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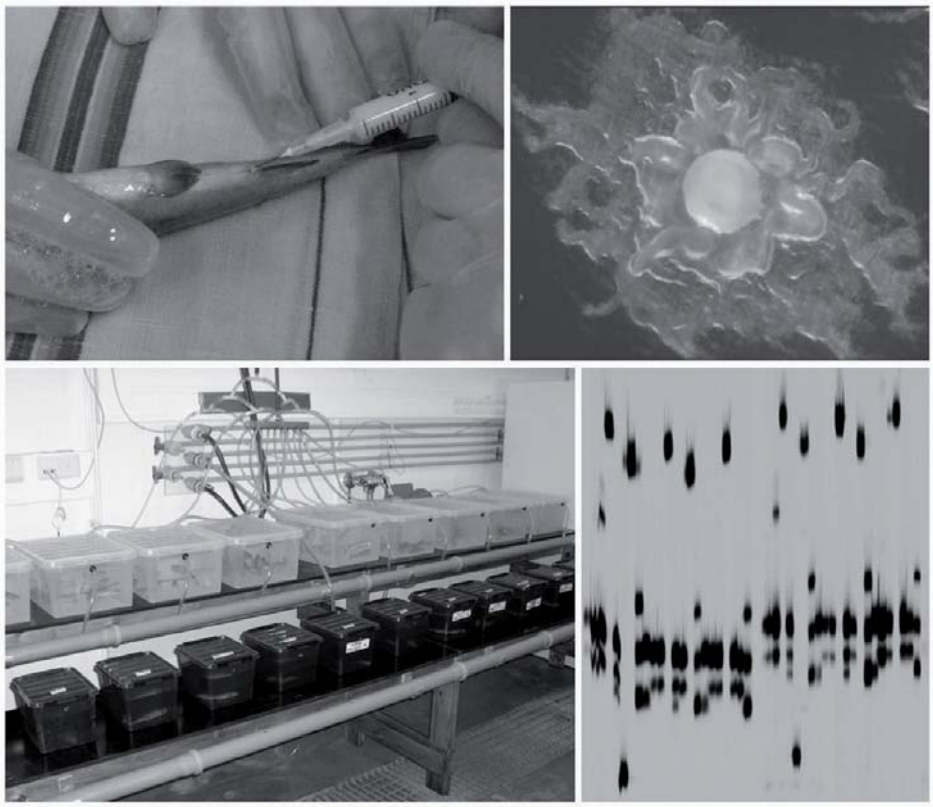
Heidi Kunttu

Characterizing the Bacterial Fish Pathogen *Flavobacterium columnare*, and Some Factors Affecting its Pathogenicity



JYVÄSKYLÄN YLIOPISTO

JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 206

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Ylistönrinteellä, salissa YAA303
toukokuun 8. päivänä 2010 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Mathematics and Science of the University of Jyväskylä,
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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2010

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Jyväskylä Studies in Biological and Environmental Science
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Department of Biological and Environmental Science, University of Jyväskylä

Cover pictures by Heidi Kunttu

URN:ISBN:978-951-39-3867-3
ISBN 978-951-39-3867-3 (PDF)

ISBN 978-951-39-3853-6 (nid.)
ISSN 1456-9701

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ABSTRACT

Kunttu, Heidi
Characterizing the bacterial fish pathogen *Flavobacterium columnare*, and some factors affecting its pathogenicity
Jyväskylä: University of Jyväskylä, 2010, 69 p.
(Jyväskylä Studies in Biological and Environmental Science
ISSN 1456-9701; 206)
ISBN 978-951-39-3867-3 (PDF), 978-951-39-3853-6 (nid.)
Yhteenveto: Kalapatogeeni *Flavobacterium columnare* -bakteerin ominaisuuksia ja patogeenisuuteen vaikuttavia tekijöitä
Diss.

Flavobacterium columnare is a bacterial pathogen of fresh water fish causing lethal columnaris disease worldwide. At Finnish fish farms, columnaris disease has occurred since the early 1980s, and is a serious problem in salmonid farming in Finland. Currently, the only effective treatment is with antibiotics. Factors related to the pathogenicity of *F. columnare*, transmission of columnaris disease and the immunity of salmonids to columnaris infections are poorly known. *F. columnare* strains isolated from Finnish fish farms were characterized geno- and phenotypically, virulence and transmission experiments were conducted, and the efficacy of two immunostimulants against columnaris infections in rainbow trout (*Oncorhynchus mykiss* Walbaum) fingerlings was evaluated. Finnish *F. columnare* strains constituted a genetically homogeneous group that differed from strains isolated from other parts of the world and tolerated narrower pH and salinity ranges than *F. columnare* type strain (NCIMB 2248^T). Finnish strains could be divided into seven genetic groups, which can co-occur during the same outbreak at fish farms. Among the genetic groups, four colony types were formed. Formation of rhizoid colonies is suggested to be a prerequisite for virulence of *F. columnare*. Colony types may have a role in the infection process and persistence of *F. columnare* at fish farms. The bacterium was able to survive in fresh water and also as a saprophyte, and to use saprophytism as a transmission strategy. These properties, together with co-occurrence of different genetic groups, may promote the continuous nature of outbreaks at fish farms and lead to development of highly virulent *F. columnare* strains. Immunostimulation was effective in boosting innate immune defence parameters of rainbow trout, but was inefficient in protecting fish against *F. columnare* infections. This suggests that antibody-mediated immunity is also essential in protecting fish from columnaris disease.

Keywords: Colony type; columnaris disease; *Flavobacterium columnare*; immunostimulation; saprophytism; transmission; virulence.

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

Responsibilities of Heidi Kunttu in the articles of this thesis: I was responsible for the molecular laboratory work performed in paper I and took part in planning the experiments and writing the article with co-authors. All the experiments in II–V were planned, conducted and the articles written jointly with the co-authors.

I Suomalainen, L.-R., Kunttu, H., Valtonen, E. T., Hirvelä-Koski, V. & Tiirola, M. 2006. Molecular diversity and growth features of *Flavobacterium columnare* strains isolated in Finland. Diseases of Aquatic Organisms 70: 55-61.

II Kunttu, H. M. T., Suomalainen, L.-R., Jokinen, E. I. & Valtonen, E. T. 2009. *Flavobacterium columnare* colony types: Connection to adhesion and virulence? Microbial Pathogenesis 46: 21-27.

III Kunttu, H. M. T., Jokinen, E. I., Suomalainen, L.-R. & Valtonen, E. T. 2010 *Flavobacterium columnare* colony types: Studies on chondroitin AC lyase activity and adhesion inhibition. Manuscript.

IV Kunttu, H. M. T., Valtonen, E. T., Jokinen, E. I. & Suomalainen, L.-R. 2009. Saprophytism of a fish pathogen as a transmission strategy. Epidemics 1: 96-100.

V Kunttu, H. M. T., Valtonen, E. T., Suomalainen, L.-R., Vielma, J. & Jokinen, E. I. 2009. The efficacy of two immunostimulants against *Flavobacterium columnare* infection in juvenile rainbow trout (*Oncorhynchus mykiss*). Fish & Shellfish Immunology 26: 850-857.

ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AHL	acylated homoserine lactone
AO	Anacker and Ordal
ARISA	automated ribosomal intergenic spacer analysis
bp	base pair = 3.4 Å
CFU	colony forming unit
ECP	extracellular product
FCS	foetal calf serum
HBSS	Hanks's balanced salt solution
HMB	hydroxymethylbutyrate
IAM	Institute of Applied Microbiology
IFO	Institute for Fermentation
ISR	intergenic spacer region (synonym to ITS)
ITS	internal transcribed spacer (synonym to ISR)
LH-PCR	length heterogeneity PCR
LPS	lipopolysaccharide
NCIMB	National Collection of Industrial, Marine and Food Bacteria
OD	optical density
OMP	outer membrane protein
OTC	oxytetracyclin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PMA	phorbol 12-myristate 13-acetate
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
rrn operon	ribosomal RNA operon
rRNA	ribosomal RNA
RT	room temperature
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSCP	single strand conformation polymorphism
SSH	suppressive subtractive hybridization

1 INTRODUCTION

1.1 Columnaris disease at fish farms

Columnaris disease is an infectious bacterial disease of freshwater fish leading annually to severe financial and material losses at fish farms world wide. The disease is caused by the *Flavobacterium columnare* bacterium and its symptoms include gill necrosis, fin and jaw erosion, and skin lesions. *F. columnare* infects several fish species from catfish and tropical aquarium fish to salmonids in the warm water period (Snieszko 1974, Bernardet 1989, Decostere et al. 1998, Wagner et al. 2002). Columnaris disease transmits by contact or by propagules shed into the water (Groff & LaPatra 2000, Welker et al. 2005). Young fish are more susceptible to *F. columnare* than older fish (Fujihara et al. 1971), but healthy, older fish with previous columnaris infection can be carriers of the disease (Pacha & Ordal 1970, Becker & Fujihara 1978, Suomalainen et al. 2005a). Transmission of *F. columnare* is known to be enhanced by mechanical injuries to fish, high temperature and rearing density, and high amount of organic matter in water (Morrison et al. 1981, Decostere et al. 1999a, Suomalainen et al. 2005b).

Although *F. columnare* exists in natural water habitats and can be isolated also from wild fishes (Rickard et al 2003, Revetta et al. 2005, Olivares-Fuster et al. 2007a), it mainly causes columnaris disease under fish farming conditions. Recently, however, infections caused by *F. columnare* have been reported in a wild perch (*Perca fluviatilis*) population (Morley & Lewis 2009). In Finland, the number and severity of columnaris outbreaks have increased since the first observations of the disease in 1980s (Rimaila-Pärnänen. 2003, Eskelinen 2009, Pulkkinen et al. 2010). The reasons behind this apparent increase in pathogenicity of *F. columnare* are unclear. At present, columnaris disease is the most remarkable fish disease affecting salmonid smolt production at Finnish fish farms (personal communications with representatives of Finnish fish farming industry). Epidemiological factors under fish farming conditions together with increased summer temperatures may favour development of

more virulent *F. columnare* strains and have been suggested to be the reason behind the more pathogenic disease (Pulkkinen et al. 2010).

Various methods have been tested and used to cure and prevent infections by *F. columnare*. Chemical bathing has proven somewhat effective in controlling and treating columnaris disease (Altinok 2004, Darwish et al. 2008). Although use of probiotics, i.e. microbes beneficially affecting the health of the host by competing with pathogenic bacteria, in aquaculture has been studied widely (for reviews see Gatesoupe 1999, Verschuere et al. 2000, Irianto & Austin 2002), there are only few publications on their effect on *F. columnare* infections. Some competing bacteria, like *Aeromonas hydrophila* and *Citrobacter freundii*, have been shown to decrease the infection success of *F. columnare* (Wakabayashi 1991). On the other hand, *Pseudomonas* sp. did not affect the infectivity, even though promising results for this bacterium as a growth inhibitor of *F. coumnare* were obtained *in vitro* (Suomalainen et al. 2005a). Therefore, the only practical and effective method for treating columnaris disease in Finnish aquaculture is antibiotic treatment with oxytetracyclin (OTC), which has to be administered repeatedly during summer months to control fish mortality. Thus, there is a risk of development of antibiotic resistant *F. columnare* strains at fish farms. Moreover, use of antibiotics in fish farming has been shown to lead to antibiotic resistance among other bacteria associated with farms (Schmidt et al. 2000, Miranda & Rojas 2007). Therefore, alternative treatment methods need to be studied.

There is evidence that fish are able to mount an antibody response against *F. columnare* (Fujihara & Nakatani 1971, Bernardet 1997, Grabowski et al. 2004), and recently a modified live vaccine has been developed against columnaris disease in channel catfish (Shoemaker et al. 2007). This vaccine has also proven efficient in feed-trained largemouth bass fry (*Micropterus salmoides floridanus*) against natural exposure to *F. columnare* (Bebak et al. 2009). However, the effectiveness of the vaccine in salmonids is not known as it is not licensed for use in these species or within the EU.

1.2 *Flavobacterium columnare*

Although there had been earlier difficulties in isolating *F. columnare* from diseased fish, development of improved culture methods has enabled routine isolation and study of the pathogen under laboratory conditions (Shieh 1980, Decostere et al. 1997, Tiirola et al. 2002a). Several phenotypic properties of *F. columnare* have been used in recognition. It is a Gram negative rod, grows on low nutrient media, tightly adheres on agar and forms yellowish colonies with a convoluted centre and rhizoid edges (Bullock et al. 1986, Bernardet & Grimont 1989, Durborow et al. 1998). Colonies absorb congo red, and flexirubin type pigments are present (Bernardet & Grimont 1989). In older cultures, spherical, degenerative cell forms, spheroplasts, may occur. Optimal growth temperature for *F. columnare* is between 20 and 25 °C, but some strains are known to grow at

37 °C. The bacterium is able to multiply at salinity up to 0.5 %. *F. columnare* exhibits gliding movement, and produces catalase and cytochrome oxidase. It is able to use glucose as a nutriment and hydrolyses chondroitin sulphate and casein, but not cellulose, chitin or starch (Bernardet & Grimont 1989, Shotts & Starliper 1999).

The taxonomic history of *F. columnare* is complicated. The bacterium was first described by Davis (1922), who named it *Bacillus columnaris* based on its column-like growth form on fish tissue. Two decades later, Ordal & Rucker (1944) isolated the bacterium and classified it as *Chondrococcus columnaris* based on the apparent production of microcysts and fruiting bodies. Garnjobst (1945), however, showed the absence of both microcysts and fruiting bodies, reclassified the bacterium to the genus *Cytophaga* and renamed it *Cytophaga columnaris*. In the beginning of the 1970s, the name of the genus was changed to *Flexibacter* and the name of the bacterium to *Flexibacter columnaris* (Leadbetter 1974). The present name, *Flavobacterium columnare*, was given by Bernardet et al. (1996), as these authors reclassified some bacteria belonging to the group *Cytophaga-Flavobacterium-Bacteroides* according to phylogenetic studies based on molecular techniques.

