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Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome II: serological studies

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ABSTRACT: The possibility of serological differentiation between isolates of *Flavobacterium psychrophilum* was analyzed by ELISA and slide agglutination. Twenty-five Danish isolates and 20 isolates from other European countries were studied using polyclonal rabbit antisera and whole-cell preparations. Unabsorbed as well as reciprocally absorbed antisera and purified Ig preparations derived from the antisera were included. Most of the isolates originated from clinical outbreaks of rainbow trout fry syndrome (RTFS) or coldwater disease (CWD), but some were isolated from asymptomatic fish or from other fish species with different disease signs. The ELISA showed the existence of different serotypes most distinctly, but slide agglutination supported the ELISA results. Three serotypes were found among the isolates studied: 1 major serotype (serotype Th) represented most of the Danish isolates and isolates from other European countries; 2 minor serotypes (serotypes Fd and Fp^T) also occurred. Serotype Th could be further divided into a major subtype, Th- 1, and a minor subtype, Th-2. Serotype Fp^T was defined by the type strain *F. psychrophilum* NCIMB 1947^T, and seemed to include mostly isolates from asymptomatic fish or from fish species other than rainbow trout.

KEY WORDS: *Flavobacterium psychrophilum* · Rainbow trout fry syndrome · Coldwater disease · Serotypes · ELISA · Slide agglutination

INTRODUCTION

Since the 1940s, a disease named peduncle disease (Davis 1946, cited by Bullock et al. 1971), low temperature disease (Borg 1960) or (bacterial) coldwater disease (B(CWD)) (Wood & Yasutake 1956, Holt et al. 1989) has affected fry and fingerlings of coho salmon *Oncorhynchus kisutch* in the USA. Rainbow trout *O. mykiss* and several other salmonids may also be affected (Bullock et al. 1971, Schachte 1983, Amos 1985, Holt 1987). During the past 10 yr, a disease known as rainbow trout fry syndrome (RTFS) has spread over most of Europe causing serious losses in rainbow trout hatcheries among fry and fingerlings (Weis 1987, Bernardet et al. 1988, Lehmann et al. 1988, Baudin-Laurencin et al. 1989, Lorenzen et al. 1991, Bruno 1992, Santos et al. 1992, Sarti et al. 1992, Toranzo & Barja 1993).

Whereas RTFS is typically characterized by internal pathological signs such as splenomegaly and anaemia (Bernardet et al. 1988, Baudin-Laurencin et al. 1989, Lorenzen et al. 1991, Santos et al. 1992), signs of CWD are usually external and include skin and muscle lesions on the flank or in the peduncle area (Davis 1946, Wood & Yasutake 1956, Borg 1960, Wood 1974, Holt 1987). Larger, chronically infected fish may develop scoliosis, lordosis (Conrad & DeCew 1967) and abnormal swimming behaviour (Kent et al. 1989, Meyers 1989).

At low water temperatures (<7–8°C) such as are encountered in winter signs very similar to those described for CWD in USA have also been observed in Europe among fingerling/table-size rainbow trout (10 to 300 g) (Weis 1987, Lehmann et al. 1988, Dalsgaard & Hørlyck 1990, Bruno 1992, Santos et al. 1992, Lorenzen et al. 1997) as well as among other reared salmonid species (O. Thoresen, Norway, V. Hirvelä-Koski & T. Pohjanvirta, Finland, pers.

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comms.). Other non-salmonid fish species have been reported as susceptible, although exhibiting somewhat different pathological signs (Lehmann et al. 1991).

Recently, the same bacterium, *Flavobacterium psychrophilum* (previously *Flexibacter psychrophilus/Cytophaga psychrophila*; Bernardet et al. 1996), was shown to be involved in both CWD and RTFS (Bernardet & Kerouault 1989, Santos et al. 1992, Lorenzen et al. 1997), with the recent fulfilments of Koch's postulates for RTFS (Evensen & Lorenzen 1996). In phenotypic studies, isolates from Denmark and other European countries proved to be very similar to strains from the USA and France studied by others. Comparative plasmid analyses, however, demonstrated differences among the European isolates and between American and European isolates (Holt 1987, Lorenzen et al. 1997). As the plasmid profiles revealed only a few plasmids to be present, some other characters were needed for distinguishing between isolates in epidemiological studies.

Using slide agglutination with rabbit antisera and whole-cell preparations, Pacha (1968) and Holt (1987) found no serological differences among 10 and 28 American isolates, respectively. Macroscopic tube agglutination and reciprocal absorption, however, demonstrated at least 2 different serotypes among 6 of the American isolates studied by Holt (1987). Wakabayashi et al. (1994) demonstrated 2 serotypes using absorbed antisera and O-antigen preparations of *Flavobacterium psychrophilum* in microtiter agglutination tests: serotype O-1 comprising isolates from coho salmon in Japan and USA, and serotype O-2 comprising isolates from ayu *Plecoglossus altivelis* and rainbow trout in Japan. Pacha & Porter (1968) and Bullock (1972) found *Flavobacterium columnare* and *F. psychrophilum* to differ clearly from each other as well as from non-pathogenic cytophagas based on slide-agglutination of whole cells.

The present work was undertaken to search for possible serological differences among the European isolates of *Flavobacterium psychrophilum* despite the phenotypic uniformity reported thus far for the organism (Lorenzen et al. 1997). The type strains *F. psychrophilum* NCIMB 1947^T and *F. columnare* NCIMB 2248^T, both isolated in the USA, were included for comparison. The results of Holt (1987) and Pacha (1968) indicated that slide agglutination using unabsorbed antisera and whole cell preparations might not be sufficient for distinguishing between different serotypes. Therefore, the present study utilized both an enzyme-linked immunosorbent assay (ELISA) and the slide agglutination test, the reactants being whole cell preparations and unabsorbed as well as absorbed antisera.

