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NOTE

Characteristics of *Flexibacter psychrophilus* isolated from Atlantic salmon in Australia

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ABSTRACT: *Flexibacter psychrophilus* (syn. *Cytophaga psychrophila*) was isolated in Tasmania, Australia, from farmed Atlantic salmon *Salmo salar* with moderate to severe erosion of the fins; there was no evidence of skin lesions. The mortality level in the population of affected fish was less than 0.01% wk^{-1}, but the morbidity level was in excess of 80%. The phenotype of the Australian isolates is in good agreement with strains from Europe and North America and differs only in that the Australian isolates produced a brown pigmentation on tyrosine agar and did not hydrolyse Tween 80. The growth *in vitro* of all isolates was inhibited by acriflavine, ampicillin, and oxolinic acid at concentrations in excess of 0.5 µg ml^{-1} and by oxytetracycline at 1.56 µg ml^{-1}; none of the isolates were inhibited by sulphamethazine or trimethoprim at 25 µg ml^{-1}.

KEY WORDS: *Flexibacter psychrophilus* · Fin rot · Atlantic salmon *Salmon salar* · Antibiotic sensitivity

Filamentous bacteria belonging to the *Flexibacter-Cytophaga* Complex (FCC) are well recognized fish pathogens, with marine and freshwater species capable of causing disease. *Flexibacter columnaris* has been reported as a pathogen of freshwater fish throughout the world (Austin & Austin 1987), while accounts of *F. maritimus* infection of sea farmed fish have been made in Japan (Wakabayashi et al. 1986), Scotland (Bernardet et al. 1990), and Spain (Alsina & Blanch 1993). *Cytophaga psychrophila*, re-classified as *F. psychrophilus* (Bernardet & Grimont 1989), is the agent of coldwater disease in salmonids and may cause a spectrum of pathologies ranging from ulcerative skin necrosis to systemic infection involving major body organs. *F. psychrophilus* is the cause of significant levels of mortality in coho salmon *Oncorhynchus kisutch* hatcheries of the USA and Canada (Holt et al. 1993) and is also recognized as the aetiological agent of rainbow trout *O. mykiss* 'fry mortality syndrome' in France, Denmark, the United Kingdom, Germany, Spain, and Finland (Bernardet & Kerouault 1989, Lorenzen et al. 1991, Austin 1992, Bruno 1992, Santos et al. 1992, Dalsgaard 1993, Toranzo & Barja 1993, Wiklund et al. 1994). Other FCC bacteria associated with fish disease are *F. ovolyticus*, a pathogen of Atlantic halibut *Hippoglossus hippoglossus* eggs and larvae (Hansen et al. 1992), *C. johnsonae*, a putative pathogen of barramundi *Lates calcarifer* (Carson et al. 1993), and *C. aquatilis* (Strohl & Tait 1978). In addition, unidentified FCC bacteria have been described as causes of fish disease in the USA (Kent et al. 1988), Scotland (Mudarris & Austin 1989), and Germany (Hilger et al. 1991). The majority of reports of FCC bacteria causing disease are derived from the northern hemisphere. This report describes the characteristics of *F. psychrophilus* isolated from Atlantic salmon in Australia, a biogeographically isolated region in the southern hemisphere.

Materials and methods. Diseased Atlantic salmon *Salmo salar* were obtained from a commercial fish hatchery in Tasmania, Australia, operating with flow-through tanks fed with river water Fish had a body weight between 44 and 47 g and were held at a stocking density of 23 to 25 kg m^{-3}. At the time of sampling, the water temperature was 5°C and had ranged between 2 and 9°C over the previous 3 mo. Routine grading for size 2 mo prior to sampling and low water temperatures were the only identifiable stress factors (Elliott 1981) preceding evidence of disease.

A total of 30 fish with eroded fins and/or tails were examined. The presence of pathogens other than FCC bacteria was determined by aseptically collecting

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kidney tissue by pipette and culturing on Blood Agar Base No. 2 (CM271, Oxoid, Basingstoke, UK) supplemented with 7% (v/v) defibrinated sheep's blood. Eroded fins and tails were sampled by scraping the margin of the lesion with a sterile scalpel blade; the collected material was then inoculated onto freshwater Ordal's agar (FOA) (Anacker & Ordal 1959). Agar plates were incubated at 14°C for up to 10 d.

