Diagnosis of flavobacteriosis by direct amplification of rRNA genes

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ABSTRACT: A broad-range bacterial PCR method with universal 16S rDNA targeting primers and bacterial cultivation was used to identify the putative pathogen in flavobacterial outbreaks. Restriction fragment length polymorphism (PCR-RFLP) analysis and sequencing of the partial 16S rDNA PCR products of 10 skin samples and 10 representative isolates derived from the same fish specimens revealed differences between direct molecular and cultivation-based analysis. *Flavobacterium columnare*-like sequences dominated in the direct molecular analysis in most cases, whereas most of the isolates belonged to a phylogenetically heterogeneous group of flavobacteria clustering with *F. hibernum. F. columnare* was isolated in only 1 outbreak. The possible explanations for the different results may be attributable to difficulties in the plate cultivation procedure of external flavobacterial samples. During plate cultivation, the dominating *Flavobacterium* species can be masked by saprophytic species of the same genus or other genera, or the growth of flavobacteria can be completely inhibited by antagonistic bacteria such as *Pseudomonas*. Direct analysis of the prevailing 16S rDNA sequences avoids the problems with cultivation and may thus be preferable for the diagnosis of flavobacterial diseases. When isolating flavobacteria from external samples, serial dilution of the sample before plating can improve the results.

KEY WORDS: Broad-range PCR amplification · *Flavobacterium* · Identification · Antagonism

INTRODUCTION

Diseases associated with *Flavobacterium* spp. cause worldwide economic losses in fish-farming. During the 1990s these diseases were also diagnosed in salmonid fish-farming in Finland (Koski et al. 1993, Wiklund et al. 1994, Rintamäki-Kinnunen et al. 1997). Accurate and prompt identification of the suspected fish pathogen can ensure correct treatment and provide a valid basis for effective management decisions. However, the primary isolation of flavobacteria is problematic in many cases and has long impeded investigation of the pathogenesis of *Flavobacterium* species (Anderson & Norton 1991, Dalsgaard 1993, Shotts & Starliper

1999). Flavobacteria are considered ubiquitous in the fish-farm environment (Austin & Austin 1987). As the disease first affects external tissues, the causative agent may be difficult to separate from the natural microbial fauna of the fish skin.

Broad-range PCR amplification of 16S rDNA with universal bacterial primers and subsequent sequence-analysis of cloned products is a widely used method in molecular microbial ecology. The sequences can enable the identification of microbes to the genus or species level (Fox et al. 1980, Woese 1987). Successful trials using PCR amplification and direct sequencing from DNA of tissue samples have also been undertaken in human clinical diagnostics (e.g. Wilson et al. 1990, Chen et al. 1994, Rantakokko-Jalava et al. 2000). A large-scale study of over 500 human clinical samples in the mid-1990s showed that this approach was supe-

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rior to bacterial culture in some cases, but it was rather slow and labour-intensive at that time (Rantakokko-Jalava et al. 2000). Nowadays, as more advanced sequencing techniques have become available, broad-range PCR amplification combined with direct sequence-analysis may offer a fast, cost-effective and accurate identification method in the analysis of bacterial diseases. However, as far as we know, the approach has not previously been applied in fish disease diagnostics.

The aim of this study was to evaluate the broad-range bacterial PCR technique in the diagnosis of flavobacteria from fish skin samples in comparison with the plate cultivation procedure. The analyses were performed on duplicate samples from the same fish ulcers. The results show that the 2 methods often differ in their outcome. After bacterial cultivation with the classical plate-streaking method, the growth of the flavobacterium that showed dominance in the molecular analysis was masked by non-flavobacterial species or a group of flavobacteria that clustered phylogenetically with *Flavobacterium hibernum*. Bacterial antagonism in plate cultures is also presented and discussed in connection with bacterial isolation.