MATERIALS AND METHODS

Bacterial isolates. The bacterial isolates included in our study are listed in Table 1. Isolates 1 to 25 were collected from clinical outbreaks of either CWD or RTFS in Danish fish farms during 1990–93. Isolates a to t were received from other European countries and originated mostly from rainbow trout, like the Danish isolates. However, some of the non-Danish isolates were collected from other fish species having signs of disease different from CWD and RTFS. The type strains *Flavobacterium columnare* NCIMB 2248^T (Fc^T) and *F. psychrophilum* NCIMB^T (Fp^T) were also included in the study.

Cultivation. The bacteria were cultivated at 15 to 17°C on modified Anacker & Ordal's medium (Anacker & Ordal 1955), henceforth referred to as Anacker & Ordal's agar/broth enriched (AOAE/AOBE) (Lorenzen 1993).

Bacteriological identification. Identification of the isolates was based in part on the phenotypic characters described by Bernardet et al. (1990) and has been detailed in a previous study (Lorenzen et al. 1997). Briefly, these features included morphology and pigmentation of the colonies, morphology and motility of the bacteria in wet mount preparations, reactivity of colonies with the API ZYM gallery, presence of catalase, cytochrome oxidase and flexirubin, and the ability to absorb Congo red, and to hydrolyze starch and tyrosine.

Rabbit antisera. The antisera used in the study are listed in Table 2. Antisera were produced against 2 Danish isolates, no. 5 (~Th or 911209-1) and no. 14 (~Fd or 900530-4/2), and named anti-Th and anti-Fd, respectively. Antisera were also produced against the type strains Fp^T and Fc^T and named anti-Fp^T and anti-Fc^T, respectively. Isolate Th (911209-1) originated from the first documented outberak of RTFS in Denmark and anti-Th was the first antiserum produced. Isolate Fd (900530-4/2), which originated from a later clinical outbreak, did not agglutinate with anti-Th and was therefore used for producing another antiserum, anti-Fd. The antisera were raised according to the method of Lancefield et al. (1975) with the following modifications. Bacteria were grown in AOBE for 2 to 3 d, centrifuged 4187 × *g* for 45 min, washed 3 times in 0.1 M phosphate-buffered saline (PBS) pH 7.2, and inactivated by resuspension in 0.3% phosphate-buffered formalin at 4°C for at least 48 h. Before injection, the suspension was centrifuged at 4187 × *g* for 45 min and the bacteria resuspended in PBS. The concentration was adjusted by spectrophotometer (520 nm) to approximately 1×10^8 cells ml^{-1}. New Zealand white rabbits were given 9 intravenous injections of this suspension in the ears over a period of 3 wk. The first

Table 1. Isolates of *Flavobacterium psychrophilum* included in the study

Code	Isolate	Source: species	Tissue	Clinical signs	Geographic origin	Year
Fc^T	*F. columnare* NCIMB 2248^T	Chinook salmon	Kidney	–	Washington, USA	1955
Fp^T	*F. psychrophilum* NCIMB 1947^T	Coho salmon	Kidney	CWD	Washington, USA	Unknown
1	910611-1	Rbt*, fry	Spleen	Costiasis[a]	Denmark	1991
2	911209-2 (NCIMB 13384)	Rbt, fry	Spleen	RTFS	Denmark	1990
3	910803-1	Rbt, fry	Kidney	RTFS	Denmark	1991
4	910516-1	Rbt, fry	Spleen	IPNV[b]	Denmark	1991
5 (Th)	911209-1 (NCIMB 13383)	Rbt, fry	Spleen	RTFS	Denmark	1990
6	910619-1	Rbt, fry	Spleen	RTFS	Denmark	1991
7	910614-2	Rbt, young	Skin	CWD	Denmark	1991
8	911126-2	Rbt, young	Spleen	CWD	Denmark	1991
9	911126-3	Rbt, young	Skin	CWD	Denmark	1991
10	911009-3	Rbt, young	Skin	CWD	Denmark	1991
11	910614-3	Rbt, young	Kidney	CWD	Denmark	1991
12	910614-5	Rbt, young	Eye	CWD	Denmark	1991
13	900406-1	Rbt, young	Kidney	CWD	Denmark	1990
14 (Fd)	900530-4/2	Rbt, fry	Intestine	RTFS	Denmark	1990
15	930210-1	Rbt, breeding fish	Eye	(CWD)[c]	Denmark	1993
16	930223-2	Rbt, young	Kidney	CWD/RTFS	Denmark	1993
17	930305-1	Rbt, breeding fish	Kidney	(CWD)[c]	Denmark	1993
18	930310-1	Rbt fry	Spleen	RTFS	Denmark	1993
19	930324-1	Rbt, young	Kidney	CWD	Denmark	1993
20	930407-1	Rbt, young	Spleen	CWD/RTFS	Denmark	1993
21	930413-1	Rbt, young	Skin	CWD	Denmark	1993
22	930427-2	Rbt, fry	Spleen	RTFS	Denmark	1993
23	930610-1	Rbt, fry	Spleen	RTFS	Denmark	1993
24	930611-2	Rbt, fry	Spleen	RTFS	Denmark	1993
25	930616-1	Rbt, fry	Spleen	RTFS	Denmark	1993
a	UPI 293[1]	Rbt, fry	Spleen	RTFS	Dorset, UK	1992
b	WIL 293[1]	Rbt, fry	Spleen	RTFS	Devon, UK	1992
c	PT 4.1[2]	Rbt, fingerling	Spleen	RTFS	Spain	1992
d	16/90[3]	Tench**	Spleen	Haemorrhage	Germany	1990
e	TG 28/86[4]	Rbt, young	Blister	CWD	Touraine, France	1986
f	Tours 5/I[4]	Carp***	Gills	Gill injury	Touraine, France	1992
g	11 522[5]	Rbt, fry	Spleen	RTFS	France	1993
h	11 524[5]	Rbt, fry	Spleen	RTFS	France	1993
i	Fi 88/93[6]	Rbt, fry	Spleen	RTFS	Switzerland	1993
j	Fi 147/93[6]	Rbt, young	Spleen	RTFS/CWD	Switzerland	1993
k	Fi 171/93[6]	Rbt, fingerling	Spleen	RTFS[d]	Switzerland	1993
l	Fi 196/93[6]	Rbt, fingerling	Spleen	ICH[e]	Switzerland	1993
m	Fi 206/93[6]	Rbt, fingerling	Spleen	None	Switzerland	1993
n	Fi 332/92[6]	Rbt, fingerling	Spleen	RTFS	Switzerland	1992
o	255/93[7]	Brown trout****	Skin	Skin lesion	Finland	1993
p	3441/93[7]	Rbt, young	Skin	Skin lesion	Finland	1993
q	K 129-3/91[8]	Whitefish*****	Skin	Skin lesion	Finland	1991
r	K 129-4/91[8]	Whitefish	Skin	Skin lesion	Finland	1991
s	S 231[9]	Rbt, fry	Spleen	Pale gills	Northern Ireland	1993
t	386/94[10]	Brown trout, parr	Skin	Skin lesion	Norway	1994

