Random Amplified Polymorphic DNA analysis provides rapid differentiation among isolates of the fish pathogen *Flavobacterium psychrophilum* and among *Flavobacterium* species

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ABSTRACT: *Flavobacterium psychrophilum* is the agent of cold-water disease and rainbow trout fry syndrome in salmonid fish. Originally isolated in North America only, the bacterium is now also responsible for severe mortalities in many salmonid hatcheries in Europe, as well as in Japan, Chile and Tasmania. The random amplified polymorphic DNA (RAPD) technique was used to analyze the genetic diversity among a collection of 177 *F. psychrophilum* strains isolated from different fish species and in different geographical areas. Forty 10-mer primers were tested and 5 of them were selected for further analysis of the bacterial DNAs. The primers OPH 06, OPH 08, OPG 08, OPG 14, and OPG 16 generated several reproducible profiles during a preliminary screening of the whole collection of strains. Based on these results, the polymerase chain reaction (PCR) products of a selection of 60 bacterial DNAs were submitted to slow agarose gel electrophoresis for numerical analysis of the DNA fingerprintings. No correlation occurred between the combined RAPD profiles of the primers and the geographical origin of the strains, while some profiles were clearly associated with the fish species from which the strains were isolated. Another primer, OPG 10, yielded a unique RAPD profile common to all *F. psychrophilum* strains whereas the 9 other valid *Flavobacterium* species, several of which coexist in freshwater environments and may also be isolated from fish, displayed other profiles. Thus, depending on the primer used, both the typing of *F. psychrophilum* strains for epidemiology studies as well as the identification of this fish pathogen and its differentiation from related bacterial species could be achieved by using RAPD.

KEY WORDS: *Flavobacterium psychrophilum* · *Flavobacterium* spp. · Cold-water disease · Rainbow trout fry syndrome · RAPD · Epidemiology

INTRODUCTION

Flavobacterium psychrophilum (Bernardet et al. 1996) (syn. *Cytophaga psychrophila*, *Flexibacter psychrophilus*), the causative agent of cold-water disease and rainbow trout fry syndrome, was originally isolated from coho salmon *Oncorhynchus kisutch* in Washing-

ton state, USA, in 1948 (Borg 1960), and later found in chinook salmon *Oncorhynchus tshawytscha* from the same area (Anderson & Conroy 1969). In recent years, cold-water disease has also caused serious problems among rainbow trout juveniles reared in the Pacific Northwest area (R. A. Holt pers. comm.). Since the mid-eighties, the same bacterium has been responsible for high losses in rainbow trout *Oncorhynchus mykiss* fry in many European hatcheries (Weis 1987,

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Bernardet et al. 1988, Lorenzen et al. 1991, Austin 1992, Sarti et al. 1992, Toranzo & Barja 1993, Wiklund et al. 1994). In Europe, it was also isolated from European eel *Anguilla anguilla*, carp *Cyprinus carpio*, tench *Tinca tinca* and crucian carp *Carassius carassius* (Lehmann et al. 1991). In Japan, the disease has become a concern in rainbow trout, coho salmon and ayu *Plecoglossus altivelis* farms (Wakabayashi et al. 1994). Recently this bacterium was also isolated in Chile from rainbow trout fry (Bustos et al. 1995) and in Tasmania, Australia, from Atlantic salmon (Schmidtke & Carson 1995). An efficient typing method would allow an accurate comparison of strains retrieved from widely different origins and would benefit epidemiology studies of the disease.

Several other *Flavobacterium* species may be isolated from diseased freshwater fish (Bernardet et al. 1996). Some of them (*Flavobacterium columnare, F. branchiophilum, F. johnsoniae*) have been demonstrated to be pathogenic, at least in certain circumstances, while the pathogenicity of other species [*F. hydatis* (syn. *Cytophaga aquatilis*), *F. succinicans*] has not been established. As most *Flavobacterium* species are relatively fastidious and inert in many biochemical tests, the identification of *F. psychrophilum* is time-consuming and confusion with other *Flavobacterium* species may occur. A rapid and specific identification method would greatly improve the diagnosis of the conditions caused by *F. psychrophilum*.

In this study, we demonstrate that both a rapid and clear differentiation of *Flavobacterium psychrophilum* from related bacterial species, as well as an efficient intra-specific typing of *F. psychrophilum* strains, can be achieved by using Random Amplified Polymorphic DNA (RAPD) (Williams et al. 1990), depending on the primer used.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 177 *Flavobacterium psychrophilum* strains isolated over the years in different geographical areas (59 strains from France, 6 from Spain, 4 from Sweden, 22 from Denmark, 9 from Germany, 19 from Switzerland, 6 from the United Kingdom, 1 from Norway, 1 from Finland, 28 from the USA, 1 from Canada, 6 from Chile, 10 from Japan and 5 from Tasmania) were included in this study. This collection of strains was screened (see details below) and 60 of them, listed in Table 1, were included in the final study. All *F. psychrophilum* strains were grown at 18°C on Anacker and Ordal's agar (tryptone, 0.05%; yeast extract, 0.05%; beef extract, 0.02%; and sodium acetate, 0.02%; pH 7.2 to 7.4) (Anacker & Ordal 1955) supplemented to 0.5%

tryptone. Representative strains of the 9 other valid *Flavobacterium* species were included in this study and are listed in Table 2. They were grown on the same medium at 22°C.

Preparation of DNA. Each strain was collected on the agar plate, suspended in 0.9% saline and centrifuged. The pellet was resuspended in an equal volume (200 µl) of TES (50 mM Tris buffer, 1 mM EDTA, 8.56% saccharose) pH 8.00 containing 20 µg of lysozyme (Sochal, Levallois-Perret, France), and incubated at 37°C for 1 h. Fifty µl of 20% sodium dodecyl sulphate (SDS) was added to the mixture. The solution was treated 3 times with a mixture of phenol, chloroform and isoamyl alcohol (25/24/1; v/v/v) and once with chloroform-isoamyl alcohol (24/1; v/v). The DNA was precipitated by an equal volume of isopropanol, dried under vacuum, and dissolved in 200 µl of sterile Milli-Q water containing 10 µg of RNase (ribonuclease I-A from bovine pancreas, Sigma Chemical Co., St. Louis, MO, USA) and incubated at 37°C for 15 min. All DNA samples were stored at –20°C.

