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Influence of water quality and temperature on adhesion of high and low virulence *Flavobacterium columnare* strains to isolated gill arches

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Abstract

The ability of *Flavobacterium columnare* (*Flexibacter columnaris*) to attach to the gills of common carp, *Cyprinus carpio* L., was evaluated using a gill perfusion model. A comparison between a high and a low virulence strain of *F. columnare* was made and evaluated in comparison to results obtained previously with an *in vivo* model. The ion composition of the water of the organ bath in which the gills were suspended was varied and the influence on adhesion processes assessed. Experiments were carried out to examine the influence of water quality (i.e. nitrite and organic matter) and temperature on the capacity of the bacteria to adhere. It was found that the high virulence strain adhered more readily than the low virulence strain, as was found during the *in vivo* experiments. Moreover, it was observed that adhesion of the high virulence strain was enhanced by a number of factors. These were immersion of the gill in bivalent, ion-rich water, the presence of nitrite or organic matter, and high temperatures.

Introduction

Flavobacterium columnare has been recognized as a world-wide pathogen of freshwater fish. This disease was formerly called *Flexibacter columnaris*, but in 1996, it was transferred to the genus *Flavobacterium* (Bernardet, Segers, Vancanneyt, Berthe, Kersters & Vandamme 1996).

Flavobacterium columnare is the aetiological agent of columnaris disease, characterized by gill necrosis, greyish white spots on the body, skin erosion and fin rot, all occurring in varying degrees of severity. The pathogen is able to enter the bloodstream through the external lesions, causing septicaemia, although this has only been reported in a limited number of cases (Hawke & Thune 1992; Koski, Hirvelä-Koski & Bernardet 1993). Generally, outbreaks occur when the fish are stressed and temperatures are above 16 °C. Disease is favoured by elevated organic loads, crowded conditions and excessive handling (Wakabayashi 1991).

There have been numerous reports noting the difference in virulence among *F. columnare* strains (Pacha & Ordal 1963; Wakabayashi, Kira & Egusa 1970; Dalsgaard 1993). Nevertheless, there is very little information available concerning the actual factors affecting the virulence. Several experimental infections have been carried out, during which heavy mortalities have been noted (Pacha & Ordal 1963; Wakabayashi & Egusa 1972). However, few studies have attempted to evaluate the first steps of the pathogenesis of *F. columnare* (Chowdhury & Wakabayashi 1988, 1989, 1990). Hence, information concerning bacteriological events preceding disease and death are almost completely lacking.

In contrast to the majority of fish pathogens,

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artificial infection by high virulence strains of this gliding bacterium is more effective by contact exposure than by injection (Pacha & Ordal 1970). Fish kept in standing water have been shown to be more susceptible to columnaris disease than fish kept in running water. It is assumed that the standing water favours the attachment of *F. columnare* to the fish tissue. Therefore, as suggested by Toranzo & Barja (1993), further studies should be directed to determining the mechanisms of attachment, their role in the pathogenesis of *F. columnare* and the factors influencing the adhesion process.

In previous *in vivo* studies to study the early pathogenesis of columnaris disease, the high virulence *F. columnare* strain AJS 1 was found in close association with the gill tissue. This was not the case for the low virulence strain AJS 4, suggesting that ability to adhere to the gill tissue is an important initial step in pathogenesis. Moreover, an understanding of bacterial attachment and its role in the pathogenesis of disease is crucial to allow the selection of the best strategy for prevention and disease control. Del Corral (1975) and Zaldiver (1985) correlated virulence of *F. columnare* strains and the ability to adhere to epithelial cells *in vitro*. However, one should be aware that epithelial cells *in vitro* lose their polarity (Pärt & Bergström 1995). Polarity (the different properties of the apical and basolateral membranes) is a key feature of epithelial cells, and the loss of the polarity severely limits the conclusions that can be drawn from branchial or other epithelial cells in suspension. In addition, the digestion procedures involved in the preparation of cells affect surface proteins (Pärt & Bergström 1995) which may be involved in adhesion processes (Ofek & Doyle 1994a). These disadvantages may be overcome by culturing the cells and growing them as monolayers, i.e. these may re-establish their polarity and repair their surface proteins. However, gill cells in culture are restricted to respiratory epithelial cells. Chloride and mucus cells, the other two cell types that compose the gill epithelium, do not survive in culture. Additionally, the surfaces of the culture cells may eventually present different proteins from *in vivo* cells, especially in terms of distribution and accessibility of receptors. Furthermore, overall gill organization and architecture is completely lost.