Isolates were kindly provided by the following scientists:
[1]Dr Rachel Rangdale, Fish Disease Laboratory, Weymouth, UK; [2]Drs A. E. Toranzo & J. L. Barja, University of Santiago de Compostela, Spain; [3]Dr Dieter Mock, Landesanstalt für Fischerei, Nordrhein-Westfalen, Germany; [4]Dr Jean-Francois Bernardet, INRA, Jouy-en-Josas, France; [5]Dr Martine Vigneulle, CNEVA, Plouzané, Brest, France; [6]Dr Thomas Wahli, Universität Bern, Switzerland; [7]Dr Varpu Hirvelä-Koski, National Veterinary Institute, Oulu, Finland; [8]Dr Tarja Pohjanvirta, National Veterinary and Food Research Institute, Kuopio, Finland; [9]Dr Joyce McCormick, Fish Disease Laboratory, Belfast, Northern Ireland; [10]Dr Ove Thoresen, Central Veterinary Laboratory, Oslo, Norway

*Rbt: rainbow trout, **tench: *Tinca tinca*, ***carp: *Cyprinus carpio*, ****brown trout: *Salmo trutta lacustris*, *****whitefish: *Coregonus muksun*

[a]The fry suffered from an infection with *Ichthyobodo necator* (syn. *Costia necatrix*), with no signs of rainbow trout fry syndrome (RTFS)
[b]The fry were infected with the virus causing infectious pancreatic necrosis (IPNV), and showed clinical signs of IPN
[c]No signs of coldwater disease (CWD) other than blindness
[d]The fingerlings showed clinical signs of RTFS, and were also infected with *Ichthyobodo necator* and with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease
[e]The fingerlings showed very few signs of RTFS, and were recovering from an infection by *Ichthyophthirius multifiliis*

Table 2. Antigens and rabbit antisera/purified IgG fractions included in the study

Antigen	Paired rabbits (pooled)	Antiserum / purified purified IgG fraction	Titer[a]
F. psychrophilum 911209-1 (Th)	F03/F04	Anti-Th	512
F. psychrophilum 900530-4/2 (Fd)	F17/F18	Anti-Fd	256
F. psychrophilum NCIMB 1947^T (Fp^T)	F19/F20	Anti-Fp^T	128
F. columnare NCIMB 2248^T (Fc^T)	F15/F16	Anti-Fc^T	512

[a]Antisera titers were determined by microplate agglutination with the homologous antigens, the titers being the reciprocal value of the highest serum dilution showing a macroscopically visible agglutination

3 injections were 0.5 ml each and the subsequent 6 injections were 1.0 ml each. The rabbits were bled 8 d after the last injection by incision into the ear veins. Blood was centrifuged at 1157 × *g* for 30 min and the serum recovered and heated at 56°C for 30 min to inactivate complement. Antisera from 2 similarly immunized rabbits were pooled (anti-Th: F03 & F04, anti-Fd: F17 & F18, anti-Fp^T: F19 & F20, and anti-Fc^T: F15 & F16). The immune sera were stored undiluted at –20°C.

Titer: Titers of the antisera were determined by microplate agglutination modified after Davies (1990): in round-bottomed microtiter plates 2 replicates of ten 2-fold serial dilutions to 1:1024 of each antiserum were made in 50 µl PBS-1%BSA (PBS supplemented with 1% bovine serum albumin; R. Rangdale, Weymouth, UK, pers. comm.). Approximately 1 × 10^6 bacterial cells, measured as previously, of the homologous isolate suspended in 50 µl PBS-1%BSA were added to each well. Plates were incubated on an orbital shaker at 37°C for 2 h and subsequently overnight at 4°C. Titers, recorded visually against a dark background, were defined as the reciprocal value of the highest serum dilution showing a macroscopically visible agglutination.

Purification: Purification of the immunoglobulin G (IgG) fractions in the antisera was done by protein-A sepharose affinity chromatography according to the supplier's instructions (Pharmacia). Bound IgG was eluted by means of 0.03 M citric acid pH 3.0, and protein concentration was determined by spectrophotometer at 280 nm (A_{280} = 1.5 equivalent to 1 mg protein ml^{-1}). Purified IgG was biotinylated by use of N,N-hydroxysuccinimidobiotin (Sigma) as described by Tijssen (1987).

Absorption: Reciprocal absorption of antisera (for slide agglutination) and of the protein-A purified Ig fractions of the antisera (for ELISA) (Table 3) was accomplished by diluting the reagents 1:2 and 1:100, respectively, in PBS supplemented with 15 mM sodium azide, and by incubating overnight on a mill at 4°C with approximately 1 × 10^9 cells of each of the heterologous isolates per ml (Table 3). Following incubation, the bacterial suspensions were centrifuged at 4187 × *g* for 1 h and the supernatants were recovered and filtered through a 0.45 µm filter. The absorbed antisera and Ig fractions were supplemented with 1% BSA and stored at 4°C for up to several months. The antisera and Ig fractions were designated anti-Th•, anti-Fd•, anti-Fp^T• and anti-Fc^T• following absorption (Table 3). When the absorbed antisera or Ig fractions continued to show reactivity with the isolates used for absorption, other similar absorptions with the heterologous isolates were performed until this reactivity was removed. Using the reciprocally absorbed antisera on the Danish isolates 1 to 14, some cross-reactivity was observed using the slide agglutination test. Further absorptions were necessary to determine to which serogroup the isolates in question belonged. Thus, anti-Th• and anti-Fd• were absorbed with two of the cross-reacting isolates (nos. 3 and 6), and anti-Th• and anti-Fp^T• were absorbed with isolate no. 4.

