Structure of *Flavobacterium psychrophilum* populations infecting farmed rainbow trout (*Oncorhynchus mykiss*)

Krister Sundell[1], Sirpa Heinikainen[2], Pierre Nicolas[3] & Tom Wiklund[1]

[1]*Laboratory of Aquatic Pathobiology, Åbo Akademi University, Tykistökatu 6, FI-20520, Turku, Finland*

[2]*Veterinary Bacteriology Research Unit, Finnish Food Safety Authority Evira, Neulaniementie 4 FI-70210, Kuopio, Finland*

[3]*Unité Mathématique, Informatique et Génomes, INRA, Domaine de Vilvert, F-78350 Jouy-en-Josas, France*

Correspondence: ksundell@abo.fi

Abstract

Flavobacterium psychrophilum, the causative agent of bacterial cold water disease (BCWD), is currently a serious nuisance for the rainbow trout (*Oncorhynchus mykiss*) aquaculture in Finland. In absence of efficient vaccination regimes, infections with *F. psychrophilum* are mainly treated with environmentally hazardous antimicrobial agents. Despite the significant economic impact of *F. psychrophilum* on the global aquaculture industry, much remains unknown regarding the infection process of the pathogen. To shed light on the population structure of the pathogen, genetic methods such as multilocus sequence typing (MLST) have been taken into use [1]. MLST analyses of isolates collected from outbreaks of BCWD over the last three decades have revealed genotypic heterogeneity in *F. psychrophilum* infecting farmed rainbow trout in Finland. Out of the total 167 *F. psychrophilum* isolates analyzed, 22 different genotypes were identified.

It has also been reported in previous studies that *F. psychrophilum* isolated from rainbow trout can dissociate into two different morphological colony types [2], rough (R) and smooth (S) (Fig. 1), but the presence and significance of the two morphotypes in BCWD outbreaks has not been evaluated properly. The aim of the present study was therefore to examine the *F. psychrophilum* populations infecting farmed rainbow trout using different techniques. Rainbow trout from three different fish farms with BCWD outbreaks and unfertilized eggs from one hatchery were examined by agar plate culturing. Tissue samples from fish and eggs were cultured on tryptone yeast extract salts (TYES) agar plates [3], incubated at 15°C for seven days, after which the two distinct colony types were distinguished under a microscope and identified as *F. psychrophilum* by PCR.

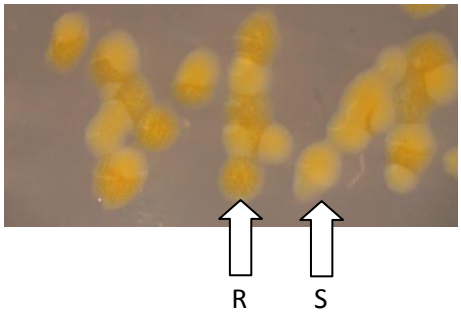

Figure 1. Rough (R) and smooth (S) colony phenotypes of *F. psychrophilum* isolated on a TYES agar plate.

A representative number of colonies (*n*=31) were sub-cultured and further characterized by plasmid analysis (NucleoSpin®Plasmid), pulsed field gel electrophoresis (PFGE) using the *sul*I restriction enzyme and antimicrobial susceptibility testing (oxolinic acid, 2 µg) by disc diffusion. Ten smooth-to-rough isolates that had been converted from S to R form *in vitro* by repeated passage in TYES broth [2] were also included in the analysis together with the parent wild-type isolates.

From five out of seven outbreaks, both rough (R) and smooth (S) colony types of the bacterium were isolated, although the sole isolation of the S type occurred in 21 out of the total 37 sampled fish individuals (Fig. 2). The concentration of *F. psychrophilum* in the unfertilized egg samples was low, corresponding to about 20–100 colony forming units ml^{-1}, equal to a concentration of 1–5 cells egg^{-1}.