Amplification conditions. Two kits of 20 arbitrary 10-mer primers each (OPG and OPH, Bioprobe, Montrevil-sous-bois, France) were evaluated for random amplification of DNAs. The technique used in this study was adapted from Meunier & Grimont (1993). Briefly, 25 µl of reaction mixture containing 25 to 50 ng of genomic DNA, 5 pmol of primer, 50 µM of each dNTP (Appligene, Illkirch, France), 0.6 U of *Taq* DNA polymerase (reference 120187C, Appligene) and 2.5 µl of 10 × polymerase chain reaction (PCR) reaction buffer was overlaid with mineral oil. The amplification reaction was performed in a DNA thermal cycler (Own E Hybaid, Teddington, England) programmed for 45 cycles of 30 s at 94°C (denaturing of DNA), 1 min at 36°C (primer annealing) and 1 min at 72°C (polymerization), with 5 min initial denaturation at 95°C and 10 min final extension at 72°C. In order to test the reproducibility of the technique, DNA amplification experiments were repeated 3 times under the same conditions. A negative control, containing no template DNA, was included in each experiment in order to detect any contaminating DNA.

Electrophoresis conditions. Gel electrophoresis was carried out by loading 25 µl samples of all 177 amplification products in submerged horizontal 1% agarose gel (Bioprobe). Gels were run for 1 h at 10 V cm^{-1} in Tris-borate buffer (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA) without cooling. Amplification products were visualized after ethidium bromide staining and photographed on a UV light transilluminator

The amplification products of 60 selected strains (see details below) generated by the primers OPH 06 and OPH 08 were also compared using special conditions of electrophoresis (3 V cm^{-1} for 8 h), improving the resolution of the patterns for computer analysis.

Table 1. *Flavobacterium psychrophilum*. Strains included in the computer analysis of their RAPD fingerprints generated by the primers OPH 06 and OPH 08

Strain	Origin	Strain	Origin
JIP 02/86	Rainbow trout *Oncorhynchus mykiss*, kidney, Picardie, France, 1986	OSU SRCo5-90	Coho salmon, kidney, Oregon, USA, 1990
JIP P02/88	Rainbow trout, kidney, Picardie, France, 1988	OSU PCP1.1	Coho salmon, kidney, Oregon, USA, 1989
		OSU NHCo4-90	Coho salmon, kidney, Oregon, USA, 1990
LVDL 1829-91	Rainbow trout, liver, Navarra, Spain, 1991	OSU CO26.BR.2.1	Coho salmon, brain, Oregon, USA, 1990
LVDL 3077-91	Rainbow trout, spleen, Navarra, Spain, 1991	OSU BC3-81	Coho salmon, kidney, Oregon, USA, 1981
		OSU NHCo2-90	Coho salmon, kidney, Oregon, USA, 1990
ISTAB AC 15/I.1	Rainbow trout, spleen, Navarra, Spain, 1996	OSU Ch8-80	Coho salmon, Oregon, USA, 1980
		OSU TM3.1	Coho salmon, kidney, Oregon, USA, 1989
SVA 31-88	Rainbow trout, muscle, Sweden, 1988	FPC 828	Coho salmon, kidney, Miyagi, Japan, 1990
SVA 120-89	Rainbow trout, muscle, Sweden, 1989	FPC 829	Coho salmon, kidney, Miyagi, Japan, 1990
SVS 911209-2	Rainbow trout, spleen, Brons, Denmark, 1990	FPC 830	Coho salmon, kidney, Miyagi, Japan, 1990
SVS 911209-1	Rainbow trout, spleen, Trehoje, Denmark, 1990	FPC 831	Coho salmon, peduncle lesion, Iwate, Japan, 1990
SVS 910614-3	Rainbow trout, skin lesion, Bregnholm, Denmark, 1991	OSU SRChF8-81	Chinook salmon *Oncorhyncus tshawytscha*, kidney, Oregon, USA, 1981
SVS 911009-3	Rainbow trout, skin lesion, Rindsholm, Denmark, 1991	DPIF 91/4043-4	Atlantic salmon *Salmo salar*, eroded fin, Tasmania, Australia, 1991
DIFR 880406	Rainbow trout, kidney, Denmark, 1990	DPIF 91/4043-8	Atlantic salmon, eroded fin, Tasmania, Australia, 1991
DIFR 900406	Rainbow trout, kidney, Denmark, 1990	DPIF 91/4043-13	Atlantic salmon, eroded fin, Tasmania, Australia, 1991
LFNW 123/89	Rainbow trout, skin lesion, Nordrhein-Westfalen, Germany, 1989	DPIF 91/4043-14	Atlantic salmon, eroded fin, Tasmania, Australia, 1991
IVP CH7/93	Rainbow trout, Bern, Switzerland, 1993	DPIF 91/4043-17	Atlantic salmon, eroded fin, Tasmania, Australia, 1991
IVP CH3/94	Rainbow trout, Bern, Switzerland, 1994		
IVP CH1/93	Rainbow trout, Bern, Switzerland, 1993	OSU AD10.1.1	Atlantic salmon, kidney, Oregon, USA, 1990
OSU Ana9.1.1	Rainbow trout, kidney, Oregon, USA, 1991	OSU CCC6-86	Cutthroat trout *Salmo clarki*, Oregon, USA, 1986
OSU 90-6 6A	Rainbow trout, spleen, Idaho, USA, 1990		
UCD 004-95	Rainbow trout, kidney, Idaho, USA, 1995	FPC 839	Ayu *Plecoglossus altivelis*, kidney, Tokushima, Japan, 1987
UCD 95-7	Rainbow trout, kidney, California, USA, 1995	FPC 837	Ayu, kidney, Iwate, Japan, 1988
UCD 146-95	Rainbow trout, kidney, Idaho, USA, 1995	FPC 838	Ayu, liver, Tokushima, Japan, 1988
UCD R3/8/95	Rainbow trout, USA, 1995	FPC 840	Ayu, kidney, Tokushima, Japan, 1987
FPC 813	Rainbow trout, kidney, Tokyo, Japan, 1992	LVDI 5/I	Carp *Cyprinus carpio*, gills, Centre, France, 1992
OSU RbS6-82	Steelhead trout *Oncorhynchus mykiss*, kidney, Oregon, USA, 1982	LFNW 16/90	Carp, spleen, Nordrhein-Westfalen, Germany, 1990
NCIMB 1947[T]	Coho salmon *Oncorhynchus kisutch*, kidney, Washington, USA	LFNW 25/90	Tench *Tinca tinca*, kidney, Nordrhein-Westfalen, Germany, 1990
NCIMB 2282	Coho salmon, kidney, Washington, USA, 1948	LVDJ XP189	Tench, kidney, Franche-Comté, France, 1992
OSU SH3-81	Coho salmon, kidney, Oregon, USA, 1981	LVDJ D 2172	Tench, Franche-Comté, France, 1995
OSU THCO2-90	Coho salmon, kidney, Oregon, USA, 1990	LFNW 131/89	European eel *Anguilla anguilla*, kidney, Nordrhein-Westfalen, Germany, 1989
OSU THCO4-90	Coho salmon, kidney, Oregon, USA, 1990		

