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Effect of *Pseudomonas* sp. MT5 baths on *Flavobacterium columnare* infection of rainbow trout and on microbial diversity on fish skin and gills

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ABSTRACT: Use of *Pseudomonas* sp. strain MT5 to prevent and treat *Flavobacterium columnare* infection was studied in 2 experiments with fingerling rainbow trout *Oncorhynchus mykiss*. In the first experiment, length heterogeneity analysis of PCR-amplified DNA fragments (LH-PCR) was used to assess the effect of antagonistic baths on the microbial diversity of healthy and experimentally infected fish. In the 148 samples studied, no difference was found between bathed and unbathed fish, and 3 fragment lengths were detected most frequently: 500 (in 75.7% of the samples), 523 (62.2%) and 517 bp (40.5%). The species contributing to these fragment sizes were *Pseudomonas* sp., *Rhodococcus* sp. and *F. columnare*, respectively. A specific PCR for detection of *Pseudomonas* sp. MT5 was designed, but none of the tissue samples were found to be positive, most likely indicating poor adhesion of the strain during bathing. LH-PCR was found to be a more powerful tool for detecting *F. columnare* in fish tissue than traditional culture methods ($\chi^2 = 3.9$, df = 1, $p < 0.05$). Antagonistic baths had no effect on the outbreak of infection or on fish mortality. *F. columnare* was also detected in healthy fish prior to and after experimental infection, indicating that these fish were carriers of the disease. In the second experiment, intensive *Pseudomonas* sp. MT5 antagonistic baths were given daily to rainbow trout suffering from a natural columnaris infection. Again, the antagonistic bacteria had no effect on fish mortality, which reached 95% in both control and antagonist-treated groups in 7 d.

KEY WORDS: *Flavobacterium columnare* · Antagonism · LH-PCR · Microbial diversity

INTRODUCTION

There has been growing interest in the development of sustainable prevention and treatment of fish diseases without the use of antibiotics. Many probiotic or antagonistic bacteria have already been identified and studied (rewieved by Gatesoupe 1999, Verschuere et al. 2000, Irianto & Austin 2002). Several antagonistic species are found within the genus *Pseudomonas* (Hatai & Willoughby 1988, Smith & Davey 1993, Bly et al. 1997, Gram et al. 1999, 2001, Spanggaard et al. 2001). According to Spanggaard et al. (2001), as many as 66% of pseudomonads isolated from fish are antagonists. We have isolated an antagonistic *Pseudomonas* sp. strain, MT5, from the skin of fingerling rainbow trout (Tiirola et al. 2002). This strain inhibited the growth of several Gram-positive and Gram-negative bacteria in agar assays, including 2 flavobacterial pathogens, *Flavobacterium columnare* and *F. psychrophilum*. In the case of *F. columnare*, bathing fish with antagonistic bacteria might be an effective way to prevent disease, since infection is generally restricted to the body surface and/or the gills, causing extensive tissue necrosis (Bernadet 1997). Furthermore, Wakabayashi (1991) and Chowdhury & Wakabayashi (1991) have reported that competitive bacteria can reduce the possibility of invasion of fish by flavobacterial pathogens. If the number of *F. columnare* cells is low,

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the pathogen may not be able to compete with the natural microbial population of the fish skin mucus and gills. It has been reported that the bacterial diversity of healthy fish reflects that present in the water environment (Nieto et al. 1984, Cahill 1990, Spanggaard et al. 2000), in which case it should be easy to manipulate the bacterial composition of fish skin by altering or increasing the amount of ambient probiotic or antagonistic bacteria. A sustainable disease management could, therefore, utilize a combination of high numbers of antagonistic bacterial cells and the poor competition ability of *F. columnare*. Successful effects of bath treatments with probiotic pseudomonads on fish mortality under experimental conditions have been reported by Smith & Davey (1993), Austin et al. (1995) and Gram et al. (1999). However, despite the antagonistic characteristics of pseudomonads *in vitro*, bathing did not have any effect on experimental infections and fish mortality in some studies (Gram et al. 2001, Spanggaard et al. 2001).

