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Isolation of *Flavobacterium*-like bacteria from diseased salmonids cultured in Chile

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Abstract

The biochemical, serological and molecular characteristics of 8-pigmented isolates phenotypically related to *Flavobacterium psychrophilum* were determined. The bacteria were isolated in 2006 from diseased Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) cultured in the South of Chile. The *F. psychrophilum* type strain ATCC 49418^T and isolate B97026, obtained from rainbow trout in UK, were included in the study as a comparison. Their potential to cause pathology in Atlantic salmon was also investigated. The 8 isolates were biochemically identical, however were completely different to *F. psychrophilum*. Slide agglutination and Dot blot allowed us to confirm that the isolates constituted a homogeneous, but distinctive serological group to the *F. psychrophilum* isolates. Similar homogeneity was observed using RAPD-PCR with identical DNA patterns obtained, regardless of the source of isolation or geographic origin. Therefore, any of the above tests can be used to discriminate these pigmented bacteria from *F. psychrophilum*. Virulence studies suggest that these isolates could be considered as a potential pathogen for salmonids, mainly in mixed cultures with *F. psychrophilum*. These fish showed typical signs of flavobacteriosis. Recent studies on the sequencing of the 16S rRNA gene, together with the phenotypic and biochemical properties obtained in this study permitted us classify these 8 isolates in the genus *Chryseobacterium.* Further molecular studies are in progress in order to know the species of these pigmented bacteria and determine the real risk for the salmonid culture.

Introduction

Flavobacterium psychrophilum is the causative agent of bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS), an important disease that is thought to affect all species of salmonid fish worldwide (see review Nematollahi et al., 2003). However, less severe losses associated with this disease have also been reported in several non-salmonid fish (Lehmann et al., 1991; Iida & Mizokami, 1996). The clinical signs in affected fish are characteristic gross lesions on the body surface in the form of ulcers, skin and muscle lesions on the flank or in peduncle area, loss of the epithelial surface and haemorrhaging in exposed dermal tissue, and sometimes necrosis on the gills and eyes (see reviews Dalsgaard, 1993; Nematollahi et al., 2003).

Detection of *F. psychrophilum* can be performed using molecular-based methods like polymerase chain reaction (PCR) (Bader & Shotts, 1998; Urdaci et al., 1998; Izumi et al., 2000; Wiklund et al., 2000), although definitive diagnosis must be supported by the isolation of the bacterium on agar which characteristic yellow-pigmented colonies are produced. We have recently isolated other yellow-pigmented bacteria associated with diseases

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in the fingerling stages of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). These microorganisms were often isolated from mixed cultures of *F. psychrophilum*, being all of them initially diagnosed as member of the *F. psychrophilum* species. The aims of the present study were to examine the biochemical, serological and molecular characteristics of these bacteria compared with those of the *F. psychrophilum* type strain, as well as their pathogenic potential in freshwater-farmed fish.

Material and methods

Biochemical characterization of the isolates

Eight isolates obtained in pure and mixed cultures from external ulcers and lesions, from severe outbreaks of mortalities in Atlantic salmon (n = 3) and rainbow trout (n = 5) in different farms located in X Region of Chile, were used in the present study (Table 1). *Flavobacterium psychrophilum* type strain ATCC 49418^T (serotype Fp^T) and isolate B97026 obtained from rainbow trout in UK (Faruk et al. 2002) were used as a comparison. Other isolate, VQ-Au1, recently obtained from the skin of infected and dead fish was chosen only for the pathogenicity studies. The bacteria were grown on Anacker Ordal (AOA; 0.5% tryptone, 0.05% yeast extract, 0.02% beef extract, 0.02% sodium acetate, pH 7.2) supplemented with 1.5% agar and incubated aerobically at 15ºC for 3–5 days. Stock cultures were maintained frozen at – 80°C in Criobilles tubes (AES Laboratory).

	Isolates present study		***Flavobacterium psychrophilum* strains**	
Characteristics	**(n = 3) [a]**	**(n = 5) [b]**	**ATCC 49418^T**	**B97026**
Host	*Salmo salar*	*Oncorhynchus mykiss*	*Oncorhynchus kisutch*	*Oncorhynchus mykiss*
	Osorno (Osorno)	Chapo lake (Llanquihue)	USA	UK
Cells form	Rods	Rods	Long filamentous rods	Long filamentous rods
Colony colour	Yellow	Yellow	Yellow	Yellow
Gram	-	-	-	-
Catalase	+	+	+	+
Oxidase	+	+	+	+
O/F	-	-	-	-
Congo red adsorption	-	-	-	-
Flexirubin pigments	+	+	+	+
Citrate Simmons	-	-	-	-
Vogues-Proskauer	-	-	-	-
Arginine decarboxylase	-	-	-	-
Lisine decarboxylase	-	-	-	-
Ornithine decarboxylase	-	-	-	-

Table 1 continued.

