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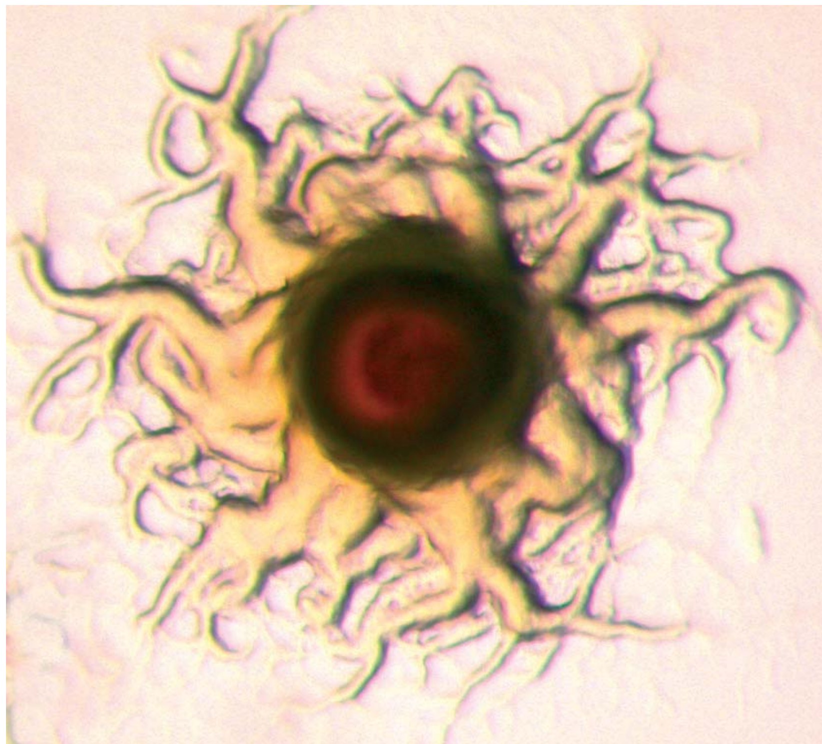
Lotta-Riina Suomalainen

Flavobacterium columnare in Finnish Fish Farming

Characterisation and Putative Disease Management Strategies



JYVÄSKYLÄN YLIOPISTO

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heinäkuun 8. päivänä 2005 kello 12.

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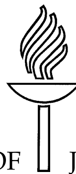

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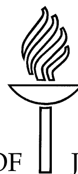

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2005

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Pekka Olsbo, Irene Ylönen
Publishing Unit, University Library of Jyväskylä

Cover picture: *Flavobacterium columnare* colony on an agar plate.
Photo by Lotta- Riina Suomalainen.

ISBN 951-39-2147-6 (nid.)
ISSN 1456-9701

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Jyväskylä University Printing House, Jyväskylä 2005

ABSTRACT

Suomalainen, Lotta-Riina
Flavobacterium columnare in Finnish fish farming: characterisation and putative disease management strategies
Jyväskylä: University of Jyväskylä, 2005, 52 p.
(Jyväskylä Studies in Biological and Environmental Science,
ISSN 1456-9701; 155)
ISBN 951-39-2185-9

The number of *Flavobacterium columnare* infections at Finnish fish farms increased significantly during the 1990s. The pathogen was studied to increase information about the characteristics of this economically important disease and to help develop more effective management strategies. The diversity of 31 *F. columnare* isolates was studied using molecular methods including 16S rDNA RFLP, RISA, AFLP and sequencing. Seven genetically different groups were found among Finnish isolates using RISA, and *F. columnare* type strain NCIMB 2248^T represented an eighth genotype. These eight genotypes were further characterised according to their growth features, virulence and virulence-related factors. Strains were highly sensitive to even low (0.1%) NaCl concentrations and pH values under 5, being unable to multiply under such conditions. Differences in virulence were also detected. High virulence was associated with the adhesion capacity, and the production of the connective tissue degrading enzyme chondroitin lyase C was significantly higher at 25 °C than at 20 °C.

PCR was a more sensitive method than agar plate cultivation for detecting *F. columnare* in both healthy and infected fish. Fish which had a previous contact with *F. columnare* were shown to remain as carriers of the disease. Such carrier fish do not, however, form a risk to the fish farming industry, since columnaris disease was not re-induced by concomitant parasitic infection or rearing-related stress. The effects of rearing density and water temperature were also studied. High water temperature was shown to be the key factor affecting the severity of columnaris outbreaks. Transmission of columnaris disease was slower at reduced rearing density, but significant changes in overall mortality between high and low density were not detected.

Putative disease management strategies were studied experimentally using rainbow trout (*Oncorhynchus mykiss*) fingerlings. Manipulation of the water quality to prevent infection was performed by bathing infected fish with 2% and 4% salt or changing the water pH to 4.5. The microbial community of fish skin and gills was manipulated by bathing fish with *Pseudomonas* sp. MT5, found to be antagonistic to flavobacteria in agar assays. None of these methods was effective against columnaris disease, although 4% salt baths decreased mortality rate of fish at the beginning of the infection.

Key words: Bath treatment; disease management; genetic diversity; fish farming; *Flavobacterium columnare*; PCR; rearing density; RISA; stress; temperature; virulence.

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I Suomalainen, L.-R., Kunttu, H., Valtonen, E.T., Hirvelä-Koski, V. & Tiirola, M.: Molecular diversity and growth features of *Flavobacterium columnare* strains isolated in Finland. Submitted manuscript.

II Suomalainen, L.-R., Tiirola, M.A. & Valtonen, E.T.: Virulence of Finnish *Flavobacterium columnare* strains. Submitted manuscript.

III Suomalainen, L.-R., Tiirola, M.A. & Valtonen, E.T. 2005: Influence of rearing conditions on *Flavobacterium columnare* infection of rainbow trout. Journal of Fish Diseases, 28: 271-277.

IV Suomalainen, L.-R., Tiirola, M.A. & Valtonen, E.T. 2005: The effect of *Pseudomonas* sp. MT5 baths on *Flavobacterium columnare* infection and on microbial diversity on fish skin and gills. Diseases of Aquatic Organisms 63: 61-68.

V Suomalainen, L.-R., Tiirola, M.A. & Valtonen, E.T.: Treatment of columnaris disease of rainbow trout - Low pH and salt as possible tools? Diseases of Aquatic Organisms, in press.

RESPONSIBILITIES OF LOTTA-RIINA SUOMALAINEN IN THE ARTICLES OF THIS THESIS

I — The idea to study genetic diversity of Finnish *Flavobacterium columnare* was mine and the study was planned together with Dr. Marja Tiirola and Prof. E. Tellervo Valtonen. I supervised the molecular laboratory work done by MSc student Heidi Kunttu. The paper was written jointly with Dr. Tiirola.

II — I planned the study and performed the experiment and also the laboratory work. I was mainly responsible for writing the article.

III — The studies included in this article were planned together with the co-authors. I performed both of the experiments and wrote the article.

IV — The studies included in this article were planned together with the co-authors. I performed the experiments and did the molecular analyses. Dr. Tiirola contributed to the perspective of the article and also participated in laboratory work and writing the paper. The main responsibility for writing was mine.

V — The study was planned with the co-authors. I performed the *in vivo* experiments and participated in the *in vitro* experiments. I wrote the article.

ABBREVIATIONS

AFLP	amplified fragment length polymorphism
bp	base pair
CFU	colony forming unit
DAPI	4'-6-diamidino-2- phenylindole
DGGE	denaturating gradient gel electrophoresis
EMBL	European Molecular Biology Laboratory
ISR	intergenic spacer region (analogous to ITS)
ITS	internal transcribed spacer (analogous to ISR)
LH-PCR	length heterogeneity analysis of PCR amplified fragments
NCIMB	National Collection of Industrial, Marine and Food Bacteria
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RISA	ribosomal intergenic spacer analysis
rRNA	ribosomal RNA
T-RFLP	terminal restriction fragment length polymorphism
TSB	tryptic soy broth

1 INTRODUCTION

1.1 Diseases in fish farming in Finland

In 2002 there were 585 fish farms in Finland, 103 of which produce fingerling fish for stocking or for further rearing. There are three types of fingerling production. Electric power companies are obliged to produce and stock salmon (*Salmo salar* L.*)* and trout (*Salmo trutta* L.) to replace the loss in the natural populations due to harnessed rivers. Government fish farms maintain brood fish and produce salmon and trout for stocking. They also maintain endangered fish species (arctic charr *Salvelinus alpinus* L., landlocked salmon *Salmo salar* m. *sebago* G., landlocked trout *Salmo trutta* m. *lacustris* L., European grayling *Thymallus thymallus* L.). Commercial fish farms produce rainbow trout (*Oncorhynchus mykiss* W.) for stocking and for further rearing in food production, but also salmon and trout for stocking purposes, as well as pikeperch (*Sander lucioperca* L.) and whitefish (*Coregonus lavaretus* L.) fingerlings.

Fish rearing in farms requires the use of high densities. A high density of susceptible hosts attracts both parasites and bacterial diseases (Rintamäki-Kinnunen & Valtonen 1996, Rintamäki-Kinnunen & Valtonen 1997, Hakalahti & Valtonen 2003) by enhanced transmission opportunities for disease agents compared to the low densities in nature. Protozoan ectoparasites, such as *Chilodonella* spp., *Ichthyobodo necator* (Henneguy) and *Ichthyophthirius multifiliis* (Fouguet), occur commonly in the summertime, and bath treatments are used to treat these infections (Rintamäki-Kinnunen & Valtonen 1997). Metazoan parasites occur mainly in earth ponds. For instance, ectoparasitic fish lice of the genus *Argulus* feed on fish skin and cause problems in juvenile and brood fish (Hakalahti & Valtonen 2003). The penetrating endoparasite *Diplostomum spathaceum* (Rudolphi) causes occasional outbreaks at fish farms where the intermediate snail host population is sometimes large (Stables & Chappell 1986, Field & Irwin 1994). However, the greatest economic losses in fish production are caused by bacterial diseases.