Strain sources:

JIP: Culture Collection of Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, Jouy-en-Josas, France

LVDL: strains provided by P. Nougayrède, Laboratoire Vétérinaire Départemental des Landes, Mont de Marsan, France

ISTAB: Culture Collection of Institut Supérieur de Technologie Alimentaire et Biotechnologie, Bordeaux, France

SVA: strain provided by A. Hellström, Statens Veterinärmedicinska Ansalt, Uppsala, Sweden

SVS: strains provided by E. Lorenzen, Statens Veterinaere Serumlaboratorium, Århus, Denmark

DIFR: strains provided by I. Dalsgaard, Danish Institute for Fisheries Research, Frederiksberg, Denmark

LFNW: strains provided by D. Mock, Landesanstalt für Fischerei Nordrhein-Westfalen, Kirchhundem-Albaum, Germany

IVP: strains provided by T. Wahli, Institute of Veterinary Pathology, Berne, Switzerland

OSU: strains provided by M. Whipple and R. A. Holt, Oregon State University, Corvallis, USA

UCD: strains provided by R. Hedrick, University of California, Davis, USA

FPC: strains provided by H. Wakabayashi, Laboratory of Aquaculture Biology, University of Tokyo, Japan

NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom

DPIF: strains provided by J. Carson, Department of Primary Industry and Fisheries, Kings Meadows, Tasmania, Australia

LVDI: strain provided by J.-L. Bind, Laboratoire Vétérinaire Départemental d'Indre-et-Loire, Tours, France

LVDJ: strains provided by M. Morand, Laboratoire Vétérinaire Départemental du Jura, Lons-le-Saunier, France

Table 2. *Flavobacterium* strains and species included in the random amplification using the primer OPG 10

Species	Strain	Origin
F. aquatile	LMG 4008^T	Deep well, Kent, England
F. branchiophilum	ATCC 35035^T	Diseased gills of yamame *Oncorhynchus masou*, Gumma, Japan
F. branchiophilum	FL-15	Diseased gills of sheatfish *Silurus glanis*, Hungary
F. columnare	JIP 39/87	Skin lesion of black bullhead *Ictalurus melas*, Ile-de-France, France
F. columnare	FPC EK28	Gill lesions of Japanese eel *Anguilla japonica*, Shizuoka, Japan
F. columnare	OSU DD3-69	Gill lesions of chinook salmon *Oncorhynchus tshawytscha*, Oregon, USA
F. flevense	DSM 1076^T	Lake Ijssel, The Netherlands
F. hydatis	DSM 2063^T	Gills of diseased salmon, Michigan, USA
F. johnsoniae	DSM 2064^T	Soil or mud, Rothamsted or Cambridge, England
F. johnsoniae	ATCC 29585	Diseased freshwater fish, Manitoba, Canada
F. pectinovorum	NCIMB 9059^T	Soil, England
F. psychrophilum	NCIMB 1947^T	Kidney of diseased coho salmon *Oncorhynchus kisutch*, Washington, USA
F. psychrophilum	JIP P02/88	Kidney of rainbow trout *Oncorhynchus mykiss*, Picardie, France
F. psychrophilum	LVDJ XP 189	Kidney of tench *Tinca tinca*, Franche-Comté, France
F. psychrophilum	OSU CCC6-86	Cutthroat trout *Salmo clarki*, Oregon, USA
F. psychrophilum	FPC 839	Kidney of ayu *Plecoglossus altivelis*, Tokushima, Japan
F. psychrophilum	OSU RbS6-82	Kidney of steelhead trout *Oncorhynchus mykiss*, Oregon, USA
F. saccharophilum	NCIMB 2072^T	River Wey, Surrey, England
F. succinicans	NCIMB 2277^T	Eroded fin of chinook salmon, Washington, USA
F. succinicans	NCIMB 2278	Lesion of chinook salmon, Snake River, Idaho, USA

Strain sources:

LMG: Culture Collection of the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium

ATCC: American Type Culture Collection, Rockville, Maryland

FPC EK28, FPC 839 and FL-15: strains provided by H. Wakabayashi, Laboratory of Aquaculture Biology, Department of Fisheries, University of Tokyo, Tokyo, Japan

JIP: Culture Collection of the Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, Jouy-en-Josas, France

OSU: strains provided by M. Whipple and R. A. Holt, Oregon State University, Corvallis, USA

DSM: Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany

NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom

LVDJ: strain provided by M. Morand, Laboratoire Vétérinaire Départemental du Jura, Lons-le-Saunier, France

Computer-assisted analysis of the DNA fingerprints. Each gel, consisting of 10 lanes of amplification products and 3 lanes of molecular weight marker (123 base pairs, bp; Gibco BRL, USA), was photographed using a Polaroid film type 665 (Polaroid, Cambridge, MA, USA). The length of lanes was about 6 cm. The banding patterns were scanned with a laser densitometer (One-Scanner, Apple Computers, Cupertino, CA, USA) and analyzed by using various programs of the Taxotron® package (Taxolab, Institut Pasteur, Paris, France). The TIFF (tagged image file format) image was searched for lanes and bands using RestrictoScan yielding migration data. Fragments sizes were interpolated from migration data using RestrictoTyper implementing the algorithm of Schaffer & Sederoff (1981). RestrictoTyper compared pairs of patterns and calculated a distance coefficient which was the complement of the Dice index. We chose to set a fixed value of 4%, indicating that 2 fragments were considered identical if their sizes did not differ by more than 4%. A distance matrix was generated for the patterns obtained with each of the primers OPH 06 and OPH 08. These distance matrices were averaged using the program Adanson and treated by the Unweighted Pair Group Method of Averages (UPGMA) (Sneath & Sokal 1973). The program generated a tree description

file which was used by the program Dendrograf to draw a dendrogram and produce an order file. The order file was used by RestrictoTyper to reorder the fragment sizes files and produce a schematic representation of the patterns. The dendrogram and the schematic graph were assembled in a single picture by using a drawing program.

RESULTS

An initial screening was carried out by testing the 40 primers on 7 representative *Flavobacterium psychrophilum* isolates. Twenty-eight primers produced clear and reproducible banding patterns, 11 of them yielding polymorphic fragments. Among these, we arbitrarily selected 6 primers for further analysis of isolates: 5 of them (OPH 06, OPH 08, OPG 08, OPG 14, OPG 16) generated different patterns among the 177 strains while the last one (OPG 10) produced an identical pattern for the 35 strains tested. Among the 177 strains in our collection, 60 strains representing the variety in geographical origin and fish host were then selected for high-resolution electrophoresis of their amplification products and computer-assisted analysis

of their DNA fingerprints. Of these, 36 represented all strains originating from fish species other than rainbow trout which were included in the whole collection. The remaining 24 strains were part of the 141 rainbow trout isolates, and originated from Europe, the USA, and Japan (17 strains, 6 strains, and 1 strain, respectively). RAPD profiles were highly reproducible when analyses were repeated, except for some variations in the intensity of a few faint PCR products.

Differentiation among *Flavobacterium psychrophilum* strains

The primer OPG 08 generated 3 different profiles. One was restricted to 1 tench isolate and another one grouped the 4 ayu isolates, while the last profile was common to all other strains. The primer OPG 14 yielded 2 distinct profiles, one grouping 12 rainbow trout isolates and the other common to all other strains tested. Seven different profiles were obtained with the primer OPG 16 (Fig. 1). All rainbow trout isolates exhibited the same profile except the strain JIP 02/86. The coho salmon isolates were divided into 2 profiles, except the strain OSU TM3.1, which displayed another profile in common with the strains isolated from Atlantic salmon, ayu, carp, tench, and steelhead trout. The European eel and cutthroat trout isolates both exhibited unique profiles.

The computer analysis of the combined schematic representations of the fingerprints obtained with the primers OPH 06 and OPH 08 generated the dendrogram presented in Fig. 2. When the primer OPH 06 was used, all isolates tested displayed 4 fragments (3 of them very close to one another) within the otherwise heterogeneous fingerprints. Another major fragment

Fig. 1 *Flavobacterium psychrophilum*. Seven different profiles detected among strains by RAPD with primer OPG 16. Lanes: 1, *Eco*R I and *Hind* III digested λ DNA molecular weight marker; 2, rainbow trout isolate JIP 02/86; 3, rainbow trout isolate JIP P02/88; 4, cutthroat trout isolate OSU CCC6-86; 5, coho salmon isolate NCIMB 1947[T]; 6, coho salmon isolate OSU BC3-81; 7, Atlantic salmon isolate DPIF 91/4043-13; 8, European eel isolate LFNW 131/89