MATERIALS AND METHODS

Fish samples. Flavobacterial outbreaks were studied in samples from 4 Finnish freshwater fish farms in central (Farm A) and northern Finland (Farms B, C and E) producing Atlantic salmon *Salmo salar* L. and rainbow trout *Oncorhynchus mykiss*, and from the research station of the University of Jyväskylä in central Finland (Farm D) culturing rainbow trout. Flavobacteriosis was characterised by erosion of fish external tissues (especially gills, fins, jaws and tails) with characteristic symptoms described by Rintamäki-Kinnunen et al. (1997). Samples were collected from outbreaks in August 2000, except for Samples D3 and D4, which were collected during January 2001. All the farms use surface water obtained from rivers and lakes above the farms. In August 2000, water temperatures varied between 17 and 20°C, and in January 2001 at Farm D the fishes had been acclimated from 1–2°C to 12°C when the disease broke out. Intensive treatment with oxytetracycline was started at Farms B and D after the disease samples had been collected. The mortality rate of the fishes remained at about 0.1 to 2% during the outbreaks. For sampling, live fishes with disease symptoms were carried in the tank water into the laboratory at the farm. They were killed by cutting their spinal cord, and sampling was performed immediately for native Gram-staining, cultivation and DNA analysis from the same ulcers. Time from collec-

tion to sampling was about 5 to 10 min. Samples were taken from the most common sites of ulcers in each outbreak (see Table 1). The sizes of the ulcers were variable (max. = 2 cm). Samples for Gram-staining and cultivation were collected with a loop, and parallel DNA samples (10 to 30 μl) were collected with a sterile scalpel. DNA samples were frozen immediately and stored at –20°C. Gram-stained samples were examined microscopically for Gram-negative filamentous rods to confirm flavobacteriosis. Isolated strains and sequences of these isolates have been marked with the prefix 'Iso' (IsoA1, IsoA2, etc.) to distinguish them from the tissue samples (A1, A2, etc.).

Bacterial strains. The strains *Flavobacterium columnare* NCIMB 2248[T] and *F. psychrophilum* NCIMB 1947[T] were kindly provided by Perttu Koski (National Veterinary and Food Research Institute, Oulu, Finland) and Jari Madetoja (Åbo Academy University, Turku, Finland), respectively. *Yersinia ruckeri* TS1 was isolated from rainbow trout (Valtonen et al. 1992). *Sporocytophaga myxococcoides* DSM 11118[T] and '*Cytophaga*' sp. DSM 3656 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Other strains used in the agar antagonism assay were obtained from HAMBI (Culture Collection of the Department of Applied Chemistry and Microbiology, Helsinki, Finland).

Bacterial cultivation and physiological tests. All the bacterial cultivations were performed at room temperature (22 ± 1°C). The tissue samples were cultivated immediately after sampling using a plate-streaking procedure on AO agar (Anacker & Ordal 1959). The plates were incubated at room temperature for 2 to 7 d before the predominant yellow-pigmented filamentous bacteria were subcultivated on an AO agar plate. However, Tissue Samples D1, D2, D3 and D4 (~20 μl) were suspended in 5 ml of R2A medium (Reasoner & Geldreich 1985) with vigorous shaking. These samples were then diluted immediately by 10-fold increments and plated on AO and R2A agar plates (200 μl on each plate). The predominant colony type from the highest growing dilution was subcultivated after 7 d of incubation. Spheroplast formation (Reichardt & Morita 1982) of flavobacteria was examined from cells grown for 2 wk in R2A medium (without agitation) by staining the samples with ethidium bromide (50 μg ml^{–1} final concentration) and then inspecting them under a Leica DMR BE fluorescence microscope. Other phenotypic tests were studied as described by Bernardet & Grimont (1989).

DNA extraction. Bacterial cells were cultivated in R2A or AO medium for 2 d. DNA was extracted by lysing the cells with Proteinase K, and were then purified by phenol-chloroform extraction and isopropanol

precipitation as described by Männistö et al. (1999). Before organic extraction, cell lysis in tissue samples was ensured by bead-milling in a cell homogenizer (Mikro-U Dismembrator, B. Braun Biotech International) with 1 wt/vol of glass beads (diameter 0.1 mm) at 1600 rpm for 3 min. The nucleic acid preparation was finally suspended in water to a total volume of 50 µl.