The diagnosis of bacterial infections is traditionally based on examination of external signs of infection and isolation of the pathogen by culture. In the case of *Flavobacterium columnare*, cultivation is a time-consuming process and can easily be biased by competitive and opportunistic bacteria present in the sample (Tiirola et al. 2002). Thus, there is a need for the development of reliable and fast tools to diagnose fish bacterial pathogens. Molecular methods, such as species-specific PCR, have already been established (Bader & Shotts 1998, Bader et al. 2003a). Length heterogeneity analysis of PCR-amplified DNA fragments (LH-PCR) (Suzuki 1998) is a method used for screening bacterial communities in different environments (Ritchie et al. 2000, Mills et al. 2003, Rogers et al. 2003, Tiirola et al. 2003). The 16S rDNA region, naturally variable in length, is amplified in the PCR reaction with fluorescently labeled universal primers, and the fragments are separated in an automated sequencer.

Our aim was to study the potential of *Pseudomonas* sp. MT5 as a tool to treat and prevent columnaris disease. The ability of the antagonistic strain to prevent *Flavobacterium columnare* infection (Expt 1) was tested in an experimental *Pseudomonas* sp. MT5 challenge in the laboratory and then by simulating naturally occurring infection process by damaging the skin mucus and exposing rainbow trout to a low concentration of *F. columnare* bacteria. The ability of the daily antagonist baths to treat an ongoing disease (Expt 2) was studied in a natural outbreak at a fish farm. The effect of *Pseudomonas* sp. MT5 antagonist baths was evaluated by measuring fish mortalities and by analyzing the microbial community structure on fish skin and gills. Previous surveys of microbial diversity of fish have been based on cultivation of bacteria (Cahill 1990, Gonzalez et al. 1999, Spanggaard et al. 2000). In this study, a DNA method, LH-PCR, was applied to screen the microbial diversity on the skin and gills of healthy fish and those with signs of columnaris disease.

MATERIALS AND METHODS

Expt 1 in the laboratory. Rainbow trout fingerlings (n = 240, average weight 5 g) were kept in 12 glass aquaria, each containing 20 fish in 16 l of water. A continuous flow of ground water with the temperature adjusted to 19.5°C was used. Fish were fed daily with commercial pellets and feces were removed daily. Before the experiment, the fish were kept in the laboratory for 3 wk. During this time, all fish were exposed to a set of stressors imitating the stress conditions at a fish farm, including exposure to *Diplostomum spathaceum* metacercariae. In the experiment, 6 randomly selected groups of fish were bathed twice (Day 2 and Day 16) with antagonistic *Pseudomonas* sp. MT5 (10^5 CFU^{-1} ml^{-1}). The bacteria were grown in 2 l of A & O broth (AOB) (Anacker & Ordal 1959) for 48 h at room temperature without shaking. Before bathing, the volume of water in the tank was reduced by 50%. The bacterial solution was poured into the aquaria, which were then refilled with water. Twenty-four h after *Pseudomonas* bathing, the fish were bathed with 3.6×10^3 CFU^{-1} ml^{-1} of *Flavobacterium columnare* (strain KE02). The skin of all fish was abraded to ensure the invasion of *F. columnare* (Madetoja et al. 2000, Bader et al. 2003b). The second exposure was given on Day 16 by again scraping the fish and exposing them to *F. columnare*. The fish were anesthetized with clove bud oil during the scraping of the skin.

Expt 2 in fish farming conditions. The effect of intensive antagonistic *Pseudomonas* sp. MT5 bathing to treat ongoing *Flavobacterium columnare* infection was studied in a natural *F. columnare* infection of rainbow trout at an inland fish farm in Central Finland during July 2003. The inflow water came from a nearby river and ambient temperature varied between 19.4 and 25.4°C during the experiment. Fish (n = 1900, average weight 2 g) with first signs of columnaris disease (grayish coloration around the dorsal fin, slight mortality) were chosen. The experiment was carried out in ten 15 l plastic aquaria (190 fish per aquarium, normal density for fish of the same size group), which were randomized between antagonistic *Pseudomonas* sp. MT5 treatment and control (5 tanks each). Antagonist-treated fish received *Pseudomonas* MT5-baths (10^5 CFU^{-1} ml^{-1}) every day for 1 wk. The control group received a sham treatment with pure AOB. Fish were not fed during the experiment but feces were removed daily. Mortality was measured daily.

