DTU Library

Occurrence of Flavobacterium psychrophilum in fish-farming environments

Madetoja, J.; Dalsgaard, Inger; Wiklund, T.

[Link back to DTU Orbit](#)

Occurrence of *Flavobacterium psychrophilum* in fish-farming environments

Jari Madetoja[1],*, Inger Dalsgaard[2], Tom Wiklund[1]

[1]Laboratory of Aquatic Pathobiology, Department of Biology, Åbo Akademi University, BioCity, Artillerigatan 6, 20520 Åbo, Finland

[2]Danish Institute for Fisheries Research, Fish Disease Laboratory, Stigbøjlen 4, 1870 Frederiksberg C, Denmark

ABSTRACT: Occurrence of *Flavobacterium psychrophilum* in fish farms and fish-farming environments was studied using agar plate cultivation, the immunoflourescence antibody technique (IFAT) and nested PCR. Characteristics of 64 *F. psychrophilum* isolates from rainbow trout *Oncorhynchus mykiss*, fish farm rearing water, ovarian fluid and wild fish were serotyped, ribotyped and compared biochemically. Virulence of *F. psychrophilum* isolates from different sources was compared by injection into rainbow trout. Additionally, the number of *F. psychrophilum* cells shed by naturally infected rainbow trout was determined. *F. psychrophilum* was detected and isolated from skin mucus, skin lesions and internal organs of diseased rainbow trout and from fish without clinical disease. The pathogen was also present in wild perch *Perca fluviatilis*, roach *Rutilus rutilus*, and ovarian fluids of farmed rainbow trout brood fish. Isolates were biochemically homogenous, excluding the capability to degrade elastin. Five different agglutination patterns with different antisera against *F. psychrophilum* were found among the isolates studied. Although several different ribopatterns were found (*Cla*I: 12 ribopatterns and *Hae*III: 9 ribopatterns), ribotype A was the most dominant. Farmed rainbow trout brood fish carried a broad-spectrum of serologically and genetically different *F. psychrophilum* in ovarian fluids. Virulence of the tested isolates in rainbow trout varied and naturally infected rainbow trout shed 10^4 to 10^8 cells fish^{-1} h^{-1} of *F. psychrophilum* into the surrounding water.

KEY WORDS: *Flavobacterium psychrophilum* · Rainbow trout · Rearing water · Ovarian fluid · Perch · Roach · Shedding rate · Serotyping · Ribotyping

INTRODUCTION

Flavobacterium psychrophilum (Bernardet et al. 1996), the aetiological agent of 'rainbow trout fry syndrome' (RTFS) and 'bacterial cold water disease' (BCWD), causes mortality in hatchery-reared salmonids (Holt 1987, Dalsgaard 1993) and ayu *Plecoglossus altivelis* (Wakabayashi et al. 1994). *F. psychrophilum* was originally isolated from diseased coho salmon *Oncorhynchus kisutch* in 1948 in North America (Borg 1960) and reported isolated for the first time in 1993 in Finland from diseased rainbow trout *O.*

mykiss (Wiklund et al. 1994). Since then, *F. psychrophilum* has caused disease among farmed rainbow trout fry and fingerlings as well as table-sized fish in Finland (Rimaila-Pärnänen et al. 1997, Madetoja et al. 2001).

The detection of *Flavobacterium psychrophilum* has traditionally been based on agar plate cultivation. However, this method is a time-consuming, laborious and non-sensitive for studying the epizootiology and transmission of *F. psychrophilum*. Hence, time-saving and more sensitive methods have been developed for the detection of *F. psychrophilum* in both fish and environmental samples, like immunofluorescence antibody technique (IFAT) (Lorenzen & Karas 1992, Madetoja et al. 2000, Madetoja & Wiklund 2002) and PCR (Toyama

*E-mail: jmadetoj@abo.fi

et al. 1994, Urdaci et al. 1998, Cepeda & Santos 2000, Izumi & Wakabayashi 2000, Wiklund et al. 2000, Madetoja & Wiklund 2002).

Flavobacterium psychrophilum has been isolated mainly from diseased and covertly infected salmonids (Holt et al. 1993, Dalsgaard & Madsen 2000). Infected living and dead fish are potential sources of *F. psychrophilum* infection that could possibly spread this pathogen in surrounding water (Madetoja et al. 2000). The shedding rate of *F. psychrophilum* by artificially infected fish has been evaluated in a previous study (Madetoja et al. 2000), but the shedding rate by naturally infected fish has not.

In addition to the detection and isolation of *Flavobacterium psychrophilum* from cultured salmonids and ayu, the pathogen has also been detected in fish farm rearing water (Rangdale 1995, Bruun et al. 2000, Schmidt et al. 2000, Wiklund et al. 2000, Madetoja & Wiklund 2002), on algae (Amita et al. 2000), in reproductive products of salmonids (Holt 1987, Rangdale et al. 1996, Brown et al. 1997, Ekman et al. 1999), in diseased cultured carp *Cyprinus carpio*, tench *Tinca tinca*, crucian carp *Carassius carassius* and in diseased wild eel *Anguilla anguilla* (Lehmann et al. 1991), in wild pale chub *Zacco platypus* (Iida & Mizokami 1996) and in a newt (Brown et al. 1997). Thus, several sources may act as a potential reservoir of infection.

Typing techniques, such as serotyping (Holt 1987, Wakabayashi et al. 1994, Lorenzen & Olesen 1997, Izumi & Wakabayashi 1999, Dalsgaard & Madsen 2000, Madsen & Dalsgaard 2000, Madetoja et al. 2001) and ribosomal RNA gene restriction (ribotyping) analysis (Cipriano et al. 1996, Chakroun et al. 1998, Madsen & Dalsgaard 2000, Madetoja et al. 2001) have been used for the characterization and differentiation of *Flavobacterium psychrophilum*. A relationship between ribotypes and host fish species and between ribotype and serotype of *F. psychrophilum* has been suggested in previous studies (Chakroun et al. 1998, Madetoja et al. 2001). However, in previous studies serological and molecular characteristics of *F. psychrophilum* originating from different sources have not been compared sufficient to clarify routes of contagion and epidemiology of this pathogen.