was present in 51 out of 60 OPH 08 profiles. The 4 Japanese strains isolated from ayu were grouped in a very distinct cluster, characterized by the absence of a major OPH 08 fragment (2100 bp) and by the presence of a unique OPH 06 fragment (1350 bp). The 5 Tasmanian Atlantic salmon isolates formed a tight cluster, while the American strain OSU AD10.1.1, originating from the same fish species, grouped with several coho salmon isolates. The coho salmon isolates were separated into 2 major clusters, with the exception of the strain OSU TM3.1, which occupied a rather isolated position in the dendrogram. This distribution of the coho salmon isolates was identical with that obtained with the primer OPG 16. Four coho isolates (3 from Chile and 1 from Canada) not included in the computer analysis displayed a fingerprint similar to that of the cluster which included the type strain NCIMB 1947. Of 24 rainbow trout isolates, 22 exhibited the same combined profile, including the strain JIP 02/86, which was differentiated by the primer OPG 16. The lack of the major 2100 bp fragment in the OPH 08 profile clearly differentiated the strains SVA 120-89 and LVDL 1829-91, but they were grouped with all other rainbow trout isolates when the primer OPG 16 was used. The primers OPH 06, OPH 08, and OPG 16 thus demonstrated the overall very high homogeneity of the strains isolated from this fish species. The only strain isolated from chinook salmon (OSU SRChF8-81) belonged to 1 of the coho salmon clusters, while the strains originating from cutthroat (OSU CCC6-86) and steelhead (OSU RbS6-82) trout occupied more isolated positions. Concerning the strains isolated from non-salmonid fish, the only strain from European eel (LFNW 131/89) grouped together with most rainbow trout isolates while the 3 strains from tench (LVDJ D2172, LVDJ XP 189, and LFNW 25/90) as well as the 2 strains from carp (LFNW 16/90 and LVDI 5/I) each exhibited distinct profiles, often characterized by the presence of unique fragments.
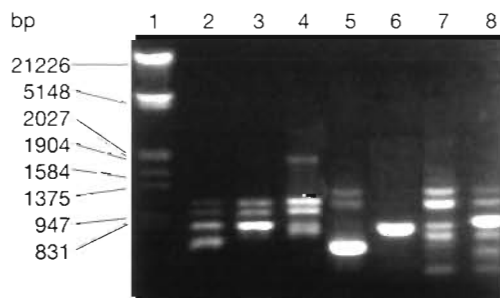
Differentiation of *Flavobacterium psychrophilum* from other valid *Flavobacterium* species

RAPD patterns obtained with the primer OPG 10 for strains and species in the genus *Flavobacterium* are shown in Fig. 3. The 35 *F. psychrophilum* strains tested exhibited the same profile, clearly different from all profiles obtained with the strains representing the 9 other valid *Flavobacterium* species (Table 2). Several strains of *F. branchiophilum*, *F. columnare*, *F. johnsoniae*, and *F. succinicans* were tested. RAPD with OPG 10 generated different profiles for each strain of the last 3 species (Fig. 3), demonstrating that this primer is also able to differentiate strains among them.
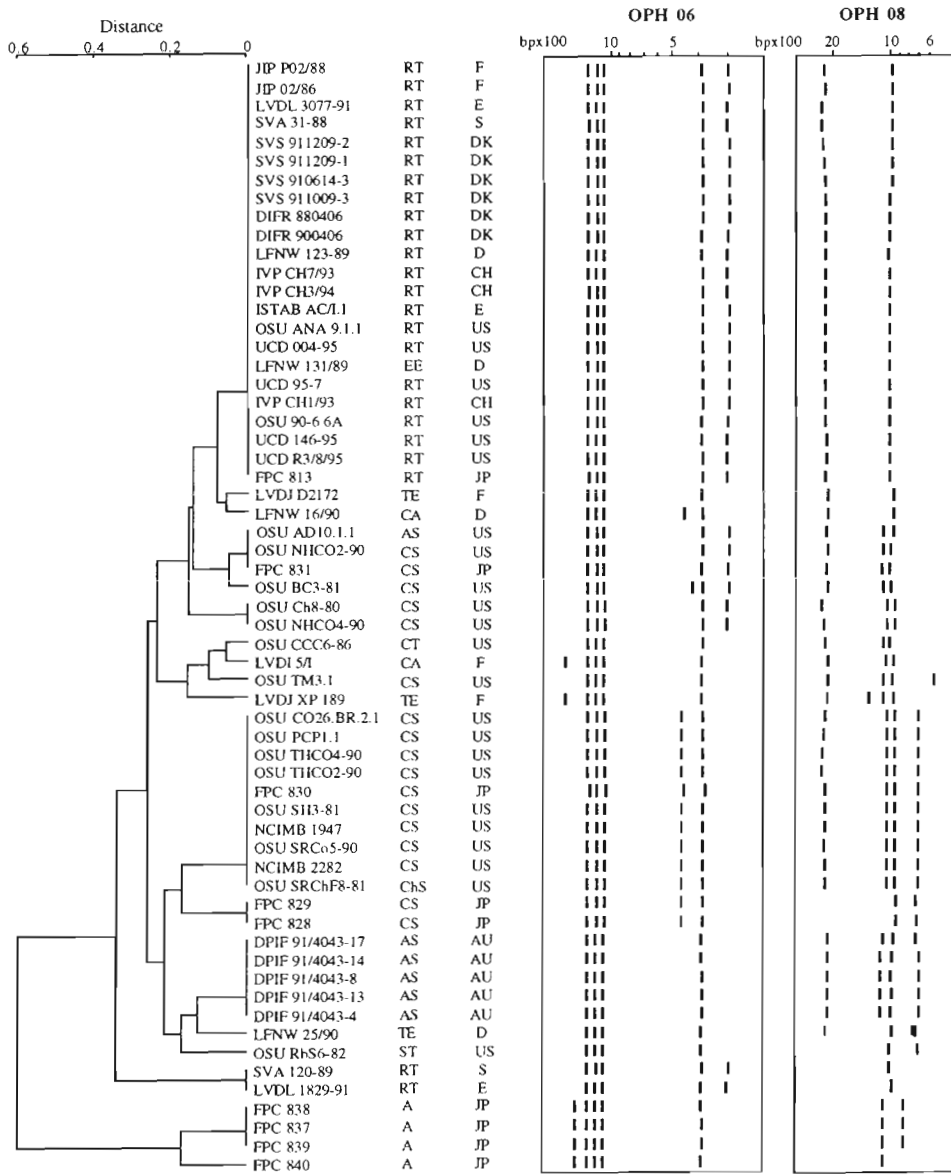
Distance
0.6   0.4   0.2   0