Sample collection and bacterial cultivation in Expt 1. The first sample (Sample 1) was taken before the start of the experiment on Day 0 (number of fish sampled = 27) and the second sample was taken from the remaining fish after Day 26, at the end of the experiment (Sample 2, n = 61). In addition to these randomly chosen fish, every individual fish with any signs of columnaris disease (lesions on the skin, erosion of the fins, gill necrosis) was examined. Parallel tissue samples for bacterial cultivation and DNA analysis were taken from skin, gills and infected tissue with a sterile scalpel and placed into sterile tubes. Samples taken for the DNA analysis were frozen immediately to –20°C. Bacterial culture was made on A & O agar (AOA) (Anacker & Ordal 1959) using the dilution cultivation method. The tissue sample (15 µl of skin mucus or uppermost gill arch) was suspended with vigorous shaking in 900 µl of AOB and diluted in a 10-fold series. From each dilution, 100 µl was spread on an AOA plate. Yellow-pigmented colonies were defined as Flavobacteria following the detection of flexirubin pigment production with 5% KOH. *F. columnare* was identified by phenotypical characteristics of the isolates (rhizoid edges of the colony, tight adherence to the agar), lack of growth in trypticase soy broth (TSB, Difco) and Congo Red (0.01% aqueous solution) absorption. LH-PCR was used to confirm the definition.

Statistical analysis. Statistical analyses were carried out by *t*-test and χ^2-test, using SPSS 11.0 software. Mortality was analyzed by comparing average percent mortality between control and antagonist-treated groups.

DNA extraction and LH-PCR. DNA was extracted by lysing cells with Poteinase K and bead-beating, and the DNA was purified using phenol-chloroform extractions and NaCl/isopropanol precipitation (Tiirola et al. 2003). LH-PCR analysis of the samples was done as previously described (Tiirola et al. 2003) using universal bacterial primers F8 (5′-AGAGTTTGATM-CTGGCTCAG-3′) (Weisburg et al. 1991) with ird700 label and Prun518 (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al. 1993). Since the LH-PCR fragment length of over 30 Finnish *Flavobacterium columnare* isolates has been confirmed to be 517 bp (authors' unpubl. results), the detection of a 517 bp PCR fragment in the LH-PCR predicted the presence of *F. columnare*.

***Pseudomonas* sp. MT5-specific PCR.** A new primer set, 16S-MT5f (5-AAGTCGAGCGGATGAAAG-3′) and 16S-MT5r (5-CTCTAGCTTGTCAGTTTAGA-3′), was designed to specifically amplify a 581 bp fragment of the 16S rDNA of *Pseudomonas* sp. MT5. Based on searches using the BLAST program (Altschul et al. 1997), one or the other of the primers had at least 1 mismatch in the last 3 bases of their 3′-ends with each of the published 16S rDNA sequences in the EMBL database (July 2004), which should terminate unspecific PCR amplification. The PCR protocol was the same as the LH-PCR protocol (Tiirola et al. 2003) using DynaZyme F501-KL polymerase (Finnzymes), except that the annealing temperature was set at 50°C, the reaction volume was 25 µl and the PCR amplification consisted of 35 cycles. The PCR protocol was tested to determine its ability to produce a correct product at varying concentrations (10, 1, 0.5, 0.25, 0.125, 0.1, 0.05, 0.025, 0.01 and 0.001 ng, DNA concentration measured with a GeneQuant RNA/DNA calculator, Pharmacia Biotech) of genomic MT5 template, extracted using the UltraClean™ microbial DNA isolation kit (Mo Bio Laboratories). Each PCR reaction also contained 1 µl of pooled mucus DNA (a pool of DNA of 10 MT5-negative samples) processed for the LH-PCR analysis.