ELISA technique. ELISA was performed using the double antibody sandwich method as described by Voller et al. (1979): flat-bottomed microtiter plates (Maxisorp Nunc-Immuno Plate, Inter Med, Teknunc, Denmark) were coated with protein-A purified Ig diluted in carbonate buffer pH 9.6 supplemented with 15 mM sodium azide. Each well received 50 µl IgG solution equivalent to 0.052 µg IgG purified from anti-Th, 0.189 µg IgG from anti-Fd, 0.062 µg IgG from anti-Fp^T, or 0.024 µg IgG purified from anti-Fc^T. The microwell plates were incubated overnight at 4°C on an orbital shaker for adsorption. Coated microtiter plates were stored at 4°C for a maximum of 4 wk in a humid chamber.

Before use, the plates were washed 3 times in PBS supplemented with 0.05% Tween 20 (PBS-T) and subsequently incubated on an orbital shaker for 1 h at

Table 3. Reciprocally absorbed rabbit antisera/IgG fractions

Antiserum/ purified IgG fraction	Isolates used for absorption[a]	Absorbed antiserum / purified IgG fraction
Anti-Th	Fd, Fp^T, Fc^T	Anti-Th•
Anti-Fd	Th, Fp^T, Fc^T	Anti-Fd•
Anti-Fp^T	Th, Fd, Fc^T	Anti-Fp^T•
Anti-Fc^T	Th, Fd, Fp^T	Anti-Fc^T•

[a]Code of isolates as in Tables 1 & 2

37°C or overnight at 4°C with bacterial cells suspended in PBS-T supplemented with 1% bovine serum albumin (PBS-T-BSA) (50 µl per well). For all of the following steps, PBS-T-BSA was used as diluent, each well receiving 50 µl, and all incubations were performed on an orbital shaker in a humid chamber, unless otherwise indicated.

After 3 more washes (PBS-T), the plates were incubated for 1 h at 37°C or overnight at 4°C with biotin-conjugates prepared from the same purified IgG as used for coating. The optimum dilution of biotinylated Ig varied according to the antiserum from which it was purified. Thus each well received 0.013 µg IgG of anti-Th, 0.025 µg IgG of anti-Fd, 0.031 µg IgG of anti-Fp^T, and 0.027 µg IgG of anti-Fc^T. Following 3 additional washings, the plates were incubated for 1 h at 37°C with peroxidase conjugated streptavidin (DAKO, Denmark) diluted 1:2000. After a final wash, all wells received the substrate H_2O_2-ortho phenylenediamine (OPD, Kem-en-Tec, Denmark) diluted in phosphate-buffered citric acid pH 5.0. The substrate was incubated for 15 min at room temperature and the reaction was stopped with 1 M H_2SO_4 (100 µl per well). Subsequently, the absorbance was measured by a Titertek Multiscan reader at a wavelength of 492 nm (A_{492}).

The concentration of IgG in the coating layer and in the biotinylated layer was adjusted to obtain a background value (A_{492}) below 0.2 and a maximum value of about 2.5 for the homologous antigen. Absorbed IgG fractions (anti-Th•, anti-Fd•, anti-Fp^T• and anti-Fc^T•) were not used for coating, but only for the biotinylated layer, and the concentration of IgG was adjusted according to the same criterion as for unabsorbed IgG fractions.

The bacteria used in the ELISA were grown in AOBE on an orbital shaker for 2 to 3 d, washed once in PBS, and suspended in PBS with 15 mM sodium azide to a density equivalent to approximately 1×10^{10} colony forming units (CFU) per ml. Occasionally, 2 to 3 d old colonies were suspended directly in PBS with sodium azide. The bacterial suspension was heated for 10 to 15 min at 55°C according to Anacker & Ordal (1959) and Holt (1987) to eliminate nonspecific aggregation. Suspensions were stored at 4°C for a maximum of 14 d or at –20°C for up to several months. For application on coated microwell plates, the heat-treated bacterial suspensions were diluted in PBS-T-BSA to an optical density of 0.2 ± 0.01 at 520 nm, equivalent to approximately 1×10^8 CFU per ml. Beginning with this concentration, seven 5-fold serial dilutions were made to a final dilution of 78125.

The following ELISA performance controls were included: (1) wells coated with purified Ig from a heterologous antiserum (anti-pike fry rhabdovirus; anti-PFR), (2) wells without bacteria, (3) wells with PBS-T-BSA in place of biotinylated antiserum in the third layer, (4) wells with biotinylated Ig from a heterologous antiserum (anti-viral haemorrhagic septicemia virus; anti-VHSV), and (5) wells without HRP-streptavidin conjugate. Control wells without bacteria were included in each test to estimate the background optical density.

For statistical comparison of the reactivities in ELISA, an index, *m*, was calculated for each isolate tested with each purified Ig-fraction. Index *m* represents the mean A_{492} of antigen dilution 1:25, 1:125 and 1:625. Comprising the central part of the ELISA curve, these 3 dilutions were considered representative of the reactivity for a given antigen with a given antibody preparation. The means of the *m*-values of different groups of isolates were compared by a modified Student's *t*-test for small samples (Campbell 1974), each group comprising isolates with similar reactivity.

Slide agglutination. The method described by Anacker & Ordal (1959) was followed with a few modifications: colonies of bacteria grown for 48 to 72 h were suspended in sodium acetate buffer (0.05 M $NaCO_2CH_3$, 0.1 M NaCl, pH 7.5) to a density equivalent to approximately 1×10^{10} CFU ml^{-1}. The suspensions were heated at 55°C for 10 to 15 min as described for the ELISA preparations, and subsequently 15 mM sodium azide was added. The preparations were stored at 4°C for a maximum of 14 d. The antisera were used undiluted and diluted 1:2, 1:4, 1:6 and 1:8 in PBS. Absorbed antisera were used undiluted. Ten µl of antiserum was allowed to react with 10 to 15 µl of the bacterial suspensions on a slide using a careful rocking motion. The reaction was recorded macroscopically against a dark background after 1 to 2 min. As a control for autoagglutination, preimmunization serum was used in an equivalent dilution.