OPH 06   **OPH 08**

Strain			
JIP P02/88	RT	F
JIP 02/86	RT	F
LVDL 3077-91	RT	E
SVA 31-88	RT	S
SVS 911209-2	RT	DK
SVS 911209-1	RT	DK
SVS 910614-3	RT	DK
SVS 911009-3	RT	DK
DIFR 880406	RT	DK
DIFR 900406	RT	DK
LFNW 123-89	RT	D
IVP CH7/93	RT	CH
IVP CH3/94	RT	CH
ISTAB AC/1.1	RT	E
OSU ANA 9.1.1	RT	US
UCD 004-95	RT	US
LFNW 131/89	EE	D
UCD 95-7	RT	US
IVP CH1/93	RT	CH
OSU 90-6 6A	RT	US
UCD 146-95	RT	US
UCD R3/8/95	RT	US
FPC 813	RT	JP
LVDJ D2172	TE	F
LFNW 16/90	CA	D
OSU AD10.1.1	AS	US
OSU NHCO2-90	CS	US
FPC 831	CS	JP
OSU BC3-81	CS	US
OSU Ch8-80	CS	US
OSU NHCO4-90	CS	US
OSU CCC6-86	CT	US
LVDJ 5/1	CA	F
OSU TM3.1	CS	US
LVDJ XP 189	TE	F
OSU CO26.BR.2.1	CS	US
OSU PCP1.1	CS	US
OSU THCO4-90	CS	US
OSU THCO2-90	CS	US
FPC 830	CS	JP
OSU SH3-81	CS	US
NCIMB 1947	CS	US
OSU SRCo5-90	CS	US
NCIMB 2282	CS	US
OSU SRChF8-81	ChS	US
FPC 829	CS	JP
FPC 828	CS	JP
DPIF 91/4043-17	AS	AU
DPIF 91/4043-14	AS	AU
DPIF 91/4043-8	AS	AU
DPIF 91/4043-13	AS	AU
DPIF 91/4043-4	AS	AU
LFNW 25/90	TE	D
OSU RhS6-82	ST	US
SVA 120-89	RT	S
LVDL 1829-91	RT	E
FPC 838	A	JP
FPC 837	A	JP
FPC 839	A	JP
FPC 840	A	JP

Fig. 2. *Flavobacterium psychrophilum*. Schematic representation of the RAPD profiles generated by the primers OPH 06 and OPH 08 for 60 isolates and the corresponding dendrogram produced by Dendrograf, a computer program belonging to the Taxotron® software package (Institut Pasteur, Paris) which uses the complement of Dice similarity coefficients. A fragment length tolerance of 4 % and average linkage were used. RT: rainbow trout; EE: European eel; TE: tench; CA: carp; AS: Atlantic salmon; CS: coho salmon; CT: cutthroat trout; ChS: chinook salmon; ST: steelhead trout; A: ayu. F: France; E: Spain; S: Sweden; DK: Denmark; D: Germany; CH: Switzerland; US: USA; JP: Japan; AU: Australia

bp
21226
5148
2027
1904
1584
1375
947
831
564

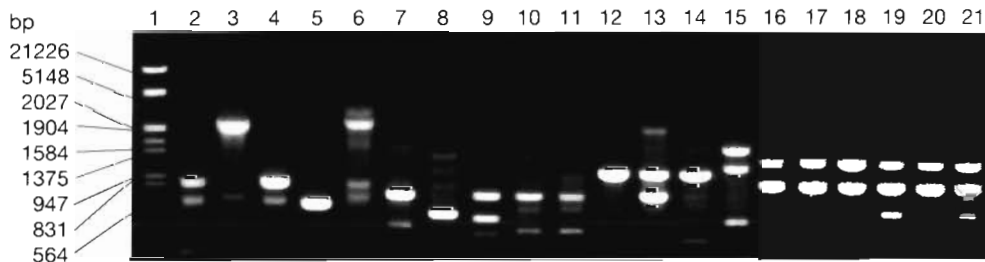1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Fig. 3. *Flavobacterium* spp. Different profiles detected among strains belonging to the 10 valid *Flavobacterium* species as determined by RAPD with the primer OPG 10. Lanes: 1, *Eco*R I and *Hin*d III digested λ DNA molecular weight marker; 2, *F. johnsoniae* DSM 2064[T]; 3, *F. johnsoniae* ATCC 29585; 4, *F. succinicans* NCIMB 2277[T]; 5, *F. succinicans* NCIMB 2278; 6, *F. hydatis* DSM 2063[T]; 7, *F. saccharophilum* NCIMB 2072[T]; 8, *F. pectinovorum* NCIMB 9059[T]; 9, *F. aquatile* LMG 4008[T]; 10, *F. branchiophilum* ATCC 35035[T]; 11, *F. branchiophilum* FL-15; 12, *F. columnare* JIP 39/87; 13, *F. columnare* FPC EK28; 14, *F. columnare* OSU DD3-69; 15, *F. flevense* DSM 1076[T]; 16, *F. psychrophilum* NCIMB 1947[T]; 17, *F. psychrophilum* JIP P02/88; 18, *F. psychrophilum* LVDJ XP 189; 19, *F. psychrophilum* OSU CCC6-86; 20, *F. psychrophilum* FPC 839; 21, *F. psychrophilum* OSU RbS6-82

DISCUSSION

This study was undertaken in order to determine whether random amplification by PCR would allow a differentiation among *Flavobacterium psychrophilum* isolates and/or between *F. psychrophilum* and other *Flavobacterium* species. Our results showed that both kinds of differentiation could indeed be achieved.

In this study, the primers OPH 06 and OPH 08 produced several different amplification profiles among a collection of 177 *Flavobacterium psychrophilum* isolates. Most geographical origins and all fish hosts were represented among the 60 strains selected for computer-assisted analysis of their resolutive electrophoresis profiles. This selection also included representatives of all different RAPD profiles detected by visual observation of rapid electrophoresis gels. No clear association could be detected between the combined patterns generated by the 2 primers and the geographical origin of the isolates, while a strong correlation occurred between some patterns and the fish host (Fig. 2).