1.3 Studying the genetic diversity of bacteria

The bacterial genome contains information which allows separation of different bacterial species, subspecies or even strains of the same species from each other. Ribosomal RNA (rRNA) genes (16S, 23S and 5S rDNA) are the most commonly used phylogenetic chronometers in bacteriology, because this genomic region is relatively conserved among prokaryotes (Fox et al. 1980). Genes coding rRNA are organized into operons (*rrn* operons) in the order 16S–23S–5S and are transcribed simultaneously (Condon et al. 1995, Liao 2000). The operons are located unevenly in the bacterial genome and the number varies between bacterial species (Klappenbach et al. 2001). The number of operons in a certain bacterial group may be an adaptation to environmental conditions and reflect the ecology of bacteria (Klappenbach et al. 2000). For comparison of bacterial species, most important are hypervariable regions of the genes in which the sequence varies maximally between closely related bacteria. In the 16S rRNA gene (1 500 bp) (bp = base pair) this kind of region lies between 60 and 110 bp (*Escherichia coli* numbering) (Giovannoni et al. 1996, Salkinoja-Salonen 2002). Moreover, based on its length the 16S rDNA provides a more practical genomic tool to study the phylogeny of prokaryotes compared to the longer 23S rDNA (2 600 bp) or less informative 5S rDNA (120 bp) (Gogarten et al. 1996). Consequently, characterization of 16S rDNA is a well-established method for the identification of species, genera and families of bacteria (e.g. Woese 1987).

Several molecular techniques have been used to study bacterial diversity and classify closely related bacteria based on their 16S rDNA sequence. Specific probes binding to 16S rDNA have been used in qualitative and quantitative

hybridization experiments in studies of microbial populations (see Giovannoni et al. 1990, Raskin et al. 1994, Amann et al. 1995). DNA-rDNA hybridization was used to classify bacteria belonging to the genera *Flavobacterium*, *Cytophaga* and *Flexibacter* (Bernardet et al. 1996), and Southern blot hybridization has been used in characterization of *F. psychrophilum* isolates isolated in Finland (Madetoja et al. 2001). Length heterogeneity PCR (LH-PCR), based on comparison of naturally varying lengths of 16S rDNA PCR products between bacterial groups, is another powerful method for analysing diversity of microbial communities (Suzuki et al. 1998, Tiirola et al. 2003). Compared to cultivation methods, LH-PCR has proven effective in detecting *F. columnare* in fish tissue (Suomalainen et al. 2005a). Bacterial communities have also been studied with restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP) analyses of 16S rDNA (e.g. Moyer et al. 1994, Lee et al. 1996, Männistö 1999, Tiirola et al. 2002b). Resolution power of RFLP is based on differences in restriction fragment profiles between different DNA sequences, and SSCP on differential movement of single stranded DNA fragments with different sequences in non-denaturing gel electrophoresis. Based on RFLP of 16S rDNA, Triyanto & Wakabayashi (1999) divided *F. columnare* strains isolated from different parts of the world into genomovars *I–III*. This analysis has since been used by others in typing *F. columnare* isolates (Michel et al. 2002, Arias et al. 2004, Darwish & Ismaiel 2005). However, compared to RFLP, SSCP has somewhat improved resolution power in studying intraspecies diversity in *F. columnare* (Olivares-Fuster et al. 2007b). The most accurate, though not the simplest, method for studying taxonomic distances of bacteria is to compare sequenced DNA regions. Sequencing of 16S DNA has been used in identification of bacteria from environmental samples, in diagnosis of flavobacteriosis in fish, and in studying genetic diversity of *F. columnare* isolates (e.g. Triyanto & Wakabayashi 1999, Tiirola et al. 2002a, Darwish & Ismaiel 2005, Revetta et al. 2005, Schneck & Caslake 2006).

Compared to 16S rDNA, analyzing a non-coding sequence between 16S and 23S genes, an internal transcribed spacer (ITS) or intergenic spacer region (ISR), provides a better approach for studying genetic differences between subspecies or strains of the same species (Garcia-Martinez et al. 1996a, Gürtler & Stanisich 1996). As no evolutionary pressure is targeted on an ITS sequence, it changes more rapidly than actual gene sequences in *rrn* operons (Leblond-Bourget et al. 1996). Both number (i.e. the number of *rrn* operons), length and sequence of ITS can vary between closely related bacteria (Gürtler & Stanisich 1996). Thus, RFLP, SSCP and sequencing of ITS have provided a better discrimination power in studying relationships of bacteria (also *F. columnare*) at intraspecies level than the same analyses of 16S rDNA (Leblond-Bourget et al. 1996, Arias et al. 2004, Darwish & Ismaiel 2005, Olivares-Fuster et al. 2007b). A comparable analysis to LH-PCR is automated ribosomal intergenic spacer analysis (ARISA) based on length differences of ITS region between closely related bacteria. ARISA is considered a rapid and effective technique in

analysing bacterial communities when fine-scale resolution is needed (Fisher & Triplett 1999).

Analyses covering the whole bacterial genome have also been used in studying genetic relationships between bacteria. One such method is amplified fragment length polymorphism (AFLP), which is based on differential restriction of genomes of related bacteria and selective PCR amplification of restriction fragments (Vos et al. 1995). Random amplified polymorphic DNA (RAPD) is also based on PCR, but primers with a randomly chosen sequence are used (Williams et al. 1990). Both AFLP and RAPD have been used in studying genetic polymorphism of *F. columnare* strains (Arias et al. 2004, Thomas-Jinu & Goodwin 2004, Olivares-Fuster et al. 2007a), and also other bacterial isolates of the same species (e.g. Garcia-Martinez et al. 1996b, Koeleman et al. 1998, Loreti et al. 2001, Hahm et al. 2003). Another technique based on restriction of genomic DNA and effective in subtyping of bacterial pathogens, pulsed-field gel electrophoresis (PFGE), has been used to characterize clinical isolates of *F. columnare* (Soto et al. 2008). A technique called suppressive subtractive hybridization (SSH) has been used in studying genomic differences between closely related bacteria (e.g. Olivares-Fuster & Arias 2008), and also between pathogenic and non-pathogenic strains of same species (e.g. Zhang et al. 2000, 2005). In *F. johnsoniae* -like bacteria, SSH has been used to detect differential expression of genes between planktic and biofilm forming stages (Flemming et al. 2009). Recently, SSH revealed genetic differences between high and low virulence *F. columnare* strains (Li et al. 2010).

1.4 Virulence factors of fish pathogenic bacteria

Bacterial pathogens have numerous ways to cause disease to their hosts. Getting contact with the host and attaching to host tissues are the most important steps in initiating the infection. After this, to utilize the host and escape the immune defence of the host, virulent bacteria excrete tissue degrading enzymes and toxins causing the disease. Thus, cell surface structures functioning as adhesion factors or having some other roles in the infection process as well as extracellular products have been studied widely in bacterial fish pathogens.

Several virulence factors have been described for bacterial fish pathogens. For example, capsular material and/or lipopolysaccharides (LPS) have been shown to be related to virulence in *Vibrio vulnificus*, and also in *Aeromonas hydrophila* and *Edwardsiella ictaluri* (Mittal et al. 1980, Yoshida et al. 1985, Amaro et al. 1997, Arias et al. 2003). The flagellum is a virulence organelle of *Vibrio anguillarum*, the flagellar sheath antigen causing the infection, and the exopolysaccharide transport system of this pathogen is required for attachment to fish skin (Norqvist & Wolf-Watz 1993, Croxatto et al. 2007). The capsule and produced cytotoxin are among the factors causing virulence of *Streptococcus iniae* (Miller & Neely 2005, Locke et al. 2007a). Other features affecting virulence

of fish pathogenic bacteria include adhesion and subsequent growth on fish mucus of *A. hydrophila*, and proteolytic activity in *Yersinia ruckeri* and *F. psychrophilum* (Fernandez et al. 2002, Álvarez et al. 2006, van der Marel et al. 2008). Also gliding motility has been shown to be related to virulence of *F. psychrophilum* (Álvarez et al. 2006).

Changes in cell surface components of a bacterium often lead to formation of different colony morphologies (e.g. Yoshida et al. 1985, Arias et al. 2003, Enos-Berlage et al. 2005). Also change in gliding motility machinery is known to lead to differential colony type formation, the phenomenon well described in *F. johnsoniae* (e.g. Chang et al. 1984, Hunnicutt & McBride 2001, Rhodes et al. 2010). Differential colony morphology formation has also been detected in *F. columnare*, but correlation to virulence has not been studied (Song et al. 1988, Bernardet 1989, Bader et al. 2005, Cain & Lafrentz 2007). Virulence mechanisms of *F. columnare* are poorly known. It has been shown that different strains of *F. columnare* exhibit differences in their virulence (Pacha & Ordal 1970, Decostere et al. 1998, Thomas-Jinu & Goodwin 2004, Suomalainen et al. 2006). Furthermore, genomovar *II* has been shown to be more pathogenic than genomovar *I* to channel catfish (*Ictalurus punctatus*) (Shoemaker et al. 2008), to which also *F. columnare* isolates belonging to different PFGE-groups showed different virulences (Soto et al. 2008). Several properties of *F. columnare* have been studied to find factors causing virulence of the pathogen. It has been suggested that adhesion on gill tissue is an important step in pathogenesis of this bacterium, and that chemotaxis to fish mucus is associated with the virulence of the pathogen (Decostere et al. 1999b, Klesius et al. 2008). In addition, activity of connective tissue degrading enzyme, chondroitin AC lyase, has been shown to be related to virulence of *F. columnare* (Suomalainen et al. 2006). It has also been detected that LPS composition of virulent *F. columnare* strains differs from that of a non-virulent mutant (Zhang et al. 2006). Extracellular and membrane associated proteases have also been characterized, but no connection to the virulence has been found (Bertolini & Rohovec 1992, Newton et al. 1997, Xie et al. 2004).

1.5 Host immune defence versus virulence mechanisms of pathogens

To avoid pathogen invasion, a host must posses a variety of defence mechanisms. The main organs having a role in immune defence of teleost fishes are the head kidney and thymus (primary lymphoid organs) (Zapata et al. 1996). The head kidney is a tissue for housing and differentiation of blood cell precursors and effective macrophage activity. The thymus is suggested to be the main source of immunocompetent T-cells, and also contains macrophages and probably B-cell precursors. The secondary lymphoid organs are gut, mucus and spleen, the role of which is trapping and processing of antigens for the immune

cells able to react to them. These tissues contain lymphocytes and macrophages, and also antibodies (Zapata et al. 1996).

In the beginning of the infection, pathogenic bacteria of fish encounter the nonspecific immune defence of the host, which fish rely on more than the specific immune defence (see below) (Anderson 1992). Nonspecific defence is based on recognition of the non-self structures of the pathogen by the host (Magnadottir 2006). The first place where a pathogen can be effectively blocked or neutralized is the mucosal surface of the skin and gastrointestinal track (Zapata et al. 1996). Phagocytosis and production of reactive oxygen species (ROS-response or respiratory burst) by phagocytes in blood and tissues present a major non-specific antibacterial defence mechanism in fish (Secombes 1996). An enzyme effective in lysing both Gram-positive and Gram-negative microbes, lysozyme, is widely distributed in serum, tissues and mucus (Yano 1996). Another bactericidal and bacteriolytic activity, complement activity, is present in plasma and body fluids in several fish species including salmonids, and has also been detected in the mucus of rainbow trout (Harrell et al. 1976). Other non-specific immune defence mechanisms of fish are cytotoxic cells, growth inhibitors, various lytic enzymes, proteases, agglutinins and pecipitins (Secombes 1996, Yano 1996, Magnadottir 2006). Specific immune defence comes into the picture in later stages of infection and during second exposure to a pathogen. This line of defence is based on recognition of specific antigens of the pathogen by cytotoxic cells and T-lymphocytes, and production of antibodies by B-lymphocytes (Kaattari & Piganelli 1996, Manning & Nakanishi 1996). Also a variety of cytokines, affecting the function of T- and B-cells and also macrophages, have a role in specific immune defence.

A pathogen must overcome the host defence mechanisms to cause the infection. To invade and colonize the host, a pathogen must be able to survive in the blood and phagocytes of fish, and be capable of deriving its growth requirements from the host (Evelyn 1996). For this, bacterial pathogens use their virulence factors by either avoiding the host immune defence or destroying host tissues. In avoidance of serum bactericidal activity (e.g. complement and lysozyme activity), cell surface proteins, LPS and polysaccharide capsular material of bacteria have a remarkable role (Evelyn 1996, Croxatto et al. 2007). Also sialic acid on the surface of some bacterial fish pathogens is known to suppress activation of the alternative complement pathway of the host (Ourth & Bachinski 1987). This phenomenon is common in pathogenic bacteria (Severi et al. 2007). Likewise, avoidance of phagocyte killing activity and survival in phagocytes is mediated largely by cell surface components giving protection against and suppressing respiratory burst activity of phagocytes (Evelyn 1996, Locke et al. 2007b). Some bacterial pathogens are also able to lyse phagocytes and escape from them by producing lytic and toxic enzymes (Bloom 1979, Evelyn 1996). Virulence factors enabling the growth of bacteria within the host include ability to compete for iron with the host and production of extracellular products (ECPs) (Miller et al. 1989, Evelyn 1996, Álvarez et al. 2008). ECPs, like hemolysins, cytotoxins, dermatotoxins, proteases, phospholipases and chondroitinases, serve to impair host defence mechanims, lyse cells and

hydrolyse host tissues facilitating the release and spread of nutrients required by the pathogens.