This study investigated the presence of *Flavobacterium psychrophilum* in fish farms and fish-farming environments by examining diseased as well as apparently healthy farmed rainbow trout, wild fish and ovarian fluid of farmed rainbow trout brood fish. The phenotypes and genotypes as well as the virulence of *F. psychrophilum* isolates from different sources were compared. Additionally, the shedding rate of *F. psychrophilum* by naturally infected farmed rainbow trout were determined.

MATERIALS AND METHODS

Isolation of *Flavobacterium psychrophilum* from rainbow trout. Rainbow trout were obtained from 1 brackish water farm and 3 freshwater farms (Farms I, II, III and IV, respectively) which were not connected with each other and which all had a previous history of RTFS or BCWD. The rearing conditions in these farms are described in Madetoja & Wiklund (2002). Fish from Farms I and IV were collected from tanks in which a few fish had signs of BCWD, but medication was not started. Fish from Farms II and III were apparently healthy. Average weight of the studied fish obtained from Farm I (brackish water; n = 5, lateral skin lesion; n = 4, apparently healthy) and Farm II (spring water; n = 8, apparently healthy) were 25 and 13 g, respectively. Fish of 2 size classes, obtained from Farm III (spring water) were studied; average weight of table-size fish (n = 4, apparently healthy) was 349 g and fry-size fish (n = 4, apparently healthy) was 3 g. The average weight of fish (n = 7, lateral skin lesion; n = 4, apparently healthy from same tank) from Farm IV (fresh river water) was 28 g. Furthermore, 3 fish with skin lesions, average weight 1200 g, were included in the study from Farm IV. Tissue samples from spleen, kidney, skin lesion (if present) and skin mucus of all examined fish were inoculated on TYES (0.4% tryptone, 0.05% yeast extract, 0.05% $MgSO_4 \times 7H_2O$, 0.05% $CaCl_2 \times 2H_2O$, pH 7.2) agar plates (1.5%) as described by Holt et al. (1993). Plates were incubated at 15°C for 7 d.

Detection of *Flavobacterium psychrophilum* in ovarian fluid. Ovarian fluid samples from rainbow trout brood fish from spring water from Farm V (Madetoja & Wiklund 2002), were studied by the following method: aliquots of ovarian fluids from 20 brood fish were collected into sterile centrifuge tubes during routine stripping of fish and pooled. Ovarian fluids from 100 female brood fish were collected, thus forming 5 samples numbered 1 to 5. Samples were transported on ice to the laboratory for examination. The brood fish were given a therapeutic concentration (80 mg^{-1} kg fish^{-1}) of oxytetracycline (Terramycin$^{©}$, Pfizer) in the feed for 7 d, 21 d before stripping, in order to decrease possible vertical transfer of *F. psychrophilum* to the offspring. To examine the presence of *F. psychrophilum* in ovarian fluid samples, 10 µl of each sample was spread on object slides (Novakemi, 6 mm well) and studied using IFAT as described by Madetoja et al. (2000). Further, 100 µl of ovarian fluid samples were inoculated on TYES agar and tryptic soy agar (TSA, Difco) plates. Inoculated TYES agar plates were subsequently incubated at 15°C for 7 d and TSA plates at 20°C for 5 d. Ovarian fluid samples were also studied using nested PCR as described by Wiklund et al. (2000). In short, the sam-

ples were diluted 1:10 and 1:100 in sterile phosphate buffered saline (PBS) (pH 7.2). One ml of undiluted ovarian fluid and 1:10 and 1:100 dilutions were centrifuged (4500 × *g*) and the pellets washed twice with PBS, (pH 7.2). After the last wash, 20 µl of PBS were retained in the tube and 300 µl of a 5 % Chelex 100 suspension were added. Five µl of the template solution were used for the nested PCR.

Detection of *Flavobacterium psychrophilum* in wild fish. In order to study the presence of *F. psychrophilum* in wild fish, samples from perch *Perca fluviatilis* and roach *Rutilus rutilus* were examined using agar plate cultivation (TYES agar), IFAT and nested PCR. One roach and 9 perch were captured from an inlet water channel, and 3 roach and 7 perch from an outlet water channel (width 7 to 10 m, depth 1 to 3 m) of Farm IV using fish traps. Captured wild fish were killed and transported separately on ice to the laboratory for bacteriological sampling. The brain, spleen, kidney and skin mucus of fish were chosen as target organs for the detection of *F. psychrophilum* using inoculation on TYES agar and IFAT (Madetoja et al. 2000). The brain tissue and skin mucus were examined for *F. psychrophilum* using the nested PCR method as described previously. For the nested PCR, the homogenized brain was diluted 1:4, 1:10 and 1:100 in sterile PBS (pH 7.2). The mucous material (100 to 200 µl) was carefully scraped between head and tail fin from one side of the fish using a sterile scalpel and transferred into a sterile Eppendorf tube. One loop (10 µl) of undiluted skin mucus was inoculated on TYES agar or spread on an object slide for IFAT analysis. For nested PCR, the mucus samples were also diluted 1:10 and 1:100 in sterile PBS (pH 7.2). The undiluted and diluted brain and mucus samples were stored at –70°C.

Shedding rate of *Flavobacterium psychrophilum* by naturally infected rainbow trout. In order to estimate the shedding rate of *F. psychrophilum* among naturally infected fish, 10 rainbow trout, average weight 40 g, were collected from a concrete outdoor tank (4 to 5°C) with fish showing clinical signs of BCWD or fish without signs of disease. The living fish (n = 3, apparently healthy; n = 7, with skin lesions) were transferred to separate oxygenated plastic bags containing inlet water of the farm, and transported to the laboratory. In the laboratory, fish were flooded with Milli-Q water and transferred individually into plastic containers containing 1 l of *F. psychrophilum*-free well water at 4°C for 1 h. The number of *F. psychrophilum* shed by rainbow trout into the water were estimated as described by Madetoja et al. (2000). Fish were killed after the experiment and tissue samples from the spleen, kidney, lateral skin lesion (if present) and skin mucus were inoculated on TYES agar plates, as described previously.

