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Molecular diversity and growth features of *Flavobacterium columnare* strains isolated in Finland

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ABSTRACT: Columnaris disease caused by *Flavobacterium columnare* is a problem in fish farming worldwide. During the last 15 yr, outbreaks have started to emerge in Finland. *Flavobacterium columnare* Type Strain NCIMB 2248^T and 30 Finnish *F. columnare* isolates were studied using analysis of 16S rDNA by restriction-fragment length polymorphism (16S RFLP), length heterogeneity analysis of polymerase chain reaction (LH-PCR) products, automated ribosomal intergenic spacer analysis (ARISA), and 16S rDNA sequence analysis. All isolates fell into RFLP Genomovar I and had the same length in the LH-PCR analysis. Based on ARISA, 8 genetically different strains were selected for further analyses. The growth of these strains under different temperatures, NaCl concentrations, and pH values was tested. The Finnish *F. columnare* strains did not grow at NaCl concentrations >0.1% or at pH values ≤6.5, and they were susceptible to several antimicrobial agents, but not to Polymyxin B or neomycin. These findings may aid in development of methods for disease management at fish farms.

KEY WORDS: *Flavobacterium columnare* · ARISA · RFLP · 16S rRNA gene sequencing

INTRODUCTION

Flavobacterium columnare, the causative agent of columnaris disease, is a bacterial fish pathogen that was first described in 1922 (Davis 1922). The bacterium causes skin and gill infections in farmed freshwater fishes worldwide. Columnaris outbreaks started to emerge in Finland in the early 1990s (Koski et al. 1993) and have increased annually to become a serious threat to the fish farming industry (Suomalainen 2005). The causative bacterium has previously been known as *Bacillus columnaris*, *Chondrococcus columnaris*, *Cytophaga columnaris*, and *Flexibacter columnaris*, but this taxonomical confusion was resolved in 1996 when Bernardet et al. (1996) transferred the bacterium to the genus *Flavobacterium*. Currently, *F. columnare* is considered a phenotypically homogenous species when compared to other species in the *Flavobacterium/Flexibacter/Cytophaga* group (Song et al. 1988, Bernardet et al. 1996, Shamsudin & Plumb 1996, Bader & Shotts 1998a).

Several studies of the genetic variability of *Flavobacterium columnare* strains using 16S rDNA restriction fragment analyses (16S RFLP) have shown the division of the strains into 3 16S RFLP genomovars (Triyanto & Wakabayashi 1999, Triyanto et al. 1999, Michel et al. 2002). Recently, intergenic spacer region (ISR) sequencing (Arias et al. 2004), amplified fragment-length polymorphism (AFLP) fingerprinting (Figueiredo et al. 2005), and analysis by randomly amplified polymorphic DNA (RAPD) (Thomas-Jinu & Goodwin 2004) have been applied to *F. columnare* isolates. However, these studies have not covered Finnish *F. columnare* strains; indeed, the only European isolates studied have been from France. Moreover, previous studies have not characterized basic growth features or antibiotic susceptibilities of genetically different *F. columnare* types to indicate alterna-

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tives for the prevention and treatment of the disease. The use of oxytetracycline is the only method practically applied for the treatment of columnaris disease in Finland, so there is a real threat of development of resistant strains. Therefore, physiological testing and genetic typing of *F. columnare* isolates is of great importance for understanding the increasing severity of outbreaks and for designing disease management strategies. Tolerance to salinity is of special interest, since columnaris disease has not been reported from coastal areasof Finland, where the salinity of the brackish water is between 2 and 7‰. However, *F. columnare* Type Strain NCIMB 2248^T (National Collection of Industrial, Marine, and Food Bacteria) is known to grow in media with 0.5% NaCl (Bernardet & Grimont 1989).