In order to circumvent all these potential artefacts, a gill perfusion model was developed. This relatively simple model comes much closer to the *in vivo* situation, offering a means of maintaining the gill tissue intact for at least 4 h and allowing the more complex bacterium–host interactions to be studied under carefully controlled conditions. Using the gill perfusion model, the possible differences in adhesion between a high and a low virulence strain were assessed and compared with the previous results from *in vivo* studies. In addition, the ion composition and water-quality parameters of the organ bath were varied, and the influence on the adhesion of the bacterium to the gills was evaluated.

Materials and methods

Gill perfusion model

Sixteen carp, *Cyprinus carpio* L., were obtained from a carp farm (Zonhoven, Belgium) and were kept in 200-L holding tanks in filtered, recirculated tap water (20 °C) for 3 weeks prior to experimentation. The animals were fed daily with a commercial diet until 2 days before euthanasia. Skin and gills were sampled for parasitological examination. Swabs from the skin and gills were taken, and streaked onto Shieh agar supplemented with tobramycin at a concentration of 1 $\mu g\ mL^{-1}$, which is used for the selective isolation of *F. columnare* (Decostere, Haesebrouck & Devriese 1997). Plates were incubated at 25 °C for 48 h. The fish were found to be free of parasite infestations and *F. columnare* was not isolated from any fish.

The fish were anaesthetized using a stock solution of benzocaine (100 g benzocaine in 1 L of ethanol), which was administered to the water. Thereafter, the carp were injected intraperitoneally with heparin (5000 IU kg^{-1}). The fish were revived by holding them for several minutes underneath a tap so that a vigorous flow fell into the open mouth. This was also undertaken to flush loosely bound bacteria from the gills. Thereafter, the fish were immersed for 60 s in a solution of malachite green (67 mg L^{-1}). After a rinse in sterile phosphate-buffered saline (PBS), the fish were placed in a solution containing enrofloxacin for 15 min at a concentration of 10 mg L^{-1} followed by a bath in colistin (2000 IU mL^{-1}) for 15 min. The fish were then rinsed three times in sterile PBS before euthanasia and transfer to the operating table. The heart and ventral aorta were exposed by ventral dissection. A haemostat was put onto the ventral aorta (supplying the gills) to prevent the afferent

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arteries from flooding the operation field with blood when dissecting the gill arches. The opercula on both sides were removed. The ventral (afferent) end of the first two gill arches of both sides was cut and the two afferent arteries of each side were cannulated with a 20-cm-long polyethylene cannula (0.4 mm ID) to which a 1.5-cm-long stainless steel needle of 22 G was connected. The needle carried a 0.5-cm short length of polyethylene tubing (stop) on the end which was inserted into the afferent artery. A ligature (catgut 2/0) was then tied around the whole arch including the artery and the cannula was then withdrawn until the 'stop' caught on the ligature. As soon as the cannula was inserted into the afferent branchial artery, perfusion flow was begun at ≈3 mL min^{-1} $arch^{-1}$ kg^{-1} body weight. Filtered (Corning, Brussels, Belgium, 0.45 μm) and heparinized (10 IU mL^{-1}) Cortland solution was used as the perfusion fluid. The dorsal (efferent) end of the arches of both sides was then cut through and the gills were removed to a Petri dish containing Ringer solution, care being taken to avoid damage to the filaments of the holobranch. The efferent cannula was introduced into each of the efferent arteries. As before, a ligature was tied around the arches as a whole and the cannula withdrawn to the 'stop'. After cannulation, each of the gill arches was suspended separately in an aerated organ bath at constant temperature and the flow was diminished to 1.5 mL min^{-1} $arch^{-1}$ kg^{-1} body weight. A drip was used, which was set at ≈40 cm above the level of the organ bath. A schematic representation of the gill perfusion apparatus is given in Fig. 1.