Amplification of partial 16S rDNA sequences. The universal eubacterial primers 27F (5′-AGAGTTTGATC MTGGCTCAG-3′; Lane 1991) and Com2-Ph (5′-CCGT CAATTCCTTTGAGTTT-3′; Schwieger & Tebbe 1998) were used to amplify a fragment of 16S rDNA approximately 900 bp in length. In the PCR reactions, 1 µl of purified DNA was used as a template in a 50 µl PCR mixture containing 0.2 mM of dNTPs, 0.3 µM of each primer, 1 × DynaZyme reaction buffer and 2 U Dyna-Zyme F501-KL polymerase (Finnzymes). The PCR procedure included an initial denaturation step at 95°C for 5 min and 30 cycles of amplification (94°C for 30 s, 55°C for 1 min and 72°C for 3 min).

RFLP analysis of 16S rDNA. Approximately 1 µg of the PCR product was digested for 2 h with restriction enzymes *Hae*III and *Hinf*I (double-digestion, 5 U of each) (Promega). Digested PCR products were analysed by electrophoresis in a 12% polyacrylamide gel. The gel was stained for 15 min with 0.5 µg ml^{-1} ethidium bromide solution, and documented using the GelDoc2000 digital-imaging system (Bio-Rad Laboratories).

Sequencing and sequence-analysis of 16S rDNA. Subsequent sequencing of the PCR-amplified 16S rDNA product was carried out for 10 tissue samples and 10 isolates from the parallel samples. Sequencing reactions were performed using an ABI BigDye kit (Applied Biosystems) following the protocol recommended by the manufacturer, and the products were analysed using an ABI 310 DNA sequencer. Both strands of the DNA were sequenced using Primers 27F and PRUN518 (5′-ATTACCGCGGCTGCTGG-3′; Muyzer et al. 1993) for tissue samples, or 27F and Com2-Ph for bacterial isolates. Sequences were compared with the database using the BLAST software program (Altschul et al. 1997). Sequence data were compiled and a distance matrix tree was constructed by the neighbour-joining method (Saitou & Nei 1987). The topology of the tree was evaluated by the bootstrap resampling method (Felsenstein 1985) with 1000 replicates. Positions with gaps were excluded from this analysis, which was carried out using the ClustalX program (Thompson et al. 1997). The 16S rDNA sequences obtained in this study from tissue samples and isolated bacteria have been deposited in the European Molecular Biology Laboratory (EMBL) database under Accession Nos. AJ319005 to AJ319025. The 16S

rDNA gene sequence for *Sporocytophaga myxococcoides* DSM 11118^T is deposited under Accession No. AJ319026.

Agar antagonism assay. *Pseudomonas* sp. MT5 was isolated from Sample D4. Screening of the antagonism by this strain was performed with a plate assay. In the test, 50 µl of the test bacterial suspensions precultured in R2A medium for 2 d were plated onto AO and R2A plates. The plate surface was then punctured several times with a micropipette tip that had first been dipped into the suspension of fresh *Pseudomonas* sp. MT5 cultivated in R2A medium. The plates were incubated for 4 to 7 d until a confluent growth was seen on the control plates without *Pseudomonas* sp. MT5 inoculation. The inhibitory effect of *Pseudomonas* sp. MT5 was observed by looking for zones of clearing around the growth of *Pseudomonas* sp. MT5. The test strains were Flavobacterial Isolates IsoA1, IsoB1, IsoD1 and IsoD3, and Strains *Flavobacterium columnare* NCMB 2248^T and *F. psychrophilum* NCMB 1947^T. Several other strains were also tested, namely *Escherichia coli* DSM 682, '*Cytophaga*' sp. DSM 3656, *Yersinia ruckeri* TS1, *Bacillus pabuli* DSM 3036, *B. mycoides* DSM 2048 and *Rhodococcus erythropolis* DSM 43135. These strains were chosen as they represented several bacterial Gram-negative and Gram-positive groups and were available in our laboratory.