In the present study, we investigated 30 Finnish *Flavobacterium columnare* isolates obtained from disease outbreaks in northern and central Finland, in order to select representative strains for further analysis. The molecular diversity of these strains was studied using 16S rDNA RFLP, LH-PCR (length heterogeneity analysis of polymerase chain reaction products, Suzuki et al. 1998), and ARISA (automated ribosomal intergenic spacer analysis, Fisher & Triplett 1999). Using this information, 8 genetically different strains were selected for the physiological analysis. The growth patterns of 8 selected strains were studied under different temperatures, salt concentrations, and pH, as well as the susceptibility of the strains to various antibiotics, to gather information for developing potential disease management strategies.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. Thirty *Flavobacterium columnare* strains isolated from disease outbreaks at Finnish fish farms from 1993 to 2003 were obtained for this study (Table 1). The strains were stored frozen at –70°C in enriched AO-broth (Bernardet & Keroault 1989) supplemented with 10% glycerol and 10% bovine serum. For DNA extraction and growth measurements, the strains were grown in AO-broth for 24 h with constant agitation (120 rpm). For extraction of DNA, the cells were lysed with Proteinase K, and the DNA was purified by phenol-chloroform-isoamyl alcohol extractions and precipitated with NaCl and isopropanol, as described previously (Tiirola et al. 2003).

LH-PCR and ARISA. The partial 16S rRNA gene was amplified using the universal bacterial primers fD1 with ird700 label and Prun518 (Table 2), yielding a PCR product of 517 bp. The reaction mixture contained 0.3 mM dNTPs, 0.3 µM of each primer, 1 × DynaZyme buffer, and 2 U DynaZyme II DNA-polymerase (Finnzymes, Espoo, Finland). The total reaction volume in each PCR tube was 40 µl, with 1 µl of template DNA (20 to 50 ng). The PCR included an initial denaturation step at 95°C for 5 min, 30 cycles of amplification (94°C for 30 s, 55°C for 60 s, 72°C for 180 s), and a final extension step at 72°C for 15 min. For ARISA, the univer-

Table 1. *Flavobacterium columnare.* Strains used in this study. Information regarding the year of isolation, fish farm, and fish species is given for each isolate. *Strains studied for their 16S rDNA sequence, growth characteristics, and antibiotic susceptibility

Isolate/ strain code	Fish farm	Fish species	Watercourse	ARISA group
NCIMB 2248^T		Chinook salmon	Washington, USA	F*
3294/95	1[a]	Trout	River Oulujoki	A*
10819/96	1[a]	Arctic charr	River Oulujoki	A
8128/97	1[a]	Arctic charr	River Oulujoki	B*
3147/98	1[a]	Trout	River Oulujoki	A
1277/99	1[a]	Trout	River Oulujoki	A
1179/01	1[a]	Brown trout	River Oulujoki	A
2390/02	1[a]	Salmon	River Oulujoki	A
8239/97	2[a]	Rainbow trout	River Iijoki	C*
9528/97	3[a]	Rainbow trout	River Torniojoki	C
1991/94	4[a]	Rainbow trout	River Oulujoki	A
1468/99	5[a]	Brook trout	River Oulujoki	A
1199/00	6[a]	Rainbow trout	River Kymijoki	A
1397/00	7[a]	Rainbow trout	River Oulujoki	D*
1820/02	8[a]	Rainbow trout	River Vienan Kemijoki	A
2287/02	9[a]	Trout	River Lestijoki	A
2559/93	10[a]	Salmon	River Simojoki	A
Ke/02	11[b]	Salmon	River Kemijoki	E*
Mo/02	12[b]	Salmon	River Oulujoki	A
Ke/03	13[b]	Salmon	River Kemijoki	C
Os/03	14[b]	Salmon	River Kemijoki	G*
Ra/03	15[b]	Salmon	River Iijoki	E
Htan4/03	16[c]	Rainbow trout	River Kymijoki	A
Htan5/03	16[c]	Rainbow trout	River Kymijoki	H*
Htan6/03	16[c]	Rainbow trout	River Kymijoki	H
Mo/03	17[c]	Trout	River Oulujoki	E
Htku1/03	16[c]	Pikeperch	River Kymijoki	H
Htku2/03	16[c]	Pikeperch	River Kymijoki	E
Lauh/03	18[c]	Grayling	River Kymijoki	A
BA1972/03	19[a]	Rainbow trout	River Oulujoki	A
FK2/03	11[b]	Salmon	River Kemijoki	E