The first two gill arches of both the left and right side from each fish were used in adhesion tests. Dissection and cannulation of the four gill arches was carried out within 12 min. Each gill arch was placed separately in an organ bath. Perfusion was carried out for 30 min to allow the gills to recover from the most acute effects of the procedure. Thereafter, gills were used in adhesion tests.

Flavobacterium columnare strains

Two *F. columnare* strains were used, strains AJS 1

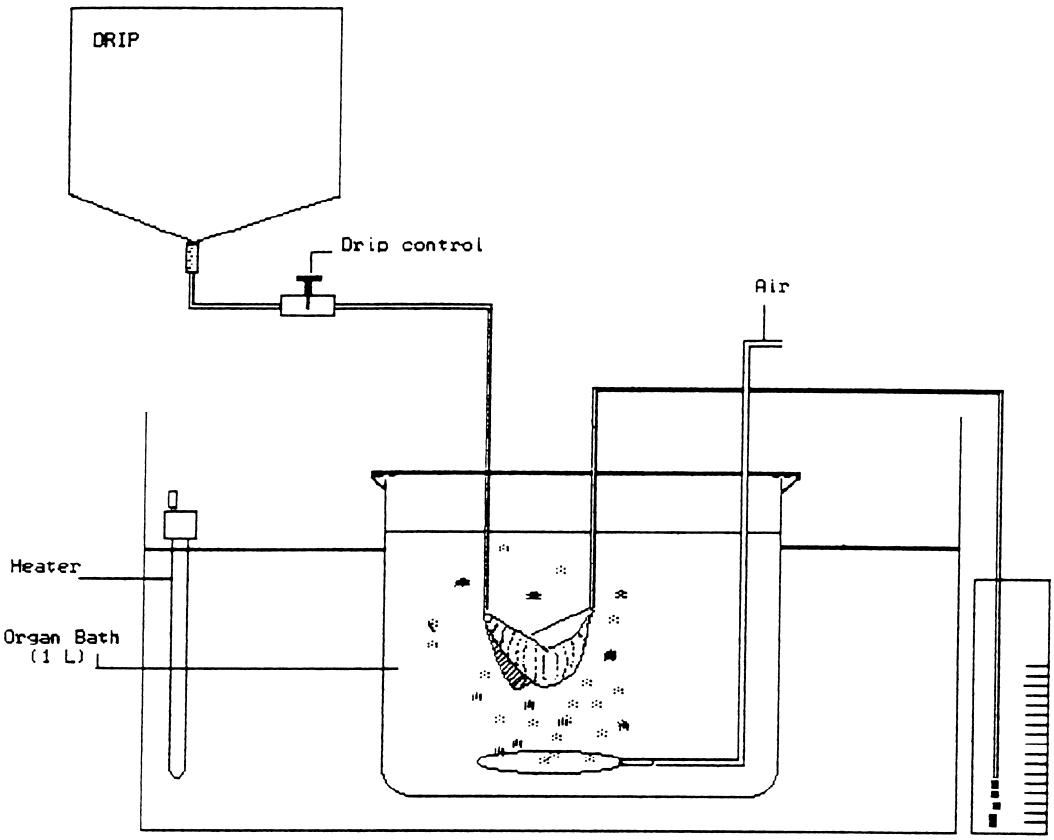

Figure 1 Schematic representation of the gill perfusion apparatus.

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and AJS 4. Both strains were isolated in Belgium. Strain AJS 1 was obtained from a black molly, *Poecilia sphenops* L., with focally bleached and ulcerated skin. The fish originated from an aquarium with an appreciable daily mortality rate. Strain AJS 4 was isolated from a black molly whose skin around the dorsal fin was covered with grey patches. Only a low mortality rate was noted among the animals in the aquarium from which the fish was taken. Results of experimental studies in black mollies proved that strain AJS 1 was a high-virulence strain, whereas strain AJS 4 was had a low virulence (Decostere, Haesebrouck & Devriese 1998). Additionally, the use of an immunoperoxidase technique, capable of demonstrating the bacteria attached to the skin and gills, clearly proved that the high virulence strain AJS 1 was much more capable of adhering to the gills than strain AJS 4, the latter displaying very little if any adhesion capabilities. These findings strongly indicate that virulence is correlated with the ability to adhere to the gill tissue.

