Adhesion and biofilm production of different *Flavobacterium psychrophilum* colony phenotypes

Jenny Norrgård & Tom Wiklund

Laboratory of Aquatic Pathobiology, Åbo Akademi University, Tykistökatu 6, 20520 Turku, Finland

Correspondence: twiklund@abo.fi

Abstract

We have previously shown that *Flavobacterium psychrophilum* occur as two morphological phenotypes, smooth (S) and rough (R) [1]. On agar plates, these two phenotypes can be readily separated and they do not change from one type to another during cultivation on solid media. In broth, the rough phenotype shows a uniform growth mode resulting in a turbid solution, while cells of the smooth phenotype auto-agglutinate at the bottom of the tube. In broth, rough phenotype is stable, while the smooth phenotype is unstable and gradually changes to the rough type. Both phenotypes can be isolated from diseased fish. However, the biological significance of these two types is so far unknown. Although the cells of the two types are known to show different physiological characteristics [2], the adhesion and biofilm production have not been previously examined separately.

The adhesion of three S and three R phenotype isolates of *F. psychrophilum* to polystyrene (microtiter plates) was evaluated under different environmental conditions (temperature, water hardness) or to polystyrene coated with rainbow trout mucus. All adhesion experiments were done in fresh water, with a natural hardness of 4 °dH, obtained from a lake in SW Finland. The pH of the water was adjusted to 7.2. The water was filtered to remove particles and autoclaved. In order to examine the influence of water hardness on the adhesion, three different media with ºdH 4, 14 and 28 were prepared [3]. The adhesion of the cells to the well surface was measured using a crystal violet staining method [4], with some modifications. Briefly, bacterial suspensions (100 µl) were added in quadruplicate to the microplate and incubated at 15 ºC for 1 h. Following washing with 0.5 % NaCl, the adhered cells were stained with 0.1% crystal violet solution for 45 min at room temperature. After washing, the stain bound to the adhered cells was subsequently released with 96 % ethanol and the absorbance measured at 595 nm. Skin mucus was collected from rainbow trout body surface, diluted with sterile filtered fresh water and centrifuged twice (10 ºC, 14 000 *g*, 10 min) in order to exclude cellular and particular matter. After the supernatant was filter-sterilized, the mucus from all rainbow trout was pooled and diluted to a protein concentration of 0.5 mg mL^{-1} and kept at –70 ºC until needed. Wells of a microtiter plate was coated with mucus over night at 4 ºC, washed and the adhesion of the bacteria to the coated wells was subsequently assayed.

The biofilm production on polystyrene surfaces was evaluated in microtiter plates both under nutrient poor conditions using autoclaved lake water and under nutrient rich conditions using fish feed as nutrients. The bacterial growth was measured spectrophotometrically at 520 nm or by dilution and plate count (CFU).

The results clearly showed that smooth cells adhered more readily than rough cells to the tested polystyrene surfaces suggesting the presence of different adhesion mechanisms or adhesion molecules on the cell surface of the two phenotypes (Fig. 1). The adhesion was highest at 15 ºC and decreased at 5 ºC and 25 ºC. However, statistical differences were not always observed between the adhesion at different temperatures. This temperature optimum is reflecting the temperature for natural disease outbreaks, however the results could also be explained by an acclimatization of the bacteria to the standard incubation temperature (15 ºC) used in our laboratory. The results also indicated that high water hardness (ºdH 28) had a significant ($p < 0.05$) negative influence on the adhesion of smooth isolates to polystyrene, but no trends were observed for rough cells. These results suggest that the presence of Ca^{++} and Mg^{++} ions in the water negatively affects the adhesion efficiency of the smooth cells to inert surfaces.

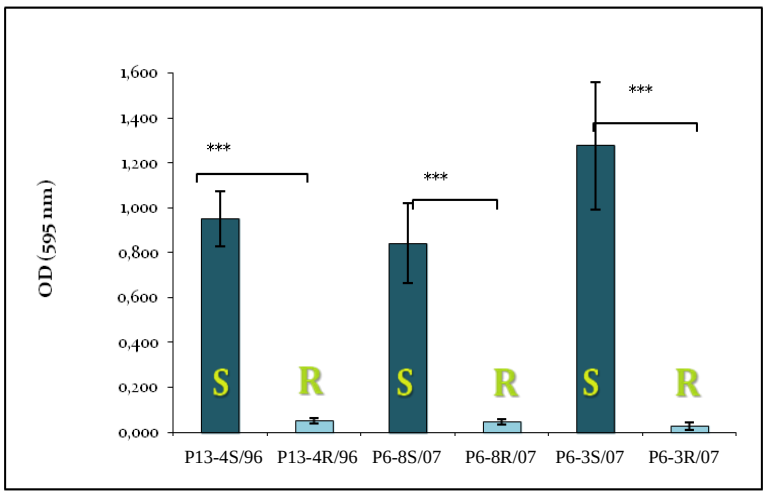

Figure 1. Adhesion of smooth (S) and rough (R) *F. psychrophilum* isolates, onto microtiter plate wells. Values are averages of four observations ± S.D. Significance: ***, P< 0.001.

A significantly increased adhesion of smooth cells was observed to surfaces coated with rainbow trout mucus, compared to non-coated surfaces. Cells of only one rough variant showed increased adhesion to mucus. An increased adhesion to rainbow trout mucus will facilitate the introduction of the bacteria to the fish. However, the results do not give any indication of the role of possible mucus antibodies on the adhesion process.

Cells of both phenotypes used nutrients from fish feed for growth, but the growth was clearly inhibited after 3 days of incubation. Both S and R phenotypes showed identical biofilm formation capacities in wells with and without fish feed, producing a significantly higher number of cells under nutrient rich conditions (fish feed) compared to nutrient poor conditions (only lake water).

The results clearly showed that smooth phenotype isolates have an advantage over the rough phenotypes concerning adhesion to inert surfaces, but both phenotypes were able to form biofilms. Nutrient conditions in fish farms due to continuous addition of fish feed might be high enough to produce dense *F. psychrophilum* biofilms on fish tanks and farming equipment.

References

[1] Högfors-Rönnholm E., Wiklund T., 2010. Phase variation in *Flavobacterium psychrophilum*: characterization of two distinct colony phenotypes. Disease of Aquatic Organisms, 90:43–53

[2] Högfors-Rönnholm E., Wiklund T., 2010. Hemolytic activity in *Flavobacterium psychrophilum* is a contact-dependent, two-step mechanism and differently expressed in smooth and rough phenotypes. Microbial Pathogenesis, 49:369–375

[3] Hinton A.Jr., Holser R., 2009. Role of water hardness in rinsing bacteria from the skin of processed broiler chickens. International Journal of Poultry Science, 8:112–115

[4] Álvarez B., Secades P., Prieto M., McBride M.J., Guijarro J.A., 2006. A mutation in *Flavobacterium psychrophilum* tlpB inhibits gliding motility and induces biofilm formation. Applied and Environmental Microbiology, 72:4044–4053