Cloning and sequencing. PCR products selected for sequencing were ligated into the pGEM-T easy vector (Promega) and used to transform JM109 *Escherichia coli* cells by electroporation. Plasmids were extracted using the Sigma GenElute miniprep kit and prepared with the SequiTherm excel kit II (Epicentre Technologies) for bidirectional sequencing using sequencing primers T7 and SP6 and a LiCor DNA 4200 automated sequencer (Li-Cor BioTech).

Nucleotide sequencing accession numbers. The partial 16S rDNA sequences obtained from the samples were analyzed and edited using AlignIR 1.1 software (Li-Cor) and compared with the EMBL database using the BLAST program (Altschul et al. 1997). The sequences were submitted to the EMBL database under accession numbers AJ631284 to AJ631301 and AJ632132 to AJ632134.

RESULTS

In Expt 1, antagonistic *Pseudomonas* sp. MT5 baths had no effect on fish mortality following challenge with *Flavobacterium columnare* (Fig. 1). In total, 19 moribund fish with lesions on the skin or gill necrosis were seen during the experimental outbreak, 42% of which were from the antagonist-treated group. No signs of columnaris disease were observed before the experiment in Sample 1 (n = 36 fish) and no *F. columnare* isolates were obtained. In the LH-PCR analysis, however, the 517 bp fragment indicating the presence of *F. columnare* was seen in 41.7% of fish in Sample 1. During the second sampling at the end of the experiment (Sample 2, n = 61), there were no clinical signs of columnaris disease, but *F. columnare* was isolated from 5 of 61 sampled fish (8.2%) and LH-PCR detected the *F. columnare* fragment in 7 fish (11.5%), all from the antagonist-treated group (Fig. 2). The connection between the 517 bp fragment and *F. columnare* was

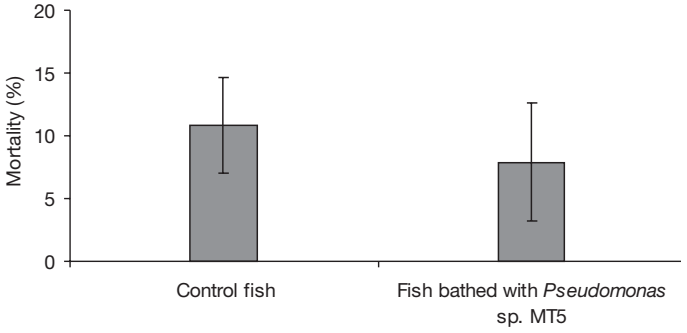

Fig. 1. *Oncorhynchus mykiss* infected with *Flavobacterium columnare*. Average percent mortality (±SE) of infected rainbow trout (control fish and fish treated with antagonistic *Pseudomonas* sp. MT5 baths) in Expt 1

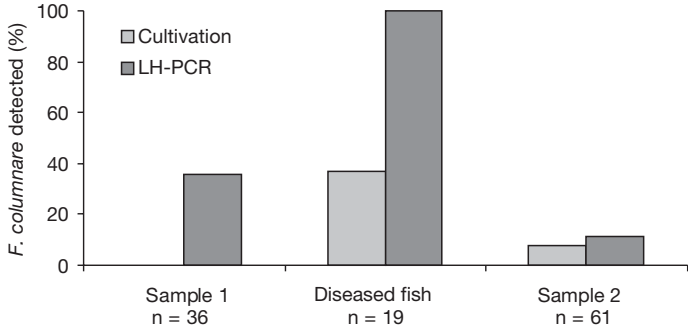

Fig. 2. *Oncorhynchus mykiss* infected with *Flavobacterium columnare*. Proportion of healthy (Samples 1 and 2) and diseased fish exhibiting *F. columnare*, detected by cultivation and LH-PCR methods, in Expt 1

Table 1. *Oncorhynchus mykiss* infected with *Flavobacterium columnare*. Occurrence of *F. columnare* in experimentally infected, moribund and dead rainbow trout, detected by bacterial cultivation and LH-PCR. +: *F. columnare* detected; –: no detection of *F. columnare*; C: control; A: antagonistic bath with *Pseudomonas* MT5