1.6 Influence of fish farming conditions on disease outbreaks

In addition to the host-pathogen relationship, the environment also influences the outcome of infection, affecting both host and pathogen. Infectious diseases occur when susceptible fishes are exposed to pathogens under certain environmental stress conditions, such as temperature changes, eutrophication, metabolic products of fishes, and pollution (Snieszko 1974) (Fig. 1). In intensive fish culture, the effect of these stressors is enhanced due to unnaturally high host densities making fish more susceptible to infections by various pathogens. Crowding and handling stress can have a suppressive effect on the innate immunity of fish, which increases the probability of infections (Magnadottir 2006). In addition, skin abrasions are common in farmed fish which makes the fish more vulnerable to infections (e.g. Madetoja et al. 2000, Moyer & Hunnicutt 2007). Also parasitic infections occurring simultaneously with bacterial infections may enhance the invasion of bacterial pathogens (Cusack & Cone 1986, Rintamäki-Kinnunen et al. 1997, Bandilla et al. 2006, Pylkkö et al. 2006). Furthermore, treating parasitic infections with chemicals (e.g. formalin bathing to cure infections by the protozoans *Ichthyobodo negator* and *Ichthyophthirius multifilis*) may damage fish skin and increase risk of bacterial infections (Madsen & Dalsgaard 1999).

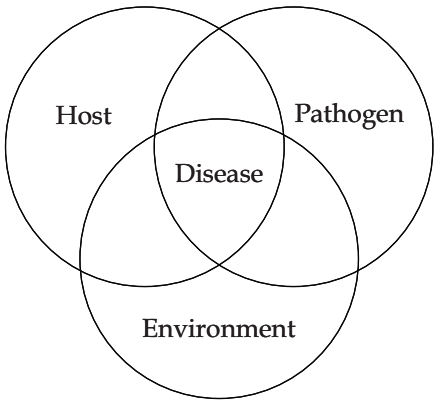

FIGURE 1 Infectious disease occurs when a susceptible host is exposed to a virulent pathogen under appropriate environmental conditions. Redrawn from (Snieszko 1974).

High host densities at fish farms enhance transmission of pathogens (Ogut et al. 2005). In a dense host population the virulence of pathogens is expected to be higher when transmission rate is high (Anderson & May 1982, Andre &

Hochberg 2005). The aqueous environment allows pathogen propagules to transmit long distances even if the infectious source is immobilized, which increases the fitness of the pathogen (Ewald 1994, Day 2002). The fitness is increased further if the pathogen is able to survive long periods outside the host. For example, *F. psychrophilum* is able to survive for almost a year and maintain its infectivity for at least seven days in fresh water (Madetoja et al. 2003). Pathogens transmitting trough water phase may be more virulent than pathogens transmitting via direct host contact (Ewald 1994). Transmission via water is especially favourable in environments where host densities are low, but it can also be beneficial in the farming environment where the probability of encountering a new host and successful transmission is enhanced by high host densities. Moreover, the multiple infections occurring at fish farms (Madetoja et al. 2001, Madsen et al. 2005), e.g. simultaneous infection of the host by genetically different pathogen strains of the same species, may enhance the transmission further (Read & Taylor 2001). There is evidence that pathogen competition during multiple infections favours faster host exploitation by the pathogen, thus selecting for more virulent strains, and finally leading to evolution of higher virulence of the pathogen (e.g. Gandon et al. 2001, Wargo et al. 2007). However, host resistance and genetic properties play a key role in the outcome of within-host competition and thus in the evolution of parasite virulence (de Roode et al. 2004).

In addition to living fish, dead fish and fish feed can serve as reservoirs of bacterial pathogens, even though transmission of the disease from those resources has not been confirmed (Wakabayashi 1991, Madetoja et al. 2000). In nature, any fish with disease symptoms are quickly predated by piscivorous fish or birds, which effectively reduces transmission of diseases. At fish farms natural predation does not exist, which enhances the transmission success of pathogens. Pathogenic bacteria that are able to form biofilms may use this property to survive in the face of changing environmental conditions and, as the circumstances become more favourable to infection, as a source of transmission (Hall-Stoodley & Stoodley 2005). Indeed, fish pathogenic *F. columnare* and *F. psychrophilum* are known to form biofilms (Rickard et al. 2003, Revetta et al. 2005, Álvarez et al. 2006) which might also serve as an infection source at the fish farms as well and influence the prolonged and repeated infections.

1.7 Curing and preventing diseases at fish farms and enhancing immune defence of fish

In fish farming, mortality of fish is controlled by vaccination, antibiotics and bathing with various chemotherapeutics. Vaccination is useful for prophylaxis of vibriosis (caused by *V. anguillarum*), enteric redmouth disease (*Y. ruckeri*) and furunculosis (*A. salmonicida* subs. *salmonicida*) (Sakai 1999), and experiments on

vaccination of fish against e.g. *A. hydrophila* and *F. psychrophilum* have given promising results (Rahman & Kawai 2000, Crump et al. 2005, Dumetz et al. 2007, Högfors et al. 2008, LaFrentz et al 2008, LaPatra et al. 2010). However, development of vaccines against intracellular pathogens, like *Renibacterium salmoninarum* (causing bacterial kidney disease, BKD), is more challenging (Sakai 1999). Curing and preventing the infections of young fish is especially problematic for fish farmers (Ellis 1988). Due to their small size, vaccination of juvenile fish by injection is troublesome. Oral, spray or bath vaccination can be used, but these methods usually are not as effective as injection. Moreover, exposure of developing immune system of young fish to a foreign protein can induce a state of unresponsiveness to the antigen later in life. At the early life stages, disease resistance of fish consists mainly of non-specific defence mechanisms (phagocytes, soluble factors). Specific defence functions (lymphocytes, antibodies), especially immunological memory, is not developed fully to give fish a long term resistance in infections. However, the best way of immunization and its effect on immune response always depends on fish species, the size (not only the age) and condition of fish, and also on environmental factors.

Immunostimulants can also enhance the immune defence of fish (e.g. Anderson 1992, Sakai 1999, Bricknell & Dalmo 2005, Dalmo & Bogwald 2008). Immunostimulants are used in aquaculture to improve growth of fish and resistance to pathogens under stressful conditions. A variety of substances of animal (like extracts from marine animals), plant (soybean protein) and microbial (β-glucans, LPS) origin, as well as synthetic compounds [levamisole, hydroxymethylbutyrate (HMB)] and diet components (vitamins), can be used to stimulate the immune system of fish. Innate defence mechanisms are those affected by most immunostimulants, but the specific immune system is also known to be enhanced. Immunostimulants can be easily applied orally to young fish, and treatments can be scheduled to the times when disease outbreaks are expected. Fish can be treated with immunostimulants also by bathing/immersion, and intraperitoneal or intramuscular injections.

There is only one report concerning immunostimulation of fish against columnaris disease (Suomalainen et al. 2009). In that study, promising results were obtained using a product AlkoSel® (Lallemand Animal Nutrition, Blagnac, France), an inactivated yeast (*Saccharomyces cerevisiae*) containing selenomethionine. However, experimental studies have shown that in various fish species microbial glucans have given protection against bacterial pathogens such as *A. hydrophila*, *A. salmonicida*, *E. ictaluri*, *E. tarda*, *V. anguillarum*, *V. salmonicida*, *Y. ruckeri* and *S. iniae* (Robertsen et al. 1990, Chen & Ainsworth 1992, Anderson & Siwicki 1994, Selvaraj et al. 2005, Misra et al. 2006, Russo et al. 2006). Immunostimulants can thus reduce the losses caused by diseases in aquaculture. They may not, however, be effective against all infections (Sakai 1999).

2 AIMS OF THE STUDY

Factors affecting the severity of columnaris disease in Finnish salmonid fish farming are not well understood. Especially the reasons behind *F. columnare* becoming more pathogenic in Finland are not known. The only currently available treatment is use of antibiotics, which causes a risk of development of antibiotic resistant strains both at farms and among bacteria in the aquatic environment.

The main objectives of this thesis were to obtain more insight into the factors affecting pathogenicity of *F. columnare* and to evaluate the efficacy of immunostimulation as an alternative for antibiotic treatments. The following questions were set:

- Are there genetic differences between *F. columnare* strains isolated from outbreaks at Finnish fish farms, and what are the growth requirements of Finnish *F. columnare* strains? (I)

- How do different colony morphology types formed by Finnish *F. columnare* strains differ from each other, and is there a connection between virulence and colony morphology? (II, III)

- What is the transmission strategy of *F. columnare*, is *F. columnare* shed or columnaris disease transmitted at different rates from living and dead fish, and how long is *F. columnare* able to survive outside the host? (IV)

- Is it possible to improve the first line immune defence of juvenile rainbow trout against columnaris diseasse by using immunostimulants? (V)

In order to answer these questions, the studies reported in I−V were carried out using *F. columnare* strains isolated from columnaris outbreaks at Finnish fish farms as a pathogen and juvenile rainbow trout as host.

3 SUMMARY OF MATERIALS AND METHODS

3.1 Bacterial strains

Thirty *Flavobacterium columnare* isolates were obtained from 18 Finnish fish farms from disease outbreaks during 1993–2003. These isolates, together with *F. columnare* type strain NCIMB 2248^{T} (Table 1), were used in the experiments in I–V. Isolates were stored at –70 to –80 °C in 500 µl stocks of enriched Anacker and Ordal (AO) (Bernardet & Kerouault 1989) (I) or Shieh medium (Shieh 1980) (II–V) containing 10 % of glycerol and 10 % of bovine serum (I) or foetal calf serum (FCS, Gibco, BRL Co.) (II–V). For the experiments, bacterial stocks were thawed and inoculated into AO (I, II) or Shieh medium (II–V), and grown at room temperature (RT) under constant agitation (100–120 rpm) to a late log phase (23–24 h). Optical density (OD) of bacterial cultures at 595 nm were measured using a micro plate reader (Multiskan Plus, Labsystems, Vantaa, Finland), and the number of bacteria was expressed as colony forming units (CFU) ml^{-1} based on a previously determined OD/CFU relationship.

TABLE 1 *Flavobacterium columnare* strains used in the experiments in I–V. Year of isolation, fish farm number and fish species are given for each strain. After genetic grouping of the strains with ARISA (I), strains marked with an asterisk (*) were used as representatives of the genetic groups in further studies in I–V. For simplicity, the representative strains are referred in the body text as strains A-H.

Isolate/strain code	Fish farm	Fish species	Watercourse	Genetic group
NCIMB 2248^{T}		Chinook salmon	Washington, USA	F *
3294/95	1 [a]	Trout	The River Oulujoki	A *
10819/96	1 [a]	Arctic charr	The River Oulujoki	A
8128/97	1 [a]	Arctic charr	The River Oulujoki	B *
3147/98	1 [a]	Trout	The River Oulujoki	A
1277/99	1 [a]	Trout	The River Oulujoki	A

TABLE 1 Cont.

1179/01	1 [a]	Brown trout	The River Oulujoki	A
2390/02	1 [a]	Salmon	The River Oulujoki	A
8239/97	2 [a]	Rainbow trout	The River Iijoki	C *
9528/97	3 [a]	Rainbow trout	The River Tornionjoki	C
1991/94	4 [a]	Rainbow trout	The River Oulujoki	A
1468/99	5 [a]	Brook trout	The River Oulujoki	A
1199/00	6 [a]	Rainbow trout	The River Kymijoki	A
1397/00	7 [a]	Rainbow trout	The River Oulujoki	D *
1820/02	8 [a]	Rainbow trout	The River Vienan Kemijoki	A
2287/02	9 [a]	Trout	The River Lestijoki	A
2559/93	10 [a]	Salmon	The River Simojoki	A
Ke/02	11 [b]	Salmon	The River Kemijoki	E * (I)
FK2/03	11 [b]	Salmon	The River Kemijoki	E * (II–V)
Mo/02	12 [b]	Salmon	The River Oulujoki	A
Mo/03	12 [b]	Trout	The River Oulujoki	A
Ke/03	13 [b]	Salmon	The River Kemijoki	C
Os/03	14 [b]	Salmon	The River Kemijoki	G *
Ra/03	15 [b]	Salmon	The River Iijoki	E
Htan4/03	16 [c]	Rainbow trout	The River Kymijoki	A
Htan5/03	16 [c]	Rainbow trout	The River Kymijoki	H *
Htan6/03	16 [c]	Rainbow trout	The River Kymijoki	H
Htku1/03	16 [c]	Pikeperch	The River Kymijoki	H
Htku2/03	16 [c]	Pikeperch	The River Kymijoki	E
Lauh/03	18 [c]	Grayling	The River Kymijoki	A
Ba1972/03	19 [a]	Rainbow trout	The River Oulujoki	A

[a] Isolated by Finnish Food Safety Authority (formerly: National Veterinary and Food Research Institute), Oulu regional unit, Finland

[b] Isolated by Dr. P. Rintamäki, University of Oulu, Finland

[c] Isolated by Dr. L.-R. Suomalainen, University of Jyväskylä, Finland

3.2 Fish and experimental setup of bath challenge (II, V)

Rainbow trout (*Oncorhynchus mykiss*, Walbaum) fingerlings with no previous contact with *F. columnare* originated from a fish farm in Central Finland. The fish were kept in a wet laboratory in treated bore hole water in 250-l flow-through tanks at 16 °C and fed with commercial trout feed (Nutra Parr, Skretting, Norway). Oxygen concentration of the water was monitored continuously, and was near full saturation (> 80 %). The fish were acclimated to laboratory conditions for at least 3 weeks before the experiments. Water temperature for bacterial challenge was increased gradually to 25 °C during a seven day period. For exposures to *F. columnare* bacteria and immunostimulation experiments, the fish, mean wight 6.0 g (II) or 4.7−11.4 g (V), were transferred to an experimental wet laboratory. Bath challenges were done in 10-l plastic aquaria, 22−25 fish in each, at 25 °C under constant aeration

with water containing 5.0−6.5 × 10^6 ml^{-1} of virulent *F. columnare* strain C (IV) (see Suomalainen et al. 2006) or strain-colony type variant of *F. columnare* (II) (see section 3.5 and Table 2). Fish exposed to Shieh broth served as negative controls. All the treatments were replicated 3−6 times. After a 2–h exposure, the fish were transferred into clean 10-l (II) or 50-l (V) flow-through aquaria (25 °C) and the mortality was monitored.