Plasmid analysis. Plasmid analysis was performed as previously described (Lorenzen et al. 1997).

RESULTS

Bacterial isolates

All isolates were identified as *Flavobacterium psychrophilum* according to their phenotypic properties as detailed previously (Lorenzen et al. 1997).

Antisera

The titer of the antisera used in our study ranged from 128 to 512 as determined by microtiter agglutination (Table 2).

Slide agglutination

The results of the slide agglutination studies using unabsorbed antisera are not presented as these results were not meaningful. High levels of cross-reactivity were observed unless the antisera were diluted at least 1:6, and this dilution gave a reactivity too faint to read with certainty.

The results of the slide agglutination studies using reciprocally absorbed antisera are presented in Table 4. Most isolates (27 out of 45) agglutinated with antiserum anti-Th•; some (8) reacted with antiserum anti-Fd•, and some (8) reacted with anti-Fp^T•. The 4 isolates from other European countries reacting with anti-Fd• also reacted with anti-Fp^T• (g, k, m, q). Two isolates could not be placed, either due to autoagglutination (p) or lack of reactivity (r). None of the isolates reacted with anti-Fc^T•, and likewise Fc^T did not react with any of the absorbed antisera raised against isolates of *Flavobacterium psychrophilum*.

Among the isolates reacting with anti-Th•, 5 isolates reacted with anti-Fd•, 2 isolates reacted with anti-Fp^T•, and 4 isolates reacted with both anti-Fd• and anti-Fp^T•. Following absorption of anti-Fd• and anti-Fp^T• with selected cross-reacting isolates (nos. 3 & 6, and 4, respectively), this cross-reactivity was removed without removing the reaction with the homologous isolates, Fd and Fp^T, respectively. By contrast, if antiserum anti-Th• was absorbed with the selected cross- reacting isolates, the reactivity to the homologous antigen, Th, was not retained. Therefore, the cross-reacting isolates were considered more closely related to isolate Th than to the isolates Fd or Fp^T.

Table 4. Slide agglutination and ELISA results for 25 Danish isolates and 20 isolates from other European countries and 2 type strains (Fp^T & Fc^T) using reciprocally absorbed antisera (agglutination) or purified IgG preparations (ELISA)

Agglutination, serotype \ ELISA, serotype		Th• (27)••		Fd (8)	Fp^T (3)	?••• (7)	Fc^T
		Th-1 (18)	Th-2 (9)				
Th[b] (27)	Th (16)	[a] 5 (Th), 7, 8, 9, 10, 11, 12, 16, 18, 20, 22, 24, 25, j, n	13				
	Th-Fd (5)[c]	i	3, 6, 19, b				
	Th-Fd-Fp^T (4)[d]		23, c, e, h				
	Th-Fp^T (2)[e]	4, a					
Fd (8)	Fd (4)			2, 14 (Fd), 15, 21			
	Fd-Fp^T (4)[f]			g, k, m, q			
Fp^T (8)					1, d, t, Fp^T	17, f, l, o, s	
?•••• (2)						p, r	
Fc^T							Fc^T

•The serotypes Th (including the subtypes Th-1 and Th-2), Fd, Fp^T and Fc^T are labelled according to the bacterial isolate used for immunization of the rabbits. The serotypes are mainly defined by the reactivity pattern in ELISA, except for serotype Fp^T, which also includes isolates that do not react in ELISA. The subtypes of Th, Th-1, and Th-2 are defined by the reactivity pattern in ELISA only

••Number of isolates belonging to the serotype. For serotypes Th and Fd the homologous antigens are included in the number of positively reacting isolates

•••Serotype could not be determined by ELISA. The isolates did not react with any of the purified IgG preparations containing antibodies against *F. psychrophilum*, anti-Th•, anti-Fd• or anti-Fp^T•

••••Serotype could not be determined. The isolates either strongly auto-agglutinated or did not react with any of the 3 absorbed antisera against *F. psychrophilum*, anti-Th•, anti-Fd• or anti-Fp^T•

[a]Numbers and letters refer to the code for the isolates listed in Table 1

[b]For serotypes Th and Fd, the agglutination studies revealed 4 and 2 different reactivity patterns, respectively, defined by the amount of cross-reactivity (Th, Th-Fd, Th-Fd-Fp^T, Th-Fp^T and Fd, Fd-Fp^T, respectively)

[c]Subgroup of Th, including isolates reacting with anti-Th• and with anti-Fd•

[d]Subgroup of Th, including isolates reacting with anti-Th• and with anti-Fd and anti-Fp^T•

[e]Subgroup of Th, including isolates reacting with anti-Th• and with anti-Fp^T•

[f]Subgroup of Fd and Fp^T, including isolates reacting with anti-Fd• and with anti-Fp^T•

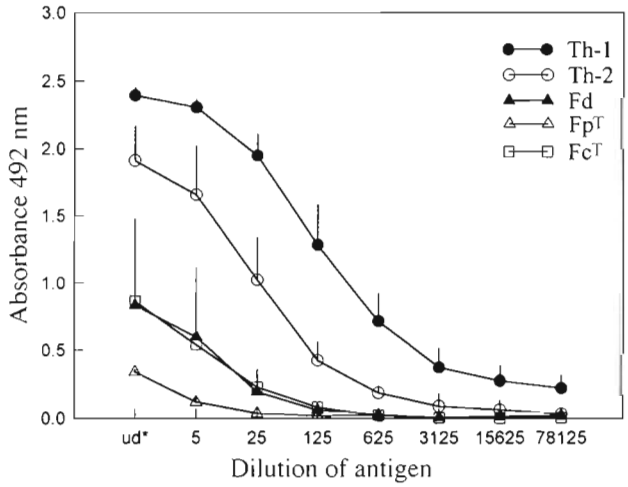

Fig. 1. Results of ELISA studies with Danish isolates 1 to 14 and with the 2 type strains, Fp^T and Fc^T, using non-absorbed, purified IgG preparation anti-Th. Each serotype is illustrated by the mean + SD of the A_{492} values of the isolates defining the serotype. Except for subtype Th-2, the homologous isolates are included in each serotype. Serotype Fc^T included 1 strain (Fc^T), serotype Fd included 2 isolates (nos. 2, 14(Fd)), serotype Fp^T included 1 isolate (no. 1) and the type strain Fp^T, serotype Th-1, included 8 isolates (nos. 4, 5(Th), 7, 8, 9, 10, 11, 12), and serotype Th-2 included 3 isolates (nos. 3, 6, 13). *Undiluted bacterial suspension, corresponding to OD = 0.2 (520 nm). See text for further details