At present, the flavobacterial pathogens *Flavobacterium columnare* (Koski et al. 1993) and *F. psychrophilum* (Wiklund et al. 1994) are the most important bacterial disease causing organisms in Finnish fish. Other important bacterial pathogens in Finland are *Aeromonas salmonicida* subsp. *salmonicida* causing furunculosis, atypical *Aeromonas salmonicida* subsp. *achromogenes* (aAS), *Listonella anguillarum* (vibriosis), *Yersinia ruckeri* (enteric red mouth disease, ERM), *Renibacterium salmoninarum* (bacterial kidney disease BKD) and *Pseudomonas anguilliseptica* (Rahkonen & Westman 1990, Wiklund & Bylund 1990, Rintamäki & Valtonen 1991, Valtonen et al. 1992). The impact of these diseases on the economy of fish farming is lower because control methods are well established in the country.

Some abiotic and rearing-related factors present at fish farms may predispose fish to diseases. High water temperature during summer can make the multiplication of microbes faster as it also stresses the fish. High organic matter in the water (e.g. excess feed, faeces) can serve as growth media for fish pathogenic bacteria (Decostere et al. 1999b). Handling and transferring of fish creates stress, but also might break the mucus layer of the skin which acts as a first line barrier against diseases, making fish more susceptible to bacterial invasion.

Both pathogenic bacteria and parasites may co-occur at fish farms during summer (Rintamäki-Kinnunen 1997, Rintamäki-Kinnunen et al. 1997). Parasite-enhanced bacterial invasion in fish has been reported in fish farming conditions (Cusack & Cone 1986, Rintamäki-Kinnunen & Valtonen 1997, Ravichandran et al. 2001) but to my knowledge only a few papers based on experimental evidence have been published (Busch et al. 2003, Pylkkö et al. in press). Parasitic infections can subject fish to microbial infections in two ways. Ectoparasites may enhance the invasion of microbes by disrupting skin mucus and subjecting fish to microbes. On the other hand, penetrating endoparasites (e.g. *Diplostomum spathaceum*) or blood sucking parasites (e.g. *Argulus* sp.) may act as vectors carrying pathogens directly into the tissues of the host (Cusack & Cone 1986, Ahne 1985, Pylkkö et al. in press). Skin damage has been shown to enhance the invasion of flavobacterial pathogens when the mucus layer was removed or damaged before exposure to bacteria (Madetoja et al. 2000, Bader et al. 2003a).

At fish farms host mortality is kept low by vaccination (furunculosis and vibriosis), antibiotic treatments and bath treatments. However, bath treatments can themselves subject fish to secondary infections and bacterial invasion. Formalin bathing, for example, is routinely used for eradication of the protozoan parasites *Ichthyobodo necator* and *Icththyphthirius multifiliis*, but it also facilitates the invasion of flavobacterial pathogens (Madsen & Dalsgaard 1999). Considering all these factors, a fish farm is a favourable environment for disease-causing organisms. Diseases are transmitted easily and the fish population always offers susceptible individuals for pathogens.

1.2 Flavobacterium columnare

1.2.1 Occurrence at Finnish fish farms

At present, flavobacterial diseases are the leading cause of fish mortality in Finnish fish farming. In 2002, the National Veterinary and Food Research Institute diagnosed 30 fish farms positive for flavobacterial diseases: 10 farms for *F. psychrophilum* (causing cold water disease and rainbow trout fry syndrome) and 20 farms for *F. columnare* (columnaris disease) (Statistics of National Veterinary and Food Research Institute in Kalaterveyspäivä 2003). The number of *F. columnare* -infected farms was double that in 2001. However, these statistics do not cover all fish farms, so the actual number of infected farms may be considerably higher. Furthermore, the diagnosis of flavobacterial diseases by the traditional agar plate cultivation method is unsure and can lead to false negative results (Tiirola et al. 2002).

Columnaris outbreaks at farms occur in sequence throughout the warmest summer months, when the water temperature rises above 20°C. The frequency of columnaris disease outbreaks can be seen in data collected in 2001-2004 from a fish farm in northern Finland where salmon and trout yearlings are produced (Table 1). Columnaris disease occurred in both age groups of salmon (summerlings and yearlings), and during the warm summers 2002 and 2003 columnaris disease was re-established in a majority of tanks. Fish gained no immunity against the disease: 98.2% of the tanks holding fish in age group 0 (summerlings) were repeatedly treated for columnaris diseases in summer 2002, and the same group was severely infested (100 %) again during the summer 2003.

TABLE 1 The occurrence of columnaris disease in salmon (*Salmo salar*) fingerlings represented as the proportion of tanks treated with antibiotics and proportial number of antibiotic treatments per tank (number of treatments/number of treated tanks). The data were collected from a fish farm in northern Finland.

Year	Age group	% of tanks treated	Proportial number of treatments per tank
2001	0	59	1
	1	91	1
2002	0	98	1.6
	1	86	1.7
2003	0	93	1.1
	1	100	1.5
2004	0	3	1
	1	43	1

1.2.2 Epizootiology and clinical signs of columnaris disease

Columnaris disease probably occurs in all reared fish species, salmonids and non-salmonids, with a wide geographical distribution. Columnaris disease is one of the most important diseases affecting salmonid yearling production in Finland (Statistics of National Veterinary and Food Research Institute in Kalaterveyspäivä 2003) and catfish production in the USA (Durborow et al. 1998). Ornamental fish kept in aquaria are also susceptible to the disease (Decostere et al. 1998, Decostere & Haesebrouck 1999, Michel et al. 2002, Tripathi et al. 2005). High water temperature, crowding, stress and poor water quality have all been shown to induce columnaris outbreaks (Fish & Rucker 1943, Ordal & Rucker 1944, Johnson & Brice 1952, Wakabayashi & Egusa 1972, Holt et al. 1975, Becker & Fujihara 1978, Wakabayashi 1991, Decostere et al. 1999b).

It has been suggested that fish having recovered from *F. columnare* infection may remain as carriers of the disease, shedding the pathogen into the environment and serving as a reservoir for the disease in a fish population (Pacha & Ordal 1970, Becker & Fujihara 1978, Bernardet 1997). However, the onset of the disease may require stressful conditions or heavy concomitant parasitic infection, because healthy fish are generally not susceptible to columnaris disease in normal conditions (Vogel 1958, Durborow et al. 1998). On the other hand, fish infected previously with *F. columnare* have been shown to develop immunity (Fujihara & Nakatani 1971, Grabowski et al. 2004) which, however, is not seen at Finnish fish farms (Table 1). Whether or not stressful conditions or parasitic infection can actually induce columnaris disease in carrier fish is still uncertain, but the effect of stress in exposing fish to other microbial pathogens is well documented (e.g. Sniezko 1974, Anderson 1990, Salonius & Iwama 1993, Hietala et al. 1995, Iguchi et al. 2003).

Columnaris disease is usually restricted to the external surfaces of fish (Bernardet 1997, Tripathi et al. 2005). Although in some cases *F. columnare* can also be isolated from inner organs, skin and gills are the most reliable tissues for isolation (Bader et al. 2003a, Welker et al. 2005). The reasons for this are unclear. The most typical symptom is the "saddleback" lesion: erosion and necrosis of the skin tissue around the dorsal fin, which sometimes even reach the spine. Extensive necrosis of the gill tissue is also common. Erosion of jaw and tail can be seen, but in some acute cases fish may die before any of the symptoms are observed. The progression of the disease in fish is fast; fish will usually die within a few hours of the first visible symptoms. Extensive damage of the skin causes severe loss of osmotic regulation and electrolyte homeostasis, which eventually causes death to the fish (Tripathi et al. 2005).

1.2.3 Taxonomy and biochemical characteristics

The causative agent of columnaris disease was first described in 1922, when the disease had caused outbreaks in fish reared in a US Biological Station by the Mississippi River, Fairport, Iowa (Davis 1922). The pathogen itself was not isolated, but it was seen in wet mounts and was named as *Bacillus columnaris* based on the rod shape of the bacterial cells and the column-like accumulation of cells in infected tissue. The bacterium was isolated in 1944 (Ordal & Rucker 1944). Since then the taxonomy of *F. columnare* has been confusing. Ordal and Rucker (1944) suggested that the pathogen produced microcysts and fruiting bodies and classified it as *Chondrococcus columnaris*. Garnjobst (1945) found no evidence of microcyst formation and transferred it to the genus *Cytophaga* under the name *Cytophaga columnaris*. In 1974 the genus was changed to *Flexibacter* and the name "*Flexibacter columnaris*" was given (Leadbetter 1974). Finally, in 1996, Bernardet et al. (1996) defined the bacterium as *Flavobacterium columnare* based on molecular phylogenetic analyses of the isolates.

Flavobacterium columnare forms yellow rhizoid colonies that adhere tightly to an agar surface (Fig. 1). Yellow coloured bacterial colonies are a result of production of flexirubin type pigment. Cells are Gram-negative, long slender rods (0.5 µm x 5-10 µm). Characteristic gliding motility of the cells can be detected from fresh cultures using the hanging drop method (Frerichs 1984). According to Bernardet (1989) and Bernardet & Grimont (1989) *F. columnare* grows in media supplemented with 0.5% NaCl but not with 1% NaCl. Differential biochemical characteristics of the two flavobacterial pathogens occurring in Finland, *F. columnare* and *F. psychrophilum*, are presented in Table 2.

