B067. The cells were grown on a filter paper on top of a Shieh agar plate and were visualised under HR-SEM. **Panel A**: Biofilm of the Rhizoid morphotype covered with an extracellular layer. **Panel C**: A closer view of 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.

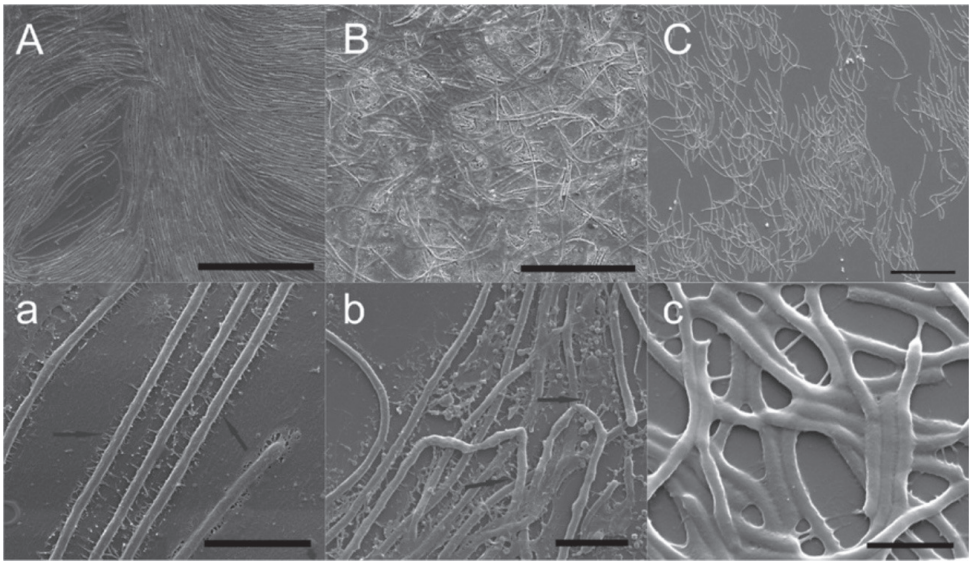

Figure 3 Internal structure of colonies formed by Rhizoid, Rough and Soft morphotypes of *F. columnare* B067. *F. columnare* cells were grown between a glass slide and Shieh agar and visualised under HR-SEM. **Panel A**: Rhizoid morphotype, **Panel B**: Rough morphotype and **Panel C**: Soft morphotype. 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

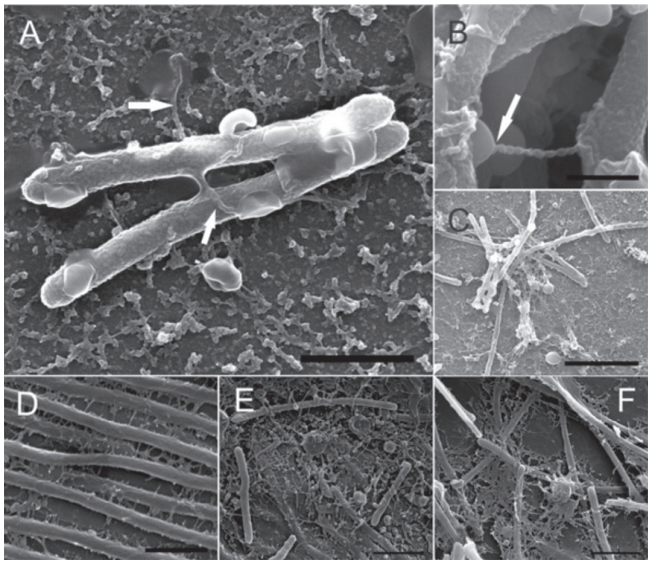

Figure 4 Surface structures and vesicles observed in *F. columnare* B067 cultured in liquid and on agar. Panels A to C represent the planktonic cells, and **panels D to F** show cells grown on agar. **Panel A**: A typical view of the planktonic cells of the Rhizoid morphotype. Vesicles and rope-like 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).

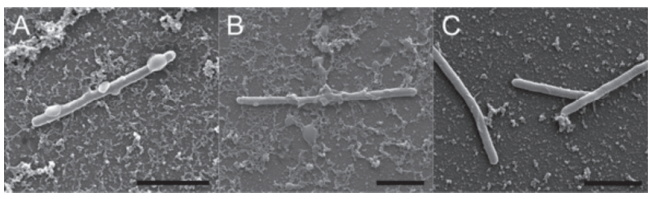

Figure 5 Planktonic cells of Rhizoid, Rough and Soft morphotypes of *F. columnare* B067. A typical view of the cells grown in liquid medium visualised under HR-SEM of the three morphotypes. Large vesicles were typical on the surface and surroundings of the **A)** Rhizoid and **B)** Rough morphotypes.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

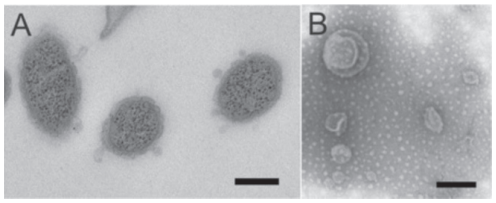

Figure 6 OMVs of Rhizoid morphotype *of F. columnare* B067. **Panel A**: Outer membrane vesicles visualised under TEM from the thin sections of the cells of the *F. columnare*; and **Panel B**: Purified vesicles under TEM. In the thin sections, the size of the observed vesicles was under 100 nm, whereas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