ELISA studies

The results of the ELISA studies are presented in Figs. 1 & 2 and in Tables 4 & 5. The performance controls showed that there was no nonspecific reactivity in the ELISA system used (data not shown).

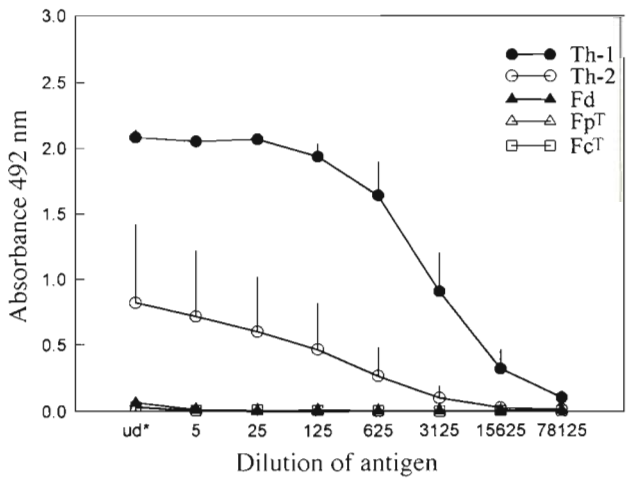

Fig. 2. Results of ELISA studies with Danish isolates 1 to 14 and with the 2 type strains, Fp^T and Fc^T, using reciprocally aborbed purified IgG preparation anti-Th*. Notation as in Fig. 1

The results presented in Figs. 1 & 2 are based on studies with the 2 type strains and isolates 1 to 14, and with anti-Th and anti-Th*, respectively. Among the 14 Danish isolates, 8 (nos. 4, 5, 7, 8, 9, 10, 11, 12) including the homologous isolate (no. 5, Th) reacted strongly with anti-Th (denoted Th-1; Fig.1, Table 4). One isolate (no. 2) in addition to the homologous one (no. 14, Fd) reacted strongly with anti-Fd; 1 isolate (no. 1) in addition to the type strain (Fp^T) reacted strongly with anti-Fp^T, whereas only Fc^T reacted strongly with anti-Fc^T (Table 4). Three isolates (nos. 3,6,13) did not react strongly with any of the antibody preparations studied, but showed a moderate reactivity with anti-Th (therefore denoted Th-2; Fig. 1, Table 4). An isolate was considered strongly reacting if its values of A_{492} in dilutions 1:25, 1:125, and 1:625 on average constituted at least 50% of the values of A_{492} obtained with the homologous isolate.

To compare the curves (Fig. 1) representing the strongly (including the homologous isolate) and the weakly reacting isolates, the means of the *m*-values for isolates with strong and weak reactivity were compared by using a *t*-test. Consequently, for the comparison, each curve was represented by a single mean and standard deviation (Table 5, shown only for anti-Th). In the case of anti-Th, the mean and standard deviation of the 3 moderately reacting isolates (Th-2) were also calculated. For each purified antibody preparation (anti-Th, anti-Fd, anti-Fp^T, and anti-Fc^T) the strongly reacting isolates differed signif-

Table 5. Statistical testing (*t*-test) of the ELISA results obtained with unabsorbed antiserum anti-Th, the Danish isolates 1 to 14 and 2 type strains (Fp^T and Fc^T). The ELISA results are illustrated in Fig. 1. Notation as in Fig. 1

Serotype (no. of isolates)	Mean, SD of values of *m*[a]	*t*, Th-1[b]	*t*, Th-2[c]
Th-1 (8)	1.3178, 0.2153		5.66•••
Th-2 (3)	0.5456, 0.1434	5.66•••	
Fd (2)	0.0923, 0.0618	7.65•••	4.06••
Fp^T (2)	0.0247, 0.0071	8.12•••	4.87••
Fc^T (1)	0.1097	5.29•••	2.63

••$p < 0.05$; •••$p < 0.01$

[a] *m* is an index defined by the mean of the absorbances of dilution 1:25, 1:125, and 1:625 for each isolate within a serotype (see 'Materials and methods' for further explanation). The means and standard deviations (SD) included here represent the means and SDs of the *m*-values of the isolates within a serotype

[b] *t*, Th-1 indicates the result of testing the absorbances (*m*-values) of the 8 isolates within serotype Th-1 against the absorbances obtained for the other serotypes

[c] *t*, Th-2 indicates the result of testing the absorbances of the 3 isolates within the subtype Th-2 against the absorbances obtained for the other serotypes

icantly ($p < 0.05$) from the weakly reacting isolates (Table 5, only presented for anti-Th). For anti-Th, the moderately reacting isolates (Th-2) also differed significantly from the strongly (Th-1) as well as the weakly reacting isolates (Table 5). Based on the strong reactivities, the 14 isolates studied fell into 3 groups with 1 of the groups consisting of 2 subgroups. Each isolate belonged to only 1 group, henceforth referred to as serotype. According to this scheme, Fc^T belonged to its own serotype. The serotypes are referred to according to the name of the homologous isolate of the antibody preparation defining the group. Serotype Th comprise 2 subtypes, subtype Th-1 consisting of the isolates reacting strongly with anti-Th, and subtype Th-2 consisting of the isolates reacting moderately with anti-Th.