3.3 Genomic analyses of *Flavobacterium columnare* strains (I)

DNA of *F. columnare* strains was isolated using proteinase K for cell lysis, phenol-chloroform-isoamyl alcohol extraction for purification, and NaCl and isopropanol for precipitation of DNA (Tiirola et al. 2003). In order to study genetic properties of *F. columnare* strains isolated from Finnish fish farms and to detect genomic differences between them, LH-PCR analysis (Suzuki et al. 1998) of partial and RFLP analysis (Weisburg et al. 1991, Triyanto & Wakabayashi 1999) of whole 16S rDNA were conducted (see Table 1). ARISA (Fisher & Triplett 1999) of 16S-23S ITS region was also done, according to which the Finnish strains could be divided into seven genetic groups, the type strain representing the eighth group (Table 1). Sequence analysis of the 16S rDNA was conducted on the representative strains of the genetic groups. These repesentatives were also used in further studies.

3.4 Growth features of *F. columnare* strains (I)

The effect of growth environment on representatives of different genetic groups of Finnish *F. columnare* strains was tested *in vitro* (Table 1). The growth rate of bacteria was measured at different temperatures (17.0, 19.0, 22.0, 25.0 and 27.4 °C), salt (NaCl) concentrations (0, 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1 %), and pH (6.2, 6.5, 6.8, 7.4, 8.0, 8.6 and 9.2). An antibiotic susceptibility test to ten antimicrobial agents (ampicillin, erythromycin, gentamycin, nitrofurantoi, neomycin, Polymyxin B, streptomycin, tetracycline, trimethoprim-sulpha and florphenicol) was carried out as described by Michel et al. (2002).

3.5 Experiments on colony morphology types formed by *F. columnare* strains (II, III)

Four different colony morphology types (colony types 1–4) are formed among *F. columnare* strains on Shieh agar plates under laboratory conditions (Table 2, Fig. 2 in section 4.2). Formation of colony types was followed in more detail in

successive Shieh broth and Shieh agar cultures, and also in AO broth and agar cultures. To study further the colony types formed on Shieh agar, molecular level studies as well as studies on adhesion, chondroitin AC lyase activity, and virulence were carried out. For clarity, a certain colony type formed by a strain belonging to a certain genetic group is designated a strain-colony type variant, e.g. variant D4, in which the capital letter refers to the genetic group and the number to the colony type formed by the strain (Table 2).

TABLE 2 Colony types (1–4) formed on Shieh agar in laboratory conditions by *Flavobacterium columnare* type strain (NCIMB 2248^T) and Finnish *F. columnare* strains belonging to different genetic groups. A colony type formed by a strain belonging to a certain genetic group is called a strain-colony type variant, in which the capital letter refers to the genetic group and the number to the colony type formed by the strain. The variants were used in the experiments in II and III.

Genetic group of the strain	Original colony type formed by the strain	Colony types formed in subcultures	Strain-colony type variant
A	1	1	A1 [a]
B	3	3	B3 [a, d]
C	1	1	C1 [a, b, d]
D	2	2	D2 [a]
		4	D4 [a]
E	1	1	E1 [a, b, c, d]
		2	E2 [a, b, c, d]
F (NCIMB 2248^T)	4	4	F4 [a]
G	2	2	G2 [a, b, c, d]
		4	G4 [a, b, c, d]
H	2	2	H2 [a, b, c, d]
		4	H4 [a, b, c, d]

[a] Variant used in the molecular level and polystyrene adhesion experiments in II
[b] Variant used in the gill adhesion experiment in II
[c] Variant used in the virulence experiment in II
[d] Variant used in the chondroitin AC lyase activity and all adhesion experiments in III

3.5.1 Genomic analyses and whole cell proteins (II)

Strain-colony type variants were analyzed using ARISA (I, II) to determine whether differential colony type formation by the strains affected the ARISA grouping. Possible whole genome differences were analyzed by AFLP (Vos *et al.* 1995), which detects genetic variation between the colony types also in other parts of the genome. Finally, whole cell proteins of the variants were extracted and protein compositions were compared using SDS-PAGE (Tan et al. 1997).

3.5.2 Adhesion on polystyrene (II, III)

Adhesion capacity of all the strain-colony type variants, with *Escherichia coli* strain JM109 as a reference bacterium was tested on 96-well polystyrene plates (Immuno Plate Maxisorp, Nunc Co., Denmark) (II). Bacterial cells were incubated on the plates at RT for 2 h, and relative adherence was determined using a modification of the method of Álvarez et al (2006), which is based on measuring the absorbance of dissolved crystal violet from stained adhered bacteria. The growth of bacteria was monitored by measuring the OD of unstained bacteria incubated on another plate (growth control bacteria).

In III, adhesion of variants B3, C1, E1, E2, G2, G4, H2 and H4 was tested at 5, 15, 20 and 25 °C using the same method as described above. When determining the adhesion capacities, the growth of bacteria was taken into account by multiplying the absorbance of dissolved stain with a coefficient calculated from the OD values of growth control bacteria after 2 h of incubation.

3.5.3 Adhesion on gill tissue of rainbow trout (II)

The outermost gill arches (mean weight 30 mg) were taken from 12 rainbow trout fry (mean weight of fish 23 g) and exposed to broth cultures of *F. columnare* variants C1, E1, E2, G2, G4, H2 and H4. The gill arches were incubated on a petri dish with bacterial cultures for 2 h at RT under constant agitation (50 rpm), and the gill arches were homogenized in phosphate-buffered saline (PBS). Serial dilutions were made from the homogenate and plated on Shieh agar containing tobramycin, 1 μg ml^{-1} (Decostere et al. 1997). After 2 days incubation at RT the number of colonies were counted and the adhesion capacities of the variants determined as CFU ml^{-1} values.

3.5.4 Inhibition of adhesion (III)

To determine whether there are differences between adhesion molecules on the cell surfaces of *F. columnare* colony types, adhesion of variants B3, C1, E1, E2, G2, G4, H2 and H4 was tested on polystyrene after modifying the bacterial surface with four different substances: hyaluronidase causing hyaluronic acid breakdown; antibacterial substance polymyxin B which binds acidic polysaccharides, e.g. bacterial LPS by formign complex with lipid A moiety (Morrison & Jacobs 1976); sodium metaperiodate oxidizing adjacent hydroxyl groups; and proteolytic enzyme proteinase K. Bacteria with no modifying substance were used as untreated controls. Bacteria were incubated for 30 min at RT (12 min at 25 °C for proteinase K) with the substances after which the adhesion capacitiy was determined as above taking into account the growth of bacteria. Adhesion inhibition percentage of each treatment for each variant was calculated by comparing the treated bacteria with their untreated controls.

Adhesion of the variants B3, C1, E1, E2, G2, G4, H2 and H4 on polystyrene after incubation with six sugars was tested to determine possible differences between the colony types in specificity to an adhesion receptor on a fish surface.

Bacteria were incubated with N-acetylneuraminic acid, N-acetylglucosamine, mannose, fucose, glucose and galactose, and the adhesion capacities were determined as above taking into account the growth of bacteria. Adhesion inhibition percentage of each treatment for each variant was calculated.

As growth of variants treated with sodium metaperiodate and N-acetylneuraminic acid decreased during the adhesion experiment, the toxicity of these substances on *F. columnare* was tested on C1 and E1.

3.5.5 Chondroitin AC lyase activity (III)

Degradation of chondroitin sulphate (ChS, chondroitin sulphate C, Sigma Chemical Company, Ill. USA), i.e. chondroitin AC lyase activity of the variants B3, C1, E1, E2, G2, G4, H2 and H4, was determined as described previously (Teska 1993, Suomalainen et al. 2006) at 5, 15, 20 and 25 °C. Briefly, bacterial cultures were first pre-incubated on 96-well plates at the four temperatures for 75 min. The substrate was then added to the bacteria, suspensions incubated for 15 min at the temperatures of pre-incubation, and turbidity of wells (as OD at 595 nm) measured with a plate reader. Protein concentrations in bacterial suspensions were determined with Bio-Rad Protein Assay Kit (Bio-Rad, Inc.) using bovine serum albumin (BSA) as a standard. Chondroitin AC lyase activities were expressed as ChS lysed per bacterial protein in a time unit (μg mg^{-1} min^{-1}).

3.6 Saprophyte experiment (IV)

Six rainbow trout (mean weight 16.0 g) were exposed to 5.0×10^6 CFU ml^{-1} of *F. columnare* strain C in 15-l plastic tanks. Three of the fish were killed before the exposure and the other three immediately after it. Three control fish, killed before the exposure, were exposed to sterile Shieh broth. After the exposure, fish were transferred individually into 3-l tanks containing static well water (20 °C). The tanks were replaced with tanks containing fresh water twice a day during the first 3 days, and once a day thereafter. Bacterial shedding was measured from the water by plate counting immediately after removal of the fish and expressed as CFU ml^{-1}.

3.7 Transmission experiment (IV)

Five rainbow trout ("donor fish") (mean weight 16.0 g) were exposed to *F. columnare* strain C as described in section 3.6 and three control fish were exposed to sterile Shieh broth. After the challenge, the fish were transferred individually to separate tanks receiving fresh well water (flow rate 6.5 l h^{-1}, 25 °C). Each tank was connected to another tank by water flow downstream (for

experimental setup, see Fig. 1 in IV), and an uninfected rainbow trout ("recipient fish 1") was placed in this tank. After the donor fish died, the water flow to the recipient 1 was closed and redirected to a new tank containing a new recipient fish (recipient 2) for 24 h. After this, water flow was again disconnected and recipient fish 3 was connected to the donor fish for the next 24 h. The connection between donor fish and recipient fish 3 was closed after 24 h exposure, and recipient fish 4 was connected to the donor fish for the following 24 h. Survival of the recipient fish was monitored for one week. Bacterial shedding from the living, and later dead, donor fish was measured from the water at 12 h intervals by plate counting and expressed as CFU ml^{-1}.

3.8 Survival in water (IV)

Survival of *F. columnare* strain C in water was studied using sterilized distilled and lake water. Bacterial culture (1 ml) was inoculated to water (29 ml) to adjust final bacterial concentration to 8.8×10^6 CFU of bacteria ml^{-1} in triplicate 50-ml sterile tubes. The tubes were kept in a dark cabinet at RT. The survival of bacteria in the water was monitored by plate counting on days 0, 1, 3, 7, 10, 14, 21, 35, 50, 62, 93 and 149 after inoculation.

3.9 Immunostimulant experiments (V)

Three trials were carried out to study the effects of administration of immune stimulatory agents on the immune status of rainbow trout fingerlings and resistance of fish against columnaris disease. In Trial 1 the fish received a formulated diet containing yeast β-glucan, in Trial 2 the fish were intraperitoneally injected with yeast β-glucan, and in Trial 3 the fish were fed with pelleted trout feed with added leucine metabolite hydroxymethylbutyrate (HMB). At the end of all the trials, functional immune parameters of the fish were determined and the fish bath challenged with *F. columnare*.

3.9.1 Trial 1: Feeding with yeast β-glucan

The fish (mean weight at start 8.8 g) received a formulated diet with varying concentrations of yeast β-glucan (Macrogard, Biotec Pharmacon ASA, Norway): 0.0, 0.2, 0.6 or 1.8% of feed. For each concentration of glucan four replicate 50-l glass aquaria (n = 25 fish in each, 18 °C) were set up. The fish were fed with glucan-supplemented feed for 21 days, and sampled for determination of their innate immune functions on days 7, 14 and 21 (n = 8 fish in each treatment level and time). The remaining fish (n = 66 fish per treatment) were bath-challenged on day 22.

3.9.2 Trial 2: Injection with yeast β-glucan

The fish (mean weight at start 11.4 g) were injected with yeast β-glucan. Based on results from a pilot study (for details of the pilot study, see V), fish received intra peritoneally 1 mg of glucan in 100 ul of sterile PBS, or PBS only as a control. Ten replicate 50-l glass aquaria (n = 17 fish in each, 18 °C) were set up for both treatments. Innate immune function parameters were determined on day 11 (n = 18 fish in each treatment), and the rest of fish (n = 152 fish per treatment) were bath-challenged on day 14 post injection.

3.9.3 Trial 3: Feeding with β-hydroxy-β-methylbutyrate (HMB)

The fish (mean weight at start 4.7 g) received feed supplemented with 0, 25 and 50 mg kg^{-1} body weight day^{-1} of β-hydroxy-β-methylbutyrate (HMB, Metabolic Technologies, Inc., USA), a leucine metabolite. The fish were placed in 4 replicate aquaria (n = 40 fish in each, 16 °C) per treatment level. The fish were fed 21 days after which innate immune parameters (n = 8 fish in each treatment) were assayed. Rest of the fish (n = 152 per treatment) were bath-challenged on day 22.

One group of fish (n = 40) received a 21-day prophylactic course of OTC (125 mg kg^{-1} body weight day^{-1}) in order to study the effectiveness of the antibiotic treatment against the strain C of *F. columnare* used for infections in all the trials. These fish were challenged simultaneously with HMB-fed fish.

3.9.4 Determining the immune status of fish

At the end of the trials, immune parameters representing the innate (nonspecific) branch of defence were determined: plasma lysozyme activity, plasma complement bacteriolytic activity, ROS-production by whole blood and isolated head kidney leukocytes. Fish were anesthetized and a blood sample taken. Plasma was separated and frozen (–70 °C) until analyzed. The head kidney was removed and homogenized in heparinised Hanks's balanced salt solution (HBSS) supplemented with 2 % FCS (Gibco). The head kidney leucocytes were isolated with a two-step Percoll density gradient (Pharmacia LKB Biotechnology AB, Sweden) using a modification of a procedure published earlier (Harrell et al. 1976), and the number of leucocytes was adjusted.