Characteristics	Isolates present study		*Flavobacterium psychrophilum* strains	
	(n = 3) [a]	(n = 5) [b]	ATCC 49418^T	B97026
Host	*Salmo salar*	*Oncorhynchus mykiss*	*Oncorhynchus kisutch*	*Oncorhynchus mykiss*
	Osorno (Osorno)	Chapo lake (Llanquihue)	USA	UK
Growth at (°C)				
0	-	-	-	-
4	+	+	+	+
15	+	+	+	+
28	+	+	-	-
37	-	-	-	-
Growth with NaCl (%)				
0	+	+	+	+
1.0	+	+	+	+
1.5	+	+	w	w
3	+	+	-	-
6	-	-	-	-
Growth on				
Trypticase soy agar	+	+	-	-
Nutrient agar	+	+	+	+
R2A	+	+	+	+
Marine agar 2216	-	-	-	-
Tryptone yeast extract agar	+	+	+	+
Columbia blood agar	+	+	-	-
MacConkey agar	-	-	-	-
Enzyme activities				
DNase	w	w	-	-
Urease	-	-	-	-
ß-Galactosidase	-	-	-	-
Hydrolysis of				
Aesculin	+	+	-	-
Gelatine	+	+	-	+
Starch	-	-	-	-
Tween 80	-	-	w	w

[a], VQ– 2206s; VQ–4836s and VQ–6316s
[b], VQ–106r; VQ–5916r; VQ–5926r; VQ–5946r and VQ–5966r

Table 1. Differential phenotypic characteristic of *Flavobacterium*-like isolates from known *F. psychrophilum* strains included in this study. Number of isolates are show in parenthesis; +, Positive; –, Negative; w, weakly positive.

All isolates were identified using phenotypical properties, standard bacteriological tests (MacFaddin, 1984), and other biochemical tests (Bernardet et al., 2002; Avendaño-Herrera et al., 2004a). Biochemical reactions tested included: colony morphology and pigmentation, cell morphology, gliding motility, Gram-staining, cytochrome oxidase, catalase reaction (3% H_2O_2), oxidation/fermentation reactions, Voges-Proskauer test, lysine descarboxylase, ornhitine descarboxylase, arginine descarboxylase, presence of cell wall-associated flexirubin type pigments and absorption of Congo red. Hydrolysis of the following substrates was determined using AOA as basal medium: gelatin, agar, starch, Tween 80, casein, DNA, blood and tyrosine. The growth temperature range was tested from 0 to 42°C on AOA. Growth in the presence of 0 to 10% (w/v) NaCl was also determined. Growth was detected on MacConkey agar, Simmons' citrate agar, blood agar, R2A agar, Marine agar 2216, Nutrient agar, Trypticase Soy Agar and Triptone yeast extract agar. Further biochemical analysis were carried out using API 20E and API ZYM (bioMérieux) strips, according to the manufacturer's instruction with the exception of the incubation temperature, which was fixed at 15°C.

Antimicrobial tests were applied by disc diffusion method on Mueller-Hinton agar (MHA) with 1% NaCl, as well as the dilute versions of MHA medium, as recommended by the Clinical and Laboratory Standards Institute (CLSI 2006) for use with *F. psychrophilum*. The chemotherapeutic reagents used (Oxoid, (micrograms per disc)) were: amoxicillin (25), trimethoprim-sulfamethoxazole (1.25/23.75), enrofloxacin (5), florfenicol (30) and oxytetracycline (30). The diameter of each zone inhibition was determined after 48, and, if necessary, 72 h of incubation at 15°C. Reference strain *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658^T was used as a control.

Identification of the isolates by PCR

Chromosomal DNA was extracted using Insta-Gene Matrix (Bio-Rad) for pure bacterial cultures according to the manufacturer's instructions. All PCR amplifications were done using the species-specific primer designed by Urdaci et al. (1998) and the commercial kit Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech), which included all the reagents needed for the PCR reaction (buffer, nucleotides and *Taq* DNA polymerase), except the specific primers and DNA template. All amplifications were carried out in a PXE 0.5 Thermalcycler (Thermo Electron Corporation), and the products were electrophoresed in a 1.5% (w/v) agarose gel visualized with ethidium bromide (Bio-Rad) and photographed under UV light. A 100-bp DNA ladder (Invitrogen) was used as a molecular mass marker. The presence of a single band of 1088 base pair (bp) was considered to be positive for identification of *F. psychrophilum*.

