In vitro and *in vivo* adhesion of *Flavobacterium columnare*

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Abstract

Flavobacterium columnare is the causative agent of the columnaris disease, which is recognized as one of the main pathogens of fresh water fishes. Early studies [1] indicated that *F. columnare* presents a broad genetic heterogeneity within the species, and isolates can be classified into three different genomovars based on the 16S rDNA gene restriction fragment length polymorphisms (16S rDNA-RFLP), 16S rDNA sequencing, and DNA-DNA hybridization. It has been reported that genomovar II isolates are more virulent to blue and channel catfish than genomovar I strains [2].

Since bacterial attachment is a prerequisite step for host colonization and subsequent pathogenesis, we hypothesized that genomovar II stains may adhere more efficiently to fish skin and mucus than genomovar I strains. In the first study, we evaluated the dynamics of adhesion of genomovar I and II strains to the epithelial tissue of gills in Channel catfish after exposure to *F. columnare* by bath immersion. Briefly, a total of 180 pathogen-free catfish fingerlings with a mean initial weight of 4.2 g were challenged with an overnight culture of *F. columnaris* strain ARS-1 (genomovar I) and BGFS-27 (genomovar II) for 0.5 h at a dose of about $5x10^6$ CFU/ml. At 0.5, 1, 6, 12, and 24 h post challenge, gill sample were excised, homogenized and *F. columnare* cells were enumerated by plate-counting. Also, samples after 0.5 h post challenging were examined with scanning electron microscopy to visually assess if there was a difference between the two genomovar strains (fixed in 10% neutral buffered formalin for 48 h and dehydrated in hexamethyldisilazane (HMDS) for 1 h).

Results showed that during the first 0.5 to 1h, there was not a significant difference in adhesion to gill tissue between the two strains (Fig. 1). However, at 12 to 24 h post-challenge, the number of BGFS-27 cells from gill samples was significantly higher than those from ARS-1. We concluded that ARS-1 cells were cleared from the gills while BGFS-27 was able to persist at a higher number in gills. Result of SEM for 0.5 h post challenge sample shows no difference between the two strains (Fig. 2).

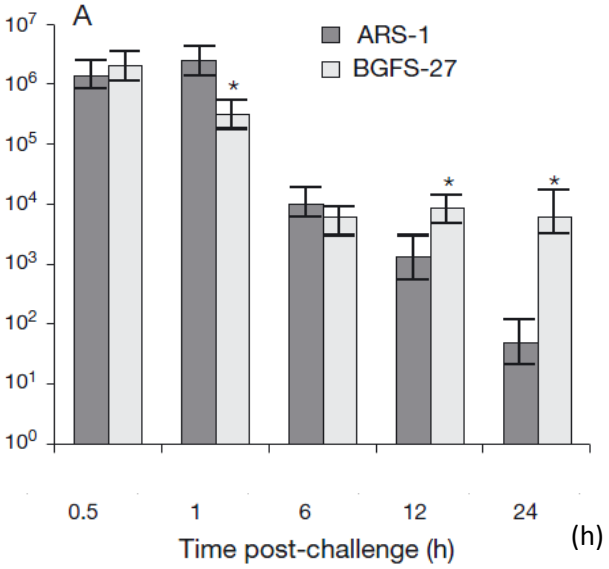

Figure 1. Mean (±SE) number of *Flavobacterium columnare* cells adhered to channel catfish *Ictalurus punctatus* gill during the time course of the study. Cell counts are colony forming units (CFU) per gram of tissue on a logarithmic scale. * indicates significant difference ($p < 0.05$).

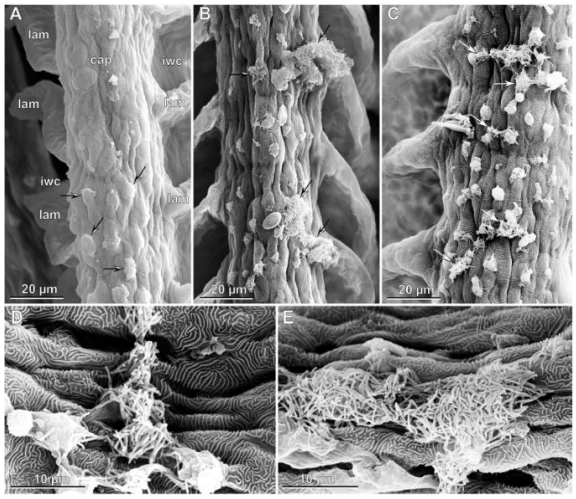

Figure 2. Gill of channel fish *Ictalurus punctatus*. Panel A: control. Panel B and C: Aggregation of cells of strain BGFS-27 and ARS-1 on gill filaments. Panel D and E: higher magnification view of adhered cells.

