

Adhesion dynamics of *Flavobacterium columnare* to channel catfish *Ictalurus punctatus* and zebrafish *Danio rerio* after immersion challenge

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ABSTRACT: The adhesion dynamics of *Flavobacterium columnare* to fish tissues were evaluated *in vivo* by immersion challenge followed by bacterial plate count and confirmatory observations of gill-adhered bacterial cells using scanning electron microscopy. Adhesion of *F. columnare* genomovar I (ARS-1) and II (BGFS-27) strains to skin and gill of channel catfish *Ictalurus punctatus* and gill of zebrafish *Danio rerio* was compared. At 0.5 h post-challenge, both strains adhered to gill of channel catfish at comparable levels (10^6 colony forming units [CFU] g^{-1}), but significant differences in adhesion were found later in the time course. Channel catfish was able to effectively reduce ARS-1 cells on gill, whereas BGFS-27 persisted in gill beyond the first 24 h post-challenge. No significant difference was found between both strains when adhered to skin, but adhered cell numbers were lower (10^3 CFU g^{-1}) than those found in gill and were not detectable at 6 h post-challenge. Adhesion of BGFS-27 cells to gill of zebrafish also occurred at high numbers ($>10^6$ CFU g^{-1}), while only $<10^2$ CFU g^{-1} of ARS-1 cells were detected in this fish. The results of the present study show that particular strains of *F. columnare* exhibit different levels of specificity to their fish hosts and that adhesion to fish tissues is not sufficient to cause columnaris disease

KEY WORDS: Adhesion · Columnaris disease · *Flavobacterium columnare* · Host–pathogen specificity

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INTRODUCTION

Flavobacterium columnare is a Gram-negative bacterium belonging to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group and the causative agent of columnaris disease (Bernardet et al. 1996). It has a cosmopolitan distribution in temperate and tropical latitudes and has been isolated from an ecologically and phylogenetically diverse spectrum of wild-caught, aquaculture-reared, and ornamental freshwater fishes (Triyanto & Wakabayashi 1999, Schneck & Caslake 2006, Olivares-Fuster et al. 2007). Given the nexus of appropriate host and environmental factors, columnaris disease can be economically devastating for the

channel catfish *Ictalurus punctatus* (Rafinesque, 1818) aquaculture industry where in-pond mortality rates among adults and fingerlings can reach 60 and 90%, respectively (Plumb 1999).

Flavobacterium columnare presently is classified into 3 distinct genomic groups or genomovars (Triyanto & Wakabayashi 1999), with genomovar II being more virulent to catfish than genomovar I (Shoemaker et al. 2008). Recently, Klesius et al. (2008) reported that genomovar II isolates were more strongly chemotactic to channel catfish mucus than genomovar I isolates. In addition, Shoemaker et al. (2008) showed a positive correlation between virulence and

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adherence in channel catfish fry when challenged with *F. columnare*. It is plausible that genomovar II strains could more efficiently adhere to the epithelial tissues and mucus coverings of catfishes such that intense infections and subsequent manifestation of columnaris disease are more likely. However, the relationship between adhesion of *F. columnare* cells and virulence is not clear at this point with some studies supporting a positive correlation (Decostere et al. 1999a,b) while others found no such association (Kunttu et al. 2009). Herein, we studied the dynamics of adhesion of genomovar I and II strains in channel catfish and zebrafish *Danio rerio* (Hamilton, 1822) after exposure to *F. columnare* by immersion bath. We included zebrafish as a common experimental organism that is susceptible to columnaris disease (Moyer & Hunnicutt 2007).