Identification and characterization of *Flavobacterium psychrophilum*. Bacteria forming yellow colonies typical for *F. psychrophilum* on TYES agar plates were identified using an esculin tube test (Madetoja et al. 2001), TSA cultivation and PCR (Wiklund et al. 2000). An isolate's ability to degrade gelatine (0.6 %), casein (0.5 %) and elastin (0.1 %) was studied on TYES agar plates supplemented with respective substrates (Madsen & Dalsgaard 1998). Reactions were interpreted as positive if a clearing zone surrounded the colonies. The plates were incubated at 15°C for up to 10 d. Production of catalase was determined using a fresh bacterial culture and 3 % hydrogen peroxidase and, if necessary, microscopical confirmation of the reaction. Cytochrome oxidase reaction was determined using filter paper soaked with SpotTest™ Oxidase Reagent (Difco). The reaction was read within 30 s. Isolates identified as *F. psychrophilum* were further characterized and differentiated using serotyping and ribotyping as described by Dalsgaard & Madsen (2000) and Madsen & Dalsgaard (2000). A 1 kb DNA ladder (Life Technologies, Gibco BRL) was used as a size marker in the ribotyping analysis. *F. psychrophilum* from rearing water of Farms II, III, IV and V (see Table 1) had been previously isolated (Madetoja & Wiklund 2002) and included in the present characterization studies.

Fish and challenge experiment. In order to examine the virulence of the characterized *Flavobacterium psychrophilum* isolated from rainbow trout, wild fish, ovarian fluid and rearing water, 13 isolates were used to challenge rainbow trout that were obtained from a commercial freshwater fish farm in Finland without previous history of infection with *F. psychrophilum*. These fish were kept in a 0.225 m³ fibreglass tank with a continuous flow of well water of 12°C. Prior to the experiment the fish were acclimated for 10 d in similar tanks. Fish were fed ad libitum with a commercial feed (Ewos) throughout the experiments.

Pure cultures of *Flavobacterium psychrophilum* were stored in TYES broth at –70°C. For the challenge experiment, bacteria were thawed and 30 µl of bacterial suspension was transferred into 5 ml TYES broth and incubated for 2 d at 15°C with shaking. The cells were harvested and washed once with sterile PBS (pH 7.2) by centrifugation at 2500 × *g* for 15 min at 4°C. After the final centrifugation, cells were re-suspended in sterile PBS and immediately used in the experiments. The number of bacteria (expressed as colony forming units, CFU) in the suspension was determined using 10-fold serial dilutions and drop-plate technique on TYES agar.

Fish were anaesthetized using benzocaine (ethyl *p*-aminobenzoate, 40 mg l⁻¹, Sigma) before injection. Rainbow trout (average weight 9 g) were injected sub-

Table 1. *Flavobacterium psychrophilum.* Characteristics of the pathogen isolated from rainbow trout, wild fish, ovarian fluid samples and water (n = 38). RT: rainbow trout; P: perch: –: negative reaction; +: positive reaction; *: auto-agglutination; NR: not restricted

Farm	Isolate	Source	Elastinase	Serotype	Ribotype *Cla*I	*Hae*III
I	L1/00	Skin lesion, RT[1]	–	Fp[T], Th	B	B
I	L2/00	Skin lesion, RT[1]	–	Fp[T], Th	B	B
I	L3/00	Skin mucus, RT	–	Fp[T]	A	A
I	L4/00	Skin lesion, RT[1]	–	Fp[T], Th	B	B
I	L5/00	Skin mucus, RT	–	Fp[T], Th	B	B
I	L6/00	Skin lesion, RT[1]	–	Fp[T], Th	B	B
I	L7/00	Skin mucus, RT	–	Fp[T], Th	B	B
I	L8/00	Skin mucus, RT	–	Fp[T], Th	B	B
II	K1/98	Spleen, RT	+	*	A	A
II	K4/00	Kidney, RT[a]	+	Fp[T]	A	A
II	K7/00	Spleen, RT[a]	+	Fp[T]	A	A
II	K5/00	Spleen, RT[b]	+	Fp[T], Fd	A	A
II	K6/00	Kidney, RT[b]	+	Fp[T], Fd	A	A
II	K8/00	Spleen, RT[c]	+	Fp[T], Fd	A	A
II	K9/00	Kidney, RT[c]	+	Fp[T], Th	B	B
IV	ST1/00	Skin lesion, RT[1]	+	Fd	A	A
IV	ST2/00	Skin lesion, RT[1]	+	Fd	A	A
IV	ST3/00	Kidney, RT[1]	+	Fd	A	A
IV	ST4/00	Skin lesion, RT[1]	+	Fd	A	A
IV	ST5/00	Skin lesion, RT[1]	+	Fd	A	A
IV	ST6/00	Skin lesion, RT[1]	–	Fp[T], Th	F	D
IV	ST7/00	Kidney, RT[1]	+	Fd	A	A
IV	ST8/00	Skin lesion, RT[1]	+	Fd	A	A
V	H1/00	Ovarian fluid sample 3, RT	–	Fp[T], Th	B	F
V	H2/00	Ovarian fluid sample 3, RT	–	Th, Fd	B	H
V	H3/00	Ovarian fluid sample 4, RT	–	Fp[T]	E	G
V	H4/00	Ovarian fluid sample 5, RT	+	Fp[T], Fd	A	E
IV	A6/00	Skin mucus, P[2]	–	Fp[T]	E	G
IV	A7b/00	Skin mucus, P[2]	–	Fp[T]	E	G
IV	A9/00	Skin mucus, P[2]	+	Fp[T]	I	I
II	K2/99	Tank water	+	Fp[T]	A	A
II	K3/99	Tank water	+	Fp[T]	A	A
III	M1/99	Tank water	–	*	B	H
III	M2/99	Inlet water	–	Fp[T]	H	A
IV	ST9/00	Pond water	–	Fp[T]	L	NR
V	H5/00	Inlet water	–	Fp[T], Fd	J	NR
V	H6/00	Tank water	–	Fp[T], Fd	J	NR
V	H7/00	Tank water	–	Fp[T]	K	NR

[1]Diseased fish. [2]Wild fish. [a–c]Same letter indicates isolates from 1 fish

cutaneously (s.c.) with 1.9 to 6.6×10^7 CFU fish^{-1} of the different isolates (20 fish isolate^{-1}) in 50 µl PBS. As control groups, 20 fish were injected with either 4.7×10^7 CFU fish^{-1} of the virulent strain T1-1 of *Flavobacterium psychrophilum* (Madetoja et al. 2000) in 50 µl PBS or only 50 µl PBS. After injection, the fish were marked using fin clipping and transferred into 3 tanks (5 groups tank^{-1}, see Table 4), and kept in conditions as described above. The water temperature during the experiment was 12 ± 1.6°C. Dead fish from the tanks were collected daily and samples from spleen, kidney and skin lesions (if present) were inoculated on TYES agar plates, which were incubated at 15°C for at least 7 d. Tissue samples from the survivors (1 to 5 fish from each group) were also inoculated on TYES agar. The identity of bacteria forming yellow colonies on the agar plates was tested using slide agglutination and elastinase production test (Madetoja et al. 2001).