Serological characterization

Antisera against two representative isolates obtained from rainbow trout and Atlantic salmon, codified as VQ-5926r and VQ-6316s respectively, were prepared by intravenous injections of rabbits with formalin-killed cells (10^9 cells ml^{-1}) according to Sørensen and Larsen (1986). To evaluate the antigenic cross-reactivity among the *Flavobacterium*-like

bacteria and *F. psychrophilum* species, all serological tests included polyclonal antiserum raised against ATCC 49418^T and strain B97026, which were also prepared in this study. Serological assays were carried out according to Avendaño-Herrera et al. (2004a), using whole cell preparation and heat stable O-antigens of each isolate as well as the *F. psychrophilum* type strain ATCC 49418^T, and B97026. The serological relationship between isolates was determined using agglutination (Toranzo et al., 1987), with strong and rapid agglutination considered positive. Dot blot analysis was also conducted as denoted by Cipriano et al. (1985). Controls were made with PBS and serum from non-immunised rabbits. Only a reaction similar to that exhibited by the homologous strain was scored as positive.

RAPD-PCR analysis

The RAPD reactions were performed using Ready-To-Go™ RAPD analysis beads (Amersham Pharmacia Biotech) following the protocol described by Avendaño-Herrera et al. (2004b). These commercial beads have been optimised for RAPD-PCR reactions and, as with the PCR beads above, contained buffer, nucleotides and *Taq* DNA polymerase; the only reagents added to the reaction were template DNA (1 µl), 100 pmol of respective RAPD primers (supplied in the kit) and water to make the reaction up to a volume of 25 µl. Amplification products were separated by horizontal electrophoresis and photographed under UV light. Data analysis on the resulting RAPD profiles was performed using GelCompar II software (Applied Maths). The computed similarities among strains were estimated by means of the Dice coefficient (S_d) (Dice 1945). A dendrogram was produced on the basis of the unweighted average pair group method (UPGMA).

Virulence tests in fish

Infectivity trials were conducted using healthy Atlantic salmon (average weight 8–10 g) obtained from a hatchery with no history of flavobacteriosis located in the central of Chile. To make sure that they were free from *F. psychrophilum* and other freshwater pathogens, the fish samples (gills, mucus, skin and kidney) were subjected to standard microscopical and bacteriological examination, and also analysed by PCR (Urdaci et al., 1998). Fish were allocated at a rate of 15 fish per 10-L tank with freshwater, aerated and acclimatized for 72 h prior to bacterial challenge. Representative *Flavobacterium*-like VQ-6316s and *F. psychrophilum* VQ-au1 were chosen for the trial. Virulence assays were performed by immersion challenge according to Avendaño-Herrera et al. (2006). Three types of trials were conducted by direct inoculation with a bacterial suspension of 5 x 10^6 cells ml^{-1} into sterile freshwater: a) isolate VQ-6316s; b) *F. psychrophilum* VQ-au1 and c) bacterial mixtures of the both microorganisms (2.5 x 10^6 cells ml^{-1} of each strain). Fish without bacterial challenge were included as control. All trials were maintained in closed system at 15 ± 1°C with a pH ranging from 7.6 to 7.8, and oxygen 7.8 mg L^{-1} using a 24L:0D light regime. The fish were fed daily at 1.5% body weight and the water in each tank was changed once every-other-day to remove the faecal matter.

Dead fish were removed from tanks daily for a 28 days period and subjected to microbiological analysis to confirm re-isolation of