RESULTS

Analysis of putative flavobacterial pathogens by broad-range bacterial PCR and bacterial isolation

The direct microscopic examination of 27 bacterial samples collected from skin ulcers showed dominance of thin Gram-negative flavobacteria-like rods. However, yellow-pigmented filamentous bacteria were isolated from only 15 tissue samples (Table 1). When the parallel samples were studied with the direct PCR procedure, a 16S rDNA PCR-product was obtained from 18 samples. In the remainder of the samples, the PCR technique was unsuccessful despite several trials. Flavobacterial isolates were obtained from 5 PCR-negative samples, and flavobacterial isolation was not successful from 8 PCR-positive samples; 10 samples were positive and 4 samples were negative in both the analyses (bacterial PCR and flavobacterial isolation). PCR products of the 10 tissue samples and corresponding isolates (marked with the prefix 'Iso') were subjected to RFLP analysis and sequencing of the 16S rDNA. PCR-RFLP was used in preliminary analysis to examine product homogeneity and the possibility of direct sequencing. Double-digestion of the PCR products with restriction enzymes *Hae*III and *Hinf*I yielded

Table 1. Success of flavobacterial cultivation and broad-range bacterial PCR assay from 27 fish skin and tissue samples collected from flavobacterial outbreaks on 5 fish farms (A–E) in August 2000. +: flavobacteria was isolated from samples or PCR product was obtained from direct amplifications; –: no flavobacteria isolated or no PCR product obtained. Sample code is shown when used in sequence analysis. [a]Samples collected January 2001

Farm	Fish species and age (yr)	Fish	Site	Cultivation of flavobacteria	Direct PCR amplification
A	*Oncorhynchus mykiss* (0+)	1	Gills	+ IsoA1	+ A1
		1	Skin lesion	+	–
		2	Gills	–	+
		3	Tail	–	+
		3	Pectoral fin	+ IsoA2	+ A2
		4	Dorsal fin	–	+
		5	Ventral fin	+	–
		6	Dorsal fin	+	–
B	*Salmo salar* L. (1+)	7	Gills	–	+
		8	Gills	–	–
		9	Gills	+	–
		10	Gills	+	–
		11	Gills	+ IsoB1	+ B1
		12	Gills	–	+
		13	Gills	–	+
		14	Gills	–	–
C	*Salmo salar* L. (1+)	15	Gills	–	+
		16	Jaw	+ IsoC1	+ C1
		17	Tail	+ IsoC2	+ C2
		18	Tail	–	+
D	*Oncorhynchus mykiss* (0+)	19	Tail	+ IsoD1	+ D1
		20	Tail	+ IsoD2	+ D2
		21[a]	Jaw	+ IsoD3	+ D3
		22[a]	Jaw	+ IsoD4	+ D4
E	*Salmo salar* L. (1+)	23	Jaw	–	–
		24	Jaw	+ IsoE1	+ E1
		25	Jaw	–	–

2 clear RFLP profiles from the samples, and some profiles from tissue samples and corresponding isolates did not match (Samples A2, C2 and D2; Fig. 1). Sequence-analysis of the partial 16S rDNA showed that *Flavobacterium columnare*-like sequences dominated in 6 of the 8 late-summer samples (Table 2). These samples were derived from all 5 fish farms studied and from different tissues (gills, jaws, fins and tails). The sequences were clear, thus showing that the PCR products were mostly homogeneous in each tissue sample.