RESULTS

Isolation of *Flavobacterium psychrophilum* from rainbow trout

Flavobacterium psychrophilum was isolated from skin lesions of 4 out of 5 fish with visible lateral skin lesions and from skin mucus of all 4 apparently healthy fish without lesions from Farm I. *F. psychrophilum* was isolated only from internal organs of 4 out of 8 apparently healthy fish obtained from Farm II. *F. psychrophilum* was present in skin lesions and mucus and internal organs in 8 out of 10 fish with skin lesions, but bacteria were not isolated from apparently healthy fish (n = 4) obtained from Farm IV (see Tables 1 & 4). *F. psychrophilum* was not isolated from rainbow trout obtained from Farm III.

Detection of *Flavobacterium psychrophilum* in ovarian fluid

Flavobacterium psychrophilum was detected and isolated in 3 out of 5 ovarian fluid samples using TYES agar cultivation, IFAT and nested PCR (Table 1). Pure culture of yellow colonies occurred on TYES agar, cultivated from one ovarian fluid sample. The number of *F. psychrophilum* in this sample was estimated to be 390 CFU ml^{-1}. The average total numbers of bacteria in ovarian fluid samples were 350 and 390 CFU ml^{-1}, estimated on TYES agar and TSA, respectively.

Detection of *Flavobacterium psychrophilum* in wild fish

The results of the detection of *Flavobacterium psychrophilum* in wild fish tissue using different methods are shown in Table 2. *F. psychrophilum* was detected

using nested PCR in skin mucus sample of 1 perch captured from the inlet water channel, and in brain samples of 2 roach out of 10 fish examined (roach and perch) and in skin mucus samples of 7 (2 roach, 5 perch) out of 10 fish captured from the outlet water channel. *F. psychrophilum* was observed using IFAT in skin mucus samples from 1 roach from the inlet water channel and in all 10 fish captured from the outlet water channel. *F. psychrophilum* was isolated on TYES agar from skin mucus samples of 3 perch, which were captured from the outlet water channel (Table 1). The skin mucus samples contained PCR inhibitory substances, but diluting the samples 1:10 or 1:100 reduced the inhibition impact (Table 2).

Shedding rate of *Flavobacterium psychrophilum* by naturally infected rainbow trout

The shedding rate of *Flavobacterium psychrophilum* by 3 apparently healthy rainbow trout, kept individually in experimental water, varied between 4.2×10^4 and 1.1×10^7 cells fish^{-1} h^{-1}. The number of *F. psychrophilum* shed into the water by 7 naturally infected rainbow trout with skin lesions varied between 4.2×10^4 and 1.7×10^8 cells fish^{-1} h^{-1}. *F. psychrophilum* was isolated from external and/or internal tissues of all experimental fish (n = 20 isolates) and experimental water (n = 6 isolates; Table 3).

Identification and characterization of *Flavobacterium psychrophilum* isolates

All isolates showing negative reaction in an esculin tube test, absence of growth on TSA plates, and positive PCR results were identified as *Flavobacterium psychrophilum* (n = 64; Tables 1 & 3). The isolates degraded gelatine and casein, and were catalase and cytochrome oxidase positive. Of the 64 *F. psychrophilum* isolates 42 degraded elastin.

Table 2. *Flavobacterium psychrophilum* infecting *Perca fluviatilis* and *Rutilus rutilus*. Detection of *F. psychrophilum* from perch and roach tissues using TYES, IFAT and nested PCR. The fish were captured in the inlet and outlet water channels near Farm IV. Results are expressed as number of fish positive for *F. psychrophilum*/number of fish examined. ND: not done

	Brain	Spleen	Kidney	Skin mucus
Inlet				
TYES	0/10	0/10	0/10	0/10
IFAT	0/10	0/10	0/10	1[c]/10
Nested PCR[a]	0/10	ND	ND	0/10
Nested PCR 1:10	0/10	ND	ND	1[c]/10
Nested PCR 1:100	0/10	ND	ND	1[c]/10
Outlet				
TYES	0/10	0/10	0/10	3[c]/10
IFAT	0/10	0/10	0/10	10[b,c]/10
Nested PCR[a]	2[b]/10	ND	ND	0/10
Nested PCR 1:10	1[b]/10	ND	ND	7[b,c]/10
Nested PCR 1:100	1[b]/10	ND	ND	7[b,c]/10

[a]Brain samples were diluted 1:4, skin mucus samples were undiluted. [b]Roach. [c]Perch

Serotyping

In the slide-agglutination tests, 5 different reaction patterns among the *Flavobacterium psychrophilum* isolates studied were observed (Tables 1 & 3). Eighteen isolates out of 64 (28%) agglutinated with 2 of the cross-absorbed antisera, and 2 isolates showed spontaneous agglutination. These 20 isolates were not serologically typed with the antisera used. The Fd serotype was the most common among the isolates and 30 out of 64 isolates (47%) examined agglutinated with anti-Fd serum. Variations in the agglutination of different isolates isolated simultaneously from 1 fish with different antisera were also observed.