Complement activity was determined using a modification of a method published earlier (Nikoskelainen et al. 2004). Plasma (0, 1, 2 and 4 ml) and PBS were mixed to a volume of 50 µl and suspended with an equal volume of suspension of *Escherichia coli* KL12 pEGFL LucAmp indicator bacteria (containing a reporter gene for luciferase) on microplates. After incubation for 90 min, D-luciferin (Sigma) was added and luminescence measured with Victor^2 1420 Multilabel Counter (Wallac, Finland). The luminescence data were converted to bacterial viability percentage and bacteriolytic activity was calculated.

Plasma lysozyme activity was determined with a turbidometric microplate assay (Jokinen et al. 2003) using a suspension of *Micrococcus lysodeicticus* (Sigma) as the substrate. The turbidity (OD at 450 nm) in wells was monitored for 30 min at 1 min intervals with a Victor^2 1420 Multilabel Counter. The plasma lysozyme activity was expressed as Units ml^{-1} (U ml^{-1} = 1000 × Δ OD min^{-1} ml^{-1}).

Production of ROS by whole blood and isolated head kidney leukocytes was determined at 24 °C on 96-well microplates. The reaction was stimulated with phorbol 12-myristate 13-acetate (PMA), enhanced with luminol, and measured using a modification of a chemiluminescence method (Nikoskelainen et al. 2004, Markkula et al. 2005). One hundred µl of isolated head kidney leucocytes, or heparinised whole blood diluted 1:300 with HBSS, was dispensed on plate, luminol (Sigma) added, and the reaction started with PMA (Sigma). The luminescence was measured (Plate Chameleon, Hidex, Finland), and the peak luminescence value determined.

4 RESULTS AND DISCUSSION

4.1 Finnish *Flavobacterium columnare* strains (I)

4.1.1 Genetic properties

The Finnish *F. columnare* strains did not differ from each other or from the *F. columnare* type strain according to LH-PCR analysis of partial 16S rDNA or 16S rDNA RFLP analysis. All the strains yielded a 517 bp PCR product in LH-PCR, which may indicate that this length is characteristic for the pathogen. All the strains also had similar restriction profiles with each other (results not shown). Thus, according to the classification based on 16S rDNA RFLP analysis by Triyanto and Wakabayashi (1999), Finnish *F. columnare* strains together with the type strain belong to the genomovar *I* among genomovars *I–III* of *F. columnare* strains isolated from different parts of the world. So far all the *F. columnare* strains isolated from Europe have belonged to genomovar *I* (Triyanto & Wakabayashi 1999, Michel et al. 2002). Furthermore, most of the Finnish strains and the type strain have been isolated from salmonids. Based on the results of Michel et al. (2002) there is little chance that the genomovar *II* would infect any salmonid fish. Host specificity of *F. columnare* genomovars was also detected by Olivares-Fuster et al. (2007a), who reported an association between genomovar *I* and threadfin shad (*Dorosoma pretenense*), and between genomovar *II* and two catfish species, channel catfish (*I. punctatus*) and blue catfish (*I. furcatus*). Genomovar *III* includes strains isolated from ayu fish (*Plecoglossus altivelis*) in Japan and channel catfish in the USA (Triyanto & Wakabayashi 1999, Darwish & Ismaiel 2005).

Based on ARISA profiles, Finnish *F. columnare* strains could be divided into seven different groups, with the type strain representing an eighth group (genetic groups A–H, see Table 1). ARISA reveals the number of 16S–23S ISR regions of different length, which, however, does not always correlate with the total number of ISR copies in the bacterial genome. Among Finnish strains, one to three ISR regions of different length were detected. Arias et al. (2004) used

sequence analysis of ISR region to type *F. columnare* isolates from USA and Brazil belonging to genomovars *I* and *II*. They detected four genomic groups among the strains, but the result was based on only one copy of the ISR sequence from each strain. Sequencing may give a misleading result if the number of the ISR sequences is not taken into account. Thus, ARISA and RFLP of ISR are more convenient subtyping methods, as the results are repeatable and not affected by the number of ISR copies. Using these methods Darwish & Ismaiel (2005) could subdivide the strains isolated from the USA and belonging to genomovars *I* and *II* into two groups in each genomovar.

Most of the Finnish *F. columnare* strains fell into genetic group A, while there was only one strain in each of the genetic groups B, D and G. Strains belonging to genetic groups A, C, E and H were isolated from different salmonid species, and one strain belonging to the genetic group H also from non-salmonid species. This was not surprising, because *F. columnare* strains belonging to genomovar *I* have previously been isolated from both salmonids and non-salmonids (Triyanto & Wakabayashi 1999, Michel et al. 2002, Olivares-Fuster et al. 2007a). More interesting was that three genetic groups were isolated from a disease outbreak at the same farm. A similar phenomenon has been reported by others, as different *F. columnare* genomovars and *F. psychrophilum* ribotypes have been isolated from the same outbreak or at the same time from broodfish and fry (Triyanto et al. 1999, Madetoja et al. 2001, Madsen et al. 2005). Because *F. columnare* exists in natural waters, the presence of several genotypes might be a common phenomenon for this bacterium and may contribute to the continuous nature of the outbreaks (Rickard et al. 2003, Revetta et al. 2005, Pulkkinen et al. 2010). Furthermore, the co-existence of genetically different pathogen strains may lead to increased virulence (e.g. Read & Taylor 2001), and indeed the genetic groups of Finnish *F. columnare* strains have been shown to differ from each other in virulence (Suomalainen et al. 2006).

The 16S rDNA sequence analysis of representatives of the genetic groups showed a genetic proximity of the Finnish strains; the 1329 bp continuous sequences assembled and used to create a neighbour-joining tree revealed a highly homologous group of Finnish strains that differs from the strains isolated from other parts of the world. Finnish strains cluster with the strain IFO 15943^T that is derived from the type strain NCIMB 2248^T (IAM 14301) (Nakagawa et al. 2002), and should thus cluster with the type strain. This unexpected difference could be explained by an error in sequencing or by the existence of multiple 16S rDNA copies with sequence differences in the type strain. In a study by Tiirola et al. (2002a), 16S rDNA sequences amplified directly from diseased fish clustered with the *F. columnare* type strain, but formed a separate group, which is a similar result as obtained in the present study.

4.1.2 Growth features

Representative strains of 7 genetic groups were able to grow at all the temperatures tested (17.0−27.4 °C), but in each strain there were differences in growth rates between the temperatures. Although in most of the strains the growth rate increased with temperature, there were differences between the strains at different temperatures. Variation in growth ability at different temperatures has been observed between different *F. columnare* strains in other studies, and some strains have been shown to grow at temperatures over 30 °C (Bernardet 1989, Decostere et al. 1998). In only two of the Finnish strains (E and H) did the growth rate start to decrease above 25 °C, which indicates that in the Finnish strains the optimal growth occurs in the same temperature region (25–30 °C) as in *F. columnare* strains isolated from other parts of the world (Decostere et al. 1998). High water temperature is associated with higher mortality during columnaris infection (Snieszko 1974), although this may not be a consequence merely of an increased growth rate. For example, according to Suomalainen et al. (2005b) transmission of *F. columnare* is also higher at elevated temperatures.

Salt concentration and pH had significant effects on the growth rate of the Finnish strains. The strains were able to grow at pH 6.8 to 9.2, but the growth was inhibited in more acidic conditions. The growth rate decreased with increasing salt concentration and growth stopped at a concentration of 0.1 %, at which only strain A showed some growth. There were also differences between the strains in other salt concentrations. The type strain, however, was not affected by the salt concentration and its growth was inhibited only at pH 6.2. Based on these results, and due to the external nature of the columnaris disease (Bernardet 1997), acidic or salt bathing could provide a potential prevention or treatment method for *F. columnare* infection. Indeed, rearing fish in salinities between 3 and 9 ‰ was found to inhibit columnaris infection (Altinok & Grizzle 2001), and columnaris disease has not been reported to occur in seawater or in brackish water areas (salinity between 2 and 7 ‰) around Finland. In an experimental infection with Finnish *F. columnare* strains, however, neither acidity nor salinity gave protection against the infection, although salinity did significantly decrease the transmission rate of the columnaris disease (Suomalainen et al. 2005c). Thus, the use of antibiotics for curing columnaris infections at Finnish fish farms is still the most effective option. Tetracycline, and florfenicol if tetracycline-resitant strains should occur, is recommended as the treatment by The Ministry of Agriculture and Forestry in Finland (Anon. 2009). Use of antibiotics is also supported by the results in I, as Finnish strains were sensitive to most of the antimicrobial agents tested. However, the observed intermediate resistance of strains A, E, G and H to neomycin, and intermediate resistance of strain A and complete resistance of strains C, E, G and H to Polymyxin B predict possibility of emergence of resistant *F. columnare* strains in the future also to other antibiotics. Therefore it is

of great importance to study other possible treatment methods for columnaris disease to be used at Finnish fish farms.

4.2 Colony types formed by Finnish *F. columnare* strains (II, III)

Four different colony morphologies (colony types 1–4) are formed among Finnish *F. columnare* strains on Shieh agar in laboratory conditions (Table 2 in section 3.5, Fig. 2). Colony type 1 forms rhizoid colonies and is flat with a yellow centre. Colony type 2 is hard, more orange in colour, non-rhizoid or only slightly rhizoid, and has irregular edges and a convex growth form. Colony type 3 has round edges and a smooth, yellowish appearance. Colony type 4 is white or light yellow and smooth with an irregular shape. Differential colony type formation also occurred on AO agar, but the appearance of colonies differed to some extent from those formed on Shieh agar. For example, the colonies formed on AO agar were more spreading and grew so close to each other that they could not be clearly separated. The difference between colonies formed on these two media may be caused by the lower amount of nutrients in AO medium compared to Shieh medium. Colony types formed on Shieh agar were chosen for further studies.

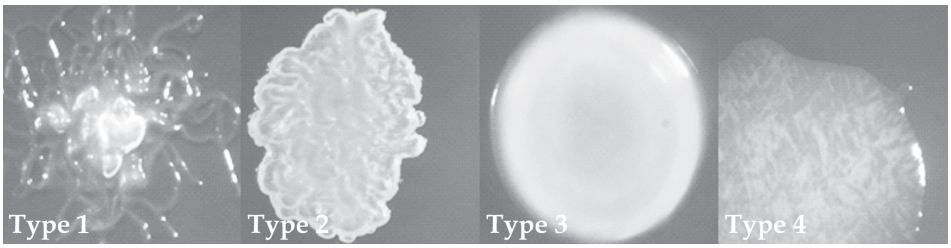

FIGURE 2 Colony types 1–4 of *Flavobacterium columnare* formed on Shieh agar plate cultures in the laboratory. Diameter of the colonies varies between 1 to 6 mm.

Formation of different colony morphologies between *F. columnare* strains and also in subcultures has been detected previously (Song et al. 1988, Bernardet 1989, Bader et al. 2005), but no studies have been conducted to clarify the reasons behind this phenomenon. Overall, change in colony morphology is not a rare phenomenon; it occurs in many human and animal bacterial pathogens. For example, colony morphology variation between translucent and opaque colony types is observed in both *Vibrio parahaemolyticus* and *V. vulnificus*, whereas *Mycobacterium avium* represents smooth and rough colony types (Simpson et al. 1987, Kansal et al. 1998, McCarter 1998). Closer relatives of *F. columnare*, *F. succinicans*, *F. johnsonae* and *F. psychrophilum*, have also been reported to form different colony morphologies (Anderson & Ordal 1961, Chang et al. 1984, Bernardet & Kerouault 1989).

4.2.1 Genetic basis of differential colony formation (II)

No differences in ARISA, AFLP or whole cell protein profiles were detected between the different colony type variants of the same strain, which suggests that no changes in genomic sequence or protein composition are involved in the colony type change in *F. columnare*. However, these methods provide a limited survey of the whole genome and of gene expression products. Using, for instance, SSCP and SSH, and studying outer membrane proteins (OMP) or cell surface LPS might have given some differences between the colony types (e.g. Zhang et al. 2000, Zhang et al. 2006, Olivares-Fuster et al. 2007b). Different colony morphologies of *F. psychrophilum* have been shown to differ in their whole cell protein, OMP and ECP profiles, and the rough mutant of *Edwardsiella ictaluri* differs from its smooth parent in LPS composition (Arias et al. 2003, Högfors-Rönnholm et al. 2009). Differential colony formation of both *V. vulnificus* and *V. parahaemolyticus,* on the other hand, correlates with the production of capsular polysaccharide (CPS) (Yoshida et al. 1985, Enos-Berlage & McCarter 2000).

Several factors could lead to the formation of different colony morphotypes by *F. columnare*. One is a phenomenon called phase variation. Phase variation was thought to be a random event occurring in a bacterial population producing subpopulations of different phenotypes as a consequence of heritable genomic changes, like DNA inversions, recombinational deletions or differential DNA methylation (Henderson et al. 1999). Variation is considered antigenic variation if the change results in antigenically distinct subpopulations and is involved in avoidance of immune selection (van der Woude & Bäumler 2004). However, growing evidence suggests that phase variation is not always a random event, but rather a response to an environmental change and caused by change in gene expression allowing bacteria to adapt the surrounding environment (van der Woude 2006). Change is caused by a reversible on/off switch in the expression of one or more genes, but irreversible phase variation has also been detected (van der Woude & Bäumler 2004, Hilton et al. 2006). In the present study, change of colony morphology in *F. columnare* was found to be irreversible.

Change in gene expression can be triggered by intra- or extracellular activators or inhibitors, such as temperature or signalling molecules received from the environment (Ratiner 1999, van der Woude & Bäumler 2004). These molecules can be related to bacterial quorum sensing, which has a central role in phase variation. Quorum sensing is a chemical signalling system used by bacterial populations in response to cell density to control a variety of functions via regulation of gene expression. It is suggested that, within a host, quorum sensing bacteria delay the production of virulence factors until the population is large enough to cause an infection (Bassler 1999). Besides chemicals produced by bacteria, molecules received from a host can also affect behaviour of bacterial populations (Daniels et al. 2004, Gonzalez & Keshavan 2006). Quorum sensing in *V. cholerae* is known to function in the infection process and in control of virulence gene expression (Zhu et al. 2002, Higgins et al. 2007).