Stock suspensions of the two strains were stored at 70 °C. After thawing, the bacteria were grown for 24 h at 30 °C in Shieh broth (Shieh 1980). Subsequently, subcultures were made and incubated for 20 h at 25 °C with gentle shaking. The cultures were checked for purity and the number of colony forming units (cfu) was determined by plating ten-fold dilutions on Shieh agar. Shieh agar was prepared as Shieh broth, but with the addition of 10 g L^{-1} agar (Agar Noble, Difco, Detroit, MI, USA).

Experimental design

A first experiment (experiment I) was carried out to investigate possible differences in adhesion abilities between the high- and low-virulence *F. columnare* strains. Therefore, the first two gill arches of both the left and right side of a carp were each placed in a separate organ bath of 1 L Ringer solution at 28 °C and perfused as described above. Strains AJS 1 and AJS 4 were added to the baths containing the first left and right gill arches, respectively. The final titre of *F. columnare* in each organ bath was 10^7 cfu mL^{-1}. Then 80 mL of sterile Shieh broth was added to the organ bath of the second left gill arch. Nothing was added to the organ bath of the second right gill arch. Both second gill arches served as controls. After inoculation, gill arches were incubated for 60 min. During the exposure period, the perfused gills were observed macroscopically for the presence of excessive mucus and/or formation of threads. A Gram stain was made of the threads. After the incubation period, the gill arches were processed for examination of adhesion as described below. The experiment was conducted four times.

In a second experiment (experiment II), the influence of the ion composition of the water on the adhesion of *F. columnare* was examined.

The first two right and left gill arches of a carp were prepared as described above and each incubated in an organ bath at 28 °C. These organ baths contained Ringer solution (first left gill arch), distilled water (first right gill arch), distilled water with 0.03% NaCl (second left gill arch) or distilled water with 0.03% NaCl, 0.01% KCl, 0.002% $CaCl_2 2H_2O$ and 0.004% $MgCl_2 6H_2O$ (formulated water) (second right gill arch). After 30 min of perfusion, all arches were inoculated with *F. columnare* strain AJS 1. The final concentration of *F. columnare* in each organ bath was 10^6 cfu mL^{-1}.

During the course of the exposure period, the gills were observed macroscopically. Again, a Gram stain was made of the threads. The gill arches were incubated for 60 min and then processed for examination of adhesion as described below. The experiment was conducted four times.

Finally, experiments were carried out to determine the influence of water quality and water temperature on the ability of *F. columnare* to adhere to the branchial epithelium (experiment III). The first two gill arches of both the left and right side of two carp were each placed in an organ bath containing Ringer solution and perfused as described above. The temperature of the bath of the first left gill arch of both carp was 17 °C. All other arches were incubated at 28 °C. Nothing was added to the bath of the second left gill arch. Then 5 mg L^{-1} nitrite was added to the bath of the first right gill arch of both carp and 2 g L^{-1} organic material was added to the bath of the second right gill arch. The latter consisted of a mixture of food residues and faeces collected from the bottom of a fish tank. The slurry was sterilized (121 °C, 15 min) before addition to the organ bath. After 30 min of perfusion, all gill arches of the first carp were inoculated with *F. columnare* strain AJS 1 and those of the second carp with *F. columnare* strain AJS 4. The final titre of *F. columnare* in each bath was 10^6 cfu mL^{-1}. The gill arches were incubated for 60 min, and thereafter, processed for examination of adhesion as described below.

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During the course of perfusion, the gills were observed macroscopically. A Gram stain was made of any occurring threads. The experiment was conducted four times.

Processing of the gills for examination of adhesion

After the incubation period, the perfused gill arch was cut into three equally sized pieces. A randomly selected piece was washed three times in square Petri dishes containing 40 mL of sterile PBS at 25 °C and then placed into a preweighed vial containing 2 mL of sterile PBS. The vial was weighed to the nearest 0.01 g. The contents of the vial was placed in a sterile mortar. The gill was macerated and ten-fold serial dilutions of the gill slurry were made in PBS. Fifty microlitres of each dilution was inoculated onto Shieh agar. For each dilution, three plates were used. After 24 h of incubation at 25 °C, viable counts were made. Consequently, the number of cfu g^{-1} of gill tissue was calculated.