Ribotyping

Based on the results of a previous study (Madetoja et al. 2001), *Cla*I and *Hae*III restriction enzymes were

Table 3. *Flavobacterium psychrophilum* isolated from *Oncorhynchus mykiss* and water. Characteristics of *F. psychrophilum* isolated from rainbow trout (n = 20, avg. weight 40 g) and experimental water (n = 6) after the shedding rate study. The fish were obtained from Farm IV. Water samples were taken from plastic containers where individual fish were kept for 1 h. +: positive reaction; −: negative reaction

No. of isolates	Source	Elastinase	Serotype	Ribotype	
				*Cla*I	*Hae*III
23	Skin mucus[a,b], lesions, spleen, kidney, water	+	Fd	A	A
1	Skin mucus[a]	−	Fp[T]	C	H
1	Skin mucus	−	Fp[T]	E	G
1	Skin mucus[b]	+	Fp[T], Fd	D	C

[a,b]Same letter indicates isolates from 1 fish

Dis Aquat Org 52: 109–118, 2002

Table 4. Mortality of *Oncorhynchus mykiss* (avg. weight 9 g), injected with selected isolates of *Flavobacterium psychrophilum* (n = 13) and *F. psychrophilum* strain T1-1. RT: rainbow trout; P: perch; s.c.: subcutaneously; –: negative; +: positive; NR: not restricted

Isolate	Tank no.	Source	Challenge dose 10^7 CFU fish^{-1} s.c.	Mortality 7 d post challenge (%)	Mortality 14 d post challenge (%)	Mortality 28 d post challenge (%)	Elastinase	Serotype	Ribotype *Cla*I	Ribotype *Hae*III
L5/00	3	Skin mucus, RT	3.6	85	100	100	–	Fp^T, Th	B	B
K4/00	3	Kidney, RT	1.9	5	45	70	+	Fp^T	A	A
ST3/00	1	Kidney, RT	2.5	25	90	90	+	Fd	A	A
A6/00	2	Skin mucus, P	4.9	0	5	10	–	Fp^T	E	G
A9/00	2	Skin mucus, P	3.2	25	40	40	+	Fp^T	H	I
H1/00	1	Ovarian fluid sample 3, RT	4.7	90	100	100	–	Fp^T, Th	B	F
H2/00	1	Ovarian fluid sample 3, RT	6.6	100	100	100	–	Th, Fd	B	H
H3/00	2	Ovarian fluid sample 4, RT	5.1	0	15	20	–	Fp^T	E	G
H4/00	2	Ovarian fluid sample 5, RT	5.3	95	100	100	–	Fp^T, Fd	A	E
M2/99	3	Inlet water	2.3	50	55	55	–	Fp^T	H	A
ST9/00	1	Pond water	6.1	0	5	5	–	Fp^T	L	NR
H5/00	3	Tank water	3.2	10	10	10	–	Fp^T, Fd	J	NR
H6/00	2	Tank water	1.9	80	80	80	–	Fp^T, Fd	J	NR
T1-1	3	Madetoja et al. (2000, 2001)	4.7	95	100	100	+	Fp^T, Th	C2[a]	H2[a]
Control	1		0	0	0	0				

[a]Identical profile with ribotype B/B (*Cla*I/*Hae*III)

selected for the ribotyping analysis. A total of 12 different ribotypes were found among 64 *Flavobacterium psychrophilum* isolates using *Cla*I (A–L) and 9 ribotypes among 60 studied isolates using *Hae*III (A–I) (Tables 1 & 3). Ribotype A was the most dominant among these, and 40 out of 64 examined isolates (63%) belonged to this ribotype, restricted using *Cla*I, and 40 out of 60 isolates (67%) using *Hae*III. Ribotype B was the second most common among the isolates (*Cla*I: 11 out of 64; and *Hae*III: 8 out of 60 examined). Ribotype B was the most dominant among *F. psychrophilum* isolates from Farm I (*Cla*I and *Hae*III: 7 out of 8 examined), and Ribotype A among the isolates from Farm II (*Cla*I and *Hae*III: 8 out of 9 examined) and Farm IV (*Cla*I: 30 out of 38; and *Hae*III: 30 out of 37 examined). *F. psychrophilum* isolated from rearing water of Farms III and V belonged to Ribotypes B, H (*Cla*I) and H, A (*Hae*III), and J, K (*Cla*I), respectively. *Flavobacterium psychrophilum* isolates from wild perch, belonged to 2 ribotypes (*Cla*I: E, H and *Hae*III: G, I). *Flavobacterium psychrophilum* isolated from ovarian fluid samples formed a heterogenous group (*Cla*I: A, B, E and *Hae*III: E, F, G, H). Variations in the ribopatterns of the different isolates isolated simultaneously from 1 fish were also observed (Tables 1 & 3). Examples of different ribotypes of *F. psychrophilum* using *Cla*I are shown in Fig. 1.
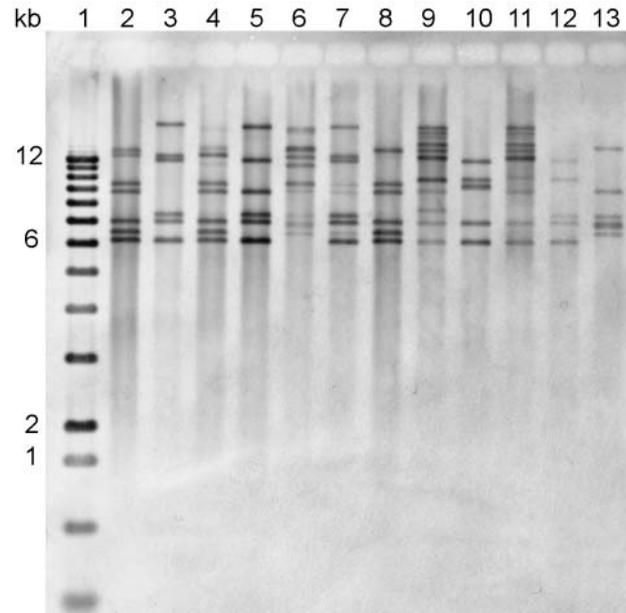

Fig. 1. Examples of different *Cla*I patterns of *Flavobacterium psychrophilum* isolates. Lanes: (1) 1 kb molecular size marker indicated as kilobase (kb) on the left; (2) Ribotype A; (3) B; (4) C; (5) D; (6) E; (7) F; (8) G; (9) H; (10) I; (11) J; (12) K; (13) L