[a]Isolated by the National Veterinary and Food Research Institute, Oulu regional unit
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[c]Isolated by L.-R. Suomalainen, University of Jyväskylä

Table 2. *Flavobacterium columnare*. Primers for the polymerase chain reaction used in genetic analysis of Finnish isolates

Primer	Direction	Sequence	Source
fD1	Forward	5′-AGA GTT TGA TCC TGG CTC AG-3′	Weisburg et al. (1991)
rD1f	Forward	5′-GGC TGG ATC ACC TCC TT-3′	Weisburg et al. (1991)
23Sr	Reverse	5′-GGG TTB CCC CAT TCR G-3′	Borneman & Triplet (1997)
Prun518	Reverse	5′-ATT ACC GCG GCT GCT GG-3′	Muyzer et al. (1993)
Com2Ph	Forward	5′-AAA CTC AAA GGA ATT GAC GG-3′	Schwieger & Tebbe (1998)
FvpF1	Forward	5′-GCC CAG AGA AAT TTG GAT-3′	Bader et al. (2003)
FvpR1	Reverse	5′-TGC GAT TAC TAG CGA ATC C-3′	Bader et al. (2003)

sal primers rD1forward and 23Sr (with ird700 label) were used (Table 2), amplifying a fragment of approximately 700 bp. The reaction mixture was as described above. PCR included an initial denaturation step at 94°C for 2 min, 35 cycles of amplification (94°C for 30 s, 52°C for 30 s, 72°C for 30 s), and final elongation for 15 min. The LH-PCR and ARISA were performed with a LI-COR 4200 automatic sequencer (LI-COR BioTech) in a 6% Long Ranger denaturating polyacrylamide gel (FMC Bioproducts). Quantity One software (Bio-Rad Laboratories) was used for analyzing the fragment-length data.

16S rDNA restriction analysis. The 16S rRNA gene of *Flavobacterium columnare* was amplified with the primers fD1 and rD1. Then, 10 µl of PCR product was digested with the restriction enzymes *Msp*I and *Hae*III (MBI Fermentas). The digested samples were electrophoresed in a 12% native polyacrylamide gel electrophoresis system (Sambrook et al. 1989), followed by ethidium-bromide staining and UV-illumination.

Cloning and sequence analysis. Nearly full length 16S rDNA of 7 selected strains representing different ARISA genotypes were sequenced in 3 overlapping segments. The gene was amplified with 3 primer sets (primers fD1/Prun518, same protocol as in LH-PCR; Table 2). For primer pairs FvpF1/FvpR1 and Com2Ph/FvpR1 (Table 2), PCR amplification included an initial denaturation step at 94°C for 10 min, 30 cycles of amplification (95°C for 30 s, 59°C for 30 s, 72°C for 60 s), and the final extension step at 72°C for 6 min. The PCR products were cloned into TA-vector pGEM-T-easy (Promega) and electroporated into *Escherichia coli* JM109 cells. Plasmids were extracted with a Sigma GenElute kit and sequenced bidirectionally with ird-labelled primers with Excel SequiTherm Kit II (Epicentre Technologies) using a LI-COR 4200 sequencer. The 16S rDNA sequences obtained from the *Flavobacterium columnare* strains were submitted to the EMBL (European Molecular Biology Laboratory) database under Accession Numbers AJ831824 to AJ831830. The sequences were analyzed using AlignIR software (LI-COR). Phylogenetic trees were created by a neighbor-joining method using Clustal X software. Alignment positions with gaps were not included in the calculations. The topology of the tree was evaluated by the bootstrap resampling method with 1000 replicates.