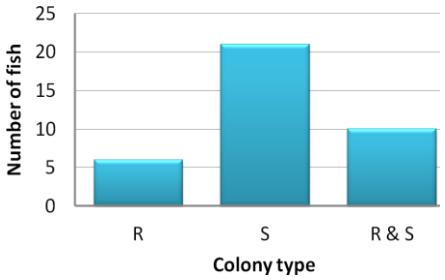

Figure 2. Number of only rough (R), smooth (S) and co-occurring R and S (R & S) colony types isolated from 37 rainbow trout with BCWD.

The results from the antimicrobial susceptibility testing showed that oxolinic acid resistant cells were isolated from each outbreak (Tab. 1). The two colony types were either susceptible or not susceptible to oxolinic acid, indicating that both resistant and sensitive cells could be isolated from a single disease outbreak. No correlation between colony type and resistance towards oxolinic acid was found and the smooth-to-rough converted isolates shared the same resistance pattern together with the parent isolates.

Farm	Outbreak	Rough	Smooth	Smooth-to-rough
1	A	Sensitive	Resistant	Not converted
	B	Resistant	Sensitive	Sensitive
2	A	Resistant	Resistant	Resistant
	B	Notisolated	Resistant	Resistant
3	A	Resistant	Resistant	Not converted
	B	Notisolated	Resistant	Resistant
	C(eggs)	Sensitive	Resistant	Resistant

Table 1. Oxolinic acid susceptibility of rough, smooth and smooth-to-rough converted *F. psychrophilum* isolates from BCWD outbreaks and eggs.

The plasmid pattern was partly associated to the colony type, showing identical or completely different patterns for the R and S types isolated from the same outbreak. In farm 1 and 3, the R types showed a different plasmid pattern compared to the S isolates, while the two colony types from farm 2 shared the same pattern. The smooth-to-rough converted isolates retained their plasmids and no correlation between the plasmid profile and oxolinic acid resistance was found.

The PFGE profiles were analyzed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) and a similarity dendrogram was constructed using UPGMA and Dice similarity coefficient. The isolates from the three different fish farms showed genetic homogeneity indicated by the band similarity exceeding 94% between most of the tested isolates. R and S types coincidentally isolated from the same fish individual sometimes showed

indistinguishable PFGE patterns and in some cases different. *F. psychrophilum* isolated from eggs had either a similar or a differing PFGE pattern compared with the isolates infecting fish in the respective farm.

Our results showed a high prevalence of oxolinic acid resistant isolates, although the use of this antimicrobial has been banned in Finnish aquaculture since 2001. The results also demonstrated an apparent connection between the PFGE profile and oxolinic acid susceptibility, resistant isolates being genetically more homogeneous than the sensitive isolates. The overall high prevalence of resistant isolates in the examined fish farms and the close genetic relatedness between them could be a result of natural selection of genotypes resistant to oxolinic acid. It is possible, that earlier heavy use of oxolinic acid has led to selection of resistant isolates in the studied farm environments. Since resistance to first generation quinolone antimicrobials is mainly chromosomally mediated, it could be expected that the level of resistance to oxolinic acid can persist in farm environments over long periods. Because R and S types of *F. psychrophilum* involved in BCWD outbreaks may differ in their antimicrobial resistance, it would be advisable to test both types for antimicrobial susceptibility when considering the proper treatment strategy. The inability to control resistant *F. psychrophilum* cells could lead to repetitive disease outbreaks.

In our study, the concentration of *F. psychrophilum* cells isolated from unfertilized egg samples was extremely low, suggesting that vertical transmission is not the most important factor maintaining the infection pressure in fish farm environments. Nevertheless, some of these *F. psychrophilum* isolates were genetically very close to those isolates causing disease in the same farm, indicating that outbreaks in rainbow trout fry may originate from infected eggs.

In conclusion, the study showed that within a BCWD outbreak, *F. psychrophilum* cells with different morphology, plasmid content, antibiotic susceptibility and PFGE pattern could be isolated, corroborating both genotypic and phenotypic heterogeneity within the bacterial population infecting farmed rainbow trout. In addition to improving the possibility of pathogen survival under stressful environmental conditions, diversity within an infective bacterial population might complicate the control of BCWD.

References

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