Fig. 1 shows the reactivity pattern against anti-Th of Fc^T and of isolates of the 3 serotypes Th (1 & 2), Fd and Fp^T, represented among isolates 1 to 14. Serotype Fp^T also included the homologous strain Fp^T. Reciprocal reactivity patterns were found using the other purified IgG- preparations (anti-Fd, anti-Fp^T and anti-Fc^T), except that serotype Th-2 in these cases could not be differentiated from serotype Th-1 (results not shown). Using anti-Fc^T, there was essentially no reactivity with any of the isolates of *Flavobacterium psychrophilum* studied (data not shown).

Fig. 2 shows the reactivity pattern with anti-Th of the same 14 isolates and 2 type strains following reciprocal absorption. It is evident that the reactivities of the 8 isolates within serotype Th-1 and the 3 isolates within serotype Th-2 were retained, whereas the reactivities of the weakly reacting isolates belonging to serotype Fd and Fp^T, and of Fc^T were removed. Similarly, for the other 3 antibody preparations studied, only the reactivities of the strongly reacting isolates were retained following reciprocal absorption (results not presented). The absolute values in Figs. 1 & 2 cannot be directly compared, as the tests were not performed simultaneously.

Absorption of anti-Th• with 3 isolates (nos. 3, 6, and 13) belonging to serotype Th-2 removed the reactivity to serotype Th-2. However, the reactivity to serotype Th-1 was retained though slightly lowered (data not shown).

Inclusion of the other 11 Danish isolates and the 20 isolates from other European countries in the ELISA studies apparently did not change the frequency of the 3 serotypes markedly (Table 4). Thus out of a total of 45 isolates serotypes Th Fd, and Fp^T contained 27, 8, and 3 isolates, respectively. One Danish isolate (no. 17) and 6 isolates from other European countries (f, l, o, s, p, r) could not be related to any of the serotypes. Six isolates in addition to nos. 3, 6 and 13 were found to belong to subtype Th-2 (19, 23, b, c, e, h).

Comparison of our ELISA results with those obtained by slide agglutination (Table 4) showed that the reactivity patterns were very similar: (1) except for 3 isolates (4, a, i) the isolates of serotype Th-1 in ELISA all reacted only with antiserum anti-Th• in slide agglutination; (2) 8 of the 9 isolates of serotype Th-2 in ELISA reacted in slide agglutination with antiserum anti-Fd• (3, 6, 19, b) or with antisera anti-Fd• and anti-Fp^T• (23, c, e, h) in addition to anti-Th•, but were considered more closely related to serotype Th based on the absorption studies discussed previously; (3) the 4 Danish isolates of serotype Fd in ELISA reacted only with antiserum anti-Fd• in slide agglutination, whereas the 4 isolates from other European countries also reacted with anti-Fp^T•; (4) the 3 isolates of serotype Fp^T and 5 of the 7 isolates that could not be related to any serotype by ELISA agglutinated with antiserum anti-Fp^T•. Two isolates (p, r) could not be placed by either method.

In Table 6, the distribution of the isolates according to serotype is compared with previous results of plasmid analyses on the same 25 Danish isolates and 20 isolates from other European countries (Lorenzen et al. 1997). This comparison revealed that most isolates of serotype Th and Fd had a plasmid of 3.2 kb, whereas isolates belonging to serotype Fp^T generally had different plasmid profiles.

Table 6. Size of plasmid(s) according to serotype in 25 Danish isolates and 20 isolates of *Flavobacterium psychrophilum* from other European countries and the 2 type strains Fp^T and Fc^T

Size of plasmid(s) (kb)[a]	Serotype[b] Th	Fd	Fp^T	?[c]	Fc^T
3.2	3, 5 (Th), 6, 7, 8, 9, 10, 11, 12, 13, 16, 18, 19, 20, 22, 23, 24, 25, a, b, c, e, h, i, j, n	2, 14 (Fd), 21, g, k, m	s, t	p	
2.4			Fp^T		
0	4	q	1, d, l, o	r	Fc^T
5.7 + 2.2			f		
3.2 + 2.6		15	17		

[a]Molecular weight in kilobases (kb)
[b]Numbers and letters refer to the Danish and foreign isolates, respectively, listed in Table 1
[c]Serotype not determined

DISCUSSION

The present study revealed that, among 25 Danish isolates and 20 isolates of *Flavobacterium psychrophilum* from other European countries primarily originating from diseased rainbow trout, at least 3 distinct serotypes could be demonstrated. One of the serotypes, Th, included the majority of the isolates, with the remainder being of 2 minor serotypes, Fd and Fp^T.

The ELISA technique and slide agglutination test generally supported each other, in that results were very similar using either technique, i.e. isolates that in ELISA reacted with 1 of the 3 purified IgG preparations (anti-Th, anti-Fd & anti-Fp^T) also reacted with the corresponding antiserum in slide agglutination. The opposite was, however, not always the case due to cross-reactivity in slide agglutination. In ELISA, no cross-reactivity among the 3 serotypes was observed following reciprocal absorption. Therefore the ELISA was considered more appropriate for defining the serotypes. However, slide agglutination was useful for defining serotype Fp^T, which ELISA did not differentiate consistently.

Absorptions of purified IgG fractions and antisera were performed at different dilutions, which might have resulted in a better absorption of the former than the latter. The criteria, however, for accepting the result of the absorptions were similar as described in 'Materials & methods' and therefore the results of slide agglutination should have paralleled closely those obtained by ELISA. ELISA and slide agglutination, however, are based on 2 different principles, the former relying on the simple binding of antibodies to antigens, the latter on the ability of antibodies to cross-link antigens. Therefore exactly congruent results cannot always be expected. In addition, the antibody preparations used for the 2 techniques in the present study were not identical, even if originating from the same sera: for agglutination, a low dilution of absorbed serum was used, whereas high dilutions of the IgG fraction of serum were used for ELISA. Also, agglutination may partly rely on IgM-antibodies (Roitt 1984), which have lower specificity but higher avidity compared to IgG, which could explain the somewhat broader specificity of the agglutination test compared to ELISA.