A second piece of the perfused gill arch was placed in 4% phosphate buffered formaldehyde for 24 h. Afterwards, the tissues were paraffin-embedded, sectioned at 5 μm and stained with haematoxylin and eosin (H & E) and Giemsa. The sections were examined by light microscopy (using oil immersion at ×500 magnification).

In addition to bacteriological and histological examination, the perfused gill arches of the first experiment were also sampled for scanning electron microscopy (SEM). Therefore, a third piece was immediately fixed in cacodylate buffer (0.1 M, pH 7.3) containing 2.5% glutaraldehyde and 2% paraformaldehyde. It was post-fixed in 1% (w/v) osmium tetroxide in distilled water. After this, the gill arch was dehydrated in alcohol and acetone for subsequent critical point drying in liquid carbon dioxide, glued with carbon cement on aluminium stubs, sputtered with a gold layer, and examined with a Philips 501 SEM (Phillips, Eindhoven, The Netherlands).

Statistical analysis

Differences in cfu g^{-1} gill tissue within each experiment were compared using the one-way analysis of variance (ANOVA) (computer program STATISTIX 4.1, Analytical software). In the first experiment, the values for the high- and low-virulence strains were compared. Within the second and third experiments, for each individual *F. columnare* strain, each value was compared with the value obtained when Ringer (28 °C) was used as bathing fluid. A significance level of 0.05 was used.

Results

Experiment I: adhesion of *F. columnare* strains AJS 1 and AJS 4

The gill arches suspended in cultured broth of *F. columnare* strain AJS 1 displayed white-yellowish threads on top of the primary lamellae from 30 min after exposure to the pathogen (Fig. 2). When a Gram stain was made of these threads, masses of long, slender Gram-negative bacteria were observed. Examination by means of a phase contrast microscope revealed long, gliding bacteria.

However, the gill arches challenged with cultured broth of strain AJS 4 showed very little if any filament-like structures.

Results of the bacteriological examination of gills exposed to strain AJS 1 and AJS 4 are given in Table 1. The number of bacteria associated with the gills was significantly higher for strain AJS 1 than for strain AJS 4 ($P < 0.05$).

A similar trend was apparent upon histological examination of gills. Holobranchs challenged with cultured broth of strain AJS 1 had filamentous bacteria occurring in aggregates associated with the gill epithelium (Fig. 3). No filamentous bacteria were observed in sections of gill arches exposed to strain AJS 4 or control gill sections.

When *F. columnare* strain AJS 1 was used for challenge, the adherence to the gill tissue of long, slender, rod-shaped bacteria occurring in microaggregates was evident upon SEM examination (Fig. 4). Bacteria were not observed by SEM on the tissue of the control gills or on the gill tissue challenged with strain AJS 4.

Experiment II: influence of ion composition

In the present study, all gill arches were inoculated with strain AJS 1. Gill arches suspended in Ringer solution or in the formulated water displayed white-yellowish threads on the tips of the primary lamellae, as described in experiment I. Gill arches placed in organ baths with distilled water or distilled water with 0.03% NaCl displayed markedly fewer and shorter threads.