The *Flavobacterium columnare*-like bacterium (IsoD1) was cultivated from only 1 sample (Fig. 2), and no rhizoid colonies that could belong to *F. columnare* were seen in other plate cultivations during the primary isolation procedure. It is noteworthy that the primary cultivation of Sample D1 was performed by plating the serially diluted sample. Isolate IsoD1 had a close sequence identity to the type strain of *F. columnare* (98.8% sequence identity in 807 bp overlap). The sequences from Cold-water Samples D3 and D4 from the jaws of rainbow trout were very similar to that of *F. psychrophilum*, as were Bacterial Isolates IsoD3 and IsoD4. When the 16S rDNA sequences (approximately 700 bp long) of 7 other isolates were compared with the EMBL sequence database, it was clear that the isolates were members of the genus *Flavobacterium* and
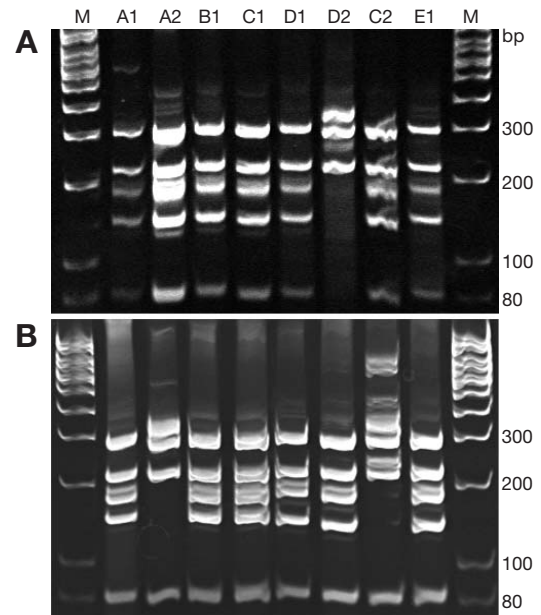

Fig. 1. PCR-RFLP patterns of the 900 bp PCR product of partial 16S rDNA genes (A) from fish tissue samples and (B) from the corresponding flavobacterial isolates. The samples were digested with restriction enzymes *Hae*III and *Hinf*I (double-digestion). Lane M is a molecular weight marker. The 2 samples from the cold-water period (Samples D3 and D4 and corresponding Isolates IsoD3 and IsoD4) yielded the same restriction enzyme patterns as Sample D2

Table 2. Phylogenetic positions of partial 16S rDNA sequences directly amplified from fish samples. Identity, Overlap: percentage identity with best match and length of overlapping sequence, respectively; [T]: type strain

Sample	Best match in EMBL database	Identity (%)	Overlap (bp)
A1	*Flavobacterium columnare* ATCC 49513	99.3	444
A2	'*Cytophaga*' sp. DSM 3657	98.0	442
B1	*F. columnare* ATCC 49513	99.8	444
C1	*F. columnare* ATCC 49513	99.8	444
C2	*F. columnare* ATCC 49513	99.3	444
D1	*F. columnare* ATCC 49513	99.3	444
D2	*F. psychrophilum* NCMB 1947[T]	100.0	401
D3	*F. psychrophilum* NCMB 1947[T]	98.3	459
D4	*F. psychrophilum* NCMB 1947[T]	98.7	462
E1	*F. columnare* ATCC 49513	99.8	444

clustered with *F. hibernum* and '*Cytophaga*' sp. DSM 3657 (earlier incorrectly described as the type species of *Sporocytophaga cauliformis* [Gräf & Stürzenhof-ecker 1964]). These 7 isolates shared similar phenotypic characteristics (colonies were flat, spreading, non-adherent, with filamentous margins on AO agar; colonies were yellow due to flexirubin-type pigments; gliding motility was active in broth medium; acid was produced aerobically from starch and esculin; cytochrome oxidase was produced; Congo-Red test was negative). '*Cytophaga*' sp. DSM 3657 (EMBL Accession No. M93151) was one of the closest relatives of Isolates IsoA1, IsoA2, IsoB1 and IsoC1 (96.1 to 97.6% sequence identity). Three other isolates, IsoC2, IsoD2 and IsoE1, were more closely related to *F. hibernum* ATCC 51468[T] (Accession No. L39067), with 94.8 to 96.1% sequence identity. All 10 isolates formed spheres (spheroplasts) with diameters of 1 to 10 µm after 1 to 2 wk cultivation in R2A, as revealed by fluorescence microscopy of cells stained with ethidium bromide.