Treatment	Fish number	*F. columnare* isolation	517 bp peak intensity in LH-PCR (%)
C	1	–	100
	2	–	100
	3	–	100
	4	–	100
	5	+	28.4
	6	–	36.2
	7	–	39.6
	8	–	100
	9	+	84.7
	10	–	83.8
	11	–	100
A	12	+	100
	13	–	38.2
	14	+	5.5
	15	–	59.1
	16	+	26.6
	17	–	100
	18	+	100
	19	+	100

confirmed by cloning and sequencing samples from diseased (accession number AJ631286) and healthy fish (AJ631284 and AJ631285). Among the diseased fish, *F. columnare* was isolated from only 7 fish (37%), but it was detected by LH-PCR from all fish (Fig. 2, Table 1). Cultivation success was not connected to the relative amount of *F. columnare* present in the sample. The difference in *F. columnare* detection from infected fish between the 2 methods was statistically significant ($\chi^2 = 3.9$, df = 1, $p < 0.05$). The survival of the antagonistic *Pseudomonas* sp. MT5 could not be estimated with LH-PCR analysis, since the fragment length of the strain (523 bp) is a very common length among pseudomonads, and was already seen before *Pseudomonas* sp. MT5 bathings.

The MT5-specific PCR using the primer set 16S-MT5f and 16S-MT5r yielded a single product from genomic MT5 DNA at annealing temperatures between 43 and 59°C. For the final protocol, 50°C was chosen for the annealing temperature. This protocol detected 25 pg of pure *Pseudomonas* sp. MT5 DNA in a reaction mixture to which 1 µl of fish mucus DNA had been added. All Expt 1 samples which produced a peak at 523 bp, characteristic for the MT5 strain, in LH-PCR (altogether 25 samples) were analyzed by the MT5-specific PCR protocol. None of these samples contained enough *Pseudomonas* sp. MT5 DNA to yield a positive signal.

LH-PCR results were obtained from 148 tissue samples from the beginning (n = 56) and the end of Expt 1 (n = 58), and from diseased fish (n = 34). Amplified fragment lengths ranged from 465 to 571 bp (Fig. 3). Bacterial diversity, determined as number of fragments detected, changed during the experiment. In Sample 1, taken before the experiment, 42 fragment lengths were present. In Sample 2, taken after the experiment, the diversity had decreased to 36 fragments in antagonist-treated fish and to only 21 fragments in control fish. *Flavobacterium columnare* was not detected in the control fish of Sample 2. The most prevalent fragment sizes detected were 500, 523 and 517 bp, which on average accounted for 68% of total peak intensities (Fig. 3, Table 2). Since we wished to know which bacteria might have contributed to these fragment sizes, we sequenced 21 clones containing these PCR fragments (1 clone from a diseased fish and 20 from a healthy fish). Clones obtained from the fish with no signs of columnaris disease in Sample 2 con-