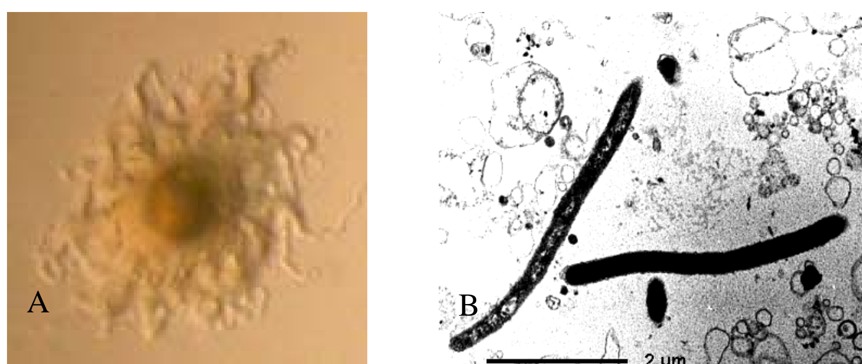

FIGURE 1 Typical *Flavobacterium columnare* colony shape on an agar plate (A) and cell morphology by transmission electron microscopy (B) (picture taken by H. Reunanen).

TABLE 2 Some characteristics of flavobacterial fish pathogens *Flavobacterium columnare* and *F. psychrophilum* (according to Bernardet & Grimont 1989, Bernardet et al. 1996, Madetoja 2002)

	F. columnare	*F. psychrophilum*
Morphology of colonies	Rhizoid, strongly adherent to agar, convex	Round with entire margins, flat
Gram staining	-	-
Gliding motility	+	+ (weak)
Flexirubin type pigments	+	+
Congo red absorption	+	-
Production of catalase	+	+
Growth in TSB	-	-
Growth at 15 °C	+	+
Growth at 25 °C	+	-
Growth at 37 °C	+/-	-
Growth with 0.5 % NaCl	+	+
Growth with 1.0 % NaCl	-	-

In the primary culture of *F. columnare* from an infected fish, rhizoid and tightly adherent colonies are usually isolated. However, during several subcultivations in the laboratory the characteristics of the colonies may change. Less rhizoid and atypical colonies are formed, eventually leading to a complete loss of typical colony shape. This had been noticed by Bernardet (1989) in *F. columnare* type strain NCIMB 2248^T (ATCC 23463) which was first isolated in 1972 and stored in the culture collection. The loss of adherence may severely influence the virulence of *F. columnare*, because adherence to fish tissues has been show to correlate with virulence in some *F. columnare* strains (Decostere et al. 1999a).

In older liquid cultures of *F. columnare*, atypical cell forms can be detected. *F. columnare* cells turn into round spheroplasts, which can be seen by microscopy (Bernardet & Grimont 1989). The significance of spheroplasts to the ecology of the bacterium is unknown. These cell forms might be connected to the survival strategy of the species (Garnjobst 1945) or they may be typical forms of degrading cells.

1.2.4 Diagnosis of columnaris disease

Basically, four kinds of methods can be used to detect *F. columnare* in infected fish: (a) isolation of the causative agent (agar plate cultivation) and biochemical characterization of the isolates, (b) direct microscopic techniques, (c) specific antibodies and (d) DNA-based techniques. The practical diagnosis of fish diseases relies strongly on traditional cultivation-based methods and biochemical characterization of bacterial colonies. Cultivation, however, is an uncertain and time-consuming method to detect flavobacterial pathogens, especially *F. columnare*, in fish (Tiirola et al. 2002). Other methods can be used to detect bacterial cells directly from infected tissue but also to characterize the

bacterial isolates. These methods are faster and give more reliable results, but so far they have not been validated as an official method in disease diagnosis in Finland. Therefore they are usually applied only for research purposes.

F. columnare does not grow on tryptic soy agar (TSA) used generally in fish disease diagnostics. Therefore nutrient-poor media such as AO-agar (Anacker & Ordal 1955) and Shieh medium (Shieh 1980) are typically used to isolate because this pathogen. Several days after primary culture, yellow pigmented colonies start to appear and rhizoid edges of the colonies can usually be seen. The identification of yellow pigmented colonies is slow due to the slow growth rate of the bacterium. Pure cultures have to be subcultivated from primary isolates and it takes several days before new colonies can be seen. Biochemical testing of the isolates is also time-consuming.

Columnaris disease is an epidermal disease (Bernardet 1997, Tripathi et al. 2005) and when the cultivation is made from surfaces of fish, contamination can rarely be avoided. Therefore competitive and opportunistic bacteria growing on the agar plate can inhibit the growth of *F. columnare* (Wakabayashi 1991, Tiirola et al. 2002). This problem can be solved by using dilution cultivation, whereby the amount of opportunistic bacteria is reduced in each dilution so that in the final dilution only the dominant species remains (Tiirola et al. 2002). The probability of pure culture can also be increased by using an antibiotic supplement such as tobramycin in the growth medium (Decostere et al. 1997). However, it is not known if all *F. columnare* strains can grow with tobramycin.

Direct microscopic techniques include direct observation of flavobacterial cells in infected tissue, which is a practical method for routine disease monitoring performed regularly at fish farms. Using this method, *F. columnare* can not be discriminated from other long, rod-shaped bacteria, but it can be used in preliminary diagnosis. During acute infection, high numbers of bacterial cells are present in the infected tissue. Wet mounts of infected skin or gills show haystack–like masses of bacterial cells, bacterial columns typical of *F. columnare*. Tissue scrapings can also be fixed on microscopic slides and Gram-stained when the bacterial cells become more visible.

An immunofluorescense method for diagnosing *F. columnare* has been developed by Lorenzen and Karas (1992). The method is relatively easy and fast, and it can detect minor cell counts directly from an infected tissue. Combined with immunohistochemistry, information on the location of the pathogen cells in the tissue can be obtained. If a universal stain for all bacterial cells (for example DAPI) is included in addition to specific staining, total bacterial counts can be determined as well as the proportion of the pathogen in the bacterial community. This method can be applied, for example, for detecting pathogens in water samples (Madetoja et al. 2000). Even though this information can be useful, the calculation of bacterial cells by this method is time-consuming and may be appropriate only for research purposes.

DNA-based methods include specific PCR (Toyama et al. 1996, Bader & Shotts 1998, Bader et al. 2003b, Welker et al. 2005), broad range PCR (Tiirola et al. 2002) and *in situ* hybridization (Tripathi et al. 2005). Species-specific PCR is

the most suitable method for routine screening of multiple samples because it is easy and results can be obtained quickly. Reliability and sensitivity of specific PCR (compared to cultivation) is high, even 30 CFU in one mg of tissue can be detected (Welker et al. 2005). However, PCR may suffer from contamination and false positive results if it is not performed carefully. In broad range PCR universal bacterial primers are used, and the PCR products can be applied for bacterial community analysis (such as DGGE, T-RFLP or LH-PCR) or sequencing. These methods provide additional information on the actual microbial diversity in the sample and the proportion of pathogen in it. However, this is also time consuming and requires specialized equipment such as automated sequencers. Therefore it is more suitable for research purposes. *In situ* hybridization, like immunofluorescense, allows the specific localisation of *F. columnare* cells. It is quite laborious for routine use, but, if it is combined with histopathology (Tripathi et al. 2005), it can provide useful information on infections in general.

1.2.5 Treatment and prevention of columnaris disease at fish farms

Columnaris disease is most effectively treated with antibiotic drugs. In Finland, mainly tetracyclines are used. However, the use of antibiotics is problematic for two reasons. First, development of antibiotic-resistant strains due to repetitive antibiotic treatments is a real risk. This has already been seen in Sweden and Denmark, where tetracycline resistant strains have emerged in *F. psychrophilum* (Schmidt et al. 2000, Ekman 2003). Second, residues of antibiotics can diffuse into the environment downstream from fish farms and resistance can develop in environmental bacteria (e.g. Schmidt et al. 2000). Bathing fish with disinfecting chemicals is an option, because columnaris infection is initially restricted to epidermal surfaces. Bath treatments kill bacteria on fish surfaces and in the water. Chloramine-T, potassium permanganate, benzalkonium chloride, hydrogen peroxide, copper sulphate and Diquat® (herbicide) have all been tested and found to be somewhat effective against columnaris disease under experimental conditions (Jee & Plumb 1981, Wakabayashi 1991, Speare & Arsenault 1997, Thomas-Jinu & Goodwin 2004a). These substances, however, can be harmful for the fish and the fish farm personnel. Therefore, because of the growing risk of antibiotic resistance and the use of harmful chemicals, alternative more ecological and economical methods are needed for prevention and treatment of columnaris disease.

Manipulation of the composition of the bacterial community of the fish could be used in prevention of columnaris disease. Wakabayashi (1991) reported that the presence of competitive bacteria such as *Aeromonas hydrophila* and *Citrobacter freundii* reduced the ability of *F. columnare* to invade fish. These health promoting microbes, which hamper the invasion of pathogens or stimulate the fish immune system, are defined as probiotic bacteria. Probiotic bacteria (usually viable, but in some cases also dead) can be administrated to fish by bathing or as a feed supplement. The mechanism by which these bacteria hamper the growth of pathogenic bacteria is usually antagonism, and

more specifically competition for iron or production of inhibitory substances. Probiotic bacteria have already been tested but not used in practise in prevention and treatment of fish diseases caused by *Saprolegnia* sp., *Aeromonas salmonicida*, *Yersinia ruckeri* and *Vibrio* species (Smith & Davey 1993, Bly et al. 1997, Gram et al. 1999, Robertson et al. 2000, Nikoskelainen et al. 2001, Spanggaard et al. 2001, Lategan & Gibson 2003, Lategan et al. 2004, Hjelm et al. 2004) and other species pathogenic to fish (reviewed by Gatesoupe 1999, Verschuere et al. 2000, Irianto & Austin 2002). These studies encourage research on more ecological and natural ways to prevent fish diseases. Recently our research group isolated an antagonistic *Pseudomonas* strain that effectively prevented the growth of *Flavobacterium psychrophilum* and *F. columnare* but also other fish pathogenic bacteria in agar assays (Tiirola et al. 2002).