Quorum sensing has not been studied in *F. columnare*, but in *F. psychrophilum* no evidence of quorum sensing was detected when the acylated homoserine lactone (AHL) -based signalling system was examined (Bruhn et al. 2005). However, several other quorum sensing systems are known in addition to AHL-based signalling, such as autoinducer 2 and 3, cyclic dipeptides, bradyoxetin and diffusible signal factor (Bassler 1999). Moreover, quorum sensing systems and/or signals have been found in some fish pathogenic bacteria, such as *V. anguillarum*, *A. hydrophila*, *Aeromonas salmonicida, Vibrio harveyi*, *Edwardsiella tarda* and *Y. ruckeri* (Milton et al. 1997, Swift et al. 1997, Freeman & Bassler 1999, Morohoshi et al. 2004, Bruhn et al. 2005). Thus, the possible involvement of quorum sensing system in regulating colony morphology and virulence of *F. columnare* can not be excluded.

Colony type variation may occur also in a less controlled manner. Clonal microbial populations contain cells with phenotypic heterogeneity (i.e. variable degrees of virulence, resistance to antimicrobial treatments, and capacity to differentiate) with certain genes being expressed non-uniformly across the population (Avery 2006, Dubnau & Losick 2006). This allows bacterial pathogens to adapt to environmental changes with selection favouring the subpopulation of the cells best fitting the circumstances. It is possible that various colony types are constantly expressed by *F. columnare*, and that environmental conditions (in the laboratory and probably also in the host) determine the colony morphology favoured in different situations.

What is the biological meaning of differential colony morphology formation by *F. columnare*? From fish challenged with strain-colony type variant E1, colony types 2 and 4 in addition to colony type 1 were isolated from primary cultures. This suggests that different colony types may have different roles in the infection process of columnaris disease, but whether they are formed by phase variation, quorum sensing or environmental selection of existing subpopulations can not be evaluated. However, it is known that, especially in an animal host, the host environment can drive selection against a particular phenotype of a bacterium, which can result in a different phenotype for cells cultured in the laboratory and those isolated from the host (van der Woude & Bäumler 2004). Indeed, both phase variation and quorum sensing are considered important tools of pathogenic bacteria to evade innate and acquired immune defence mechanisms during colonization or infection of animal host (Bassler 1999, van der Woude & Bäumler 2004, Gonzalez & Keshavan 2006, van der Woude 2006), because adaptation of pathogen to growth and survival within the host, and transition to and from external reservoir, require regulation of expression of virulence factors (Miller et al. 1989).

Results in II suggest that formation of different colony types in *F. columnare* occurs as a response to a change in growth environment, and is a consequence of a change in gene expression or selection of bacterial cells expressing favoured genes rather than a mutation in a genome. Gene expression was not determined in the present study, but SSH could have been used for that (Flemming et al. 2009, Garbeva & de Boer 2009). However, one effect of environmental change, a change in nutrient conditions, could be seen

in this study as different colony formation on AO and Shieh agars. This has also been detected in *Vibrio vulnificus* and *Neisseria gonorrhoeae*, in which the change in colony morphology was caused by phase variation as a response to starvation (Stohl & Seifert 2001, Chatzidaki-Livanis et al. 2006). Furthermore, it has been suggested recently that *Flavobacterium johnsoniae* like isolates exhibit distinct phenotypes and genotypes which are associated with the ability to form biofilms, and that gene expression is significantly altered when the isolates switch from the free-living to the attached lifestyle (Flemming et al. 2009). It is possible that differential colony formation by *F. columnare* may aid the bacterium to persist at fish farms and cause the infection in favourable conditions. However, suggested mechanisms and reasons behind production of different colony types need to be verified in the future.

4.2.2 Relationship between virulence (II), adhesion (II, III) and chondroitin AC lyase activity (III) of the colony types

A relationship between colony morphology variation and virulence has been shown in many pathogenic bacteria (see e.g. Simpson et al. 1987, van der Woude & Bäumler 2004). For example, fish pathogenic *V. vulnificus* bacteria with translucent colony type are less virulent than bacteria exhibiting opaque colonies, and isolates of human pathogen *M. avium* forming rough colonies are more virulent than isolates with smooth colonies (Yoshida et al. 1985, Kansal et al. 1998).

Finnish *F. columnare* strains belonging to different genetic groups have been shown to differ from each other in their virulence (Table 3). In II, it was shown that only the strain-colony type variant E1 forming rhizoid colonies was highly virulent in the challenge experiment while the other variants were of low virulence (Table 3). Variants B3 and C1 have previously been shown to be of low and high virulence, respectively (own unpublished data). Rhizoid or spreading growth form is an indication of gliding motility in *F. columnare*, and also in its close relatives *F. johnsoniae* and *F. psychrophilum* (Chang et al. 1984, Bernardet & Kerouault 1989, Bernardet et al. 1996, Staroscik et al. 2008). Gliding motility has been studied extensively in *F. johnsoniae*, and disruption of any gene involved in gliding has been shown to lead to formation of non-spreading colonies (e.g. McBride 2004, Rhodes et al. 2009). Furthermore, in a mutant strain of *F. psychrophilum,* the loss of gliding motility leads to non-spreading colonies and decreased virulence (Álvarez et al. 2006). This mutant, however, was also deficient in extracellular proteolytic activity. Högfors-Rönnholm et al. (2009) recently isolated a spontaneously–formed, non-motile, non-virulent and caseinase-negative variant of *F. psychrophilum*. In the present study neither proteolytic nor caseinase activity was studied, and it is not known how the change in colony morphology is associated with these properties of *F. columnare*.

Virulence of the strain-colony type variants was not related to their adhesion capacity on polystyrene or gill tissue (see Table 3). Variants forming colony type 2 were the most adherent on polystyrene, followed by rhizoid

colony type 1, then by type 3 and finally by variants forming type 4. On gill tissue, the most adherent variant was G4. This may indicate that the adhesion factors of *F. columnare* in polystyrene adhesion are different from those in gill tissue adhesion. On the other hand, *F. columnare* bacteria in the aquatic environment are able to form biofilms on inorganic materials (Rickard et al. 2003, Revetta et al. 2005), and it has been suggested that pathogenic bacteria with environmental reservoirs use the same adhesion mechanisms when interacting with different substrates (Tarsi & Pruzzo 1999). Thus, it is justified to use polystyrene adhesion as an indicator of the general adhesion capacity of *F. columnare*.

The relationship between virulence and adhesion capacity of *F. columnare* has also been studied earlier. No connection was found between adhesion on crude mucus-coated slides and virulence of the Finnish strains (Suomalainen et al. 2006). On the other hand, Bader et al (2005) reported that a less virulent *F. columnare* mutant forming smooth colonies was significantly less adherent both on skin and gill tissue of channel catfish (*I. punctatus*) than the wild type strain, which formed hard colonies with finger-like projections and which may have resembled our rhizoid colony type 1. Also Decostere et al. (1999a) have shown a connection between adhesion on gill tissue and virulence of an *F. columnare* strain.

Decostere et al. (1999a) reported that adhesion on gill arches by a highly virulent *F. columnare* strain is enhanced by increased temperature. This result contradicts that reported in III, as none of the variants was the most adherent at the highest temperature tested (25 °C), although temperature had a significant effect on the adhesion of all the variants. Moreover, the most virulent variants C1 and E1 (see Table 3) were not more adherent than other variants at any temperature. Result from the adhesion and virulence experiment on Finnish *F. columnare* strain-colony type variants indicate that, even though adhesion capacity is essential to initiate the columnaris infection, it is not the main factor defining virulence in *F. columnare*. Also the porperty of forming rhizoid colonies is needed to achieve high virulence.

No trends in chondroitin AC lyase activity profiles were observed between the temperature and the variants forming the same colony type, and only E1 and E2 showed increasing activity as the temperature increased. This contradicts the previous studies, in which chondroitin AC lyase activity of *F. columnare* increased along with the temperature (Teska 1993, Suomalainen et al. 2006). A different assay procedure may account for this discrepancy. Suomalainen et al. (2006) showed that chondroitin AC lyase activity is connected to the virulence of the genetic groups of Finnish *F. columnare* strains. When the average lyase activities at four temperatures were considered in III, two groups could be clearly separated: C1, E1, E2, G2 and G4 with high chondroitin AC lyase activity, and B3, H2 and H4 with low activity (Table 3). However, only C1 and E1 have been defined as high virulence variants whereas B3, E2, G2, G4, H2 and H4 are of low virulence. Based on this it is suggested that decreased virulence of a strain-colony type variant within a genetic group

is not a consequence of a decreased chondroitin AC lyase activity, but a result of a change in colony morphology of a strain to a non-rhizoid type. Even though chondroitin AC lyase activity has been shown to be related to the virulence of *F. columnare*, it solely is not enough to cause high virulence in *F. columnare*, but also rhizoid growth form is required.

TABLE 3 Summary of the representatives of genetic groups and strain-colony type variants of *Flavobacterium columnare* (letter refers to genetic group and number to colony type), and their virulence, adhesion capacity on polystyrene and chondroitin AC lyase activities (I–III). For comparison *F. columnare* type strain (NCIMB 2248^T) is included to the table. + low, ++ intermediate, +++ high, ND not determined; mortality caused by low virulence variant = 0 – 19 % and high virulence variant = 60 – 100 % (own unpublished data, Suomalainen et al. 2006, II); adhesion on polystyrene (absorbance at 595 nm): high ≥ 0.035, 0.020 < intermediate < 0.035, low ≤ 0.020 (II); chondroitin AC lyase activity (chondroitin sulphate lysed bacterial protein^{-1} time unit^{-1}, µg mg^{-1} min^{-1}): high ≥ 500, 200 < intermediate < 500, low ≤ 200 (III).

Genetic group of the strain	Virulence of the studied strain	Strain-colony type variant	Virulence of the strain-colony type variant	Adhesion on polystyrene of strain-colony type variant [c]	Chondroitin AC lyase activity of strain-colony type variant [d]
A	+++ [a]	A1	ND	+++	ND
B	+ [a]	B3	+ [a]	+	+
C	+++ [b]	C1	+++ [a]	++	+++
D	+ [b]	D2	ND	+++	ND
		D4	ND	+	ND
E	+++ [b]	E1	+++ [c]	++	+++
		E2	+ [c]	+++	++
F (NCIMB 2248^T)	+ [b]	F4	ND	+	ND
G	+++ [b]	G2	+ [c]	+++	+++
		G4	+ [c]	+	+++
H	+ [b]	H2	+ [c]	+++	+
		H4	+ [c]	+	+

[a] own unpublished data
[b] (Suomalainen et al. 2006)
[c] II
[d] III

4.2.3 Inhibition of adhesion (III)

Association with a host is mediated by bacterial cell surface adhesion molecules binding on specific receptors on the host tissue. These adhesion structures have been studied in pathogenic bacteria by inhibiting the adhesion by treating bacteria with sugars that mimic host receptors or with agents that modify cell surface (e.g. Simon et al. 1997, Hamilton et al. 1998, Wang & Leung 2000, Thomas & Brooks 2004). Treatment of *F. columnare* with sodium metaperiodate, incubation of bacteria with glucose, N-acetylglucosamine, galactose and sucrose, and treatment of gill tissue with sodium metaperiodate have been shown to reduce the adhesion of bacteria on the gill tissue of carp (*Cyprinus carpio* L.) (Decostere et al. 1999c). Also in *F. psychrophilum*, treating bacteria with sodium metaperiodate and trout kidney phagocytes with N-acetylneuraminic acid, decreased the association of *F. psychrophilum* with the phagocytes (Wiklund & Dalsgaard 2003). In another study, hemagglutination activity of *F. psychrophilum* was inhibited by treating bacterial cells with proteinase K and N-acetylneuraminic acid (Møller et al. 2003). It was concluded that both *F. columnare* and *F. psychrophilum* bind to host cells with a lectin-like bacterial surface structure, and that a major component of a host cell receptor is of carbohydrate nature, N-acetylneuraminic acid in case of *F. psychrophilum*.

In III, the effect of cell surface modification on adhesion capacity of the variants was not clearly related to the colony type. Only colony type 4 variants G4 and H4 reacted similarly to all treatments. In most of the variants, modification of the cell surface with polymyxin B, sodium metaperiodate and proteinase K significantly decreased the adhesion on polystyrene. However, sodium metaperiodate was found to be toxic to *F. columnare*, which was seen both from growth in plate cultures and turbidity measurements on microplates. Reduced growth of *F. columnare* after sodium metaperiodate treatment has also been detected earlier, and treatment has been shown to affect the viability of *Actinobacillus pleuropneumoniae* as well (Decostere et al. 1999c, Van Overbeke et al. 2002). The decrease in adhesion after polymyxin B treatment suggests that the adhesion molecules of *F. columnare* strain-colony type variants may be LPS or some other molecules of a carbohydrate nature (see Morrison & Jacobs 1976). It has been shown earlier that LPS composition differs between high and low virulence strains of *F. columnare* (Zhang et al. 2006). In the present study, polymyxin B did not decrease the adhesion of low virulence G4 and H4, which may be due to a weak adhesion capacity of these variants or to different adhesion molecules compared to the variants forming other colony types. Decrease in adhesion after proteinase K treatment, on the other hand, would suggest that the adhesion molecule is of a protein nature. However, it is probable that there are several types of adhesion molecules in *F. columnare* strain-colony type variants which are both of carbohydrate and protein nature. Still, the adhesion mechanisms of variants forming colony type 4 may be similar to each other and differ from those of other colony types. As no differences in whole cell protein SDS-PAGE patterns were detected between the strain-colony type variants in II, difference in adhesion between colony type 4 and other

colony types may be caused by differential cell surface carbohydrate composition.