Isolation and antagonism of *Pseudomonas* sp. MT5

Clear evidence of bacterial antagonism in the agar plate cultivation was obtained by plating serial dilutions of Sample D4. A plated aliquot corresponding to 100 nl of the original sample yielded 5 light-coloured non-flavobacterial colonies with a diameter of 4 to 8 mm on AO agar (Fig. 3A) after 1 wk incubation. The 10-fold dilution yielded several hundreds of flavobacterial colonies (Fig. 3B), and 100-fold dilution 60 flavobacterial colonies. The same phenomenon (an increase in colony number at higher dilutions) was also seen on parallel R2A plates, although the inhibition of flavobacteria

was not as complete, but mottled around the colonies of antagonistic bacteria. The antagonistic strain was isolated and characterised by sequencing and physiological testing. Identification of the strain with API 20E and API 50 CHE indicated that the strain had physiological characteristics of *Pseudomonas fluorescens* (data not shown). The partial 16S rDNA sequence (724 bp long) of the strain was most similar to the 16S rDNA sequence of *P. jessenii* type strain (98.6% sequence identity). The strain was designated *Pseudomonas* sp. MT5.

Pseudomonas sp. MT5 had an antagonist effect against a wide variety of Gram-positive and Gram-negative strains, inhibiting the growth (Fig. 3C) of all 12 strains tested on the AO plate except *Bacillus mycoides*. The number of plated bacterial cells and plate composition had some effect on the antagonism. The inhibition was stronger and the inhibitory zone was larger on AO agar than on R2A agar. On AO agar the zone of inhibition of flavobacterial strains varied
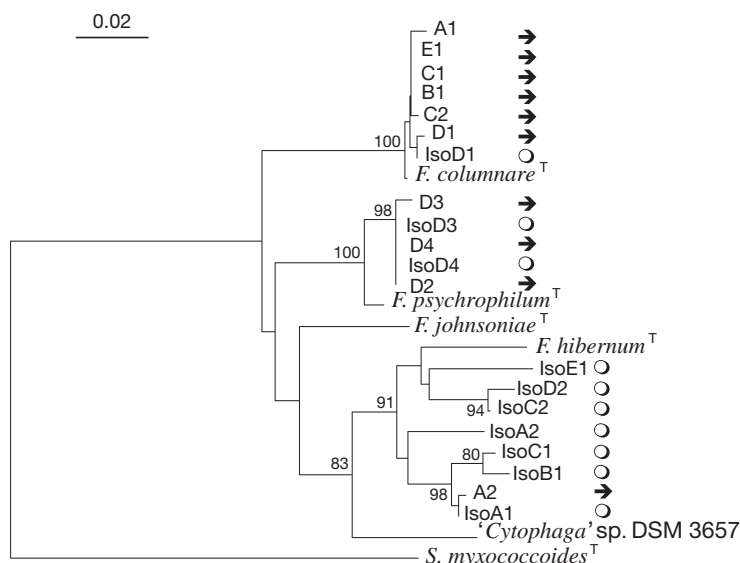

Fig. 2. Phylogenetic tree of 16S rDNA sequences from tissue samples (arrows) and from parallel flavobacterial isolates (circles) in comparison with flavobacterial reference strains and *Sporocytophaga myxococcoides*, which is included as an outgroup. The tree was calculated from 417 bp aligned positions using the neighbour-joining method. Reference sequences used for construction of the dendrogram were ABO10951 (*Flavobacterium columnare* NCMB 2248[T]), AY034478 (*F. psychrophilum* NCMB 1947[T]), L39067 (*F. hibernum* ATCC 51468[T]), M93151 ('*Cytophaga*' sp. DSM 3657), M59051 (*F. johnsoniae* ATCC 17061[T]) and AJ319026 (*Sporocytophaga myxococcoides* DSM 11118[T]) ([T] = type strain). Values above branch nodes are bootstrap percentages (only values greater than 75% are shown). Scale bar represents 2% estimated sequence divergence