1.3 Genetic characterization of bacteria

Genetic typing of bacterial populations can be based on phylogenetic chronometers (specific biological molecules that are homologous in all bacterial species) or analysis of the whole genome. Ribosomes, for example, are homologous molecules and ribosomal subunit-coding genes (16S, 23S and 5S rDNA, where S refers to sedimentation rate in an ultracentrifuge) are widely used in phylogenetic studies. In procaryotic genomes, these ribosomal genes are arranged as operons, where 16S, 23S and 5S rRNA genes are lined up (Fig. 2) allowing efficient transcription. Some bacterial strains can carry multiple copies of the operon in their genomes ensuring fast transcription to ribosomes (Gurtler & Stanisich 1996). The 16S rRNA gene is a commonly used chronometer because it contains both conserved and variable regions and is also a moderately well conserved molecule despite long phylogenetic distances (Brock et al. 1994). Therefore the 16S rRNA gene is also widely used in microbial community analyses. At the beginning of February 2005, 193745 16S rRNA gene sequences were found in the EMBL database with a search term "DE: 16S ! spacer" , where DE is an abbreviation for "description" and search terms were separated by "!" defined as "but not" (http://srs.ebi.ac.uk/).

Between ribosomal 16S and 23S genes lies the intergenic spacer region (ISR, synonymous with internal transcribed sequence ITS). This region consists mainly of noncoding DNA and thus it is subjected to low evolutionary pressure. In strains with multiple copies of rRNA operons, considerable length heterogeneity between ISR sequences has been detected (Gurtler & Stanisich 1996) which makes this chronometer suitable for typing different strains within one species. Ribosomal intergenic spacer analysis (RISA) is a common name for methods studying differences in ISR regions. Differences can be detected by sequencing, fragment analysis or hybridization.

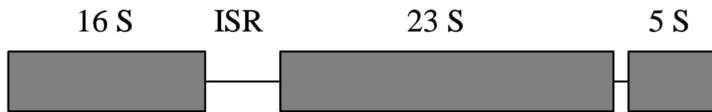

FIGURE 2 Organisation of ribosomal genes in prokaryotes.

Genetic typing also includes methods that fingerprint the whole genome of the strain. Suchs methods are, for example, genomic RFLP and hybridization analysis, randomly amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP). These methods are more sensitive than 16S sequence analysis, because the heterogeneity of the whole genome is screened, covering both conserved and variable DNA.

Genetic characterization of *Flavobacterium columnare* isolates provides information on the genetic diversity of the bacterium and also on the epidemiology of columnaris disease in outbreaks. 16S rDNA restriction fragment analysis (RFLP) has been used to distinguish different *F. columnare* genomovars in several studies and has shown the division of *F. columnare* into three genomovars (Toyama et al. 1996, Triyanto et al. 1999, Triyanto & Wakabayashi 1999, Michel et al. 2002, Arias et al. 2004). Also analysis of non-coding DNA sequences such as ISR (Arias et al. 2004, Figueiredo et al. 2005), and methods fingerprinting the whole genome such as RAPD and AFLP (Arias et al. 2004, Thomas-Jinu & Goodwin 2004b) have been applied to *F. columnare*. RAPD analysis was able to segregate *F. columnare* into groups according to the fish species of origin as well as according to length of the bacterial cells (Thomas-Jinu & Goodwin 2004). Arias et al. (2004) found several ISR sequences within one genomovar, indicating subdivision within previously described genomic groups. None of these previous studies, however, have covered Finnish *F. columnare* isolates, and the only European strains studied so far have been from France. Therefore the epidemiological nature of *F. columnare* infections and the genetic diversity of strains in Finland are not known.

2 OBJECTIVES

Flavobacterium columnare is currently the largest threat in salmonid fish production in Finland. The objective of this study was to answer basic questions regarding the pathogen to understand why columnaris disease has become such a problem at Finnish farms. In this thesis I also aimed to find more natural and less harmful ways to prevent and treat columnaris disease, because the treatment of the disease mainly relies on antibiotics and the use of harmful chemicals.

Specific aims of study:

1) In previous studies regarding genetic features, virulence or other characteristic of *F. columnare*, Finnish isolates have been ignored. However this information is critical to understand the epidemiological nature of columnaris diseases at fish farms. I aimed to characterise Finnish *F. columnare* isolates by means of genetic diversity, growth requirements and virulence.

2) The roles of factors present at fish farms which possibly induce the occurrence of columnaris disease are poorly known. Especially the effect of stress, concomitant parasitic infections and rearing density needs to be understood. In this thesis I describe some factors related to fish rearing that contribute to columnaris outbreaks.

3) Currently, the diagnosis of columnaris disease is based on agar plate cultivation, which is a slow and unreliable method to detect *F. columnare* in fish because of the slow growth of the pathogen and contamination from fish surfaces during the cultivation process. I applied PCR-based bacterial community analysis to compare the reliability of PCR and cultivation in disease diagnostics and to describe the role of *F. columnare* in the bacterial community of both infected and clinically healthy fish.

4) Because of the risk of development of antibiotic resistance in *F. columnare*, putative ecological disease management strategies for prevention and treatment of columnaris disease at fish farms are needed. Bathing of fish with chemicals can be used, but often these chemicals are harmful to the fish or the fish farming personnel. I aimed to find more ecological disease management strategies using the results obtained from the growth characteristic studies.

3 MATERIALS AND METHODS

3.1 *Flavobacterium columnare* isolates

A total of 30 *Flavobacterium columnare* isolates (Table 3) obtained from disease outbreaks in Northern and Central Finland during 1993-2003 and the type strain NCIMB 2248^T were subjected to *in vitro* and *in vivo* analyses regarding genetic diversity, growth characteristics, virulence (mortality in fish associated to bacterial strain) and virulence related factors (adhesion and degrading enzyme production), tolerance to different salt concentrations and pH and antimicrobial susceptibility.

TABLE 3 *Flavobacterium columnare* strains used in genotypic analysis. The strains used in *in vitro* and *in vivo* experiments are marked with a reference to the original publication.

Isolate/ Strain code	Fish farm	Fish species	Water course	Genotype (RISA)
NCIMB 2248T	Washington, USA	chinook salmon		F [I, II, V]
3294/95	1[1)]	trout	River Oulujoki	A [I, II, V]
10819/96	1[1)]	arctic charr	River Oulujoki	A
8128/97	1[1)]	arctic charr	River Oulujoki	B [I, II, V]
3147/98	1[1)]	trout	River Oulujoki	A
1277/99	1[1)]	trout	River Oulujoki	A
1179/01	1[1)]	brown trout	River Oulujoki	A
2390/02	1[1)]	salmon	River Oulujoki	A
8239/97	2[1)]	rainbow trout	River Iijoki	C [I, II, V]
9528/97	3[1)]	rainbow trout	River Torniojoki	C
1991/94	4[1)]	rainbow trout	River Oulujoki	A
1468/99	5[1)]	brook trout	River Oulujoki	A
1199/00	6[1)]	rainbow trout	River Kymijoki	A
1397/00	7[1)]	rainbow trout	River Oulujoki	D [I, II, V]
1820/02	8[1)]	rainbow trout	River Vienan Kemijoki	A
2287/02	9[1)]	trout	River Lestijoki	A
2559/93	10[1)]	salmon	River Simojoki	A
Ke/02	11[2)]	salmon	River Kemijoki	E [III, IV]
Ke/03	11[2)]	salmon	River Kemijoki	C
FK2/03	11[2)]	salmon	River Kemijoki	E

continues…

Mo/02	12[2)]	salmon	River Oulujoki	A
Mo/03	12[2)]	trout	River Oulujoki	E
Os/03	13[2)]	salmon	River Kemijoki	G[I, II, V]
Ra/03	14[2)]	salmon	River Iijoki	E[I, II, V]
Htan4/03	15[3)]	rainbow trout	River Kymijoki	A
Htan5/03	15[3)]	rainbow trout	River Kymijoki	H[I, II, III, V]
Htan6/03	15[3)]	rainbow trout	River Kymijoki	H
Htku1/03	15[3)]	pikeperch	River Kymijoki	H
Htku2/03	15[3)]	pikeperch	River Kymijoki	E
Lauh/03	16[3)]	grayling	River Kymijoki	A
BA1972/03	17[1)]	rainbow trout	River Oulujoki	A

[1]) Isolated by National Veterinary and Food Research Institute, Oulu
[2]) Isolated by Dr. P. Rintamäki-Kinnunen, University of Oulu
[3]) Isolated by L.-R. Suomalainen, University of Jyväskylä.

3.2 In vitro experiments

3.2.1 Genetic analysis of *Flavobacterium columnare* isolates (I, II)

The genetic diversity of the 30 Finnish *F. columnare* isolates obtained from disease outbreaks during 1993-2003 was studied using molecular methods (Paper I). The *F. columnare* type strain NCIMB 2248^T was also included in all studies as a reference strain. Genomovar classification, based on restriction analysis (RFLP) of the 16S rRNA gene according Triyanto & Wakabayashi (1999), was performed using restriction enzymes *Msp*I and *Hae*III. More detailed intraspecies analysis was targeted on the intergenic spacer region (ISR). Length and copy number of ISR was determined for each isolate in RISA. Phylogenetic analysis was based on sequencing of nearly full length 16S rDNA and AFLP analysis of the genome (performed according to Vos et al. 1995 with some modifications). Finally, the occurrence of plasmids was studied using extraction methods described by Kado & Liu (1981), Ginsted & Bennet (1988) and Gerhardt et al. (1994), and also by pulsed field gel electrophoresis (II).

3.2.2 Characteristics of *Flavobacterium columnare* in vitro (I, II, V)

The growth rate of *F. columnare* at different pH values (6.2 - 9.2), temperatures (16 – 24 °C) and NaCl concentrations (0.0 - 1.0%) was studied using Shieh medium modified by adjusting pH or NaCl addition (I). Fresh cultures were diluted (1:20) in modified Shieh medium on a microtiter plate and the optical density was measured during incubation.