This correlation was particularly clear in the case of the strains isolated from rainbow trout, probably because many strains were included in this study compared to the limited number of strains originating from other fish species. All rainbow trout isolates exhibited the same combined pattern whatever their geographical origin (California, Idaho, Oregon, USA; Japan; France; Spain; Sweden; Denmark; Germany; and Switzerland) with the exception of the strains SVA 120-89 and LVDL 1829-91 (originating from Sweden and Spain, respectively), whose patterns differed by the deletion of a major OPH 08 fragment. The fingerprints of 117 other rainbow trout isolates were compared by visual observation. When only part of the strains originating from a given country were studied by computer analysis, the other strains from the same origin were found to display the same profile. The strains isolated from trout in countries not represented among the 60 selected strains (i.e. the 2 strains from Chile, the 6 strains from the United Kingdom, the single strain from Norway, and the 1 strain from Finland) also exhibited the typical rainbow trout fingerprint. The presence of the same RAPD fingerprint among rainbow trout isolates from widely different geographical areas may be related to the long-standing international trade of rainbow trout eggs and broodfish. Vertical transmission of the disease has long been suspected. The presence of *Flavobacterium psychrophilum* in the coelomic fluid and in milt was demonstrated a decade ago (Holt 1987), but it is only recently that its presence within the egg was confirmed (Brown et al. 1997).

The 4 ayu isolates from Japan were clearly distinguished from all other strains studied by 3 different primers (i.e. OPH 06, OPH 08, and OPG 08) whereas the 2 Japanese strains isolated from rainbow trout (only 1 was included in the computer analysis) and the 4 coho salmon isolates shared common profiles with the strains from the same fish hosts in other countries. Coho salmon and rainbow trout presently cultured in Japan all originate from the USA. Ten thousand rainbow trout eggs were first introduced from California in 1877 and lesser numbers have been occasionally imported since then; large numbers of coho salmon eggs have been imported every year from the Pacific coast of the USA (and to a much smaller extent from Canada and Chile) since their first introduction in 1975 (Wakabayashi 1996). The first outbreaks of cold-water disease in coho and ayu occurred in Japan during the mid-eighties. Interestingly, the first European outbreaks were noticed at about the same time in rainbow trout reared in Germany (Weis 1987) and in France (Bernardet et al. 1988), although this fish species was introduced in Europe during the last quarter of the nineteenth century. There is no evidence that *Flavobacterium psychrophilum* existed in Japan before coho salmon and rainbow trout were introduced (H. Wakabayashi pers. comm.), although this hypothesis cannot be definitely dismissed. Therefore, it is likely that *F. psychrophilum* was introduced in Japan with infected trout or salmon eggs from the USA. The passage of the pathogen to ayu may somehow have induced a genetic modification resulting in a change in RAPD profiles. It is also possible that local bacterial strains exhibiting a particular RAPD profile indeed existed in Japan before new strains were introduced from the USA. They may have escaped detection as long as they infected wild ayu at a very low prevalence but they progressively came to induce epizootics through the intensification of ayu farming.

Few examples have been published of such a correlation between DNA fingerprints of bacteria and the animal or plant species from which they had been isolated. Different RAPD patterns were indeed displayed by *Porphyromonas gingivalis* strains isolated from humans and animals (Ménard & Mouton 1993), as well as by *Campylobacter jejuni* and *Campylobacter coli* strains isolated from poultry and other animal sources (Aarts et al. 1995), but the authors proposed no convincing explanation. Isolates of the plant pathogen *Xylella fastidiosa* were clearly differentiated by RAPD according to the species of plant from which they were isolated (Chen et al. 1995). These authors suggested that RAPD could be used to define pathotypes or subspecies among bacterial species. Several RAPD primers did not differentiate *Vibrio anguillarum* strains belonging to different serotypes and isolated from various fish species, but only a few strains were included in the study (Martinez et al. 1994). When RAPD profiles generated by 2 primers among 36 *Aeromonas salmonicida*

isolates were compared, 'no geographic, mutual or fish species associated types were found' (Hänninen et al. 1995).

Different typing methods other than RAPD have been proposed to compare *Flavobacterium psychrophilum* strains among themselves: serologic analysis (Lorenzen 1994, Wakabayashi et al. 1994); genetic analysis based on plasmid profile (Holt 1987, Lorenzen 1994) or rRNA gene restriction pattern (ribotype) (Cipriano et al. 1996); and electrophoretic pattern of proteases (Bertolini et al. 1994). Although no association could be found between fish host and either ribotype or plasmid profile, some correlation between fish host and serotype did appear in the results published by Wakabayashi et al. (1994). Two serotypes were revealed by the absorption analysis with heat-stable antigens. The serotype O-1 was common to all American and Japanese coho isolates, whereas the ayu isolates as well as the strains isolated from rainbow trout in Japan belonged to the serotype O-2 (Wakabayashi et al. 1994). These data parallel the results of our study and support the hypothesis of the American origin of the strains isolated from coho salmon in Japan. However, the question of the origin of the serotype and RAPD profiles specific to the strains isolated from ayu remains unanswered.

Comparison of our data with the protease patterns is also interesting since 20 *Flavobacterium psychrophilum* isolates among the 29 studied by Bertolini et al. (1994) were also included in our study. According to these authors, the 29 isolates were divided into 4 groups based on the presence or absence of certain proteases visualized by substrate sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A correlation was found between protease groups and fish hosts, as all 11 isolates in protease group 1 originated from coho salmon whereas all 12 isolates from other fish species belonged to protease groups 2, 3 and 4. The authors tested the virulence of representatives of each protease group in juvenile coho salmon. Some association was noticed between protease group and virulence, as most group 1 strains induced a high mortality and isolates belonging to other protease groups tended to be less pathogenic. In our study, all strains in Bertolini's protease group 1 belonged to the cluster which included the type strain whereas most strains in protease group 2 belonged to the other coho salmon cluster. Therefore, a correlation seems to exist between fish host, proteolytic activity, RAPD profile, and virulence, suggesting that several pathotypes may exist among *F. psychrophilum* isolates. However, the high virulence of protease group 1 strains was also demonstrated by Bertolini et al. (1994) on steelhead trout and 6.5 to 12% mortality also occurred when 3 groups of 25 steelhead trout were each injected sub-