Fig. 3. Effect of an antagonistic bacterium during the primary isolation of flavobacteria and in the agar plate assay. Dilution corresponding to 100 nl of Fish Tissue Sample D4 yielded 5 light-coloured non-flavobacterial colonies (A), but dilution corresponding to 10 nl of fish tissue sample yielded hundreds of yellow-pigmented flavobacterial colonies (B) on the AO agar plate. The antagonistic effect of the isolated strain *Pseudomonas* sp. MT5 was confirmed in the agar plate assay (C). Arrows indicate zone of growth inhibition of Strain IsoD4 around the points where *Pseudomonas* sp. was inoculated

from 5 mm to complete inhibition of bacterial growth on the plate area. Complete inhibition was not seen on R2A test plates. After plating serial dilutions of isolate IsoA1 on AO agar and inoculating the plates with *Pseudomonas* sp. MT5, complete inhibition of growth was observed at cell densities of 1.5×10^4 CFU flavobacterial cells plate^{-1}. On plates inoculated with 1.5×10^5 and 1.5×10^6 CFU of flavobacterial cells, the inhibitory zones were 0.6 to 2 and 0.2 to 1 cm in diameter, respectively.

DISCUSSION

This study showed that in flavobacterial diseases of salmonids a completely different picture of the putative skin pathogens can be obtained using plate cultivation compared to direct molecular analysis. Partial 16S rDNA sequences were used to determine and compare the identities of the dominant bacteria that were present in external ulcers of diseased fishes with bacteria that were cultured from the same samples. Direct 16S rDNA analysis showed that the dominating organisms in 9 of the 10 infected skin samples were closely related to 2 well-known pathogenic *Flavobacterium* species, *F. columnare* and *F. psychrophilum*, since the sequences obtained had 98 to 100% identity with the type strains of these species. For comparison, organisms sharing 16S rDNA sequence similarities of 97.0% or less usually belong to different species, as they have DNA-DNA reassociation values of <70%, a key criterion used to define species (Stackebrandt & Goebel 1994). However, different flavobacteria were isolated in most cases in the parallel cultivation procedures. This indicates that several flavobacterial species may co-exist in diseased fish samples. It is suggested that in bacterial cultivation using the plate-streaking method, some *Flavobacterium* sp. can be masked by other species of

the same genus. Based on the 16S rDNA analysis, these overgrowing organisms included isolates that were phylogenetically heterogeneous but clustered with a recently described species *F. hibernum* (McCammon et al. 1998). In a previous study (Rintamäki-Kinnunen et al. 1997), yellow-pigmented filamentous bacteria that were in most cases phenotypically similar to *F. johnsoniae* were isolated in 134 out of 247 samples following flavobacterial outbreaks at 4 salmonid fish farms in northern Finland (Farms B, C and E are included in this study). The phenotypic characteristics of these *F. johnsoniae*-like isolates (Rintamäki-Kinnunen et al. 1997) were shared by the 7 isolates IsoA1, IsoA2, IsoB1, IsoC1, IsoC2, IsoD2 and IsoE1 of this study. *F. johnsoniae* has also been isolated from farmed barramundi *Lates calcarifer* Bloch in northern Australia, and has been suggested to be a skin pathogen (Carson et al. 1993). It is possible that these *F. johnsoniae*- or *F. hibernum*-like organisms include opportunistic flavobacterial pathogens. However, in our study, it was more likely that these bacteria were growing only as saprophytes during *F. columnare*-induced disease, since only one of the tissue samples (A2) showed dominance of a bacterium of this group in the direct sequence-analysis.

Isolation of flavobacteria was unsuccessful from a number of fish samples (44%) containing filamentous Gram-negative bacteria in the microscopic examination. This difficulty in cultivating flavobacteria was also reported in the study of Rintamäki-Kinnunen et al. (1997). Other bacteria growing on the plates in the initial cultivation may have masked the growth of flavobacteria. The bacterial isolations were performed at 22°C, which may have had some adverse effect on the growth of the psychrophilic bacterium *Flavobacterium psychrophilum*. However, temperature should not have affected the cultivation of *F. columnare*. The optimum growth temperature of this species has been defined as 20 to 25°C (Bernardet & Grimont 1989), and