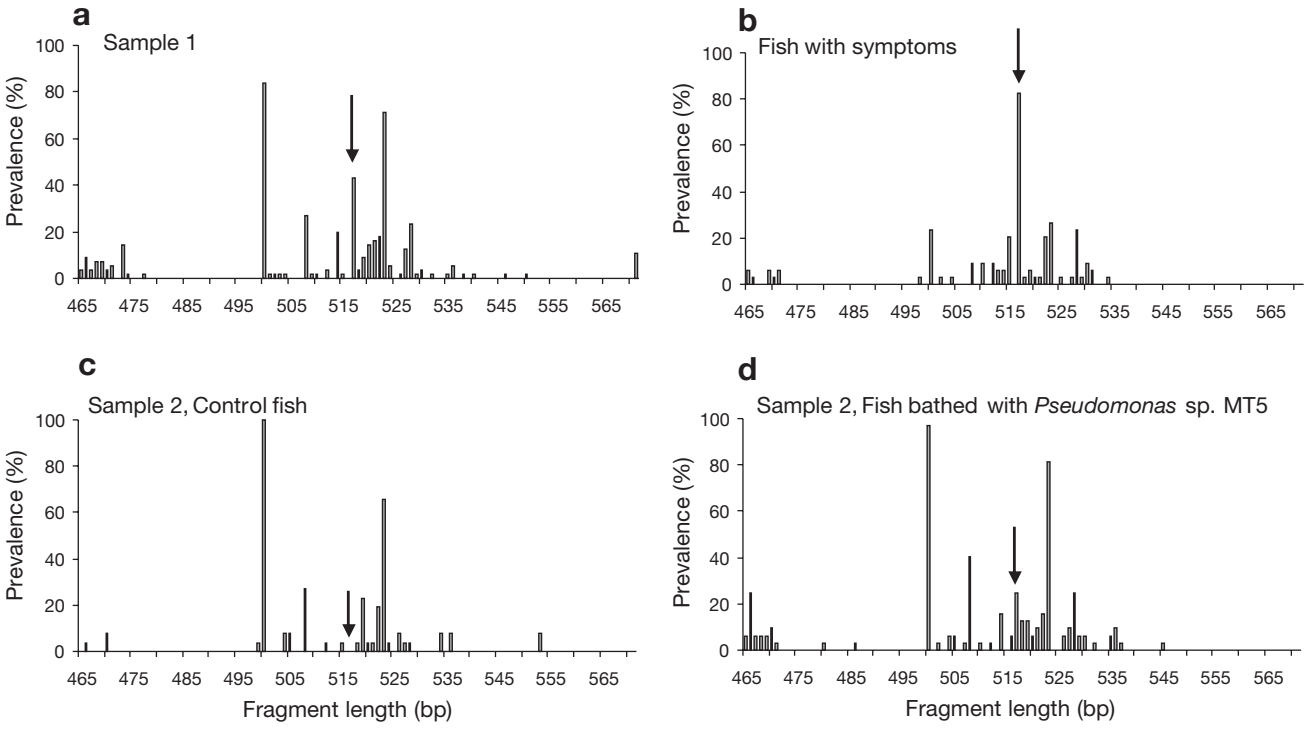

Fig. 3. Prevalence of different LH-PCR fragment-size peaks in Expt 1. The peak corresponding to *Flavobacterium columnare* is marked with an arrow. (a) Sample 1, taken on Day 0 (56 amplified tissue samples); (b) samples from fish showing symptoms of columnaris disease, collected on Days 14 to 19 (34 amplified tissue samples); (c,d) Sample 2, taken after Day 26 (58 amplified tissue samples)

tained fragments that were most similar to *Rhodococcus* sp. (4 sequences), *Pseudomonas* sp. (11 sequences) and *Flavobacterium columnare* (2 sequences) (Table 2). Three other sequences (accession numbers AJ632132 to AJ632134) were also obtained from the sample, matching closest to drinking water bacterium MB12 (99% similarity), division TM6 bacterium (90%) and *Ralstonia* sp. (98%) in the database, respectively. The sequence obtained from a diseased fish was most similar to *F. columnare*. The *F. columnare* sequences obtained from diseased and healthy fish had 13 differing bases (2.5% difference in the 517 bp sequence) and matched different *F. columnare* sequences in the database, indicating a different origin.

Table 2. Most prevalent fragment-size peaks amplified from the 148 fish tissue samples with primer pair F8 and Prun518, and the mean relative intensities of these band sizes to the total band intensity of each sample. The best match to the sequence contributing to each fragment size is shown, along with the accession number to each sequence submitted to the EMBL database

Fragment length (bp)	Accession number	Best match in EMBL database, percentage identity	Prevalence in samples (%)	Mean band intensity	SD
500	AJ631298–AJ631301	*Rhodococcus* sp. 871-AN053 99%	75.5	26.8	23.8
523	AJ631287–AJ631297	*Pseudomonas* sp. MSB2019 100%	62.2	23.1	22.9
517	AJ631284–AJ631285	*Flavobacterium columnare* B2 99% (healthy fish)	40.5	17.7	30.8
	AJ631286	*Flavobacterium columnare* ATCC23463 99% (diseased fish)			

In the natural infection (Expt 2), even intensive antagonist bathing did not decrease fish mortality. The average daily mortality rate was 11.6% in the control group and 12.0% in the antagonist-treated group. Cumulative mortality reached 95% in both treatments in 8 d, after which the remaining fish were sacrificed (Fig. 4).