MATERIALS AND METHODS

Fish husbandry. Specific pathogen free (SPF) channel catfish fingerlings (n = 180; mean weight = 4.2 g) were obtained from the School of Veterinary Medicine at Auburn University, transferred to the Aquatic Microbiology Laboratory, and stocked into 37 l aquaria at 15 fish per tank. Similarly, non-sexed adult zebrafish (n = 360, mean weight = 0.6 g) were purchased from Aquatica Tropicals (Plant City, FL, USA), transferred to the Aquatic Microbiology Laboratory, and stocked into 37 l aquaria at 30 fish per tank. Prior to stocking, 10 randomly selected fish from each species were microbiologically examined and proved culture-negative for the presence of *Flavobacterium columnare*. Channel catfish and zebrafish were fed daily to apparent satiation with AQUAMAX Grower 400 (Purina Mills) and 41% crude protein flakes (Central Garden and Pet), respectively. Each aquarium was equipped with a bio-filter and air stone, and supplied with artificial freshwater (80 ppm alkalinity, 40 ppm hardness, 0.1 ppt salinity). Temperature was kept at $27 \pm 1^\circ\text{C}$ and pH 7.8 ± 0.2 (mean \pm SE), and fish were subjected to a 12 h photoperiod. Water parameters were checked daily. Ammonia and nitrite levels were non-detectable throughout the study.

Bacterial strains maintenance. Two previously-identified strains of *Flavobacterium columnare*, ARS-1 (genomovar I) and BGFS-27 (genomovar II), were used herein (Arias et al. 2004). Both strains were originally isolated from channel catfish and experimental challenges have shown ARS-1 as a low virulence strain, while BGFS-27 is proved to be highly virulent in channel catfish (Shoemaker et al. 2008). Stock suspensions of all isolates were stored in 10% glycerol at -80°C . Strains were routinely cultured in modified Shieh

broth (Shoemaker et al. 2005) for 24 h at 26°C with gentle shaking.

Challenge experiments. A total of 4 challenge experiments were conducted, 2 with each fish species. Expts 1 and 2 used channel catfish, while Expts 3 and 4 used zebrafish. Expt 1 assessed bacterial cell adhesion to gill and skin, and Expts 2, 3, and 4 assessed bacterial cell adhesion to gill only. Each consisted of 2 treatments (ARS-1 and BGFS-27) and one control. Each treatment consisted of 3 randomized replicates (tanks). The immersion challenge was carried out as described by Shoemaker et al. (2008) with the following modifications: 15 channel catfish per treatment or 30 zebrafish per treatment were challenged with an overnight culture of the corresponding bacterial treatment (i.e. ARS-1 or BGFS-27) at approximately 5×10^6 colony forming units (CFU) ml^{-1} . Negative controls were exposed to modified Shieh broth without bacteria. After a 0.5 h challenge, fish were returned to their individual aquaria and monitored at 12 h intervals for abnormal behavior, loss of appetite, and mortality. Moribund fish were sampled for *Flavobacterium columnare* following standard protocols.

Quantification of bacterial adhesion. Immediately before challenge and at 0.5, 1, 6, 12, and 24 h post-challenge, 3 fish per replicate were euthanized with a lethal dose of tricaine methanesulfonate (MS-222; 300 mg l^{-1}) before the sinistral and dextral anterior-most gill arches were excised. Skin samples comprised 0.5 cm^2 caudal patches excised with a scalpel and taken at identical sampling intervals. To quantify adhered cells of *Flavobacterium columnare*, gill and skin samples from 3 individual fish were pooled, weighed, and homogenized into 500 μl of Shieh broth. Ten-fold dilutions from gill and skin slurries were made in Shieh broth: 100 μl of suspension was spread onto Shieh medium agar plates in triplicate and incubated at 26°C for 48 h. *F. columnare* counts (FC) based on typical yellow and rhizoid colonies were determined and expressed as the average number of CFU g^{-1} of tissue or $\log \text{CFU g}^{-1}$ tissue. From each sampling time, 10 randomly selected putative *F. columnare* colonies were purified and confirmed as *F. columnare* by specific PCR (Welker et al. 2005).

Electron microscopy. Samples for scanning electron microscopy (SEM) were taken at 0.5 h post-challenge in Expt 1. Channel catfish fingerlings (3 per treatment) were euthanized as described in the previous subsection before immersion in 10% neutral buffered formalin for 48 h. Gill arches were excised using fine forceps and scissors, gradually dehydrated in a graded series of ethanols using an automated tissue processor, transferred from 100% EtOH to a 50:50 (v:v) of 100% EtOH and hexamethyldisilazane (HMDS) for 1 h, transferred to and left in HMDS for 1 h, removed from HMDS and

air-dried for 3 h, mounted on metal stubs using 2-sided sticky tape, and sputter coated with 15 nm of gold-palladium.