Growth analysis. The growth of selected *Flavobacterium columnare* strains and the type strain was tested at different temperatures, pH values, and salt concentrations. The strains were first cultivated in 10 ml of AO-broth (pH 7.0) at 22°C, with constant agitation (150 rpm) until the culture reached 50% saturation (50% of the maximum optical density value measured at 595 nm wavelength). The effect of temperature on the growth rate was studied at 17.0, 19.0, 22.0, 25.0, and 27.4°C by adding 5 µl of bacterial culture to 250 µl of AO-broth on a microtiter plate. The effect of salt and pH was studied similarly at 22°C, but the AO-broth was supplemented with 0, 0.01, 0.02, 0.04, 0.06, 0.08, and 0.1% of NaCl or adjusted to pH 6.2, 6.5, 6.8, 7.4, 8.0, 8.6, and 9.2 (pH adjusted with HCl and NaOH), respectively. Triplicate samples were analyzed in all the experiments. The measurements of optical density at 595 nm were continued until cultures reached 30 to 50% of saturation. Differences in the growth of Finnish strains were analyzed using SPSS 11.0 software and non-parametric Kruskal-Wallis tests.

Antibiotic susceptibility tests. Susceptibility of selected Finnish *Flavobacterium columnare* strains and the Type Strain NCIMB 2248^T to antimicrobial agents was tested as described by Michel et al. (2002) using AO agar. The diameters of the inhibition zones were measured after 2 d at 21 ± 1°C. The strains were divided into 3 categories: susceptible (S), intermediate (I), or resistant (R) (NCCLS 1999). Ampicillin (≥17 mm / 14–16 mm / 13 mm), erythromycin (23 / 14–22 / 12), gentamycin (15 / 13–14 / 12), nitrofurantoi (17 / 15–16 / 14), neomycin (17 / 13–16 / 12), Polymyxin B (12 / 9–11 / 8), streptomycin (15 / 12–14 / 11), tetracycline (19 / 15–18 / 10), trimethoprim-sulpha (16 / 11–15 / 10), and florfenicol (24 / 21–23 / 20) were tested (breakpoints of S/I/R for each antibiotic given in parentheses). Commercial discs of Oxoid were used, except for florfenicol, for which Neo-Sensitabs were used (A/S Rosco).

RESULTS

All *Flavobacterium columnare* strains yielded a 517 bp PCR product in the LH-PCR analysis. Eight different ARISA profiles were observed, 3 of which (A, B, and D) were closely similar (Fig. 1). Even though it was not known if the differing bands in Groups B and D were amplified from the ISR region, these extra bands were used to group the isolates and select the strains for further studies, because the same amplification profiles were detected when the PCR was repeated. In the 16S rDNA digestion analysis, no differences between strains were detected. The Type Strain NCIMB 2248^T had a restriction profile similar to all the Finnish isolates in both digestions (not shown). This 16S RFLP digestion profile corresponded to Genomovar I in the classification created by Triyanto & Wakabayashi (1999). In the 16S rDNA sequencing, a 1329 bp continuous sequence was assembled from a representative of each ARISA group (marked with asterisks in Table 1). In the neighbor-joining tree, the Finnish *F. columnare* strains (Sequences AJ831824 to AJ831830) constituted a separate, highly homologous group, differing from strains isolated from other parts of the world (Fig. 2), but clustering with the sequence obtained from the EMBL (European Molecular Biology Laboratory) database as *F. columnare* Strain IFO 15943^T (NCIMB 2248^T).

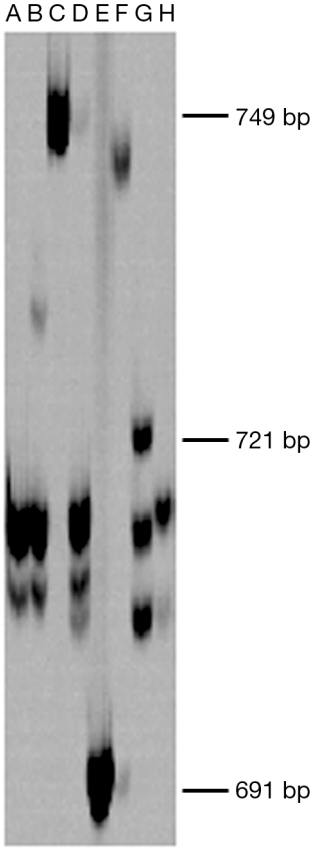

Fig. 1. *Flavobacterium columnare*. Automated ribosomal intergenic spacer analysis (ARISA) patterns (ARISA Groups A to H) of the Finnish *F. columnare* isolates and *F. columnare* NCIMB 2248^T (corresponding strain codes are given in Table 1), with a calculatory DNA ladder