DISCUSSION

Antagonism of *Pseudomonas* sp. MT5 against *Flavobacterium columnare* bacteria was found to be very strong in agar assays, preventing the

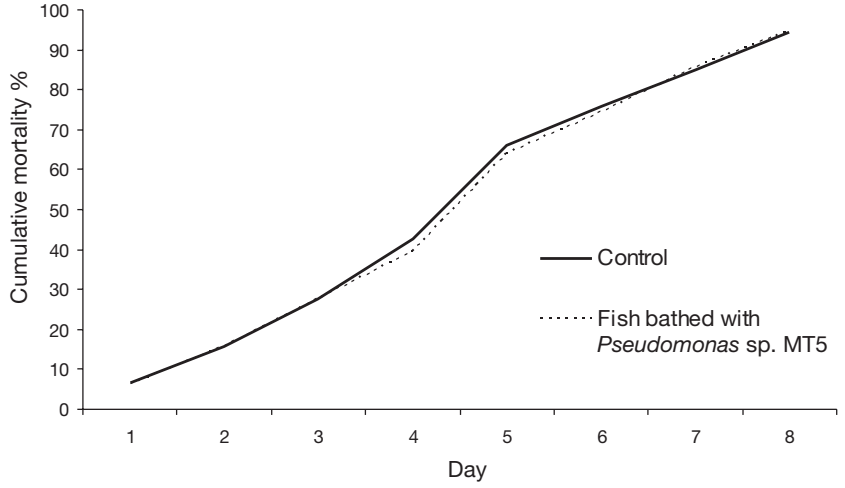

Fig. 4. *Oncorhynchus mykiss* infected with *Flavobacterium columnare*. Cumulative mortality of rainbow trout fingerlings in Expt 2, following a natural *F. columnare* infection at a fish farm. Mortality of fish with and without daily antagonistic *Pseudomonas* sp. MT5 bathings is shown

growth of 1000 times more *F. columnare* cells (Tiirola et al. 2002). However, antagonistic baths could not prevent *F. columnare* infection after experimental challenge in Expt 1 or treat the disease during an ongoing infection in Expt 2, even though, in the natural outbreak (Expt 2), the baths were given every day for 1 wk. The antagonistic abilities of a bacterial strain in agar assays are, thus, not always comparable to *in vivo* experiments (cf. Gram et al. 2001, Spanggaard et al. 2001). In Expt 1, the *F. columnare* infection was enhanced by abrading the skin mucus and challenging the fish with a low concentration (3.6×10^3 CFU^{-1} ml^{-1}) of *F. columnare* bacteria. This imitates a natural infection process, since, in Finnish fish farms, *F. columnare* infections are often preceded by ectoparasitic (*Ichthyophthirius multifiliis*, *Chilodonella* sp., trichodinids) infections (Rintamäki-Kinnunen & Valtonen 1997). One reason for the ineffective protection of fish by *Pseudomonas* sp. MT5 may have been the lack of adherence during bathing. This means that although this strain was first isolated from fish skin, re-adherence of the laboratory cultivated strain was not effective in these conditions. Antagonistic *Pseudomonas* sp. MT5 in the microbial community of fish skin and gills could not be detected directly from the LH-PCR results. The 523 bp fragment, which was one of the most prevalent fragment sizes, is also found in other *Pseudomonas* strains and species. Due to this failure of the method, a specific PCR protocol for the detection of *Pseudomonas* sp. MT5 was designed. However, the amount of MT5 DNA was under the detection level. Furthermore, LH-PCR results show that *Pseudomonas* sp. MT5 bathing did not decrease the relative amount of *F. columnare* 16S rDNA in Expt 1.