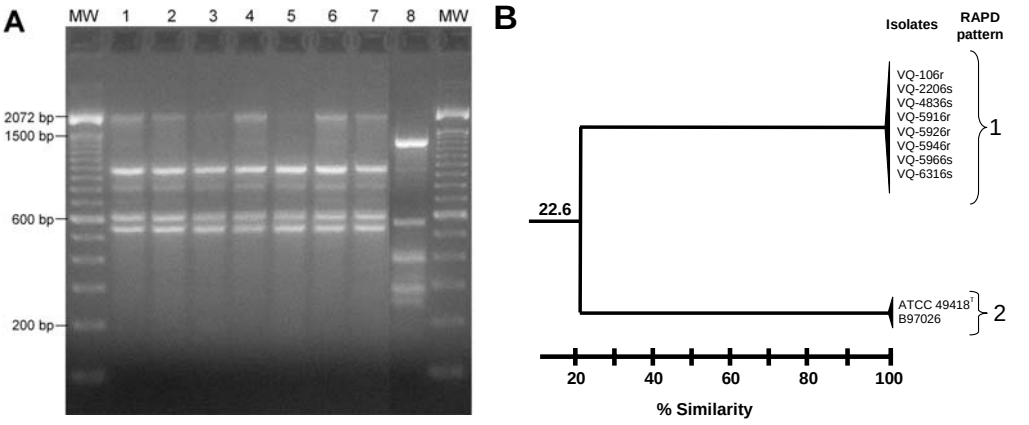

Figure 1. (A) Amplification fingerprints obtained for the isolates using RAPD with primer 5 (supplied with the kit). Lanes: MW, AmpliSize Molecular Ruler (100-bp DNA ladder, Invitrogen); 1 to 7, *Flavobacterium*-like pigmented bacteria and 8, *Flavobacterium psychrophilum* type strain. (B) Dendogram obtained using Dice similarity coefficient and unweighted pair group method average (UPGMA) analysis on the basis of RAPD profiles. Number along branches: % similarity.

the inoculated strains. Once pure cultures of the pigmented isolates were obtained, were analysed and identified by slide agglutination assay and RAPD method as described above.

Results and discussion

Biochemical homogeneity was seen among the isolates recovered from the diseased fish regardless of the host of isolation, and was very different to the *F. psychrophilum* isolates used as a comparison (Table 1). They were all Gram negative, rod-shaped, non-gliding and non-motile bacteria, catalase and cytochrome oxidase positive and were non fermentative. Colonies were smooth, shiny, circular with regular edges and yellow in colour. Growth occurred at 4–28°C and with 0–3% NaCl (this growth pattern is not typical for *F. psychrophilum*). All isolates contained a cell-wall-associated flexirubin-type pigment, but did not absorb Congo red. Growth on Columbia blood agar (a-haemolytic) and trypticase soy agar was observed, but not on MacConkey agar or Simmon's citrate medium, while the *F. psychrophilum* strains did not grow on any of these media. The isolates did not produce acetoin (Voges–Proskauer test). Gelatin, aesculin and DNA were hydrolysed, but Tween 20, tyrosine, agar, starch and casein were not. The results of the API 20E test were the same for all strains, including both *F.*

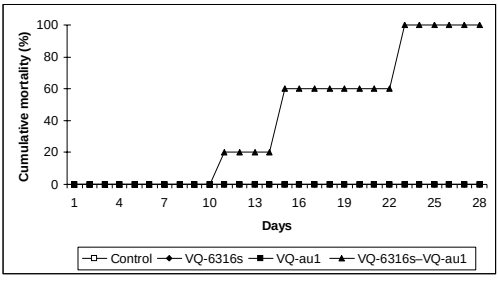

Figure 2. Percentage cumulative mortalities of Atlantic salmon challenged immersion with a bacterial suspension of 5 x 10^6 cells ml^{-1} into sterile freshwater of a) isolate VQ-6316s; b) *F. psychrophilum* VQ-au1 and c) bacterial mixtures of the both microorganisms (2.5 x 10^6 cells ml^{-1} of each strain). Fish without bacterial challenge were included as control.

psychrophilum strains (code 0006024). It is important to point out that the API 20E rendered false positive for glucose fermentation/oxidation when compared with results from traditional tube test. This difference might be due to the influence of the culture medium and/or initial inocula density (Ravelo et al., 2001).

On the other hand, results from the API ZYM test also showed complete homogeneity among all the disease isolates. Activity was not detected for melibiase, lactase, hyaluronidase, cellulose, alpha-mannosidase and alpha-fucosidase. As expected, for the *F. psychrophilum* strains, negative results were found for all the enzymes involved in carbohydrate metabolism (reactions 13 to 20 in API ZYM galleries). Therefore, the two *F. psychrophilum* strains showed classical biochemical patterns for the bacterium, as detailed in the literature (Bernardet & Grimont, 1989; Lorenzen et al., 1997).

The antibiotic sensitivities of the isolates were the same, regardless of the media used, and 48 h was required before the results could be read. All isolates were resistant to oxytetracycline and highly sensitive to the other drugs tested. This pattern of antimicrobial sensitivity could possibly be explained by the common use of these drugs in fish farms to control disease. In contrast, all *F. psychrophilum* strains were totally resistant to trimethoprim-sulfamethoxazole and highly susceptible to the remained chemotherapeutic agents tested. Moreover, scant growth occurred in 48 h, but before 72 h clear and well-defined zones of inhibition were displayed in concordance with the recommended by the CLSI (2006).

The ATCC 49418^T and Scottish isolates gave a clear product of the expected 1088 bp when PCR analysis was carried out, while products of a lower molecular size were observed for all of the disease isolates (data not shown). These results imply that the 8 isolates obtained from diseased fish do not belong to the species *F. psychrophilum*.