Statistical analysis. Differences in CFU g⁻¹ tissue within each experiment were compared using a repeated measures ANOVA procedure in the Statistical Analysis System, SAS 9.1.3 (SAS Institute). A significance level of 0.05 was accepted.

RESULTS

Post-challenge bacteriological examination of channel catfish tissues

No fish examined before challenge was positive for *Flavobacterium columnare*. Table 1 summarizes FC obtained from catfish gill and skin samples during Expt 1. FCs were on the order of 10³ CFU g⁻¹ of skin in both ARS-1- and BGFS-27-challenged catfish at 0.5 and 1 h post-challenge. At 6 h post-challenge, FCs were not detected in skin samples from any tested fish. *Flavobacterium columnare* cells adhered to gill (~10⁶ CFU g⁻¹) significantly better than to skin (~10³ CFU g⁻¹). Thirty min post-challenge >10⁶ CFU g⁻¹ of gill were counted in ARS-1- and BGFS-27-challenged fish. However, at 1 h post-challenge FC in ARS-1 infected fish were significantly higher than in fish challenged with the BGFS-27 strain. Six h post-challenge, FC in ARS-1 infected fish were reduced by 2 orders of magnitude. Differences between both strains at 6 h post-challenge were not significant. At 12 and 24 h post-challenge, the numbers of BGFS-27 cells from gill

Table 1. *Flavobacterium columnare*. Mean (±SE) *F. columnare* cells (as CFU g⁻¹ tissue) detected by bacteriological examination of channel catfish *Ictalurus punctatus* challenged with *F. columnare* during Expt 1. ND: not detected

Time (h)	Skin	Gill
Pre-challenge	ND	ND
Post-challenge with ARS-1		
0.5	1.1 ± 2.0 × 10 ³	1.4 ± 2.3 × 10 ⁶
1	2.0 ± 3.2 × 10 ³	2.5 ± 5.3 × 10 ⁶
6	ND	1.2 ± 1.5 × 10 ⁴
12	ND	1.3 ± 1.6 × 10 ³
24	ND	7.5 ± 1.8 × 10 ¹
Post-challenge with BGFS-27		
0.5	5.7 ± 2.5 × 10 ³	2.0 ± 3.8 × 10 ⁶
1	1.2 ± 3.2 × 10 ³	3.1 ± 3.2 × 10 ⁵ a
6	ND	6.0 ± 2.0 × 10 ³
12	ND	8.6 ± 1.2 × 10 ³ a
24	ND	6.8 ± 1.0 × 10 ³ a