Challenge experiment

The results of the challenge experiments (Table 4) generally indicated that isolates, which belonged to serotype Fp^T, caused lower mortality (10 to 70%) than other tested isolates of different agglutination types and the positive control strain T1-1 (90 to 100%). Mortality started 4 d post-infection and was highest at 5 to 7 d post-injection in fish. Some infected moribund fish showed dark coloration of skin and exophtalmia. Dead fish showed skin necrosis and haemorrhages at the site of injection, and the skin necrosis had progressed into open lesions in fish from Day 10 to the end of the experiments. Internally, dead fish had enlarged spleens, pale head kidneys and anaemia. *Flavobacterium psychrophilum* was isolated from spleen and/or kidney and skin ulcer of all dead fish. *F. psychrophilum* was also isolated from the spleen and kidney of 1 surviving fish (injected with isolate K4/00) 28 d after infection. The re-isolated *F. psychrophilum* indicated similar capability to elastin degradation than before challenge. Mortality was not observed in negative control fish.

DISCUSSION

The aim of the present study was to investigate the occurrence of *Flavobacterium psychrophilum* in fish farms and fish-farming environments and to compare characteristics of isolated *F. psychrophilum* originating from different sources. The pathogen was isolated from rainbow trout, skin mucus of wild fish and ovarian fluid samples of farmed rainbow trout brood fish. *F. psychrophilum* isolated from rainbow trout, from different farms, belonged mainly to a particular agglutination type and/or ribotype. In general, the isolates from the fish of each farm formed a rather homogenous group concerning production of elastinase, agglutination with different antisera and ribotype. However, isolates showing characteristics different from the majority of those from each farm were always present. The results indicated that a certain clone of *F. psychrophilum* can dominate in one fish farm, which may be important knowledge for the development of site-specific vaccines. The results of our virulence experiments indicated that several of the examined isolates caused high mortality when injected into rainbow trout. However, isolates belonging to serotype Fp^T caused lower mortality in challenged rainbow trout and thus confirm the results of previous studies (Lorenzen 1994, Madsen & Dalsgaard 1998, Decostere et al. 2000).

Flavobacterium psychrophilum isolates obtained from pooled ovarian fluids samples were serologically and genetically heterogenous. None of the 100 female fish from which ovarian fluids were collected showed any typical disease signs to *F. psychrophilum*. The isolated *F. psychrophilum* represented different serotypes and ribotypes, which may have been transmitted to the brood fish from the rearing water. One of the isolates from the brood fish showed identical ribopattern (*Cla*I: B and *Hae*III: H) and elastin degradation compared to an isolate obtained from tank water. Another ribotype (*Cla*I: E and *Hae*III: G) present in ovarian fluid was isolated from mucus of wild fish as well as mucus of rainbow trout from Farm IV. These isolates also showed identical patterns of agglutination and elastin degradation. Isolates belonging to these ribopatterns, however, were never obtained from internal organs of diseased fish. Three out of 4 isolates from ovarian fluid samples caused 100% mortality, and 1 isolate belonging to the serotype Fp^T caused lower mortality (20%) when injected into rainbow trout. The results of the present study suggest that rainbow trout brood fish might carry virulent *F. psychrophilum* and appear to be a potential source of infection. However, these bacteria did not seem to cause diseases in brood fish. *F. psychrophilum* in ovarian fluids might enhance the immunocompetence of brood fish and partly explain the elevated antibody level against *F. psychrophilum* of gravid rainbow trout observed by Wiklund & Dalsgaard (2002). In previous studies *F. psychrophilum* has been isolated from ovarian fluids of naturally infected salmonids (Holt 1987, Rangdale et al. 1996, Ekman et al. 1999). Holt (1987) observed that 1 isolate of *F. psychrophilum* from salmonid ovarian fluid caused high mortality when injected into coho salmon. However, serological and genetic characterizations of *F. psychrophilum* isolates were not done in those studies.

Flavobacterium psychrophilum was detected from brain, and detected and isolated from skin mucus of wild perch and roach without any signs of disease. The skin mucus of the fish contained PCR inhibitory substances, and thus *F. psychrophilum* detection using nested PCR failed in non-diluted skin mucus samples. *F. psychrophilum* was detected from the diluted samples containing fewer inhibitory components than the non-diluted samples, although the number of cells decreased during the dilution procedure. The elastinase-negative isolates from perch belonged to a different ribotype (*Cla*I: E and *Hae*III: G) than the elastinase-positive isolate, which showed a ribotype (*Cla*I: H and *Hae*III: I) unique among the studied *F. psychrophilum*. Ribotypes E (*Cla*I) and G (*Hae*III) were found only from 2 other isolates from skin mucus and ovarian fluid samples and discussed above. In wild fish, *F. psychrophilum* has previously been reported isolated from a diseased wild eel and pale chub (Lehmann et al. 1991, Iida & Mizokami 1996). The iso-

lates from perch belonged to serotype Fp^T and caused low or moderate mortality (10 and 40%) among rainbow trout. Our results suggested that *F. psychrophilum* was present in wild fish proximate to fish farms and may be introduced into the farms by the wild fish. Although the isolates in the present study caused mortality when high number of cells were injected into rainbow trout, it, nevertheless, is concluded that these isolates might not necessarily cause mortality under rearing conditions. A detailed analysis of ribotype, serotype and virulence of several *F. psychrophilum* isolates from wild fish are needed before a final conclusion can be reached concerning the significance of such isolates.

Flavobacterium psychrophilum obtained from the rearing water of Farm II showed reaction patterns (ribotype, agglutination and elastin degradation) identical to those of some of the isolates from rainbow trout from the same farm. In contrast, the elastinase-negative, non-typable isolate from the rearing water of Farm III belonged to an infrequently occurring ribotype, which was also found in an ovarian fluid sample. The other isolate from the rearing water of Farm III was elastinase-negative, belonged to serotype Fp^T and showed a unique ribotype H using *Cla*I. The isolates from rearing water caused low or moderate mortality in rainbow trout (5 to 55%), except for 1 isolate which caused high mortality (80%). These results suggest that certain variants of *F. psychrophilum* belonging to serotype Fp^T in rearing water most likely are not involved in disease outbreaks in rainbow trout.