What remains an open question is whether there are differences between the colony types in specificity for fish surface receptors, as no connection between inhibition of adhesion with sugars and the colony type of the *F. columnare* variant was found. The effect of sugar treatments varied between variants; some sugars significantly inhibited the adhesion of some variants, and enhanced the adherence of others. Genetic differences between the variants may have affected the outcome of the experiment. N-acetylneuraminic acid was the only sugar significantly reducing the adhesion of all the variants, but this substance was also found to be toxic to *F. columnare*. A negative effect on viability has also been seen in *E. coli* and *Haemophilus influenzae* in which accumulation of intracellular sialic acid (N-acetylneuraminic acid) inhibits growth (Vimr & Troy 1985, Johnston et al. 2007). However, in the present study, the adhesion profiles of G4 and H4 were similar after sugar treatments, which further supports the suggestion that adhesion factors of colony type 4 differ from other colony types.

4.3 Saprophytic life style and transmission properties of *F. columnare* (IV)

In aquaculture, some fish die due to bacterial and ectoparasite infections and may lie unnoticed at the bottom of the tanks or ponds (Valtonen & Keränen 1981, Rintamäki-Kinnunen & Valtonen 1997). These fish can serve as an infection source, as has been shown for a protozoan *Paraemoeba pemaquidensis* and a monogenean ectoparasite *Gyrodactylus salaris* (Douglas-Helders et al. 2000, Olstad et al. 2006). Survival of *F. columnare* outside its living host has not been studied experimentally and transmission of the bacterium is poorly understood. However, to improve information regarding the infection sources of columnaris disease at fish farms, these issues need to be clarified.

Living fish challenged with *F. columnare* (IV) shed bacteria into the water over 5 days, but *post mortem* challenged fish over 8 days. The shedding rates of fish infected *post mortem* were on average 10 times higher than in fish infected alive on days 1 to 5 (and 200 to 2000 times higher thereafter). This suggests that in addition to being a pathogen, *F. columnare* can also live as a saprophyte. Saprophytism has also been suggested for other fish pathogens, like *F. psychrophilum*, *F. branchiphilum* and *Aeromonas salmonicida*, but transmission of the disease to a living host was not confirmed (McCarthy 1977, Madetoja et al. 2000, Bernardet & Bowman 2006). In a transmission experiment described in IV, columnaris disease transmitted to a living host more efficiently from dead than from living fish. To my knowledge, this has not been previously reported in fish pathogenic bacteria. Higher transmission rate is most probably a consequence of observed higher bacterial shedding rates from dead than from living donor

fish. However, the immune status of the fish may also affect the transmission success. Indeed, despite a high shedding rate, one donor fish failed to infect the next host. It may also be that epigenetic inheritance affected the *F. columnare* bacteria shed from this donor fish (e.g. Knell et al. 1996). Strong immunocompetence may epigenetically alter the shed bacteria such that genes needed for pathogenic feeding are not expressed, the pathogen is not able to kill the host, and thus the disease is not transmitted. Colony type formation was not studied in transmission or saprophyte experiments. However, if colony types have different roles in the infection process of *F. columnare* and aid preservation of bacterium at fish farms, as suggested above, it is possible that they are also involved in transmission of columnaris disease.

Persistence of *F. columnare* in pond water at a rainbow trout farm during a columnaris outbreak has been detected (Dr. L.-R. Suomalainen, University of Jyväskylä, unpublished). The source of bacteria was most likely the decaying fish at the bottom, as incoming water contained no *F. columnare*, and one dead fish from the pond was found to be shedding thousands of *F. columnare* bacteria l^{-1} h^{-1}. The capacity for sustained survival outside fish hosts allows *F. columnare* to persist at farms during antibiotic treatment, and when fish are removed from the tanks, increasing transmission success. Water, as a physical environment, aids the transmission of the pathogen.

F. columnare was able to survive in both sterile lake and distilled water for 5 months in laboratory conditions (IV). However, the experiment was continued for 19 more months after which living *F. columnare* cells could still be isolated even though a decline in the population was detected during this period (own unpublished data). During the experiment, the studied strain C started to form type 2 colonies in addition to type 1 colonies. This may be an indication of saving metabolic resources as a response to starvation, as maintaining a rhizoid growth form may require more energy than a non-spreading growth form. It is likely that *F. columnare* uses dead bacterial cells as a nutrient source to maintain growth for a long time. *F. columnare* has also been reported to lyse *Escherichia coli* cultures and *Giardia intestinalis* cysts (Song et al. 1988, Revetta et al. 2005). This implies that *F. columnare* may be a generalist surviving in variety of freshwater environments and exploiting different hosts, possibly also transmitting from them. The ability to live in water without a host may reflect the history of *F. columnare* as a normal, non-pathogenic freshwater bacterium which also has a saprophytic way of life. It may also be that saprophytism has been used by *F. columnare* as a transition stage from a harmless aquatic bacterium to a pathogen, and has been maintained as transmission and survival strategy because of the high benefits. Indeed, living as a saprophyte will benefit *F. columnare*. Usually, pathogen fitness is decreased by pathogen-induced mortality; high reproduction rate of the pathogen may kill the host before the pathogen is able to get transmitted and reproduction of the pathogen stops when the host dies (Anderson & May 1982). This trade-off is generally believed to drive the evolution of pathogens towards intermediate levels of virulence (e.g. Frank 1996). However, if killing the host causes no costs for the pathogen, higher levels of virulence may develop.

This is probably true for *F. columnare*, as problems related to columnaris disease have significantly increased during the past 20 years at Finnish salmonid farms (Rimaila-Pärnänen 2003, Eskelinen 2009, Pulkkinen et al. 2010). At the same time, the number of antibiotic treatments has increased and symptoms of columnaris disease have become more lethal. The finding that more than one genetic group of *F. columnare* can co-exist during an outbreak at a fish farm (I) may have contributed to the columnaris infections becoming more severe. Competition between genetically different strains increases the transmission of highly virulent strains, increasing the probability of outbreaks (de Roode et al. 2005a, 2005b, Bell et al. 2006). The saprophytic lifestyle of *F. columnare* may allow increased potential for competition and, together with the ability to survive for a long time outside fish in fresh water, aid the persistence of bacteria in fish farming conditions. This may promote the evolution of highly virulent *F. columnare* strains.

4.4 Immunostimulation of rainbow trout against columnaris disease (V)

4.4.1 Effect on immune status

The first line of immune defence, determined as values of immune function parameters essential in innate protection against bacterial infection, has been previously shown to be enhanced in rainbow trout by feeding or injecting yeast β-glucan (Jørgensen et al. 1993). Other immunostimulants reportedly able to boost innate immunity of rainbow trout when given in feed include vitamin E, bacterial LPS, and several plant-derived products such as epigallocatechin-3-gallate derived from green tee, algal-based Ergosan, dietary ginger, and garlic (Peddie et al. 2002, Nya & Austin 2009a–c, Thawonsuwan et al. 2009).

In V, innate immune function parameters of juvenile rainbow trout were enhanced in response to feeding with yeast β-glucan and HMB, and intraperitoneal injection of β-glucan. Feeding the β-glucan diets for 21 days increased PMA-induced ROS-production in whole blood in a dose-dependent way, suggesting that glucan induces enhanced leukocyte killing activity or increases the number of circulating leukocytes. In plasma lysozyme activity, or in complement bacteriolytic activity, no significant changes as a response to diets were seen. Eleven days after injection of β-glucan, the levels of immune function parameters were enhanced. ROS-production in whole blood and isolated head kidney leukocytes, as well as the plasma lysozyme activity, were several-fold those of sham-injected (PBS) controls, but no effect on complement bacteriolytic activity was detected. However, the route by which the immunostimulant was given was important, as immune parameter responses to β-glucan were stronger when injected than when administered orally. This has been noted also in earlier studies (Raa et al. 1992, Duncan & Klesius 1996).

The effects of feeding HMB-supplemented diets for 21 days on ROS-production in whole blood and isolated head kidney phagocytes were dependent on the dose and differed significantly from the controls. Interestingly, HMB increased whole blood ROS-production but decreased head kidney leukocyte ROS-production, which may be caused by the increased number of leukocytes released to the circulation from head kidney as a response to immunostimulation. This phenomenon would be beneficial by increasing peripheral protection against bacteria invading the fish via skin and gills. In plasma, complement bacteriolytic activity increased with increasing dose of HMB, but lysozyme activity remained unchanged. For both β-glucan and HMB, activation of the leukocyte microbial killing mechanism was more prominent than plasma lysozyme or complement bacteriolytic activity when the stimulants were administered in feed.

4.4.2 Protection from the disease

There is evidence that immunostimulants are capable of protecting rainbow trout against bacterial infections. For instance, LPS as a feed supplement has proven effective against infections by *A. hydrophila* (Nya & Austin 2009a). On the other hand, intraperitoneal injections of dimerized lysozyme or nucleotides have been reported to protect rainbow trout from *A. salmonicida* infections (Siwicki et al. 1998, Carrington & Secombes 2007). Furthermore, it has been shown that feeding rainbow trout with an inactivated yeast (*Saccharomyces cerevisiae*) containing selenomethionine, AlkoSel®, decreases mortality of fish challenged with *F. columnare* (Suomalainen et al. 2009).

Despite the elevated innate immune defense parameters obtained in V, none of the immunostimulations gave protection to rainbow trout fingerlings against experimental *F. columnare* infection. On the contrary, among the fish fed with a diet containing 1.8 % of β-glucan, mortality was higher than among fish that received a diet with no β-glucan supplement. Similarly, the mortality of β-glucan-injected fish was higher than that of sham-injected. The fish having received HMB had slightly higher mortality than controls, but the difference between the diets was not statistically significant. However, the medication of fish with the antibiotic OTC-supplemented feed fully prevented the mortality of fish in a bath challenge with *F. columnare*, which shows the efficiency of antibiotic treatments against columnaris disease. For the appropriate function of immunostimulants, it is important to plan carefully the timing, dosage and the route of administration, as conducted in this study. The doses of the immunstimulants used in V were in the range of those used and found to be effective in previous studies (e.g. Selvaraj et al. 2005, Misra et al. 2006, Siwicki et al. 2006), and the duration of the stimulation was set to optimum in pilot studies.

The reason behind the increased mortality after immunostimulation seen in the present study is not clear, but it has been shown that overdose of immunostimulants can suppress immunity (Anderson 1992). This may be due

to activation of the immune system to work at a very high level, which exhausts metabolic reserves and leads to decreased immune defence activity in a case of infection. Indeed, immune parameters of the fish in the present study were effectively activated, but they were not effective against *F. columnare* infection. Inefficacy of immunostimulation with β-glucan has been detected also earlier against *A. salmonicida* in Atlantic salmon (*Salmo salar* L.), against *V. anguillarum* in Atlantic salmon, in rainbow trout and in turbot (*Scopthalmus maximus*), and against *E. ictaluri* in channel catfish (*Ictalurus punctatus*) (Rørstad et al 1993, Aakre et al. 1994, Thompson et al. 1995, De Baulny et al. 1996, Duncan & Klesius 1996). HMB was ineffective against *Streptococcus iniae* infection in Nile tilapia (*Oreochomis niloticus*) (Whittington et al. 2003), but, on the other hand, enhanced protection against *Y. ruckeri* and *A. salmonicida* in pikeperch (*Sander lucioperca*) (Siwicki et al. 2005, Siwicki et al. 2006).

It is known that lysozyme can work at different levels against different pathogens (Yano 1996). Thus, it may well be that in V the elevated lysozyme activity after β-glucan injection could not protect the fish against *F. columnare*. In fish with symptoms of *F. psychrophilum* infection, lysozyme activity was decreased, suggesting that flavobacteria may have a suppressive effect on the innate humoral defense mechanisms (Siwicki 2004). The complement bacteriolytic activity, on the other hand, may be inhibited by the large amount of sialic acid present on the cell surface of some virulent Gram-negative bacteria, such as *A. salmonicida* and *F. columnare* (Ourth & Bachinski 1987). This may have caused the inefficiency of elevated complement activity in infection of HMB-stimulated fish in V. The role of different colony types of *F. columnare* in avoidance of immune defense of host (see van der Woude 2006) might also be one explanation for inefficiency of immunostimulants against infection in rainbow trout. To confirm this, however, further studies are required.

Passive immunization of channel catfish has been reported to provide some protection against columnaris disease, but for resistance specific antibody formation is also essential (Shoemaker et al. 2005, Shelby et al. 2007). The successful development of a vaccine against columnaris disease in channel catfish, and the fact that older rainbow trout have developed resistance against *F. columnare*, together with the findings of the present study, indicate that successful boosting of the innate immune defence mechanisms is not alone sufficient to protect rainbow trout against columnaris disease (Fujihara & Hungate 1971, Fujihara & Nakatani 1971, Shoemaker et al. 2007); acquired antibody mediated immunity is also needed. This may be partly due to the external nature of *F. columnare* infections (Bernardet 1997, Tripathi et al. 2005). However, the character of columnaris disease in Finland may be changing, as in some cases *F. columnare* has also been isolated from the internal organs of fish in outbreaks in salmonid farms (Dr. P. Rintamäki, University of Oulu, personal communications). In earlier studies, *F. columnare* has been isolated from inner organs of experimentally infected fish (Decostere et al. 1999b, Bader et al. 2003). Hence, it may be that virulent strains are evolving the capacity to invade also

inner organs more often. This could influence also the usefulness of immunostimulants against columnaris disease in the future.

5 CONCLUSIONS

Based on their 16S rRNA gene sequences Finnish *F. columnare* strains constitute a genetically homogeneous group that differs both from the type strain and strains isolated from other parts of the world. Although Finnish *F. columnare* strains have similar temperature optima for growth, as reported previously for *F. columnare*, Finnish strains are less salt tolerant than the type strain (NCIMB 2248^T) and have a narrower pH range in which they are able to grow.

F. columnare strains isolated from fish farms in Finland can be divided using ARISA into seven genetically different groups. Strains representing different genetic groups can co-occur at the farm during one outbreak. This might be a common phenomenon for *F. columnare* and may contribute to the repeated occurrence of the outbreaks during warm water periods. The co-existence of genetically different pathogen strains may lead to increased virulence and may have contributed to columnaris disease becoming more severe in Finland during the last 20 years. Although antibiotics are still effective against *F. columnare* infections, some differences in antibiotic sensitivity between the genetic groups of Finnish strains can be detected. This may indicate a risk of development of antibiotic resistant strains in the future.