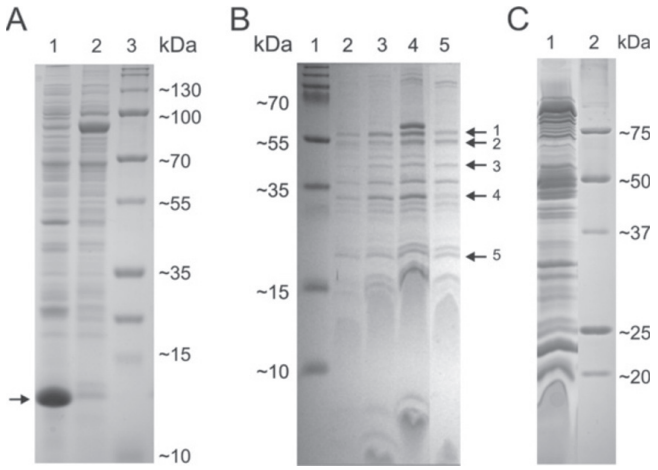

Figure 7 Protein profiles of *F. columnare* B067 morphotypes. Panel A: Extracellular protein profiles of the Rhizoid (Lane 1) and Rough (Lane 2) morphotype of the *F. columnare* strain B067 run in a 14% Tricine SDS-PAGE -gel. The protein band that was analysed further by nanoLC-ESI-MS/MS is indicated 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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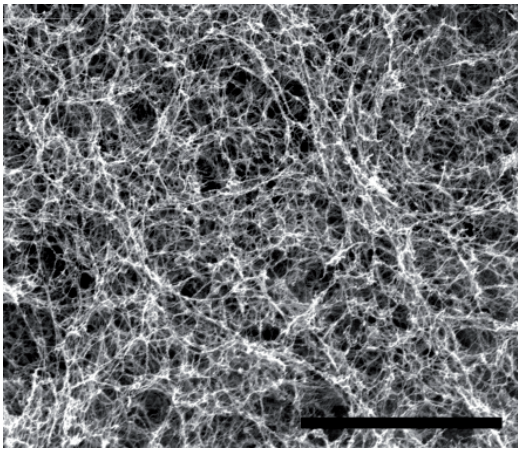
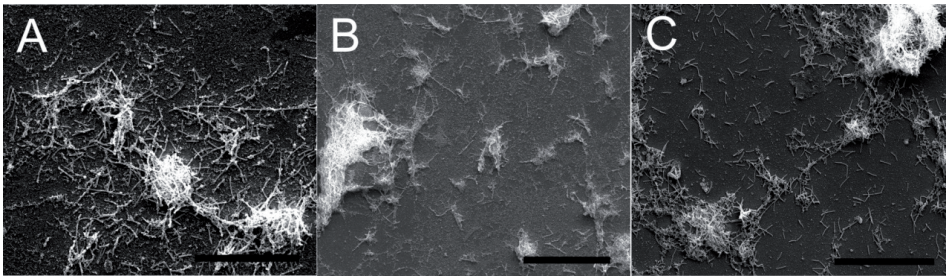
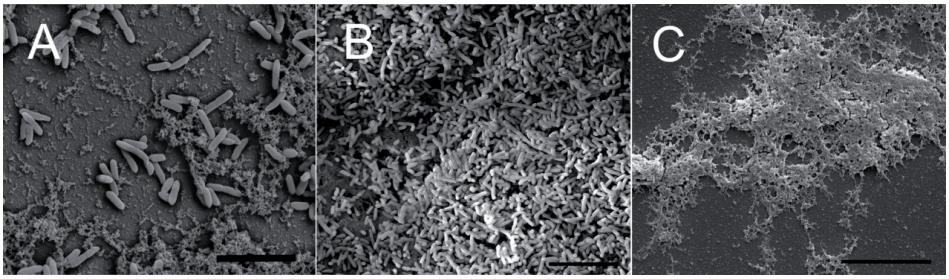


Additional file 1 (.pdf)
A view of the colony surface of the Rough morphotype of *F. columnare*
Only the extracellular material was seen on the colony surface of the Rough morphotype, and cells were not observed. The scale bar was 4 µm.



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.

II

HIGH NUTRIENT CONCENTRATION CAN INDUCE VIRULENCE FACTOR EXPRESSION AND CAUSE HIGHER VIRULENCE IN AN ENVIRONMENTALLY TRANSMITTED PATHOGEN

by

Reetta Penttinen, Hanna Kinnula, Anssi Lipponen, Jaana K.H. Bamford & Lotta-Riina Sundberg 2016

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III

GENE EXPRESSION ANALYSIS OF GLIDING MOTILITY GENES OF SPREADING AND NON-SPREADING COLONY TYPES OF *FLAVOBACTERIUM COLUMNARE*

by

Reetta Penttinen, Ville Hoikkala, Jaana K.H. Bamford & Lotta-Riina Sundberg
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