^aSignificantly (p < 0.05) different from the value for strain ARS-1

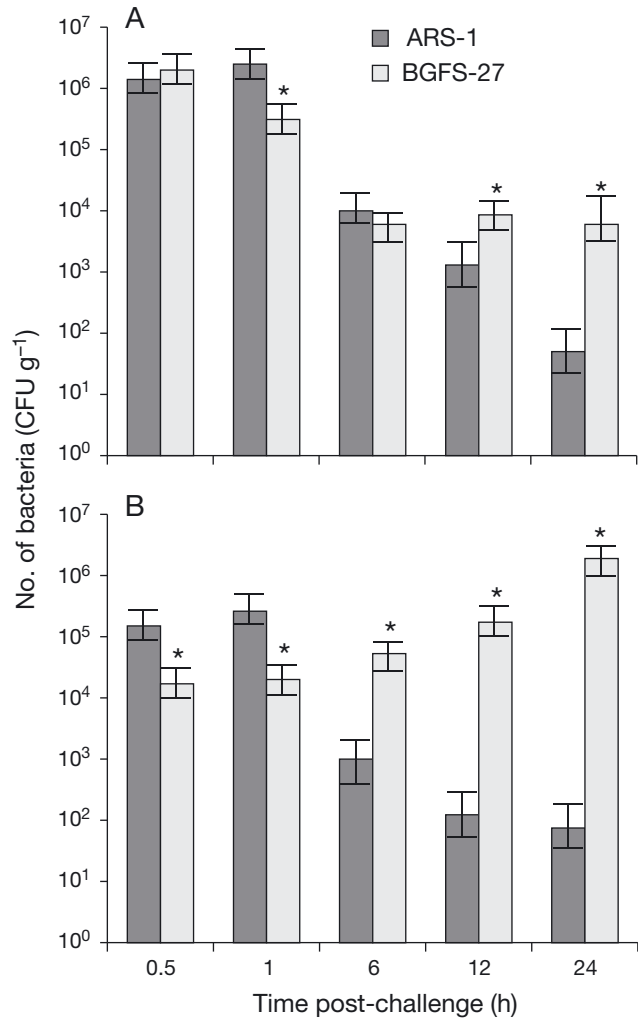


Fig. 1. *Flavobacterium columnare*. Mean (±SE) number of cells of adhered to channel catfish *Ictalurus punctatus* gill during the time course of the study. Results from (A) Expt 1 and (B) Expt 2. Cell counts are colony forming units (CFU) per gram of tissue displayed on a logarithmic scale. ARS-1 is a genomovar I strain, and BGFS-27 is a genomovar II strain. *Significantly different (p < 0.05) data when both *F. columnare* strains were compared

samples were higher than those from ARS-1. Fig. 1 shows colony counts for *F. columnare* in channel catfish over time for Expts 1 and 2. A similar trend was observed in both experiments: ARS-1 adhered to gill epithelium in higher numbers than BGFS-27 during the first 0.5 to 1 h. Ultimately, ARS-1 cells of *F. columnare* were cleared from gill, and only residual cells (<100 FC g⁻¹) were detected at 24 h. However, although BGFS-27 numbers were originally lower, these cells persisted in gill at higher numbers than ARS-1. Onset of columnaris disease using our infection model occurs from 24 to 48 h post-challenge; thus the variation observed between Expts 1 and 2 was considered normal.