cutaneously with a different ayu isolate. Similarly, Holt (1987) was able to induce severe mortality in yearling coho salmon after subcutaneous injection of *F. psychrophilum* strains isolated from coho, chinook and chum salmons, but no mortality occurred when coho salmon were injected with isolates from rainbow, brook, cutthroat and steelhead trout. In the same study some isolates from coho salmon also failed to induce any mortality. These results demonstrated that the virulence of *F. psychrophilum* isolates is not restricted to the species or to the genus of fish from which the strains were isolated. Actually, the correlation noticed by Bertolini et al. (1994) was not absolute, as 5 highly virulent coho salmon isolates belonged to the protease group 2. The virulence of *F. psychrophilum* may thus involve other mechanisms in addition to proteolytic activity, as already suggested by these authors (Bertolini et al. 1994).

The only European eel isolate included in our study (LFNW 131/89) fell within the cluster grouping most rainbow trout isolates, among others the strain LFNW 123/89 isolated from the same river in Germany and during the same outbreak (Lehmann et al. 1991), suggesting that the eel was infected by a strain originating from a rainbow trout. However, the primer OPG 16 was able to differentiate the eel isolate from all trout isolates (Fig. 1). Furthermore, the strains LFNW 16/90 and LFNW 25/90, also isolated during the same outbreak from a carp and a tench, respectively, each displayed profiles different from the typical rainbow trout pattern. Hence, *Flavobacterium psychrophilum* strains exhibiting different RAPD profiles may coexist in the course of an epizootic, but more strains should be studied in order to verify the apparent correlation with fish host noticed during the German outbreak.

A rather similar situation was found among the strains isolated in Oregon and neighbouring states. Various RAPD profiles were found among *Flavobacterium psychrophilum* strains retrieved from hatcheries belonging to 2 main groups (i.e. the lower Columbia River hatcheries and the coastal hatcheries). Over the years, many transfers of fish occurred between the hatcheries within each group, as well as much less frequent transfers between the 2 groups. Moreover, most hatcheries raise a variety of fish species such as coho and chinook salmon as well as rainbow and steelhead trout, and straying of adult fish is known to occur (R. A. Holt pers. comm.). The 2 strains isolated from chinook (OSU SRChF8-81) and Atlantic (OSU AD10.1.1) salmon were grouped together with the coho isolates and may thus originate from coho salmon reared in the same region. Indeed, the chinook isolate was retrieved from the same fish farm at which the strain OSU SRCo5-90 was isolated from coho and both strains displayed identical profiles. However, the 2 strains from

steelhead (OSU RbS6-82) and cutthroat (OSU CCC6-86) trout, also from Oregon, exhibited very distinct profiles. Thus, different RAPD profiles have been shown to occur within a region where natural and artificial transfers of fish are very frequent, and it is not clear why coho salmon isolates are divided into 2 clusters.

No conclusion could be drawn from the very homogeneous profile displayed by the 5 Tasmanian Atlantic salmon isolates, since they were all retrieved at the same time from the same fish farm and the only other Atlantic salmon isolate included in the study grouped together with coho isolates. Moreover, no strain isolated from other fish species in Tasmania was available.

Many Gram-negative yellow pigmented bacteria, several *Flavobacterium* species among them, coexist in freshwater environments and may occasionally be isolated from sick or healthy fish. Because most of these bacteria have a fastidious growth and are inert in many biochemical tests, contamination and erroneous identification may occur. Our study demonstrated that a rapid and accurate identification of *F. psychrophilum* as well as its differentiation from related species could be achieved through RAPD using the primer OPG 10 because species-specific products were amplified. Identification of *F. psychrophilum* with this technique is even possible using a single colony collected from a mixed culture on an agar plate (data not shown). Several bacterial species have also been differentiated from closely related species using particular RAPD primers. Examples among fish-pathogenic bacteria include primers generating fingerprints which clearly differentiated *Vibrio salmonicida*, *V. anguillarum*, and *V. fisheri* (Martinez et al. 1994) as well as different *Aeromonas* species isolated from fish (Miyata et al. 1995) or from widely different clinical and environmental sources (Oakey et al. 1996).

Because very few template DNA and no previous knowledge of nucleotide sequence are required, and because the whole genome is analyzed (Oakey et al. 1996), RAPD has several advantages over the other techniques that have been used alone or in various combinations for identifying *Flavobacterium psychrophilum*, such as phenotypical investigations (Holt 1987), DNA/DNA hybridization (Bernardet & Grimont 1989), serological tests revealing specific antigens (Pacha 1968, Holt 1987, Cipriano et al. 1996), whole-cell protein (Bernardet et al. 1996, Cipriano et al. 1996) or fatty acid analyses (Bernardet et al. 1996), and 16S rDNA-targeted PCR (Toyama et al. 1994). DNA/DNA hybridization is the reference technique for determining whether 2 bacterial strains belong to the same species (Wayne et al. 1987), but its use is restricted to the laboratories involved in taxonomical and phyloge-

netic studies. Phenotypic, biochemical, and serological methods of identification are usually cumbersome and time-consuming because *F. psychrophilum* is relatively fastidious and because antisera or bacterial extracts have to be prepared.

This study provides a powerful method for an efficient typing of *Flavobacterium psychrophilum* strains for epidemiological tracing. It also allows a reliable differentiation of *F. psychrophilum* from phylogenetically related bacterial species coexisting in the same environment. Until now, ribotyping was considered the only molecular typing method able to provide both identification and typing potentials (Brosch et al. 1996); it is now clear that RAPD profiles also carry both kinds of information.

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