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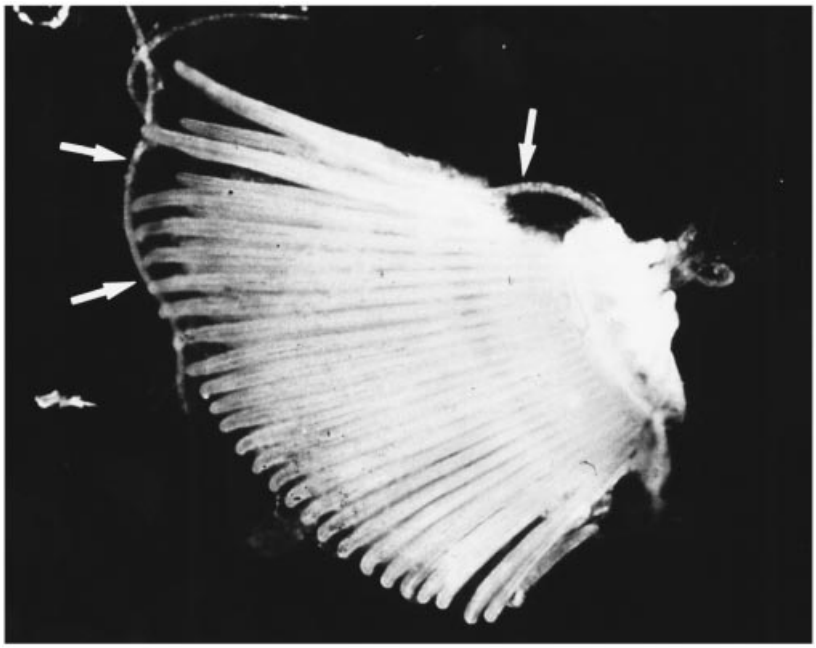

Figure 2 Gill arches suspended in an infective solution of *F. columnare* strain AJS 1 displaying white-yellowish threads on top of the primary lamellae (arrows) 1 h after the onset of exposure to the pathogen (×5).

The results of the bacteriological titrations are given in Table 1. Bacterial titres were significantly ($P < 0.05$) higher in gill arches suspended in the formulated water. Bacterial titres were significantly ($P < 0.05$) lower for gill arches suspended in distilled water with or without 0.03% NaCl.

On histological examination, association of filamentous bacteria with gill epithelium was only seen on gill arches suspended in formulated water and not in the other bath solutions.

Experiment III: influence of water quality and temperature

Gill arches exposed to strain AJS 1 and suspended in an organ bath at 28 °C displayed evidence of

Table 1 Results of the bacterial titrations of the gill arches exposed to *F. columnare* strains AJS 1 and AJS 4 in different bathing solutions. Each value represents the mean ± SE of four different experiments

Experiment	*F. columnare* strain	Exposure dose (cfu mL^{-1})	Bathing fluid	Incubation temperature (°C)	Bacterial titre (cfu g^{-1} gill tissue)*
I	AJS 1	10^7	Ringer	28 °C	$6.3 \pm 2.5 \times 10^{7a}$
	AJS 4	10^7	Ringer	28 °C	$5.4 \pm 3.5 \times 10^4$
	/	0	Ringer	28 °C	$< 5.0 \times 10^3$
II	AJS 1	10^6	Ringer	28 °C	$4.3 \pm 1.1 \times 10^5$
	AJS 1	10^6	Distilled water	28 °C	$6.3 \pm 2.5 \times 10^{3b}$
	AJS 1	10^6	Distilled water + 0.03% NaCl	28 °C	$8.8 \pm 2.5 \times 10^{3b}$
	AJS 1	10^6	Formulated water*	28 °C	$2.4 \pm 2.1 \times 10^{7b}$
III	AJS 1	10^6	Ringer	17 °C	$< 5.0 \times 10^{3b}$
	AJS 1	10^6	Ringer	28 °C	$1.7 \pm 1.0 \times 10^5$
	AJS 1	10^6	Ringer + 5 mg L^{-1} nitrite	28 °C	$3.5 \pm 1.9 \times 10^{6b}$
	AJS 1	10^6	Ringer + organic matter	28 °C	$3.3 \pm 2.1 \times 10^{7b}$
	AJS 4	10^6	Ringer	17 °C	$< 5.0 \times 10^{3b}$
	AJS 4	10^6	Ringer	28 °C	$1.0 \pm 0.4 \times 10^4$
	AJS 4	10^6	Ringer + 5 mg L^{-1} nitrite	28 °C	$1.3 \pm 0.6 \times 10^4$
	AJS 4	10^6	Ringer + organic matter	28 °C	$1.0 \pm 0.6 \times 10^4$

*Distilled water with 0.03% NaCl, 0.01% KCl, 0.002% $CaCl_2 2H_2O$ and 0.004% $MgCl_2 6H_2O$.
[a]Significantly ($P < 0.05$) different from the value for strain AJS 4.
[b]Significantly ($P < 0.05$) different from Ringer (28 °C) as bathing fluid.