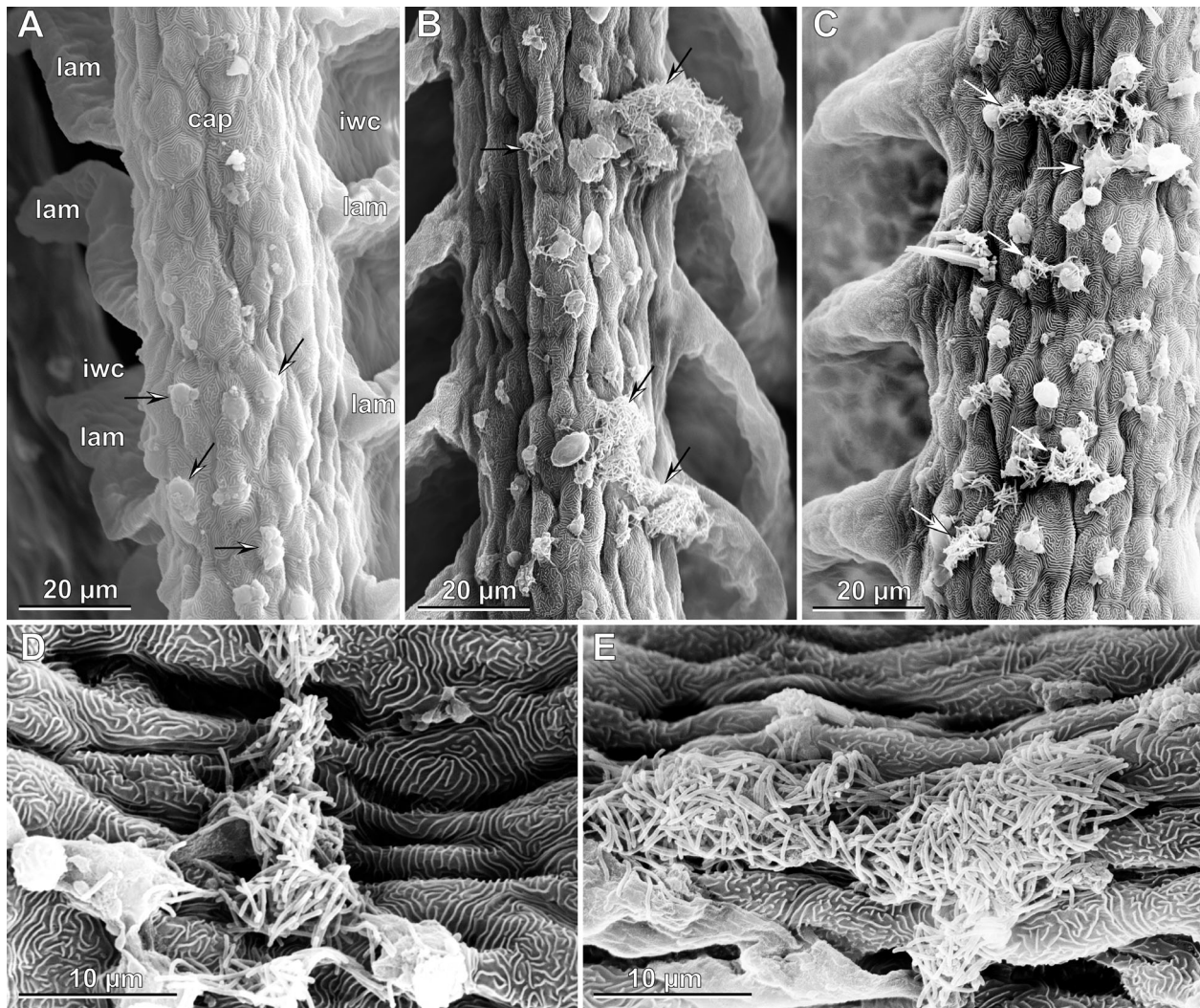


Fig. 2. *Ictalurus punctatus*. Gill of channel catfish. (A) Control, showing distribution of lamellae (lam) and interlamellar water channels (iwc) flanking the capping tissue (cap) of the gill filament. Arrows indicate the location of mucus exudate on the capping tissue of the gill filament. (B,C) Aggregates of adhered rod-shaped bacteria (arrows) presumed to be *Flavobacterium columnare*: (B) strain BGFS-27, (C) strain ARS-1. (D,E) Higher magnification view of an aggregate of cells adhered to gill: (D) strain BGFS-27, (E) strain ARS-1

Scanning electron microscopy

Gill of control channel catfish appeared normal, and a thorough search of the sampled gill arches and filaments from those control fish failed to locate a bacterial cell (Fig. 2). The capping tissue of the gill filament had a normal epidermis comprising intact epidermal cells ornamented with well-defined microridges and accompanying mucus pores with associated mucus exudate (Fig. 2A). Flanking each gill filament were normal, regularly-spaced, equally-sized gill lamellae having a smooth-surfaced respiratory epithelium (Fig. 2A). Flanking lamellae enclosed interlamellar water channels that were devoid of tissue debris or aggregates, of

bacterial cells. Regarding bacterial-challenged gill samples, qualitatively no difference between strains was observed in the numbers of adhered bacterial cells per gill arch at 0.5 h post-challenge. As with controls, the basic structural integrity of the gill filament and lamellar respiratory epithelium was intact and appeared normal, except that rod-like bacteria presumed to be *Flavobacterium columnare* were localized on the surface of each gill filament's capping tissue. Interestingly, no bacterial cell was observed within an interlamellar water channel or adhered to the surface of a lamella. Moreover, the adhered rod-shaped bacterial cells appeared to be aggregated rather than evenly distributed across the gill epithelium (Fig. 2B–E).