Temperature, salinity, and pH had significant effects on the growth of Finnish *Flavobacterium columnare* strains (Kruskal-Wallis test, $\chi^2 = 72.7$, df = 4, p = 0.000 for temperature; $\chi^2 = 68.9$, df = 4, p = 0.000 for pH; and $\chi^2 = 74.7$, df = 6, p = 0.000 for salinity) (Fig. 3). The Finnish strains were able to grow at pH values from 6.8 to 9.2, but growth was greatly inhibited at pH 6.2 to 6.5 (Fig. 3B). Growth decreased as salt concentration increased (Fig. 3C), and only the Group A strain showed any growth at a salt concentration of 0.1%. However, neither 0.1% salt concentration nor pH 6.5 inhibited the growth of the Type Strain NCIMB 2248^T. Differences in growth between strains were detected at different temperatures (Kruskal-Wallis test, $\chi^2 = 21.4$, df = 6, $p < 0.01$) and salinities (Kruskal-Wallis test, $\chi^2 = 17.3$, df = 6, $p < 0.05$).

The 8 selected *Flavobacterium columnare* strains were sensitive to all antimicrobial agents tested, except neomycin and Polymyxin B. Neomycin showed ISR Category I in the case of ARISA Group A, E, G, and H strains. For Polymyxin B, ARISA Group A showed Category I and Group C, E, G, and H strains were resistant.

DISCUSSION

The 16S rRNA gene is widely used in phylogenetic analysis and in typing bacterial strains due to its highly conserved nature. The ISR between the 16S and 23S rRNA changes more rapidly (Leblond-Bourget et al. 1996), and can be used for genotypic analysis at the intraspecies level. ARISA has, indeed, been shown to be a useful typing method for bacterial identification based on the observation that considerable length heterogeneity is apparent when the 16S to 23S rDNA spacer region is amplified from strains carrying multiple rRNA operons (Gurtler & Stanisich 1996). In the present study we used ARISA classification to identify genetically different strains for further analyses. We were able to expand the division of Finnish *Flavobacterium columnare* isolates, all belonging to Genomovar I, into 7 genetically different subgroups, as *F. columnare* NCIMB 2248^T represented the 8th subgroup. In the study of Arias et al. (2004), sequence analysis of ISR detected 4 genomic groups belonging to Genomovars I and II among isolates from USA and Brazil. However, the number of different ISR sequences present in each group was not defined. If the presence of multiple sequences is not taken into consideration, phylogenetic analysis solely based on sequence data may give contradicting results, because it is not known which copy of the ISR gene is amplified. Recently Darwish & Ismaiel (2005) designed a primer

set for amplification of the ISR region, and RFLP analysis of the PCR product revealed a subdivision of Genomovars I and II into 2 groups each. Multiple ISR sequences do not hamper RFLP analyses or ARISA analyses, since the results are repeatable.

Most Finnish *Flavobacterium columnare* isolates fell into Group A in ARISA. Interestingly, the Strains Htku1/03, Htku2/03, Htan4/03, Htan5/03, and Htan6/03 (belonging to 3 subgroups in the ARISA division) were isolated from 1 disease outbreak. The presence of several genotypes in disease outbreaks has previously been documented for *F. columnare* by Triyanto et al. (1999) and for *F. psychrophilum* by Madetoja et al. (2001). The isolation of several genetic groups from the same outbreak in this paper was due to the improved cultivation method used in isolation (dilution cultivation) and a thorough screening of the isolates during the outbreak. We suggest that the coexistence of different genetic groups in the same infection is a common phenomenon in columnaris disease, because *F. columnare* is ubiquitous in aquatic environments (Rickard et al. 2003, Revetta et al. 2005). The phenomenon can contribute to the continuous nature of outbreaks during summer or to increased virulence (e.g. Gandon et al. 2001, Read & Taylor 2001). Indeed, differences in virulence have been demonstrated in