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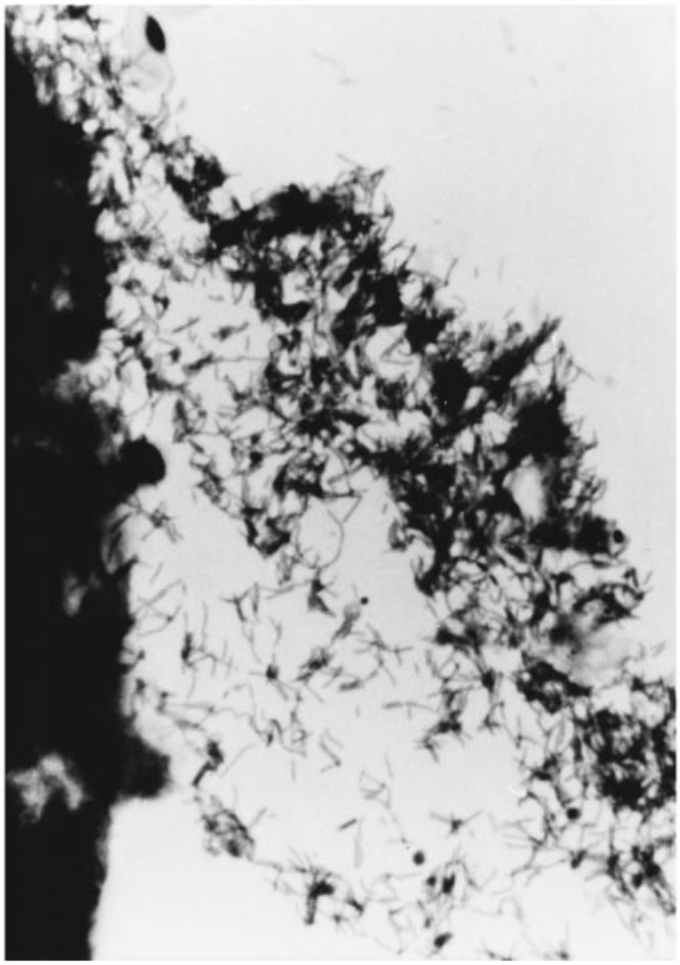

Figure 3 Cluster of *F. columnare* strain AJS 1 attached to the tip of the primary filament of an infected holobranch (Giesma, × 500).

thread formation on the extremities irrespective of the presence of nitrite or organic material. When the exposure to this strain occurred at 17 °C, only very thin and delicate filaments were observed. Filamentous structures were not observed in gill arches inoculated with strain AJS 4. When the holobranch was suspended in Ringer solution with nitrite, a marked decrease in efferent flow (from 75% to 50%) was noted from 15 min from the onset of perfusion.

With strain AJS 1, bacterial titres were significantly ($P < 0.05$) higher when organic matter or nitrite were added to the organ bath. With strain AJS 4, no significant differences were noted whether or not nitrite or organic material were added to the Ringer solution. When incubation occurred at 17 °C, the number of cfu g^{-1} gill tissue was $< 5 \times 10^3$.

Histological examination revealed filamentous bacteria localized at the tips of the primary lamellae in gill arches exposed to strain AJS 1 and incubated at 28 °C in the presence of nitrite or organic matter, but not in the other gill arches.

Discussion

Data on virulence mechanisms of *F. columnare* have not progressed much further than a description of the pathological changes associated with columnaris disease. Early studies revealed strains with different virulences, but these differences in virulence could not be related to the presence or absence of specific virulence factors.

In the present studies, a gill perfusion model was developed and used to examine the early pathogenesis of *F. columnare*. This semi-*in vivo* model offers a means of keeping the lamellar epithelium intact and maintaining the overall gill organization for several hours. In this way, bacterial adhesion to the gill tissue can be studied in detail under easily controllable conditions.