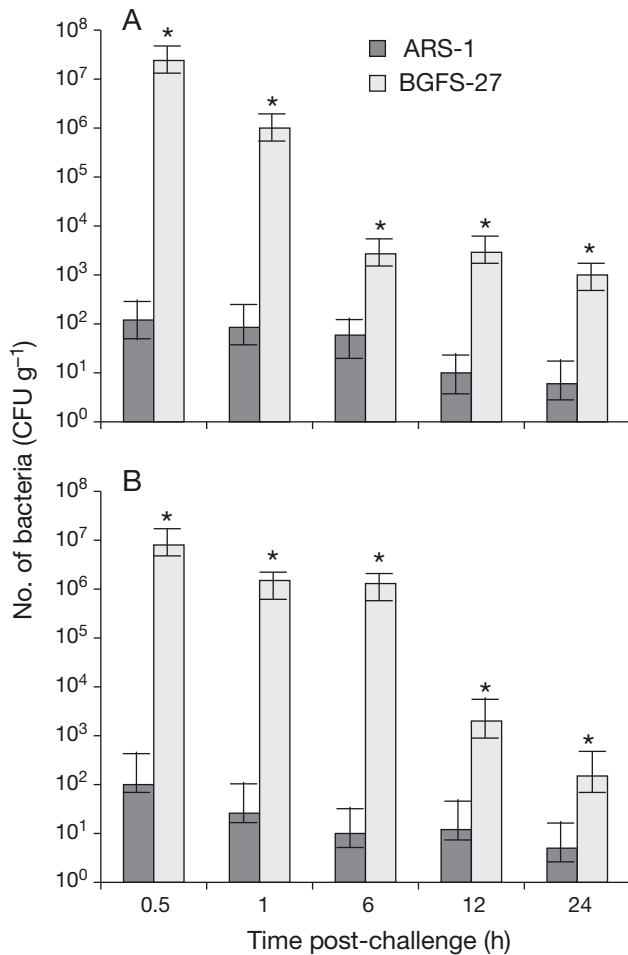


Fig. 3. *Flavobacterium columnare*. Mean (\pm SE) number of cells adhered to gill of zebrafish *Danio rerio* during the time course of the study. Results from (A) Expt 3 and (B) Expt 4. See Fig. 1 for further explanations

Flavobacterium columnare adhesion to zebrafish

No zebrafish was positive for *Flavobacterium columnare* prior to challenge. Due to the low number of *F. columnare* cells detected in channel catfish skin, adhesion to zebrafish skin was not evaluated. BGFS-27 cells adhered to zebrafish gill at a much higher rate than ARS-1 cells. High FCs ($>10^7$ CFU g⁻¹) were recovered from BGFS-27-infected fish (Fig. 3A); whereas, 100 CFU g⁻¹ were obtained from ARS-1-challenged fish 0.5 h post-challenge. Statistically significant ($p < 0.05$) differences existed between strains at all sampling points in Expts 3 and 4 (Fig. 3). Colony counts from BGFS-27 decreased over time although remained >100 CFU g⁻¹ 24 h post-challenge. No clear sign of columnaris disease was observed in zebrafish throughout the experiment.

DISCUSSION

Mucus is the first physical-immunological barrier encountered by a bacterium during the initial steps of colonization and invasion of a fish host. Fish mucus contains a variety of antimicrobial compounds such as antibacterial peptides, lysozyme, proteases, and antibodies (Ellis 1999) that may protect underlying epidermal cells from bacterial colonization via inhibition of bacterial adhesion to epithelial cells (Beachey 1981). On the other hand, bacteria possess attachment mechanisms like adhesins that have a high affinity for some components of fish mucus. Many bacterial adhesins contain carbohydrate-recognition domains (such as lectins) that bind to oligosaccharides present in mucus glycoproteins or glycolipids. Specifically, *Flavobacterium columnare* adheres to gill mucus by a lectin-mediated interaction (Decostere et al. 1999c). Klesius et al. (2008) recently proved a positive chemotactic response by *F. columnare* towards channel catfish mucus. Their results highlighted the fact that cells of *F. columnare* prefer skin and gill mucus more than intestinal mucus, which seems to correlate well with typical manifestation of columnaris disease as an external infection of skin and gill.

Several studies have evaluated adhesion of high and low virulence strains of *Flavobacterium columnare* to host tissues (Decostere et al. 1999a, Nematollahi et al. 2003, Kunttu et al. 2009), but to our knowledge this is the first study comparing strains from both genomovars. Our *in vivo* results showed significantly different attachment to channel catfish gill by *F. columnare* strains from different genomovars. Both strains adhere equally well to catfish gill, but only BGFS-27 persisted over time (previous studies have shown that this strain is particularly virulent towards catfish). At time 0, both strains showed a similar affinity for catfish gill, but BGFS-27 levels remained higher, seemingly multiplied in gill, and eventually appeared to bring about signs of columnaris disease in those fish, thus providing evidence that adhesion of *F. columnare* cells to gill in channel catfish is alone insufficient to elicit signs of columnaris disease. Hence, attachment, although necessary for invasion, cannot be directly correlated with virulence of *F. columnare*. Our *in vivo* results could explain discrepancies among various research laboratories conducting adhesion studies with *F. columnare* where only one time point was assayed (Decostere et al. 1999c, Kunttu et al. 2009). Channel catfish reduced the number of gill-adhered genomovar I cells by 4 orders of magnitude. Reduction of genomovar II cells was less effective, and in Expt 2 some channel catfish showed signs of columnaris disease. The ability of *F. columnare* to persist in host tissues may be a better indicator of potential virulence.