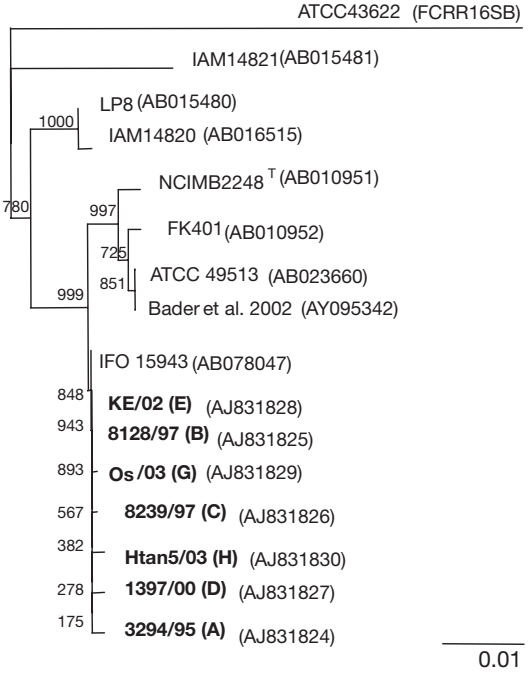

Fig. 2. *Flavobacterium columnare.* Phylogenetic tree based on neighbor-joining analysis of the 16S rDNA sequence data obtained from the representatives of Finnish *F. columnare* ARISA Groups A to H (bold type) and other *F. columnare* sequences obtained from the European Molecular Biology Laboratory database (EMBL accession numbers given in parentheses)

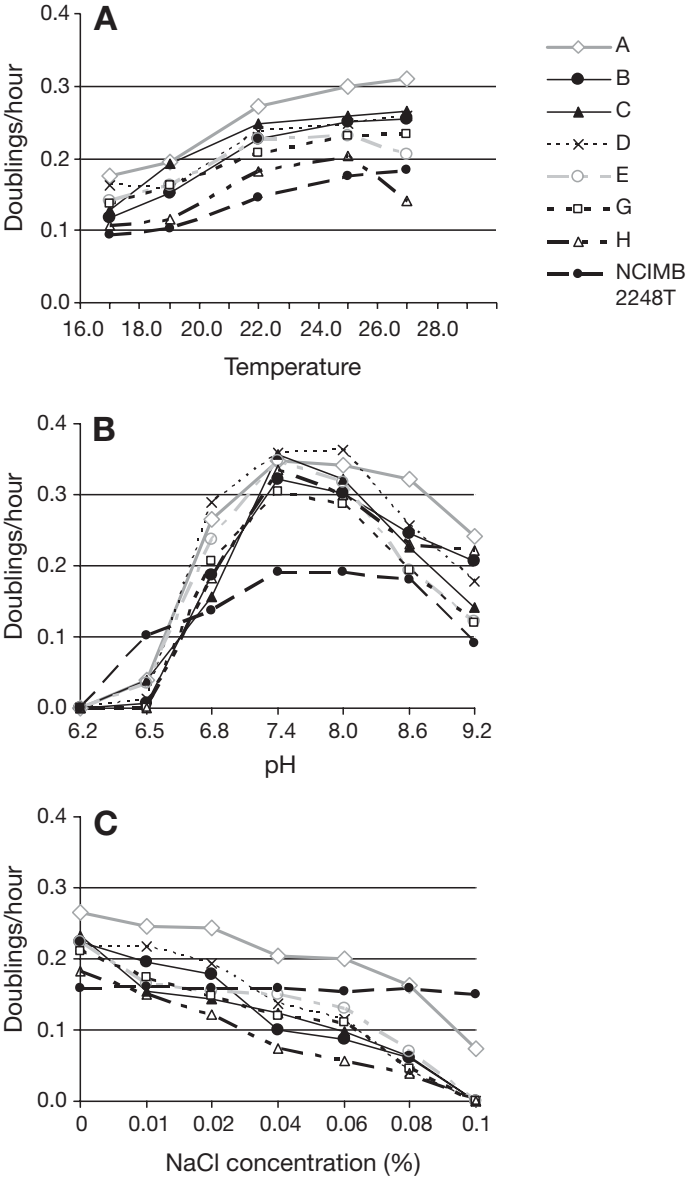

Fig. 3. *Flavobacterium columnare.* Maximum growth rates (doublings h^{-1}) of 7 selected Finnish *F. columnare* isolates and the *F. columnare* Type Strain NCIMB 2248^T (ARISA Groups A to H, Table 1) under different (A) temperatures, (B) pH values, and (C) NaCl concentrations. Corresponding strain codes are given in Table 1