The ability of low pH or high salinity treatments to eradicate *F. columnare* cells *in vitro* was studied (V). Fresh *F. columnare* culture was subjected to both 15 minutes and 1 hour treatments in 4% NaCl, pH 4.6, pH 4.8 or pH 5.0. After the exposure the number of viable cells was estimated using the plate count method.

(1987) (II). Rainbow trout mucus obtained from a freshly killed fish was fixed on the microscopic slides. The slides were incubated in fresh *F. columnare* culture and adhesion was estimated using direct cell microscope counts from Gram-stained slides. Another virulence-related feature, production of chondroitin AC lyase (a chondroitin sulphate degrading enzyme) was studied using the method described by Teska (1993). The ability of *F. columnare* to hydrolyse chondroitin sulphate C at two temperatures (20 °C and 25 °C) was measured spectrophotometrically.

3.3 Experimental infections in rainbow trout (II, III, IV, V)

3.3.1 Fish and facilities

Rainbow trout fingerlings (mean weight 1.5 - 5 g) from a commercial fish farm were used in all studies. In papers III and IV the fish had previously been infected with *F. columnare*. In papers II and V naïve fish with no previous contact with *F. columnare* were used. During the experiments fish were kept in glass or plastic aquaria with a continuous flow of water. Experiments were performed at the University of Jyväskylä (III, IV and V), at Konnevesi Research Station (II, V) and at a commercial fish farm in Central Finland (III, IV).

The most essential differences in the experimental set-ups were related to water source and temperature. In experiments III, IV and V, aerated borehole water was used. Water temperature was adjusted to 19.5 °C (III and IV). In study V two temperatures were used: 25 °C in the challenge and treatment experiment and 20 °C when studying buffering capacity of skin mucus. Studies III and IV were performed at the fish farm using river water. In study III the water temperature was adjusted with electric thermostats to 18 °C and 23 °C. Studies II and V used water from lake Konnevesi and temperature was adjusted to 25 °C with electric thermostats.

All bacterial challenges were done by bathing. *F. columnare* bacteria grown for 20 - 48 h in AOB (Anacker & Ordal 1955, III and IV) or Shieh medium (Shieh 1980, II and V). Fish were challenged in separate challenge aquaria or in the experimental tank for 20 - 30 minutes. In papers III and IV, the invasion of bacteria was enhanced by abrading the skin mucus under anaesthesia before bacterial challenge.

3.3.2 The effect of parasitic infection and rearing conditions on columnaris infection (III)

The effects of parasitic infection, stress, rearing density and water temperature on columnaris infection were studied in the laboratory and at a fish farm. Establishment of columnaris disease due to skin damage caused by the penetrating endoparasite *Diplostomum spathaceum* was studied using *F. columnare* carrier fish. Fish were exposed to newly emerged *Diplostomum*

cercariae (100 individuals/fish) collected from snails. During the experiment, fish were exposed to a set of stressors in order to induce columnaris infection. Finally, the fish skin was abraded and the fish were exposed to a low dose (3.6 x 10^3 CFU/ml) of *F. columnare* cells. Mortality of fish exposed to *Diplostomum spathaceum* and of unexposed fish was compared.

Transmission of columnaris disease at normal (100%) and reduced (25%) rearing density was studied at high (23 °C) and low (18 °C) water temperatures at a fish farm. Normal density refers to the density used for the same age and size group at the farm where the experiment was performed. The fish were challenged with the *F. columnare* strain isolated previously from the same farm and mortality of fish between the treatments was analysed.

3.3.3 Treatment and prevention of columnaris infection in vivo (IV, V)

The effects of salt (2% and 4%) and acidic baths (pH 4.6) on columnaris disease were studied using experimentally infected fish (V). Acidic and 4% salt baths were given 8 hours after the challenge and repeated every other day thereafter. The effect of a single 2% salt bath given as a preventative treatment was studied in the second experiment, where fish were bathed only once with 2% NaCl one hour after the challenge (duration 30 minutes).

The effect of previously isolated *Pseudomonas* sp. MT5, a strong antagonist against flavobacteria *in vitro* (Tiirola et al. 2002), on columnaris disease was studied in two experiments (IV). Fish were exposed to *Pseudomonas* culture prior experimental *F. columnare* challenge in laboratory conditions. The effect of these baths on the microbial diversity on fish skin and gills was monitored by bacterial community analysis and specific PCR (see below). The effect of *Pseudomonas* MT 5 was tested also in a natural outbreak at a fish farm, where *Pseudomonas* baths were administered daily.

3.4 Detection and identification of bacteria (IV)

3.4.1 Isolation and biochemical identification of *Flavobacterium columnare*

The dilution cultivation method was used in the isolation of *F. columnare* from fish tissues. This was done to reduce the number of faster growing bacteria in the samples which can harm the growth of *F. columnare* (Wakabayashi 1991, Tiirola et al. 2002). Gill or skin samples from infected and healthy fish were suspended in AO-broth and diluted in 10-fold series. Parallel plate cultivation was made from each dilution on AO-agar.

Yellow pigmented colonies with typical features of *F. columnare* (rhizoid edges, tight adherence to agar) were further characterized. The ability of bacteria to produce flexirubin type pigment was analysed with 5% KOH. Congo red absorption (0.01 % aqueous solution) was used to detect the production of

galactosamine glycan (Johnson & Chilton 1966). Absence of growth in tryptic soy broth (TSB) was also studied.

3.4.2 Polymerase Chain Reaction (PCR)

Parallel to cultivation, LH-PCR was used to screen bacterial community and detect *Flavobacterium columnare* and *Pseudomonas* sp. MT 5. Skin and gill DNA samples were taken from healthy and diseased fish. DNA from fish tissues was extracted using proteinase K lysis and bead-beating and phenol-chlorophorm extraction (Tiirola et al. 2003). Universal primers were used to amplify bacterial 16S rDNA. Diversity analysis was performed with a Li-Cor DNA 4200 automated sequencer and Quantity One software (Bio-Rad). To obtain detailed information on the species contributing to the most prevalent peak sizes three PCR products were cloned and sequenced.

A protocol for specific detection of *Pseudomonas* sp. MT5 was designed to reveal the presence of the antagonist strain in the external tissues of challenged fish. Specific detection was needed because other pseudomonads had the same fragment length of LH-PCR.

4 RESULTS AND DISCUSSION

4.1 Characteristics of Finnish *Flavobacterium columnare* isolates (I, II, V)

4.1.1 Genetic diversity

In genetic analysis of Finnish *F. columnare* isolates, no differences between isolates were detected by 16S RFLP (I). All isolates produced a restriction pattern similar to genomovar I (classification after Triyanto & Wakabayashi 1999), indicating that all Finnish isolates (as well as the type strain) belong to the same genomovar as previously studied French strains (Michel et al. 2002). Also, no differences were found in *F. columnare* fragment length in LH-PCR; all the isolates studied produced a fragment of 517 bp in length. Therefore, the amplification of a 517 bp fragment from infected fish tissue can be considered as a strong indication of presence of *F. columnare*.

Within the 30 Finnish *F. columnare* isolates representing genomovar I, 7 genotypes were detected using RISA, and *F. columnare* type strain NCIMB 2248^T represented an eighth genotype (see Table 3). RISA genotype A was predominant among the collected strains. In the study of Arias et al. (2004), sequence analysis of internal spacer region (ISR) similarly revealed a RISA subdivision within the 16S genomovar typing. Size heterogeneity was also detected by Arias et al. (2004), by Welker et al. (2005) and by Figueiredo et al. (2005). However, in these studies the number of ISR sequences per strain was not defined in detail, even though some isolates produced two bands in the PCR visible even in the agarose gel electrophoresis (Arias et al. 2004, Figueiredo et al. 2005). Among the Finnish strains we found as many as three ISR copies differing in their length within one strain. The lengths ranged from 691 bp to 746 bp. Size differences were not detectable in agarose gel electrophoresis. Whether these sequences also differ in base composition other than gaps and

insertions, is not known. In AFLP analysis, RISA genotypes F and G fell into clearly distinct groups. Also RISA genotypes C and E formed a separate branch.

A representative strain of each genotype was selected for further characterization by sequencing. The 16S rRNA gene sequence was highly homologous between the Finnish strains (sequence difference < 1%). The difference between Finnish strains and the type strain 16S sequence was 2% and the sequences obtained from the Finnish strains formed a separate branch in the phylogenetic tree when compared to other 16S rRNA sequences obtained from EMBL database. Surprisingly, there was one exception: strain IFO 15943^T was similar to the Finnish strains. This is confusing because IFO 15943^T should be the same strain as NCIMB 2248^T. The NCIMB 2248^T sequence has been submitted to the database in 1999 and the IFO 15943^T sequence in 2002. Either there are mistakes in the sequences, or else multiple ribosomal operons with differences in the 16S rRNA sequence exist in the type strain. In RISA analysis, only one band was detected in NCIMB 2248^T, but that does not exclude the possibility that several operons could be present in the genome. Whole genome RFLP and hybridization could be used to obtain further information on the actual number of operons.

The phylogenetic studies done by Bader & Shotts (1998) placed *F. columnare* ATCC 43622 close to *F. johnsoniae* in the phylogenetic tree. However, this result was false due to the use of a single sequence obtained from *F. columnare* strain ATCC 43622, which was recently revealed not to be *F. columnare* (Darwish et al. 2004). Strain ATCC 43622 has now been identified as *F. johnsoniae*, but, as mentioned, some studies have previously used it as a representative of *F. columnare* (Bader & Shotts 1998, MacLean et al. 2003).

4.1.2 Virulence

I had previously observed differences in clinical signs and progression of the disease during columnaris outbreaks (unpublished observations). In some outbreaks gill infections have mainly been observed but other symptoms have also occurred. In study II, I aimed to determine if *F. columnare* genotypes differ in their virulence and if different disease signs are connected to different genotypes. In this study I measured virulence as fish mortality after exposure to the pathogen. In an experimental study with rainbow trout I found differences in virulence between the 8 RISA genotypes: high virulence (strains B, C, E and G, 95 - 100% mortality within 84 - 114 hours post infection) and low virulence (strains A, D, F an H, maximum 25% mortality within 7 days post infection).