It should be noted that infection models for columnaris disease are difficult to standardize since one must rely on the ability of the bacterium to colonize the external surface of the host (injection models do not reproduce typical columnaris disease), and many factors such as water quality, host susceptibility and strain virulence come into play. In our laboratory, we can induce the acute form of columnaris disease quite consistently with fish showing signs of disease between 24 and 48 h post-challenge. However, in Expt 2, channel catfish started to show signs within the first 24 h post-challenge. To the best of our knowledge, conditions were comparable between Expts 1 & 2 (water parameters, fish came from the same lot and bacterial strains were grown similarly), but clearly there are other factors in play that can change the fish susceptibility to *Flavobacterium columnare*.

Interestingly, the results of the present study highlight adhesion differences between strains of different genomovars of *Flavobacterium columnare* as well as previously described host-specific associations existing between genomovars and fish species (Olivares-Fuster et al. 2007). *F. columnare* genomovar I strain ARS-1 failed to effectively adhere to zebrafish gill (<100 CFU g⁻¹), but genomovar II strain BGFS-27 adhered to zebrafish gill at even higher levels than to channel catfish gill (>10⁷ CFU g⁻¹). Numbers of BGFS-27 decreased over time such that no sign of columnaris disease was observed (no multiplication of BGFS-27 occurred in gill during the study period). In our laboratory setting, signs of columnaris disease can be instigated in both fish species challenged with either genomovar, although genomovar II strains are more virulent in general (data not shown; for a similar study see Shoemaker et al. 2008). The significant difference in adhesion properties of ARS-1 and BGFS-27 to channel catfish and zebrafish gill could explain the correlation between genomovars and fish species that is observed among some wild fishes infected with *F. columnare* (Olivares-Fuster et al. 2007).

Our scanning electron microscopy observations of *Flavobacterium columnare* in gill of channel catfish functioned merely as confirmatory, direct evidence of the presence of bacteria adhered to the gill tissue of the challenged fish, and were complementary to the plate counts. Although we examined an insufficient number of samples to confidently comment on the fine-scale site specificity of adhered cells, we nevertheless find it noteworthy that cells of *F. columnare* were restricted to the capping tissue of the gill filaments at 0.5 h post-challenge, i.e. we did not observe a cell in another site. Moreover, these adhered cells were abundant near mucus pores of the gill filament, with few cells adhered to the epithelial surface between mucus pores. These observations of *F. columnare* in

gill of channel catfish differed markedly from those of *F. psychrophilum* in gill of ayu *Plecoglossus altivelis* (Kondo et al. 2002). In that study, cells of *F. psychrophilum* were scattered across the capping tissue of the gill filament, surfaces of lamellae, and within inter-lamellar water channels of ayu at 0.5 h post-challenge (see Fig. 2 of Kondo et al. 2002). Cells were cleared from gill subsequently (see Figs. 3 to 5 of Kondo et al. 2002). As with *F. psychrophilum*, further ultrastructural studies are needed to understand site specificity and adhesion mechanisms of *F. columnare*, but our casual observations of *F. columnare* clustered about mucus pores certainly does not reject the notion of *in vivo* chemotaxis of *F. columnare* to catfish mucus.

Herein, we have demonstrated how *Flavobacterium columnare* cells, from both genomovars, adhered to channel catfish gill in high numbers within 1 h post-challenge. However, BGFS-27 (genomovar II) cells persisted at higher levels and for a longer duration. Clearly, additional virulence factors besides adhesins are required to colonize the host. Chondroitin AC lyase activity is related to virulence, and highly virulent strains have a higher activity level of this degrading enzyme (Suomalainen et al. 2006). Other suggested virulence factors for *F. columnare* are proteases and other hydrolytic enzymes (Dalsgaard 1993). Further research is needed to confirm if all genomovar II strains are indeed more effective at colonizing gill of channel catfish than genomovar I strains.

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