F. columnare (Decostere et al. 1998). However, more detailed surveys of individual outbreaks should be made to obtain additional information on the characteristics of the disease, because the factors influencing the bacterial communities behind the outbreaks may be more complicated than has previously been thought.

Restriction analysis of the 16S rRNA gene yielded identical results from all the strains studied, including the type strain. Triyanto & Wakabayashi (1999) used a

similar 16S rDNA digestion analysis to Type 23 *Flavobacterium columnare* isolates from various areas. They found 3 different genomic groups within the species. When compared to their results, the Finnish isolates belonged to Genomic Group I, along with the Type Strain NCIMB 2248^T. According to classification based on 16S rDNA digestion analysis, all European *F. columnare* strains so far studied fall into Genomovar I (Triyanto & Wakabayashi 1999, Michel et al. 2002). All the studied *F. columnare* strains also yielded a 517 bp fragment in the LH-PCR analysis, showing that this length may be characteristic for the pathogen.

In the phylogenetic tree constructed from the sequence data, the Finnish strains fell into a distinct cluster, separate from the other *Flavobacterium columnare* strains except IFO 15943^T. This IFO 15943^T sequence obtained from the EMBL database should, however, be identical to the NCIMB 2248^T sequence, since it was derived from the same strain (Nakagawa et al. 2002). The difference between these 2 sequences is most likely due to sequencing errors. Recently, *F. columnare* Strain ATCC 43622 was identified and renamed as *F. johnsoniae* (Darwish et al. 2004). Because previous authors have used this strain in phylogenetic analyses (Toyama et al. 1996, Bader & Shotts 1998a,b), we included it in our sequence analysis even though it represents a different species.

Growth of the Finnish *Flavobacterium columnare* strains was significantly influenced by temperature, increasing from 0.1–0.2 to 0.2–0.3 doublings h^{-1} when the temperature increased from 17 to 25 °C. Other studies have reported that some *F. columnare* strains can grow at temperatures >30 °C, and even at +37 °C (Bernardet 1989, Decostere et al. 1998). Although the growth of the bacteria increases at elevated temperatures, this is not the only factor increasing fish mortality at high temperatures during columnaris infection. At higher temperatures the transmission of the pathogen and mortality caused by the disease are higher (Suomalainen et al. 2005a), and the adhesion of the pathogen in fish tissues has also been shown to increase at elevated temperatures (Decostere et al. 1999).

The Ministry of Agriculture and Forestry in Finland recommends tetracycline for treatment of columnaris disease, but the use of florfenicol is accepted as an alternative antibiotic if tetracycline-resistant strains should occur. Even though the Finnish *Flavobacterium columnare* strains seem to be sensitive to tetracycline at the moment, the development of resistance can happen. As seen in this study, several other antibiotics are efficient inhibitors of *F. columnare* growth. Due to the external nature of the infection (Bernardet 1997), salt or acidic bathing could have a potential in prevention and treatment of *F. columnare* infections in ecological disease management. Altinok & Grizzle (2001) found that rearing fish in salinities between 3 and 9‰ inhibited *F. columnare* infection, and columnaris disease has not been reported to occur in seawater or in brackish water areas around Finland (salinity from 2 to 7‰), reflecting the poor growth ability evan at even low salt concentrations. However, despite the promising results *in vitro*, the success of acidic or salt bathing was shown to be limited during an experimental infection in another study (Suomalainen et al. 2005b). Therefore, antibiotic medication is still the most efficient method known for treatment of columnaris disease in Finnish aquaculture.

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