Madetoja et al. (2000) reported that artificially infected (s.c. injection) rainbow trout shed 6.8×10^3 to 3.0×10^6 cells of *Flavobacterium psychrophilum* fish^{-1} h^{-1}. These values are lower than those obtained in our present study using naturally infected rainbow trout (4.2×10^4 to 1.7×10^8 cells of *F. psychrophilum* fish^{-1} h^{-1}). The difference between the number of cells detected in the present study and that by Madetoja et al. (2000) could be due to the different weights of the fish studied. In the present study the average weight of the fish was 40 g, but in the previous study it was 1.6 g (Madetoja et al. 2000). It is thus suggested that in commercial fish farms removal of diseased and moribund fish from tanks/ponds should be done in order to decrease the number of *F. psychrophilum* in the water and thus diminish the infection pressure. However, apparently healthy, but presumably infected, fish without external signs of disease kept together in the same tank/pond with diseased fish, also shed *F. psychrophilum* into the water. *F. psychrophilum* isolated from water in the shedding rate experiment were identical (serotypes and ribotypes) to those isolates obtained from the internal organs of the experimental fish as well as from the internal organs of the fish taken directly from fish Farm IV.

Forty-two out of 64 studied *Flavobacterium psychrophilum* isolates produced elastinase and, interestingly, all elastinase-negative isolates from fish (n = 16, Tables 1 & 3) were from external tissues (skin mucus or skin lesions) or ovarian fluid. Madsen & Dalsgaard (1999) suggested that production of elastinase might be crucial for the virulence of some *F. psychrophilum* isolates. In the present study a relationship between capability of elastin degradation and high virulence of *F. psychrophilum* was not demonstrated. However, the elastinase production could be an important mechanism for the invasion of *F. psychrophilum* into the fish.

Twenty *Flavobacterium psychrophilum* isolates out of 64 examined were not typed using slide agglutination and cross-absorbed antisera against the serotypes described by Lorenzen & Olesen (1997). However, it is possible that some of these non-typable isolates might be typed using a more discriminating ELISA method (Lorenzen & Olesen 1997) and/or represent new serotypes of *F. psychrophilum*.

In the present study, elastinase-positive variants of *Flavobacterium psychrophilum* isolates belonging to serotype Fp^T were also found in the internal organs of 1 fish. The tested elastinase-positive variant caused the highest mortality of the isolates belonging to serotype Fp^T when injected into rainbow trout. According to the results of the present and previous experiments (Madsen & Dalsgaard 2000), it is suggested that certain variants of serotype Fp^T may be pathogenic for rainbow trout. The predominant serotypes Th and Fd of European *F. psychrophilum* isolates have been connected with disease outbreaks of rainbow trout (Lorenzen & Olesen 1997, Madsen & Dalsgaard 1998, 1999, Dalsgaard & Madsen 2000). In the present study isolates of serotype Fd were involved in disease outbreak at 1 farm (Farm IV, Tables 1 & 3) and isolates of serotype Th were not involved at all. *F. psychrophilum* isolates from Farm I were from skin mucus and skin lesions of rainbow trout, and most of the isolates reacted with 2 different antisera (Fp^T and Th). In a previous study (Madetoja et al. 2001) *F. psychrophilum* from Farm I were isolated from internal organs of diseased rainbow trout, and belonged to serotype Th or reacted with 2 different antisera, Fp^T and Th or Fp^T and Fd. *F. psychrophilum* isolates from Farm II showed heterogenous agglutination patterns with tested antisera.

All *Flavobacterium psychrophilum* isolates belonging to serotype Fd (n = 30) were ribotype A (*Cla*I and *Hae*III). The restriction ladders of the ribotype A (*Cla*I and *Hae*III) were identical with restriction patterns C1 (*Cla*I) and H1 (*Hae*III) previously reported by Madetoja et al. (2001), who suggested a relationship between serotype Fd and genotype F1 (ribotype C1/H1/P1), which thus is confirmed by the present study. A possible relationship between antigenicity and ribotypes of *F. psychrophilum*

isolates in previous studies has not been evaluated (Cipriano et al. 1996, Chakroun et al. 1998). However, Madsen & Dalsgaard (2000) found that 75% of studied *F. psychrophilum* isolates belonged to a certain ribotype and serotype Th or Fd.

According to the results of the present study, there seems to be different potential sources of *Flavobacterium psychrophilum* infection in fish farms. The occurrence of highly virulent *F. psychrophilum* in fish mucus and ovarian fluids indicate that transport of fish poses a significant risk of transmitting *F. psychrophilum* between farms. However, it has to be pointed out that based on the present study low-virulent isolates of *F. psychrophilum* also can be present in the mucus of wild fish, ovarian fluid of brood fish and rearing water. *Flavobacterium psychrophilum* from different farms were serologically and genetically heterogenous. Serologically and genetically different *F. psychrophilum* isolates were also found from the same farms and even from the same individual fish. Farmed rainbow trout female brood fish seemed to carry different *F. psychrophilum* in ovarian fluid. The results of the present study also support the previous findings (Madetoja et al. 2001) concerning a relationship between a certain serotype and ribotype of *F. psychrophilum* isolates.

LITERATURE CITED

Amita K, Hoshino M, Honma T, Wakabayashi H (2000) An investigation on the distribution of *Flavobacterium psychrophilum* in the Umikawa river (Japanese). Fish Pathol 35:193–197

Bernardet JF, Segers P, Vancanneyt M, Berthe F, Kersters K, Vandamme P (1996) Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1978). Int J Syst Bacteriol 46:128–148

Borg AF (1960) Studies on myxobacteria associated with diseases in salmonid fishes. J Wildl Dis 8:1–85

Brown LL, Cox WT, Levine RP (1997) Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. Dis Aquat Org 29:213–218

Bruun MS, Schmidt AS, Madsen L, Dalsgaard I (2000) Antimicrobial resistance patterns in Danish isolates of *Flavobacterium psychrophilum*. Aquaculture 187:201–212

Cepeda C, Santos Y (2000) Rapid and low-level toxic PCR-based method for routine identification of *Flavobacterium psychrophilum*. Int Microbiol 3:235–238