the temperature of the sampled fish tanks was close to the cultivation temperature in the late-summer samples. Moreover, antagonistic bacteria such as pseudomonads can also interfere with the cultivation procedure, as shown in the study. Only a few cells of *Pseudomonas* sp. MT5 were required to completely inhibit the growth of a thousand times more cells of *F. psychrophilum*-like bacteria. Although our observation was based on only 1 case (Sample D4), it is worth noting how strong an effect some single antagonistic colonies have, and how important it can be to cultivate using dilution series. Bacterial antagonism has already been reported for several *Pseudomonas* strains (reviewed by Gatesoupe 1999) and their inhibitory activity has been shown to be caused by competition for free iron by siderophores (Gram 1993, Schmith & Davey 1993, Gram et al. 1999). Whether the antagonistic properties of *Pseudomonas* sp. MT5 are also mediated by siderophores is a matter for further study. The ecological significance of pseudomonads in the bacterial communities and their possible role in protection of fish skin is also an interesting question which needs to be investigated. Interestingly, increased iron (Fe^{2+}) appears to enhance the virulence of *F. columnare* (Kou et al. 1981).

The high number (33%) of PCR-negative samples constitutes an apparent limitation of direct molecular analysis. This phenomenon is probably due to methodological problems not yet solved: the lack of sensitivity in the PCR due to the small sample size or competing non-template DNA, or PCR inhibition by some substances co-extracted in the preparation of the DNA sample. One possible explanation is also degradation of DNA by DNases, since flavobacteria, and especially *Flavobacterium columnare*, excrete enzymes that can cause rapid DNA hydrolysis (Bernardet & Grimont 1989), and extensive degradation was seen in agarose gel electrophoresis analysis of most of the DNA samples in this study.

PCR-RFLP patterns with enzymes *Hae*III and *Hinf*I have earlier been shown to be useful in characterising genotypic diversity and the 3 genomovars in *Flavobacterium columnare* strains (Triyanto & Wakabayashi 1999). Our study applied combined *Hae*III/*Hinf*I digestion. Although a valuable tool for initial characterisation of samples and for revealing the discrepancy between sequences derived from isolated bacteria and PCR-based sequences, RFLP analysis with the restriction enzymes *Hae*III and *Hinf*I did not have enough resolution to separate *F. columnare* sequences from the *F. hibernum*-clustering flavobacterial sequences. In fish-disease diagnostics, sequence-analysis may have a more universal application, since it allows comparisons with the public sequence databases, which currently include 30 000 to 40 000 16S rDNA sequences from cultivated and uncultivated organisms.

PCR-based techniques have so far been used in fish-disease diagnostics to answer specific questions with species-specific primers, as also in the diagnosis and epidemiology of flavobacterial diseases (Toyama et al. 1996, Urdaci et al. 1998, Izumi & Wakabayashi 2000, Wiklund et al. 2000). With a broad-range bacterial PCR approach, one general protocol can be applied to all bacterial diseases. Combined with sequencing, broad-range bacterial PCR is a powerful tool for identifying the dominant bacterium in a fish sample. Further optimisation of the DNA extraction procedure should also solve the problems encountered in the PCR amplification from some samples. The first 500 nucleotides from the 5' terminus of the 16S rDNA contain enough information to allow accurate assignment of bacterial sequences to the main lines of descent, and this terminus has been recommended as the region on which to concentrate (Liesack et al. 1997). This region was therefore used as a target in the present study. The PCR approach can be used with external tissue samples containing mixed bacterial populations dominated by a single species but including other strains from the same genus or other genera, as is the case in many flavobacterial infections.

The limited number of samples used in this study should be taken into account when drawing conclusions. However, it seems evident that traditional cultivation by streaking the tissue sample on a single agar plate can give a biased picture of bacterial diversity, since the results of cultivation and direct molecular analyses may vary as a function of the different amounts of fish tissue used in the analyses. The dominating flavobacteria can be more reliably isolated if the samples are serially diluted before spreading on agar plates. Further analyses with a more extensive number of samples would be useful to confirm our results and to determine which flavobacterial species are causing flavobacterial diseases in Finnish freshwater fish farms.

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