Of the Finnish *F. columnare* strains, those strain-colony type variants that form rhizoid colony type 1, which is a typical colony morphology type of *F. columnare*, are highly virulent, whereas variants forming other colony types are of low virulence. However, colony type 1 variants are not the most adherent on polystyrene, and not all variants exhibiting high chondroitin AC lyase activity are highly virulent. Thus, adhesion may not be the main virulence factor of *F. columnare*, and nor is high chondroitin AC lyase activity alone enough for the pathogen to be virulent. The property of forming rhizoid colonies, an indication of gliding motility of the bacterium, is suggested to be a prerequisite for virulence of *F. columnare*.

This study did not reveal clearly the factors behind the production of different colony morphologies by *F. columnare*. Formation of colony types most probably is a consequence of change in gene expression as a response to change in growth environment of the bacterium. It is also possible, that different colony

types are needed in different stages of the infection process of *F. columnare* in fish. The mechanism driving the selection of a certain colony type may be phase variation or quorum sensing signalling. Alternatively, various colony types may be expressed constantly in *F. columnare* populations and the best suited morphologies are selected in every circumstance. The property of differential colony morphology formation may aid *F. columnare* to persist at fish farms and cause infection in favourable conditions. These suggestions, however, need to be verified in the future.

The rate of both shedding of *F. columnare* and transmission of columnaris disease is higher from dead than from living fish. This means that in addition to a pathogenic way of life, *F. columnare* can live as a saprophyte. Thus, killing the host does not cause any costs to the pathogen and may lead to increased virulence of *F. columnare* at fish farms. Such indications have been seen lately. The bacterium is also able to survive for a long time outside the host, allowing it to persist at farms during antibiotic treatments. This increases the fitness of the pathogen further. Survival in water may be an indication of the history of *F. columnare* as a non-pathogenic freshwater bacterium, which also has a saprophytic way of life. *F. columnare* may have used saprophytism as a transition stage from a harmless water bacterium to a pathogen, and has maintained it as a survival and transmission strategy because of the benefits.

Immunostimulants, both injected and given orally, are able to increase the values of innate immune function parameters in rainbow trout juveniles. However, immunostimulations failed to protect the fish from experimental infections by *F. columnare*. One reason for this may be the external nature of columnaris disease. It is thus suggested that, in addition to boosting of innate immune defence, also acquired antibody-mediated immunity is essential in protecting fish from *F. columnare* infections. However, there are indications that virulent strains of *F. columnare* may also invade the inner organs of fish, which could influence the future usefulness of immunstimulants against columnaris disease in Finnish fish farming.

Acknowledgements

This work was carried out using the facilities of the Department of Biological and Environmental Science in the University of Jyväskylä. The study was funded by grants from Academy of Finland [Virulence Evolution project and SUNARE-project (Sustainable Use of Natural Resources)], Finnish Cultural Foundation, Central Finland Regional Fund, Ellen and Artturi Nyyssönen Foundation, Rotary Club of Savonlinna and OLVI Foundation. Travel grants were given by Biological Interactons Graduate School, European Association of Fish Pathologists and AquaTT.

First of all, I would like to express my gratitude to my supervisors Professor E. Tellervo Valtonen, Dr. Ilmari Jokinen and Dr. Lotta-Riina Suomalainen, whose expertise and support made my work possible. Not only your enthusiasm for science, but also your friendship have made the teamwork enjoyable, and given me strength and encouragement during my studies. I have been privileged to have the opportunity to work with you!

I want to thank all my co-authors, and persons who have helped me with the writing process of this thesis. For checking the language I would like to thank Professor Roger Jones. Thanks for quidance in molecular biology techniques are due, along with Lotta-Riina, to Dr. Marja Tiirola. I also appreciate the help of Professor Jouni Taskinen and Dr. Anssi Karvonen with statistical analyses. For providing most of the bacterial strains used in this study I want to thank Dr. Päivi Rintamäki (University of Oulu) and Finnish Food Safety Authority (Oulu regional unit). I am grateful to Mauno Liukkonen and Yrjö Lankinen from Savon Taimen Oy for their co-operation: for providing the fish used in the experiments and keeping me and my co-workers in touch with the field. For technical help in the laboratory I want to thank Marta Martins, Filipa Teixeira, Nina Vehniäinen, Juha Ahonen, Tarmo Halinen, Tuire Pehkonen, Anni-Maria Örmälä and Mikko Jämsen.

It has been a pleasure to belong to the aquatic parasitology research group. Sharing the common interests, and also the refreshing coffee breaks, with you have made working days pleasant.

All my dear friends, I cannot thank you enough for the great times we have had together! You have given me support, understanding and, above all, joy of life.

I warmly thank my relatives, who have encouraged me during these years. Especially I want to thank my dad, mom, and little sister Sini and her husband Aki, whose love and support have been priceless. My deepest gratitude belongs to my dear Ilkka. Thank you for standing by me, loving me, and believing in me!

YHTEENVETO (RÉSUMÉ IN FINNISH)

Kalapatogeeni *Flavobacterium columnare* -bakteerin ominaisuuksia ja patogeenisuuteen vaikuttavia tekijöitä

Flavobacterium columnare -bakteeri on maailmanlaajuisesti esiintyvä makean veden kalapatogeeni. Se esiintyy yleisesti luonnonvesissä, mutta aiheuttaa kalanviljelylaitoksilla iho- ja kidustulehduksina oireilevaa columnaris-tautia. Tauti puhkeaa lähinnä ensimmäisen ja toisen kasvukauden poikasilla lämpimän veden aikaan ja johtaa ilman antibioottihoitoa kuolemaan. Vuosittain infektiot aiheuttavat kalaviljelyelinkeinolle merkittäviä taloudellisia tappioita. Suomen kalanviljelylaitoksilla columnaris-tautia on esiintynyt 1980-luvulta lähtien, mutta tautitapaukset ovat yleistyneet huomattavasti viimeisen 20 vuoden aikana. Nykyään *F. columnare* -bakteerin aiheuttamat infektiot ovat yksi merkittävimpiä lohikalojen poikastuotantoa haittaavia tekijöitä Suomessa. Erääksi pahentuneen tautitilanteen syyksi on esitetty *F. columnare* -bakteerin taudinaiheutuskyvyn kasvua kalanviljelyolosuhteissa.

F. columnare -bakteerin patogeenisuuteen vaikuttavat tekijät sekä columnaris-taudin leviämismekanismit tunnetaan huonosti. Bakteerin kyvyllä tarttua kalan kiduksille sekä kalan sidekudosta hajottavan entsyymin, kondroitiinilyaasin, aktiivisuudella on kuitenkin osoitettu olevan yhteys bakteerin taudinaiheutuskykyyn. Lohikalojen infektioimmuniteettia columnaris-taudissa on tutkittu vähän, mutta kalojen tiedetään pystyvän tuottamaan vasta-aineita *F. columnare* -bakteeria vastaan. Hiljattain on USA:ssa kehitetty columnaris-tautirokote pilkkupiikkimonnille (*Ictalurus punctatus*), mutta sen käyttö ei ole sallittu EU:ssa, eikä sen tehoa lohikaloihin tunneta.

Suomen kalanviljelylaitoksilla tällä hetkellä vallitsevan *F. columnare* -bakteerin aiheuttaman tautitilanteen vuoksi on tarpeen saada lisää tietoa taudinaiheuttajan patogeenisuuteen johtavista syistä. On myös tärkeää kartoittaa antibioottien ohella muiden hoitomenetelmien soveltuvuutta columnaris-taudin parannuskeinoina, sillä runsaasti käytettyinä antibiootit voivat johtaa antibiooteille vastustuskykyisten eli resistenttien bakteerikantojen kehittymiseen niin kalanviljelylaitoksilla kuin niitä ympäröivissä luonnonvesissäkin. Näitä seikkoja pyrittiin selvittämään tässä väitöskirjassa tutkimalla Suomen kalanviljelylaitoksilta eristettyjen *F. columnare* -bakteeri-kantojen ja niiden muodostamien neljän erilaisen pesäketyypin eli kanta-pesäketyyppimuunnosten geneettistä monimuotoisuutta, kasvu- ja tarttumisominaisuuksia sekä näiden mahdollista yhteyttä bakteerin taudinaiheutuskykyyn. Lisäksi tutkittiin *F. columnare* -bakteerin erittymistä ja columnaris-taudin leviämistä kalasta sekä testattiin immunostimulanttien soveltuvuutta kirjolohen (*Oncorhynchus mykiss*) luonnollisen immuniteetin tehostamiseen columnaris-tautia vastaan.

Perustuen bakteerien geneettisessä luokittelussa yleisesti käytettyyn 16S rRNA -geenin sekvenssianalyysiin Suomen *F. columnare* -kantojen todettiin muodostavan geneettisesti yhtenäisen joukon, joka eroaa muulta maailmasta eristetyistä kannoista. Suomen kannat eivät sietäneet yhtä suuria kasvuympä-

ristön suolapitoisuuksia tai pH-vaihteluja kuin tutkimuksessa vertailukantana käytetty *F. columnare* -bakteerin USA:sta eristetty tyyppikanta NCIMB 2248^T. Suomesta eristetyt kannat voitiin kuitenkin jakaa seitsemään eri geneettisen ryhmään käyttäen ARISA-menetelmää, joka perustuu geenejä sisältämättömän genomialueen pituusvaihteluun läheistä sukua toisilleen olevien bakteerikantojen välillä. Eri geneettisiin ryhmiin kuuluvat *F. columnare* -kannat voivat esiintyä samanaikaisesti yhdellä kalanviljelylaitoksella, mikä voi olla syynä infektioiden toistumiseen kesäaikana. Toisistaan geneettisesti eroavien bakteerikantojen yhtäaikainen esiintyminen voi myös aiheuttaa kilpailua kantojen välillä ja johtaa taudinaiheutuskykyisempien kantojen kehittymiseen. Tämä voi olla yksi syy columnaris-infektioiden lisääntymiseen Suomessa parin viime vuosikymmenen aikana.

Tutkittaessa *F. columnare* -bakteerin kanta-pesäketyyppimuunnoksia havaittiin, että kaikkein tarttumiskykyisimmät muunnokset eivät ole taudinaiheutuskykyisimpiä. Myöskään muunnosten korkea kondroitiinilyaasiaktiivisuus ei aina ollut yhteydessä korkeaan taudinaiheutuskykyyn. Taudinaiheutuskyky oli korkea ainoastaan sellaisilla korkean kondroitiinilyaasiaktiivisuuden omaavilla muunnoksilla, jotka kasvoivat juurimaisia ulokkeita muodostavina pesäkkeinä. Tämänkaltaisen pesäkemuodostuksen on osoitettu olevan yhteydessä *F. columnare* -bakteerin liukuvaan liikkumiskykyyn. Erilaisten pesäkkeiden muodostumisen syistä ei ole varmuutta. On kuitenkin todennäköistä, että *F. columnare* säätelee eri pesäketyyppien ilmentämistä vasteena kasvuympäristönsä muutokseen. Tämä voi olla tarpeellista bakteerin säilymisen kannalta kalanviljelyolosuhteissa sekä infektion eri vaiheissa ja suojautumisessa kalan immuunipuolustukselta.

F. columnare -bakteerin havaittiin erittyvän kuolleista kaloista nopeammin kuin elävistä kaloista. Myös columnaris-taudin todettiin tarttuvan tehokkaammin seuraavaan kalaan kuolleista kuin elävistä kaloista. Lisäksi bakteeri säilyi hengissä desinfioidussa järvivedessä jopa kaksi vuotta. Tämä kertoo siitä, että *F. columnare* pystyy elämään kalanviljelylaitoksilla niin raadonsyöjänä eli saprofyyttina kuolleessa kalassa kuin vapaassa vedessäkin, jotka voivat toimia columnaris-taudin tartuntalähteinä. On mahdollista, että saprofytia on *F. columnare* -bakteerin alkuperäinen ominaisuus, joka on toiminut välivaiheena bakteerin kehittyessään harmittomasta vesibakteerista taudinaiheuttajaksi. Koska kalaisännän kuolema ei aiheuta kustannuksia *F. columnare* -bakteerille, vaan pikemminkin edesauttaa sen hengissä pysymistä kalanviljelyolosuhteissa, on elintapa saprofyyttina säilynyt bakteerilla ja voinut edistää sen muuttumista taudinaiheutuskykyisemmäksi.

Immunostimulanttien todettiin tehostavan kirjolohen luonnollista immuunivastetta, mikä määritettiin mittaamalla kalan veren ja munuaisen etuosan valkosolujen muodostamien reaktiivisten happituotteiden sekä plasman bakteerisoluja hajottavien tekijöiden (lysotsyymi-entsyymi ja komplementti) aktiivisuutta. Luonnollisen immuunivasteen tehostaminen ei kuitenkaan suojannut kirjolohta koeolosuhteissa aiheutetuilta *F. columnare* -infektioilta. Eräs syy tähän voi olla se, että columnaris-tauti esiintyy pääasiassa kalan pinnalla.

Tämän vuoksi voidaan olettaa, että luonnollisen immuunipuolustuksen tehostamisen lisäksi myös hankitulla vasta-ainevälitteisellä immuniteetilla on keskeinen rooli kalojen suojaamisessa *F. columnare* -bakteerin aiheuttamilta infektioilta. Viime aikoina bakteeria on kuitenkin saatu eristettyä yhä useammin kalojen sisäelimistä, mikä viittaisi *F. columnare* -bakteerin muuttumiseen myös sisempiin kudoksiin tunkeutuvaksi taudinaiheuttajaksi. Tämä saattaa tulevaisuudessa parantaa immunostimulanttien soveltuvuutta columnaris-taudin torjuntamenetelmänä lohikalojen viljelyssä.

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