Chakroun C, Grimont F, Urdaci MC, Bernardet JF (1998)

Fingerprinting of *Flavobacterium psychrophilum* isolates by ribotyping and plasmid profiling. Dis Aquat Org 33: 167–177

Cipriano RC, Schill WB, Teska JD, Ford LA (1996) Epizootiological study of bacterial cold-water disease in Pacific salmon and further characterization of the etiologic agent, *Flexibacter psychrophila*. J Aquat Anim Health 8:28–36

Dalsgaard I (1993) Virulence mechanisms in *Cytophaga psychrophila* and other *Cytophaga*-like bacteria pathogenic for fish. Annu Rev Fish Dis 1:127–144

Dalsgaard I, Madsen L (2000) Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. J Fish Dis 23:199–209

Decostere A, Lammens M, Haesebrouck F (2000) Difficulties in experimental infection studies with *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) using immersion, oral and anal challenges. Res Vet Sci 69: 165–169

Ekman E, Börjeson H, Johansson N (1999) *Flavobacterium psychrophilum* in Baltic salmon *Salmo salar* brood fish and their offspring. Dis Aquat Org 37:159–163

Holt RA (1987) *Cytophaga psychrophila*, the causative agent of bacterial cold-water disease in salmonid fish. PhD thesis, Oregon State University, Corvallis

Holt RA, Rohovec JS, Fryer JL (1993) Bacterial coldwater disease. In: Inglis V, Roberts RJ, Bromage NR (eds) Bacterial diseases of fish. Blackwell Scientific Publications, Oxford, p 3–23

Iida Y, Mizokami A (1996) Outbreaks of coldwater disease in wild ayu and pale chub. Fish Pathol 31:157–164

Izumi S, Wakabayashi H (1999) Further study on serotyping of *Flavobacterium psychrophilum*. Fish Pathol 34:89 – 90

Izumi S, Wakabayashi H (2000) Sequencing of *gyrB* and their application in the identification of *Flavobacterium psychrophilum* by PCR. Fish Pathol 35:93–94

Lehmann J, Mock D, Stürenberg FJ, Bernardet JF (1991) First isolation of *Cytophaga psychrophila* from a systemic disease in eel and cyprinids. Dis Aquat Org 10:217–220

Lorenzen E (1994) Studies on *Flexibacter psychrophilus* in relation to rainbow trout fry syndrome (RTFS). PhD thesis, National Veterinary Laboratory, Århus, and Royal Veterinary and Agricultural University, Copenhagen

Lorenzen E, Karas N (1992) Detection of *Flexibacter psychrophilus* by immunofluorescence in fish suffering from fry mortality syndrome: a rapid diagnostic method. Dis Aquat Org 13:231–234

Lorenzen E, Olesen NJ (1997) Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome II: serological studies. Dis Aquat Org 31:209–220

Madetoja J, Wiklund T (2002) Detection of the fish pathogen *Flavobacterium psychrophilum* in water from fish farms. Syst Appl Microbiol 25:259–266

Madetoja J, Nyman P, Wiklund T (2000) *Flavobacterium psychrophilum*, invasion into and shedding by rainbow trout *Oncorhynchus mykiss*. Dis Aquat Org 43:27–38

Madetoja J, Hänninen ML, Hirvelä-Koski V, Dalsgaard I, Wiklund T (2001) Phenotypic and genotypic characterization of *Flavobacterium psychrophilum* from Finnish fish farms. J Fish Dis 24:469–479

Madsen L, Dalsgaard I (1998) Characterization of *Flavobacterium psychrophilum*; comparison of proteolytic activity and virulence of strains isolated from rainbow trout (*Oncorhynchus mykiss*). In: Barnes AC, Davidson GA, Hiney MP, McIntosh D (eds) Methodology in fish diseases research. Fisheries Research Services, Aberdeen, p 45–52

Madsen L, Dalsgaard I (1999) Reproducible methods for

experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Org 36:169–176

Madsen L, Dalsgaard I (2000) Comparative studies of Danish *Flavobacterium psychrophilum* isolates: ribotypes, plasmid profiles, serotypes and virulence. J Fish Dis 23: 211–218

Rangdale RE (1995) Studies on rainbow trout fry syndrome (RTFS). PhD thesis, Ministry of Agriculture, Fisheries and Food, Weymouth, and University of Stirling

Rangdale RE, Richards RE, Alderman DJ (1996) Isolation of *Cytophaga psychrophila*, causal agent of Rainbow Trout fry Syndrome (RTFS) from reproductive fluids and egg surfaces of rainbow trout (*Oncorhynchus mykiss*). Bull Eur Assoc Fish Pathol 16:63–67

Rimaila-Pärnänen E, Hirvelä-Koski V, Niemi A, Seppänen J, Hernesniemi E (1997) Cold water disease in rainbow trout—case report and oxytetracycline residues after treatment (abstract in English). Suom Elainlaakaril 103: 140–144

Schmidt AS, Bruun MS, Dalsgaard I, Pedersen K, Larsen JL (2000) Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Appl Environ Microbiol 66: 4908–4915

Toyama T, Kita-Tsukamoto K, Wakabayashi H (1994) Identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal DNA. Fish Pathol 29:271–275

Urdaci MC, Chakroun C, Faure D, Bernardet JF (1998) Development of a polymerase chain reaction assay for identification and detection of the fish pathogen *Flavobacterium psychrophilum*. Res Microbiol 149:519–530

Wakabayashi H, Toyama T, Iida T (1994) A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. Fish Pathol 29:101–104

Wiklund T, Dalsgaard I (2002) Survival of *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) serum *in vitro*. Fish Shellfish Immunol 12:141–153

Wiklund T, Kaas K, Lönnström L, Dalsgaard I (1994) Isolation of *Cytophaga psychrophila* (*Flexibacter psychrophilus*) from wild and farmed rainbow trout (*Oncorhynchus mykiss*) in Finland. Bull Eur Assoc Fish Pathol 14:44–46

Wiklund T, Madsen L, Bruun MS, Dalsgaard I (2000) Detection of *Flavobacterium psychrophilum* from fish tissue and water samples by PCR amplification. J Appl Microbiol 88: 299–307