The extraordinary high level of cross-reactivity of subtype Th-2 by agglutination, and its moderate reactivity with anti-Th in ELISA might indicate that it should be considered a fourth serotype. Further studies using antiserum raised against one of the isolates within serotype Th-2 may resolve this question.

Previous plasmid analyses of the same isolates as presently studied (Lorenzen et al. 1997) revealed that bacteria from clinical outbreaks of RTFS or CWD had 1 small plasmid of approximately 3.2 kb. By contrast, isolates from fish species other than rainbow trout or from asymptomatic fish, in general, did not have any plasmids or had 2 plasmids. Interestingly, most of these latter isolates were also somewhat different serologically. They either agglutinated with anti-Fp^T or could not be placed in any of the 3 serotypes defined above by slide agglutination and ELISA (Table 6). This relationship between serological differences and plasmids suggests that part of the epitopes identified by the techniques in our study could be associated with bacterial plasmids. A possible relationship between plasmids and cell surface characteristics has been demonstrated for other bacterial fish pathogens (Toranzo et al. 1983).

None of the isolates classified as Fp^T by ELISA and/or slide agglutination originated from clinical outbreaks of RTFS/CWD. Isolates comprising serotype Th-1, Th-2 or Fd originated, with few exceptions, from rainbow trout having clinical signs of RTFS or CWD. Hence, it is possible that isolates of serotype Fp^T are less pathogenic, at least for rainbow trout. As the number of isolates within serotype Fp^T (8) was small, more studies, including challenge experiments, are needed to elucidate this matter. The type strain Fp^T, which was used for preparation of our anti- Fp^T, originated from a coho salmon from the USA with signs of CWD (Holt 1987). Several passages on artificial medium could have resulted in an attenuated strain with changed surface characteristics. It is also possible that Fp^T and serologically related isolates could be more pathogenic for coho salmon than for rainbow trout. The strains isolated by Toranzo & Barja (1993) from an outbreak of RTFS in Spain also failed to react with antiserum produced against the type strain. Similarly, Wakabayashi et al. (1994) demonstrated that while isolates (including Fp^T) from coho salmon belonged to serotype O1, isolates from ayu and rainbow trout belonged to another serotype, O2.

By use of intramuscular injection, Holt (1987) found the type strain to be highly pathogenic for yearling coho salmon and found varying degrees of pathogenicity among his other strains of *Flavobacterium psychrophilum*. However, intramuscular injection may not be the most appropriate method for testing the pathogenicity of the bacterium as this bypasses the natural host defense mechanisms of the external surfaces and facilitates entrance of the bacterium. If serotype Fp^T is less pathogenic for rainbow trout, there may be some relationship between serological properties, virulence and plasmid content. Crosa et al. (1977, 1980) demonstrated that the presence of a 50 megadalton plasmid was closely correlated with virulence of *Vibrio anguillarum*. Pathogenicity studies including different serotypes and native versus plasmid-cured isolates of *F.*

psychrophilum might elucidate any correlations among virulence, serotype and plasmid profile that exist.

Isolates from clinical outbreaks of RTFS and CWD did not differ serologically according to the results of the present study. Isolates of serotypes Th and Fd where obtained from either disease and both serotypes have been found at the same hatcheries in Denmark.

The high level of cross-reactivity in the present study using unabsorbed antiserum on whole cells for slide agglutination was in accordance with the results of Holt (1987) and Pacha (1968). Holt (1987) found 2 serotypes among 6 isolates of *Flavobacterium psychrophilum* following reciprocal absorption, but unfortunately he did not study Fp^T, making comparison of the American strains with our European isolates difficult. Likewise, following absorption, Wakabayashi et al. (1994) demonstrated serotypes O1 and O2 among isolates of *F. psychrophilum* originating from coho salmon and from ayu and rainbow trout, respectively.

Preparations of O-antigens as described by Kauffman (1944) for *Escherichia coli* were also examined by us in parallel with whole-cell preparations. However, these generally autoagglutinated and were not useful for slide agglutination. In the ELISA they showed a much lower level of reactivity compared to whole-cell preparations regardless of the antibody preparation used (data not shown). Thus, a major part of the epitopes recognized by the present antisera and purified IgG preparations in slide agglutination and ELISA, respectively, was lost using a conventional protocol for O-antigen preparation. De Jong et al. (1991) tried without success to extract LPS (of which the O-antigen is the outermost part) from *Cytophaga johnsonae* (recently reclassified as *Flavobacterium johnsoniae*; Bernardet et al. 1996) using the phenol-water procedure of Westphal & Jann (1965). *Flavobacterium johnsoniae*, *F. columnare* and *F. psychrophilum* all belong to the bacterial order Cytophagales (Reichenbach & Dworkin 1981) and probably have structural properties in common. The results of De Jong et al. (1991) indicated that the methods commonly used for extracting LPS from Enterobacteriaceae and related bacteria might not be directly applicable to bacteria belonging to Cytophagales. Wakabayashi et al. (1994) found, however, that O-antigen preparations were useful for serotyping in microtiter trays.

Using live cells, as did Pacha (1968), Pacha & Porter (1968) and Bullock (1972), we found no detectable differences compared to heat-treated cells in ELISA. But the slide agglutination test was easier to perform on heat-treated cells, because unlike live unheated cells, they did not autoagglutinate. Whole-cell preparations have also been considered convenient for the ELISA by other authors (Cumming et al. 1980, Ison et al. 1981, Elder et al. 1982, Mills et al. 1982, Bishop & Hwang 1992).

In conclusion, based primarily on ELISA and to a lesser degree on slide agglutination, and on the use of whole cells and polyclonal antibodies, the present study demonstrated 3 serotypes among 25 Danish isolates and 20 isolates from other European countries of *Flavobacterium psychrophilum*. Two of the isolates from other European countries could not be classified by either method. One serotype, Th, comprised most of the isolates, serotype Fd included few isolates, and the serotype Fp^T, defined by the type strain Fp^T, predominantly included isolates that did not originate from true outbreaks of RTFS or CWD. Pathogenicity studies, however, are necessary to elucidate any correlation among serological characteristics, virulence and plasmid profile.

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