Characterization of FCC bacteria was made using tests described previously (Carson et al. 1993) and by the use of additional procedures as outlined; incubation of all tests was made at 18°C. Cell length of 10 representative isolates was determined after 3, 7, and 17 d incubation by measuring Gram stained bacteria grown in Shieh's broth (SB) (Song et al. 1988); 10 cells per isolate were measured to calculate a mean length; a grand mean cell length was calculated for each time interval. Catalase activity was determined by the coverslip method of Taylor & Achanzar (1972) and by adding several drops of 3% (v/v) hydrogen peroxide to a dense suspension of bacteria in 1% (v/v) Tween 80 aq.; oxidase reaction was determined by the method of Kovac (Cowan 1974) and by *Dry*Slide Oxidase (Difco Laboratories, Detroit, MI, USA). Production of H_2S was detected with lead acetate strips suspended above SB supplemented with 0.01% (w/v) L-cysteine; Congo Red reaction was tested by the method of McCurdy (1969) using 0.001% (w/v) Congo Red; anaerobic growth was tested on FOA supplemented with 1.0% (w/v) $NaHCO_3$ and 1.0% (w/v) glucose (Reichenbach 1989); lecithinase production was tested on FOA supplemented with 0.5% (v/v) sterile egg yolk with precipitation of fatty acids extending from the colony margin recorded as a positive reaction. Gliding motility was determined for 10 isolates by adapting a procedure described by Perry (1973): sterile slides were coated with a thin layer of FOA, inoculated with a single central longitudinal line, and incubated in a sterile Petri dish for 3 d. A cover slip was placed directly onto the line of bacterial growth and gliding motility was then determined by examining bacteria with phase contrast microscopy and a standard focal length ×40 phase objective. The minimum inhibitory concentrations (MIC) of neutral acriflavine, ampicillin, oxolinic acid, oxytetracycline, sulphamethazine, and trimethoprim were determined by microbroth dilution in SB (Carson et al. 1993).

Results. Nearly all fish on the affected farm had eroded fins, ranging in severity from ragged margins to complete loss of the fin (Fig. 1). The most severely affected fins were the pectoral and pelvic fins and to a lesser extent the dorsal fin. Involvement of the caudal fin was infrequent but where it occurred the fin had evanesced and advancing erosion of the caudal peduncle musculature was evident. Although the level of morbidity was high, the mortality level was less than 0.01%, a level of attrition considered to be commercially acceptable. No bacteria were recovered in culture on blood agar from kidney of the 30 fish selected for examination, indicating the systemic absence of non-FCC-type bacteria, but from 27 of the fish, small deep-yellow-coloured colonies were isolated on FOA from eroded fins. The colonies were 1 to 2 mm in diameter with a smooth appearance and did not adhere to the agar; most colonies had an entire edge but a proportion had a narrow border of flat rhizoid growth. Characterization was undertaken for 20 isolates and these bacteria were identified as *Flexibacter psychrophilus* according to the attributes listed in Table 1. Attempts were made to detect gliding motility in wet preparations made from broth cultures but these were unsuccessful. Gliding was detected relatively easily by slide culture; however, even by this method, the rate of travel was slow. Motion was detected by noting the position of cells with reference to the rulings on an eyepiece graticule and making observations over a period of 5 min. Mean cell lengths of *F. psychrophilus* after 3, 7, and 17 d were 3, 4, and 7 µm, respectively. The oxidase reaction, either by Kovac's method or by *Dry*Slide Oxidase, was readily detectable in all isolates of *F. psychrophilus* and had a rate of reaction and intensity of colour similar to those of *F. columnaris*. The catalase reaction was very weak and was only detected convincingly by the coverslip method; no reaction was seen with the Tween 80 method. The MIC of selected antibacterials for 10 strains of *F. psychrophilus* are given in Table 2.

Fig. 1. *Salmo salar*, Atlantic salmon with severely eroded pectoral fin. *Flexibacter psychrophilus* was isolated from eroded fins of 27 out of 30 fish sampled. Despite a high level of morbidity amongst affected fish, mortalities were less than 0.01% wk^{-1} The coin shown has a diameter of ca 25 mm

Table 1 Phenotype of *Flexibacter psychrophilus* isolated from Atlantic salmon *Salmo salar* in Australia and of strains from Europe and North America. Data as percentage of strains positive; w: weak reaction; V: variable reaction

Test	Australian isolates	Pacha (1968)	Bernardet & Kerouault (1989)	Lehmann et al. (1991)	Holt et al. (1993)
No. of strains	20	10	7	7	28
Gliding motility	100	100	100	100	100
Catalase	100 w	100	100 w	100 w	100 w
Oxidase	100	0	100 w	100	0
Congo Red reaction	0		0	0	
Flexirubin reaction	100		100	100	100
Glucose acid	0	0	0		0
H_2S formation	0	0	0	0	0
Nitrate reduction	0	0	0	0	0
Tyrosine pigmentation	100		0	0	
Growth:					
Anaerobic	0		0	0	0
25°C	90		100	100	64
30°C	0		0	0	0
NaCl 0.5 %	100	100	100		100
NaCl 1.0 %	0	60	0		100
Hydrolysis:					
Casein	100	100	100	100	100
Chitin	0	0	0	0	0
DNA	100		100	100	
Gelatin	100	100	100	100	100
Lecithin	100		100	100	
ONPG	0		0	0	
Starch	0	0	0	0	0
Tributyrin	100	100	100	100	100
Tween 20	100		100	100	
Tween 40	100				
Tween 60	100				
Tween 80	5		100	100	
Tyrosine	100	20	100	100	V
Urea	5				