Another possible explanation for the lack of the protection after *Pseudomonas* sp. MT5 baths may be that the fish skin environment differs from that on an agar plate so radically that the antagonistic bacteria cannot function. The exact mechanism of antagonism by *Pseudomonas* sp. MT5 is unknown, but, in many cases, the antagonism of pseudomonads is based on iron-chelating siderophores (Hatai & Willoughby 1988, Smith & Davey 1993, Bly et al. 1997, Gram et al. 1999, 2001, Spanggaard et al. 2001). Finnish freshwaters are usually rich in ferrous compounds, so microbial competition for iron may not function in these locations. High levels of iron are also known to increase the virulence of *Flavobacterium columnare* (Kou et al. 1981).

Although the total microbial diversity from fish skin and gill samples was quite high (over 50 fragment lengths detected), only 2 fragments had a prevalence above 50%. The summed average peak intensity of the 3 main peaks was nearly 70% of the total peak intensity of the samples, which reflects the relative importance of these bacteria in the samples. The sequences related to these fragment sizes belonged to *Rhodococcus* sp., *Pseudomonas* sp. and *Flavobacterium columnare*. The first 2 groups are found generally in the environment. The decrease in microbial diversity between Samples 1 and 2 may be a result of rearing fish in the laboratory in well water. The bacterial diversity of the skin reflects the ambient water diversity, which may be lower in well water than in natural water.

The fish used in the laboratory trial had a history of 3 successive *Flavobacterium columnare* outbreaks prior to the experiment. Even though the infections were treated appropriately with oxytetracyclin, the markers of the pathogen were still seen in the DNA analysis of Sample 1, before the experimental infection. The LH-PCR results and the sequence obtained from healthy fish in Sample 2 show that the fish were still carriers of *F. columnare*. Sequence analysis also revealed that the *F. columnare* sequence obtained from a healthy fish (Sample 2 in Expt 1) was different (2.5% difference in the 517 bp sequence) from that obtained from a diseased fish. The strain in the healthy carrier fish most likely originated from its previous infection. In further genotypic analysis by ribosomal intergenic spacer analysis (RISA), the *F. columnare* bacteria isolated from diseased fish were, however, determined to have the same genotype as KE02 used in the experimental infection (3 isolates analyzed, data

not shown), revealing that this strain originated from the challenge.

PCR amplification of rRNA genes has been shown to be a reliable method for diagnosing bacterial pathogens in humans (e.g. Relman et al. 1992, Rantakokko-Jalava et al. 2000, Rogers et al. 2003) and in fish (Tiirola et al. 2002). Species-specific primers are used when samples are screened for known pathogens and such primers have also been developed for *Flavobacterium columnare* (Bader & Shotts 1998, Bader et al. 2003b). However, species-specific PCR may not give a real understanding of the total bacterial diversity and of changes in the microbial community, whereas universal primers can be used to amplify all bacterial rDNA. In this experiment, we obtained LH-PCR fragments indicating the presence of *F. columnare* in both healthy looking fish and fish with external symptoms of the disease. Isolation was made from only 31% of the fish that tested positive for the *F. columnare* fragment in LH-PCR. Based on these results, we propose that culture-based methods are not sufficiently reliable for diagnosing flavobacterial diseases (see Tiirola et al. 2002). In fish with clinical signs of columnaris disease, the pathogen usually dominates the microbial community of the tissue sample (Table 1, Fig. 3b). However, the success of the cultivation method was not connected to the dominance of *F. columnare* in the infected tissue, thus making cultivation very unreliable. In any case, the sample for culture should be taken from freshly deceased fish to avoid contamination by saprophytic bacteria, which may lead to false negative results when the diagnosis is based on cultivation. Screening of microbial communities in fish for pathogens by universal PCR is cost effective because the presence of the pathogen can be revealed before clinical disease occurs, and carrier fish can be detected in cases where the pathogen itself cannot be isolated. However, because LH-PCR separates sequences only by their length, several taxonomical bacterial groups can be included within 1 fragment size (Tiirola et al. 2002), which can be considered a drawback if precise information about the bacterial diversity of the sample is needed.

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