Differences in virulence are known to exist between *Flavobacterium columnare* strains, producing variance in mortality (Pacha & Ordal 1963, Dalsgaard 1993, Decostere et al. 1998). Pacha and Ordal (1963) classified the virulence of *F. columnare* into four categories: 1) High virulence (100% mortality within 24 hours), 2) moderate virulence (100% mortality within 48 hours), 3) intermediate virulence (96 hours) and 4) low virulence (more than 96 hours). According to this classification, all Finnish strains fall into the intermediate and low virulence categories. However direct comparison of virulence between

different studies is practically impossible due to different experimental conditions and fish species used.

Multiple symptoms of columnaris disease (gill inflammation, saddleback lesions, erosion of jaw and tail, grey areas on the skin) were detected in fish exposed to highly and moderately virulent *F. columnare* strains. Symptoms caused by low virulence strains were mainly saddleback lesions. This is in accordance with the findings of Pacha and Ordal (1970) who suggested, that severe gill necrosis usually occur if high virulence strain is involved.

Previous studies dealing with virulence-related factors of *F. columnare* have covered only adhesion to surfaces or production of proteases, but correlations of these two virulence mechanisms have not been studied. The slime layer of *F. columnare* probably contributes to both adhesion and gliding motility. Two kinds of slime possibly contributing to adhesion or gliding motility have been identified: acidic polysaccharide (Pate & Ordal 1967) and galactosamine glycan (Johnson & Chilton 1966). However, the acidic polysaccharide was identified only from electron microscopy of the bacterial cells, whereas Johnson & Chilton (1966) performed also biochemical analyses. I found that the adherence capacity of *F. columnare* to fish tissues (studied using crude mucus-coated microscopic slides) was strongly related to the virulence (II). This was previously reported by Decostere et al. (1999a) in a gill perfusion model, but their evidence came from only two strains, when repeatability and reliability are uncertain. In another study, adherence capability of a single virulent *F. columnare* strain was increased by high temperature and poor water quality (Decostere et al. 1999b). Also the adherence capacity of a highly virulent *F. psychrophilum* strain can be higher than that of a less virulent strain (Nematollahi et al. 2003). Recently Bader et al. (2005) developed an adhesion-defective mutant *F. columnare* strain by serial passage on ampicillin medium. They found that this adhesion defective mutant was able to produce columnaris infection if it was injected into fish. Figueiredo et al. (2005) showed that *F. columnare* isolated freshly from infected fish produced only 7% mortality when channel catfish were exposed by bathing compared to 80% mortality produced by intramuscular injection. This indicates that virulence factors other than adhesion are also important. However injection is not a natural way to produce columnaris infection because all natural defence mechanisms are bypassed.

Extensive tissue damage seen in infected fish has been explained by the production of chondroitin AC lyase by *F. columnare* (Griffin 1991, Teska 1993, Stringer-Roth et al. 2002, Figueiredo et al. 2005). This enzyme degrades mucopolysaccharides in animal connective tissues. Therefore, the production of chondroitin AC lyase is probably responsible for the pathogenity of the adhered strain. In paper II, I studied the production of the chondroitin lyase C on the eight selected *F. columnare* strains at two temperatures. Chondroitin lyase C activity was clearly temperature-dependent; activity at 25°C was significantly higher than activity at 20°C. An effect of temperature has also been reported by Stringer-Roth et al. (2002) who noticed that high temperature increased chondroitin lyase activity in isolates originating from cold water fish. It seems

that the pathogenity of *F. columnare* can be divided into two phases. In the first phase, the affinity and capability to adhere into fish play a major role in the virulence of the strain. In the second phase, adhered bacterial cells start to divide and produce lyases and proteases, which degrade fish connective tissue establishing visible signs of the disease. Production of extracellular proteases has been detected in *F. columnare* (Bertolini & Rohovec 1992), but their role in this system is yet unknown

To my knowledge, the occurrence of plasmids in *F. columnare* has not been studied. *F. psychrophilum* are known to posses at least three kinds of plasmids, which are suggested to be connected to virulence (Dalsgaard 1993, Lorenzen et al. 1997, Chakroun et al. 1998, Ashiuchi et al. 1999, Madsen & Dalsgaard 2000). In paper II, neither the seven selected Finnish *F. columnare* strains nor the type strain possessed plasmids even though four methods were applied for plasmid detection. Therefore, I suggest that virulence is not mediated by plasmids in these strains.

Virulence stability has not been studied for *F. columnare*. Virulence is known to remain stable in *F. psychrophilum* stored frozen or lyophilized but virulence was attenuated in the isolates stored at +4°C for 23 months (Michel and Garcia 2003). The bacterial strains used in the virulence study in Paper II were stored frozen and changes in virulence may have been occurred. This possible laboratory effect needs to be taken into consideration when interpreting the results. Long term storage and repeated subcultivation have prominently affected the colony morphology of *F. columnare* type strain NCIMB 2248^T, and presumably also its virulence. The type strain lacks the normal rhizoid and adherent colony morphology, which suggests the loss of gliding motility and affinity to fish tissues (II). Since the genetic characteristic of the type strain NCIMB 2248^T have remained unchanged, as shown in Paper I, the change of colony morphology is not due to a misidentification of bacterial strain or replacement due to contamination during several subcultivations.

All the strains studied in Paper II were isolated from severely infected fish and therefore considered virulent. I used a genetic basis to select representatives for virulence experiments, with one strain from each RISA group selected. Thomas-Jinu and Goodwin (2004b) suggested that virulence is a strain-specific characteristic rather than related to cell morphology or genetic group. This may well be true and variation in virulence may be found within each RISA group of Finnish *F. columnare* isolates. This may provide one explanation of why RISA genotype A is predominant among the 31 isolates studied in Paper I; strains with high virulence can exist in this group, but the one used in the experimental infection exhibited low virulence. However, the approach of selecting representative strains for the study II on a genetic basis is valid and it does not affect the results of the study.

Adaptation of strains to the local environment and water quality may affect the results in Paper II. Strain H was used in experimental infection in Paper III, where it produced higher mortality than in Paper II. In addition to the loss of virulence during storage, virulence may have been influenced by

different water quality. Strain H was originally isolated from the same fish farm where the experiment in Paper III was performed and it could have been adapted to the ambient aqueous environment.

In Paper I it was shown that even three different RISA genotypes of *F. columnare* can co-occur in one outbreak. The same phenomenon has been found by Triyanto et al (1999) and also in *F. psychrophilum* (Madetoja et al. 2001). This may have a significant impact on the nature of an untreated columnaris infection if the coexisting strains differ in their virulence. A highly virulent strain has more efficient transmission in an infected population weakened by a less virulent strain (Boots et al. 2004, Day & Proulx 2004). Two of the genotypes found in one outbreak (I) were strains with low virulence. It is possible that coexistence of genetically different, low virulence strains can increase the establishment and transmission of *F. columnare* and make the infection more severe. Indeed, increased virulence associated with competition of genetically different strains is one of the main aspects in the evolution of virulence (e.g. Gandon et al. 2001, Read & Taylor 2001).

4.2 Factors affecting columnaris disease outbreaks at fish farms (III)

Parasites are always present in freshwater fish farms and the co-occurrence of parasitic and bacterial diseases cannot be prevented. Rintamäki-Kinnunen et al. (1997) reported that 30% of fish with a parasitic infection had a simultaneous flavobacterial infection. However, interactions between parasites and bacterial diseases are poorly understood. Whether columnaris outbreaks can be induced by stress or parasites in carrier fish, serving as reservoir hosts of the disease, is an important topic. According to Bernardet (1997), immunity against columnaris disease is acquired after infection and therefore carrier fish are not likely to get the diseases again. However, this is not seen in practice in fish farms because antibiotic treatments are usually started shortly after first signs of the disease and therefore protective immunity against the disease has no time to develop.

In study III, I focused on some of the main disease-inducing factors present at fish farms to evaluate their role in enhancing the invasion and transmission of columnaris disease. The effects of parasitic infection (*Diplostomum spathaceum*), stress, rearing density and water temperature were studied. The effect of parasitic infection on the onset of columnaris disease in carrier fish was studied using a three-phased experimental design. First, the fish (shown to be carriers of columnaris disease) were exposed to parasitic infection to see if the microlesions produced by the penetrating parasites would open a route for *F. columnare*. When no signs of the disease occurred, the fish were stressed to evoke columnaris disease, which also failed to induce the disease. Finally, the fish were challenged with *F. columnare* to see if the fish with a

concomitant parasitic infection were more likely to manifest columnaris disease. No interaction between *D. spathaceum* infection and columnaris disease was found (see also Busch et al. 2003). In another study, *Diplostomum* infection was found to enhance atypical *Aeromonas salmonicida* (aAS) infection in a study using European grayling (Pylkkö et al, in press). This is most likely due to the different nature of flavobacterial infections, which occur cutaneously and do not necessarily invade fish internal organs. Therefore, a penetrating parasite might not enhance the invasion of flavobacteria, even though minor lesions in the mucus can expose fish to other pathogenic bacteria, like aAS.

In another experiment, the effects of rearing density and water temperature were studied. I found no statistical differences in mortality between two rearing densities, but the transmission of columnaris disease was faster at the normal rearing density compared to the reduced density. Lower mortality associated with reduced rearing densities has also been shown in *F. psychrophilum* (Iguchi et al. 2003). In Paper III it was also seen that water temperature in general is a key factor in the development of columnaris disease in Finland, confirming the results from previous studies done in America (Johnson & Brice 1952, Holt et al. 1975, Morrison et al. 1981). I suggest that there are at least four ways in which high temperature increases the severity of columnaris infection. First, the growth rate of *F. columnare* increases by 30% when temperature rises from 17 to 25 °C (I). Second, the adhesion capacity of *F. columnare* increases with temperature (Decostere et al. 1999b). Third, the chondroitinase activity is higher at 25 °C than at 20 °C. The fourth possible reason is related to fish response to water temperature: the optimum temperature for salmonid fish is around 16 °C and higher water temperatures create stress and therefore may render fish more susceptible to bacteria.