Our second objective was to compare the adhesion properties and biofilm formation on inert surface between genomovar I, II, and III strains.

To evaluate biofilm formation on plastic, 19 strains of representatives of three genomovars were used. Briefly, overnight bacteria culture was inoculated in modified Shieh broth in a microtiter polystyrene plate and incubated at 28 °C for 48 hours. Attached bacteria were then quantified by measuring the optical density of absorbed crystal violet by the attached cells, after an *in situ* staining procedure [3]. Overall, our results showed that there was not a significant difference in adhesion properties between genomovar I and II strains. However, we observed a considerable strain-to-strain variation.

Further, we investigated the effects of different physiochemical parameters on biofilm formation. Different hardness (12, 64, 120, 200, 360 ppm; adjusted with $CaCl_2 \cdot 2H_2O$), pH (5.8, 6.2, 6.6, 7.3, 7.9), salinity (0, 5. 7.5, 10.5, 14 ppt), and temperature (21, 28, 35 °C) were assayed and cell attachment was quantified as above. Results indicated that hardness and salinity exerted a stronger effect on adhesion properties than pH and temperature. High hardness (360 ppm), relatively low salinity (5 ppt) and moderate temperature (28 °C) favored attachment of cells to polystyrene plates (Fig. 3).

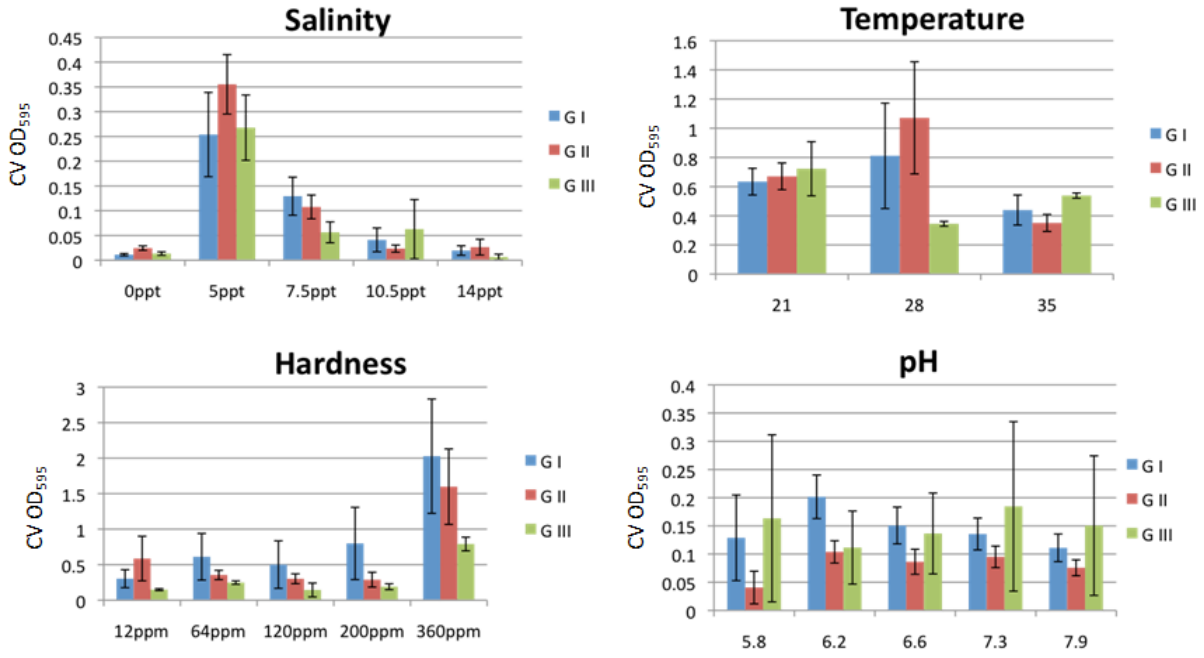

Figure 3. Biofilm formation under different physiochemical parameters

Finally, we induced biofilm formation on glass by letting *F. columnare* cells colonize a glass slide and we tracked this process by using *in situ* hybridization in combination with fluorescence microscopy. Our results showed that *F.columnare* rapidly colonizes glass. We observed that cells attached to the glass in less than 6 h post inoculation, and formed microcolonies between 12-24 h. A mature biofilm developed on glass after 48 h post-inoculation with water channels and extracellular products detected by specific stains. As with the plate-adhesion experiment, no qualitative difference in attachment between genomovar I and II strains was observed. Out data improved our understanding of how *F. columnare* colonizes surfaces and proved that this pathogen can form biofilms. We have identified some chemical parameters that influence attachment that can be used to reduce biofilm formation by this pathogen in aquaculture settings.

References

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