Serological characterization of the isolates by slide agglutination and Dot blot assays showed only a strong reaction with the antisera raised against rainbow trout and Atlantic salmon isolates (VQ-5926r and VQ-6316s), demonstrating that they belong to a same serological group (data not shown). However there was no cross–reaction with the antisera raised against the two *F. psychrophilum* isolates. The strains ATCC 49418^T and B97026 only reacted with their homologous antiserum, confirming that the pigmented isolates are antigenically distinct compared to *F. psychrophilum*. As expected no reaction with the pre-immune serum was detected.

PCR-based typing is an effective approach in the epidemiological study of diseases caused by various Gram-negative bacteria. In this study, RAPD analysis was applied to obtain genetic fingerprints of the eight isolates, and to discriminate them from the *F. psychrophilum* isolates. Initially RAPD analysis was performed using each of the six primers provided in the kit. However, only one of the six primers, oligonucleotide P5, generated reproducible patterns with an appropriate number of amplified bands. The analysis of the putative *Flavobacterium* isolates showed an identical profile among all strains with amplification bands ranging from 500 to 2000

bp, indicating genetic homogeneity between the isolates (Figure 1A). The type strain ATCC 49418^T and Scottish isolate yielded a different fingerprint ranging in size from 200 to 1300 bp, and were grouped within other cluster, with a similarity level of 22.6% (Figure 1B). RAPD fingerprinting has previously been used to distinguish between *F. psychrophilum* and numerous other closely related bacteria found in diseased salmonid fish (Crump et al., 2001).

The results of the virulence testing with *Flavobacterium*-like isolate (VQ-6316s) and VQ-au1 gave no mortalities and did not induce disease when fish were infected with the bacterium by bath. In contrast, prolonged immersion (18h) of fish with a mixture of both isolates produced the first incidence of dead fish 11 days and cumulative mortalities of 100%, 23 days after the exposure to VQ-6316s–VQ-au1 (Figure 2). It is important to denote that the timing of immersion of fish (18 h) was chosen based on our previous experience with challenge model for external pathogens (Avendaño-Herrera et al., 2006), being the most similar to the natural route of infection. Gross external lesions were seen in all fish, typical of those seen in flavobacteriosis. Microscopic examination of wet mount smears from the external lesions of dead fish revealed the presence of high quantities of long *F. psychrophilum* rods and shorter rods from *Flavobacterium*-like. A mixed culture of the two isolates was recovered from the lesions of only a few moribund fish. Based on the RAPD-PCR and confirmed by serological tests, all pure isolates obtained from the infected fish were identified as those used for the challenge. Failure to recover the bacterium on AOA from the other fish is probably due to the fastidious nature of this microorganism, as previously reported for *F. psychrophilum* (Michel et al., 1999). None of the control fish died during the experiment.

Mechanisms of virulence and condition that influence virulence of the *Flavobacterium*-like species studied are unknown, while the pathogenicity of *F. psychrophilum* infections is not yet understood, and only scant data on virulence factor are available (Nematollahi et al., 2003). We speculate that the degree of pathogenicity or expression of the pathogenic potential of disease-producing micro-organisms is increased when both isolates are mixed, since the isolates alone did not kill fish even when high concentrations were applied (data not shown). In fact, mixed infections of certain viral, bacterial, fungus or parasitic fish pathogens and *F. psychrophilum* are frequently observed in various salmonids fish species (see review Cipriano & Holt, 2005). However, so far unknown whether the attachment, colonization or subsequent protease and/or toxin released and penetration by the bacteria are triggered (Nematollahi et al., 2003). Possibly fish tissue is altered (in some way by bacteria), which facilitates bacterial infection and further invasion.

In summary, the pigmented bacteria isolated from the diseased rainbow trout and Atlantic salmon may be a potential pathogen for salmonid culture in Chile, mainly in mixed cultures with other fish pathogen such as *F. psychrophilum*. These isolates were biochemical, serological and genetically homogeneous, but were totally different to *F. psychrophilum*. Recent studies on the sequencing of the 16S rRNA gene, together with the phenotypic and biochemical

properties obtained in this study permits us classify these 8 isolates in the genus *Chryseobacterium.* In this genus there are species known to be pathogen to fish (Mudarris et al., 1994; de Beer et al., 2006), as well as other isolates not yet identified at species level, but still causing mortalities to aquatic organisms (Bernardet et al., 2005). Further molecular studies are in progress in order to know the species of these pigmented bacteria and determine the real risk for the salmonid culture.

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