These results indicate that *Flavobacterium columnare* carrier fish can become diseased again, but stress or parasitic infections may not increase the invasion of *F. columnare* cells attached on the surfaces of fish at temperatures below 20 °C. Therefore, I suggest that carrier fish are not a risk factor in fish farming at water temperatures below 20 °C, even although they may shed the pathogen into the environment. The bacterial load in the ambient water must be high to induce columnaris disease in fish at temperatures below 20 °C. The relationship between increased *F. columnare* infections and warm summers is clear. Furthermore, the mortality and severity of infections are temperature dependent. Climate warming in Europe and in Finland will make the summers warmer and longer in the future, possibly increasing the number and severity of *F. columnare* outbreaks at fish farms.

4.3 The nature of *Flavobacterium columnare* infection seen by bacterial community analysis (IV)

There are no previous studies concerning the bacterial communities of both healthy and infected fish tissues detected by PCR methods. Studies on bacterial diversity of fish have traditionally used cultivation-based methods (reviewed by Hansen & Olafsen 1999), but it has been estimated that less that 1% of bacterial species can be isolated by cultivation (e.g. Amann et al. 1995). Therefore cultivation can be considered an unreliable method in bacterial community analysis. This corresponds directly to the growing number of uncultured organism sequences in the EMBL database derived from bacterial community analyses by PCR methods (see Rappé & Giovannoni 2003). Spanggaard et al. (2000) reported that, on average, only 50% of bacteria in rainbow trout intestines were culturable when compared to direct microscopic counts, and in the study of Huber et al. (2004) the count was considerably lower (< 2%). Therefore, PCR and other DNA-based community analyses offer fast and universal tools to study bacterial diversity in otherwise difficult environments where cultivation gives biased results. Bacterial community analysis utilises broad range PCR, where bacterial 16S rRNA genes are amplified from a sample yielding a heterogenous mixture of DNA-fragments, which reflect the bacterial community present in the sample. PCR products are profiled to determine the diversity of DNA fragments, often referred as OTUs (operational taxonomical units) using DGGE, T-RFLP, cloning and sequencing (Muyzer et al. 1993, Avaniss-Aghajani et al. 1994, see also Tiirola 2002). LH-PCR (Suzuki et al. 1998) is a method differentiating heterogenous PCR products by their length. It has been applied in screening bacterial diversity in different environments such as coastal waters, soils, paper machine biofilms and clinical patient samples (Suzuki et al. 1998, Ritchie et al. 2000, Suzuki et al. 1998, Ritchie et al. 2000, Mills et al. 2003, Rogers et al. 2003, Tiirola et al. 2003). In Paper IV, LH-PCR was applied to study microbial diversity and bacterial community structure of fish skin and gills. Presence of *F. columnare* was diagnosed from the presence of 517 bp fragment in the sample, which has been confirmed to be specific for 31 *F. columnare* isolates (study I), and the identification was further confirmed by sequencing.

Bacterial community analysis revealed that fish with a previous history of columnaris disease remained as carriers even if the disease was successfully treated with antibiotics. *F. columnare*-referring fragment was present in 41.5% of the samples taken from clinically healthy fish and no isolates were obtained in parallel cultivation. Carrier status has also been suggested in earlier studies by Pacha & Ordal (1970, 1978), and it has been shown that carrier fish can shed *F. columnare* into the water (Becker & Fujihara 1978). So far, the role of *F. columnare* carrier fish in fish farming has been unclear.

LH-PCR was proven to be efficient in detecting *F. columnare* also in diseased fish. *F. columnare* was isolated from only 31% of infected fish whereas

LH-PCR detected *F. columnare* referring peak size in 100% of fish. Interestingly, the proportion of *F. columnare* in the bacterial community was not related to the success of bacterial isolation. This is probably due to inhibitory effects of the competitive bacteria during cultivation. In the diversity analysis, it was seen that *F. columnare* dominated the bacterial community of fish skin and gills during columnaris infection.

How can a pathogen like *F. columnare* with such a poor ability to compete with the other bacteria (Chowdhury & Wakabayashi 1989, Wakabayashi 1991) cause a disease and come to dominate the bacterial community? First, *F. columnare* has a strong affinity to fish tissues and it is therefore able to attach on fish surfaces fish. Second, bacterial competition on fish skin may differ from that in *in vitro* culture conditions and *F. columnare* may benefit from some interactions. Finally, *F. columnare* is known to produce bactericidal substances, which can enhance its dominance over other bacteria in infected fish (Anacker & Ordal 1959).

4.4 Treatment and prevention of columnaris infection in vivo

4.4.1 Antibiotic susceptibility (I)

At present, antimicrobial drugs are used to treat columnaris disease of fish, but regardless of this effective treatment the outbreaks tend to occur in sequence throughout the summer. The use of recurring antibiotic treatments can promote the development of antibiotic resistant strains and select for increased virulence (e.g. Porco et al. 2005). Furthermore, residues of antimicrobial drugs and other chemicals have previously spread to the environment via outflowing water, which can promote resistance in environmental bacteria (Schmidt et al. 2000). No differences in antibiotic susceptibility among the eight studied *F. columnare* isolates were found and all strains were susceptible to tetracycline and florfenicol, the two antibiotics currently used in fish farming. Resistance and intermediate susceptibility were found only to polymyxin B and neomycin.

4.4.2 Bath treatments with 4% NaCl and reduction of water pH to 4.6 (V)

Columnaris infection is usually restricted to the skin and gills of fish before the disease becomes severe and the bacteria invade the internal tissues of fish (Bernardet 1997, Tripathi et al. 2005). Therefore the use of bathing would be appropriate, especially at the beginning of an outbreak, when few or none of the signs have occurred. However, the chemicals used in treating bacterial infections can be harmful to the fish and the personnel (e.g. Nemcsok & Hughes 1988, ChemDat® 1999, Straus 2004). One of my aims in this PhD thesis was to find ecological disease management strategies to prevent and treat columnaris disease.

In the growth analysis (I), I found the Finnish *F. columnare* strains to be very sensitive to low pH and to even minor salt concentrations. The strains were not able to grow in Shieh broth supplemented with 0.1% NaCl or at pH below 6.5. *F. columnare* type strain NCIMB 2248^T, however, tolerated concentrations used in the growth assays. Poor tolerance to even low salt concentration may also explain why *F. columnare* infection occurs cutaneously; Finnish *F. columnare* strains seem to be unable to grow in physiological salt concentration. Variance between strains in growth in different salt concentrations has been detected previously, but the concentrations have varied from 0.5 to 1.0% which is higher than any of the Finnish strains can tolerate (Soltani & Burke 1994, Shamsudin & Plumb 1996). In Paper III, the ultimate tolerance to low pH (values between 4.6 and 5.0) and salt concentration 4% was studied to evaluate their disinfection effect. In these inhibition assays, at least 95% of the bacterial cells lost their viability after 15 minutes exposure to 4% NaCl or pH 4.6.

Despite the promising results of the *in vitro* tests in Paper III, salt or acidic bath treatments had no effect on the overall mortality of rainbow trout infected with *F. columnare*. Yet 4% NaCl baths significantly decreased the mortality rate of fish during the first four days of the experiment. Sensitivity of *F. columnare* to low pH values and salt has been detected previously by Soltani and Burke (1994), who reported that growth of *F. columnare* ceases in 1% NaCl and at pH below 5. Altinok and Grizzle (2001) found that rearing fish in salinities between 3 ‰ and 9 ‰ inhibited *F. columnare* infection. It is noteworthy that columnaris disease does not occur at farms in the coastal area of Finland where water salinity is between 2 ‰ and 7 ‰. In Paper V salt baths significantly reduced mortality rate, most likely by hampering the transmission of the disease by disinfecting the water of bacteria shed from the infected fish. Therefore, salt baths could be used to reduce the transmission rate of bacteria at the beginning of infections, when only a few fish show signs of the disease. Salt is already being used in the treatment of protozoan parasitic infections in fish farms, so as a preventative method it would be practical, easy and cheap to use. However, it may not offer help alternative to antibiotic treatments. Perhaps more intensive bathing (once per day) could prevent the disease more effectively, but on the other hand this would be more laborious to perform and would cause stress to the fish.

4.4.3 Bath treatments with probiotic bacteria (IV)

During recent years, a wide interest has been directed towards the use of health-promoting probiotic bacteria in the disease control in fish and other animals. Probiotic bacteria can enhance the survival of fish by stimulating the immune system or by direct resource competition with the invading pathogen. Competitive bacteria are known to inhibit the growth of *F. columnare* (Chowdhury & Wakabayashi 1989, Wakabayashi 1991) and because of the external nature of columnaris disease, fish could benefit from manipulation of the bacterial community.

Pseudomonas sp. MT5 has been previously shown to inhibit the growth of *F. columnare* and *F. psychrophilum* as well as other fish pathogenic bacteria in agar assay (Tiirola et al. 2002). However, I found that *Pseudomonas* sp. MT5 baths were not effective as a treatment or preventative method for columnaris disease of rainbow trout (Paper IV). Even the *Pseudomonas* sp. MT5 baths administered daily could not prevent mortality in infected fish. There are several studies focusing on probiotic bacteria in the treatment of bacterial fish diseases, but lack of protection has been reported (Gram et al. 2001, Spanggaard et al. 2001). Generally the probiotic characteristics of pseudomonads are based on iron chelating siderophores (Hatai & Willoughby 1988, Smith & Davey 1993, Bly et al. 1997, Gram et al. 1999, Spanggaard et al. 2001). Although the exact mechanism of antagonism is not known for *Pseudomonas* sp. MT5, it probably is competition for iron. Thus, one reason for the failure of *Pseudomonas* sp. MT5 to prevent columnaris disease may be the high amount of ferrous compounds in Finnish waters. However, this can also increase the virulence of *F. columnare* (Kou et al. 1981). Another evident reason is the poor adhesion of the strain MT5 on fish skin. Adhesion capacity is crucial for the use of probiotic bacteria, but it is not commonly studied. In Paper IV, *Pseudomonas* sp. MT 5 was not detected in tissue samples in fish bathed twice with the strain, suggesting poor adhesion capacity of the strain on the fish surfaces.