Discussion. The phenetic characters determined in this study of 20 isolates of *Flexibacter psychrophilus* are in good agreement with published data for this taxon (Pacha 1968, Bernardet & Kerouault 1989, Lehmann et al. 1991, Holt et al. 1993). Cytochrome oxidase was readily detected in the Australian isolates and this is in agreement with Bernardet & Kerouault (1989), who were able to detect this enzyme in American and European isolates. Both Pacha (1968) and Holt et al. (1993) were unable to detect oxidase activity but concluded that the different reactions for this test obtained by various investigators may be the result of using assay procedures of different sensitivities. The catalase reaction for the Australian isolates of *F. psychrophilus* was weak and this is consistent with reports made by Bernardet & Kerouault (1989) and Holt et al. (1993). None of the isolates were able to grow in FOB with 1 % NaCl although Holt et al. (1993) were able to grow their isolates at this NaCl concentration using tryptone yeast extract salts (TYES) medium. These authors note that *F. psychrophilus* grows best in Shieh's and TYES media which suggests that medium composition is important when testing growth characteristics at the limits of the physiological range for the species. The Australian isolates had some characteristics at variance with the reported phenotypes of *F. psychrophilus*. Hydrolysis of short chain length Tweens was observed for all the Australian isolates, in agreement with findings made by Bernardet & Kerouault (1989) and Lehmann et al. (1991); however, only 1 Australian isolate was able to hydrolyse Tween 80 while all the European

Table 2. Minimum inhibitory concentrations (MIC) of selected antibacterial agents for 10 strains of *Flexibacter psychrophilus* isolated from Atlantic salmon *Salmo salar* in Australia

Antibacterial	MIC (μg ml^{-1})
Acriflavine	0.20–0.39
Ampicillin	0.05–0.10
Oxolinic acid	0.05–0.10
Oxytetracycline	0.78–1.56
Sulphamethazine	> 25
Trimethoprim	> 25

and American isolates tested could degrade this substrate. The remaining difference noted was the ability of the Australian isolates to produce a brown pigmentation when growing on media enriched with tyrosine, a characteristic of *F. psychrophilus* not detected by either Bernardet & Kerouault (1989) or Lehmann (1991). The cell length of young cultures of the Australian isolates are within the range reported for the species (Holt et al. 1993) but it is interesting to note that with advancing age, cell length increases. Cell elongation with age is seen in other FCC bacteria and in particular members of the genus *Cytophaga* as defined by Reichenbach (1989).

Oxytetracycline is considered to be the antibiotic of choice for control of infection by *Flexibacter psychrophilus* in coho salmon while only partial control is achievable with sulphonamides such as sulphisoxazole and sulphamethazine (Holt et al. 1993). The MIC data for the Australian isolates of *F. psychrophilus* indicate that ampicillin and oxolinic acid are particularly active, while the MIC range of 0.78 to 1.56 µg ml^{-1} for oxytetracycline suggests that only partial control could be achieved with this antibiotic. The resistance of all the Australian isolates of *F. psychrophilus* to sulphamethazine indicates that control is unlikely to be achieved with this antibiotic and is further evidence of the unsuitability of sulphonamides for controlling infection.

The known geographic distribution of *Flexibacter psychrophilus*, once thought to be limited to North America, has increased steadily with recent reports from several European countries (Dalsgaard 1993) and Japan (Wakabayashi et al. 1991). Holt et al. (1993) predicted that *F. psychrophilus* was likely to be found in most temperate salmonid producing regions in the world, and so it is perhaps not surprising that this pathogen has now been recovered from diseased fish in a cool climate region of Australia. The origin of these Australian isolates of *F. psychrophilus* is interesting in view of the limited number of importations of salmonids. Atlantic salmon ova, conserved during shipping by the melt water of ice obtained from Wenham Lake near Boston, Massachusetts, USA (Walker 1988), were first imported to Tasmania from Great Britain in 1864. Breeding populations of Atlantic salmon failed to develop in rivers and lakes but by 1895 both rainbow trout and brown trout *Salmo trutta* had been successfully introduced and naturalized. The current Tasmanian population of Atlantic salmon for aquaculture was obtained between 1984 and 1986 as disinfected ova from disease free, landlocked fish at Gaden in New South Wales, Australia (Hortle 1988). Whether *F. psychrophilus* was naturally present in the aquatic environment of Tasmania prior to the importation of salmonids or was introduced with ova and melting ice water during attempts at naturalization is uncertain. Ribotyping or randomly amplified polymorphic DNA (RAPD) analysis of isolates from North America and Australia may help to establish the provenance of the Australian strains of *F. psychrophilus*.

The epizootiology of the pathogen in Australia contrasts with infection elsewhere, where it is seen as a cause of significant mortality in juvenile salmonids, particularly coho salmon and rainbow trout. In this first report from Australia, *Flexibacter psychrophilus* has been isolated only from Atlantic salmon with eroded fins in pre-smolt fish held under cold stress conditions. The role of the pathogen as an agent of fin erosion remains putative for the time being because no experimental challenges were undertaken in this study. The apparent lack of virulence of these Australian isolates may be a characteristic of the strains or may simply reflect the resistance of Atlantic salmon to infection with *F. psychrophilus*. Further work is required to characterize the virulence determinants of the Australian isolates and compare them with aggressins of virulent strains of *F psychrophilus* from other geographic regions.

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