Despite the promising results from *in vitro* studies concerning the use of antagonistic *Pseudomonas* sp. MT5, salt baths (4%) and acidic baths (pH 4.6), none of these treatments was effective against columnaris disease *in vivo* (IV, V). These experiments with rainbow trout (IV, V) show that promising results obtained from *in vitro* studies must be considered with caution. Fish skin mucus is a powerful buffer against ambient water quality changes and it can protect not only fish but also bacterial cells located beneath it. Therefore, as an environment, fish skin differs totally from agar plates and nutrient media. Interactions between a fish and its environment are incompletely known, making comparison between artificial (*in vitro*) and natural (*in vivo*) environments difficult.

4.5 Future aspects

More research is needed to find practical solutions in disease management of columnaris disease. The use of immunity-inducing feed supplements (immunostimulants or immunomodulators) could offer possibilities in prevention of flavobacterial diseases. The use of immunostimulants could be a practical and cheap way to prevent columnaris disease at fish farms. Furthermore, several immunostimulants are commercially available at the moment.

Also, it has been shown that fish previously infected with *Flavobacterium columnare* can develop immunity (Bernardet 1997, Fujihara & Nakatani 1971, Grabowski et al. 2004). Even though the development of immunity has not been

seen in practice in Finnish fish farming, development of vaccines is important. Vaccines have already been shown to be efficient against *F. psychrophilum* infections (Kondo et al. 2003, LaFrentz et al. 2004) but development of vaccines against *F. columnare* is still in process.

The occurrence of *F. columnare* in nature has not been studied. If this bacterium belongs to the normal bacterial community in aqueous environments, eradication of the disease from fish farms might be impossible. It is possible, however, that strains occurring in nature differ in their virulence and could be used in studying development of virulence or development of vaccines.

The significance of spheroplasts in the ecology of *F. columnare* unclear. Revealing the factors regulating their formation could open new aspects in the bacterial ecology of *Cytophaga-Flavobacterium-Bacteroides* group in general.

5 CONCLUSIONS

- Finnish *Flavobacterium columnare* isolates can be divided into at least seven genetically defined groups. The representatives of these groups differ from each others and from the type strain NCIMB 2248^T according to their virulence and growth features. Co-occurrence of different genotypes in disease outbreaks was demonstrated and this may contribute to the continuous tendency of columnaris outbreaks at fish farms. Interactions between different genotypes need to be studied further.

- Finnish *F. columnare* strains are highly sensitive to even minor salt concentrations (0.1%) and pH below 6.5. The poor tolerance to salt can explain why columnaris disease does not occur in coastal areas of Finland, where water salinity is between 2 ‰ and 7 ‰. This suggests that salt could be used as a treatment method for columnaris diseases.

- Fish remain carriers of columnaris disease after infection. However, the disease was not induced in these carrier fish by parasitic *Diplostomum spathaceum* infection and other stressors at water temperatures below 20 °C. This suggests that carrier fish are not a risk factor at fish farms in water temperatures below 20 °C.

- High water temperature is a key factor related to transmission of bacteria and to fish mortality during *F. columnare* outbreaks. Long and warm summers can make disease problems worse in Finland. Rearing fish in lower densities slows the transmission of columnaris disease, but does not affect overall fish mortality.

- Traditional cultivation method is an unreliable method in diagnosis of columnaris disease, whereas PCR is an efficient and sensitive method to detect *F. columnare* occurrence in fish tissues both in fish with symptoms and those clinically healthy.

- Because columnaris disease is usually restricted to the surface of fish, bath treatments could be used in prevention by reducing mortality rate and transmission of the disease during the first days of an outbreak. Bathing, however, does not remove the need for antibiotic treatment, which is effective since no resistant strains have yet been detected.

Acknowledgements

First of all, I would like to thank my supervisors, professor Tellervo Valtonen for introducing me the fascinating world of fish diseases, and Dr. Marja Tiirola for teaching me so many new tools for studying it. You both have given me continuous support and encouragement during my studies. Working with you has been extremely motivating and enjoyable.

Thank you to everyone who commented on my manuscripts. Colleagues in the parasitology research group have been involved in my work in many ways. Scientific themes, but also other aspects of life, have often been discussed. I received valuable comments on my papers from all of you and Anssi Karvonen also commented on the summary. My co-authors Varpu Hirvelä-Koski and MSc student Heidi Kunttu contributed greatly to the characterisation of *F. columnare* strains, which were kindly donated by the National Veterinary and Food Research Institute (Oulu regional unit) and by Dr. Päivi Rintamäki-Kinnunen. Professor Roger Jones has given valuable comments on my manuscripts and he has also kindly corrected the language in my papers and the summary.

On the practical side I would like to thank Savon Taimen Oy and Yrjö Lankinen for the views of the field and for providing fish used in the experiments. The experiments were performed at three locations. To the staff at Hanka-Taimen, thank you for your effort and help during field experiments. Thank you also for the conversations at coffee breaks, when I received first hand information from the experts. The technical staff at Konnevesi research station (Janne Koskinen, Jyrki Raatikainen and Risto Latvanen), and at the University of Jyväskylä (Juha Ahonen and Tarmo Halinen) have been of great help.

I would like to express my warmest gratitude to my mother. You have always encouraged me and supported me.

Samu, my life would not be the same without you. Our home and life together keeps me going, it brings me happiness and stability. Thank you for standing by me during all these years.

This work was funded by University of Jyväskylä (Rector's funding). I also got financial and other support from the SUNARE project (Academy of Finland), Graduate School of Biological Interactions (University of Turku) and Kemira foundation.

YHTEENVETO

Flavobacterium columnare Suomen kalanviljelyssä: karakterisointi ja mahdolliset torjuntamenetelmät

Flavobacterium columnare aiheuttaa kaloilla kuolemaan johtavaa iho- ja kidustulehdusta, jota kutsutaan columnaris-taudiksi. Tautia esiintyy yleisesti suomalaisilla kalanviljelylaitoksilla, ja tautitapaukset ovat lisääntyneet huomattavasti viimeisen vuosikymmenen aikana. Löytääkseni syitä yleistyneisiin infektioihin, selvitin suomalaisten *F. columnare* -kantojen geneettistä diversiteettiä, taudinaiheutuskykyä ja kasvuominaisuuksia. Tutkimuksissa vertailin *F. columnare* -bakteerin diagnostiikkaa bakteeriviljely- ja PCR -menetelmillä (DNA:n monistus polymeraasiketjureaktion avulla), sekä infektion vaikutusta kalan ihon mikrobiyhteisöön. Lisäksi tutkin, mitkä tekijät altistavat kalat infektioille ja mitkä olisivat mahdollisesti käytännöllisiä taudintorjuntamenetelmiä.

Tutkimuksissa selvitin 30 suomalaisen *F. columnare* -kannan geneettisiä ominaisuuksia, joiden avulla kannat tyypitettiin seitsemään eri ryhmään. Näiden seitsemän ryhmän edustajien välillä oli eroja taudinaiheuttamiskyvyssä ja kasvuominaisuuksissa. Suomalaiset kannat sietivät erittäin huonosti pieniäkin suolakonsentraatioita (< 0.1%), mutta niiden kasvu nopeutui voimakkaasti lämpötilan noustessa yli 20 °C ja kasvatusliemen pH:n ollessa välillä 7–8. Tulokset ovat suoraan rinnastettavissa kalanviljelyolosuhteisiin: columnaris-tautia esiintyy korkean veden lämpötilan aikana loppukesällä, jolloin veden pH on lähellä neutraalia tai hieman sen yläpuolella. Kun suolan ja pH:n vaikutusta tutkittiin kirjolohella columnaris-infektion aikana, ei kylvetyksillä ollut kuitenkaan vaikutusta kokonaiskuolleisuuteen. Epidemian alussa suolakylvetykset kuitenkin hidastivat kuolleisuutta merkitsevästi. Suolakylvetyksiä voitaisiin käyttää taudin ennaltaehkäisyyn. Mahdollisena taudintorjuntamentelmänä tutkin myös kalaa suojaavan probioottibakteerin vaikutusta columnaris-infektioon. Käyttämäni *Pseudomonas* sp. MT5 -kanta oli antagonistinen useille eri kalapatogeeneille maljakokeissa, mutta se ei vaikuttanut columnaris-taudin etenemiseen kirjolohella laitosolosuhteissa. Kokeessa suojabakteerikylvetyksiä annettiin päivittäin.

PCR on perinteisiin viljelymenetelmiin verrattuna luotettavampi ja nopeampi tapa columnaris-taudin diagnostiikassa. PCR-analyysin avulla havaitsin terveiden kalojen jäävän columnaris-taudin kantajiksi, vaikka tauti oli hoidettu antibioottirehun avulla. Nämä kantajakalat ovat mahdollinen riski vasta veden lämpötilan noustessa yli 20 °C. Tautia ei pystytty indusoimaan kontrolloiduissa viljelyolosuhteissa alemmassa lämpötilassa. Taudin leviämiseen kalapopulaatiossa vaikuttaa ensisijaisesti veden lämpötila. Kalatiheyttä pienentämällä voidaan taudin leviämisnopeutta hidastaa, mutta merkittäväkään tiheyden alentaminen ei estänyt taudin etenemistä oleellisesti.

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