The results of the adhesion studies using the gill

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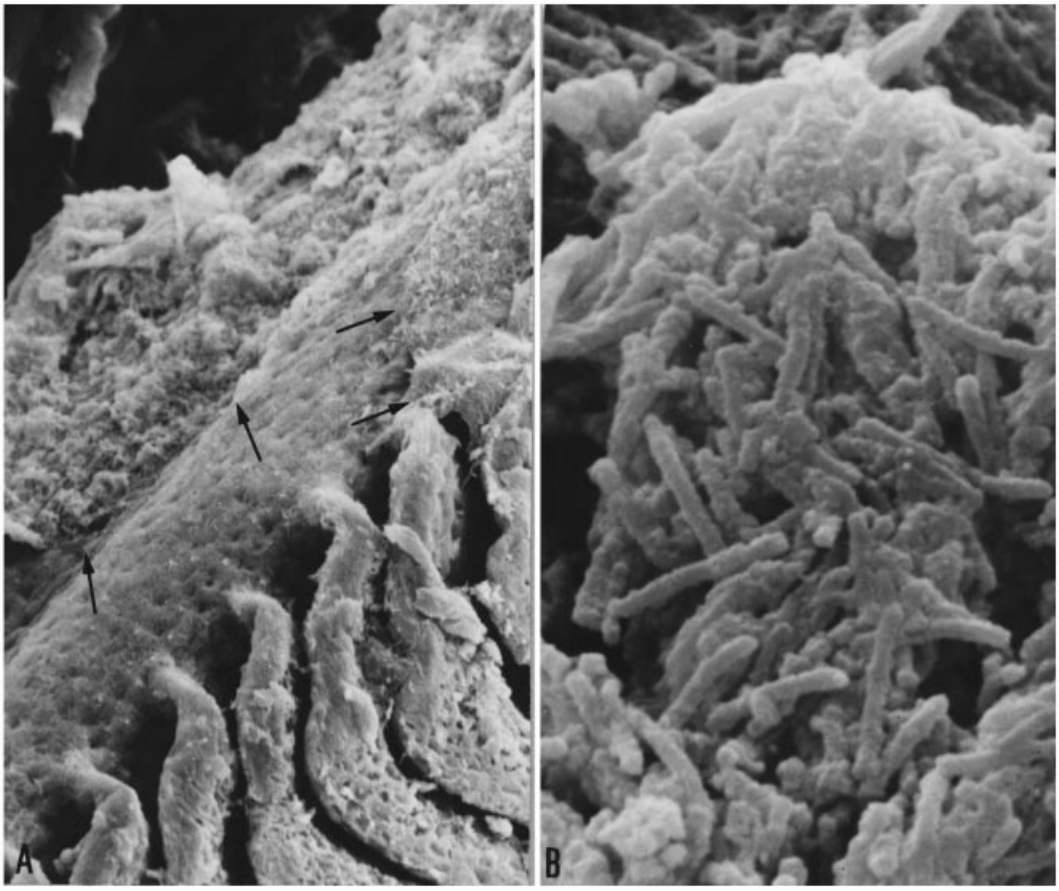

Figure 4 (A) Primary filament of a gill arch challenged with strain *F. columnare* AJS 1. Several filament and lamellar surfaces are covered with an irregular mat (arrows) (SEM, × 50). (B) The mat consists of clumps of the typical, long thin bacteria packed within mucus (SEM, × 8000).

perfusion model prove that the high-virulence strain AJS 1 has more ability to adhere to the gill epithelium than the low-virulence strain AJS 4. In many infectious diseases, the association of bacteria with host cells is considered to be the first step in establishment of bacteria at a site. There are a number of reasons to support this view (Ofek & Doyle 1994a). Firstly, bacterial adhesion enables the pathogen to withstand cleansing mechanisms operating on the surfaces. In case of the gill tissue, this means resistance to the continuous waterflow over the gill filaments and the constant turnover of the respiratory branchial epithelium and mucus. Furthermore, a concentration of nutrients (the gill surface versus the aqueous environment) may occur, offering growth advantages to the pathogen. A last advantage gained by the bacterium as a result of adhesion is that degradative enzymes are kept in close contact with the tissue. Hence, the local toxicity to the host may be enhanced. It was demonstrated that *F. columnare* produces extracellular protease(s), although no correlation with virulence has been made. Christison & Martin (1971) suggested that the slimelayer of the pathogen has a role in keeping these degradative enzymes in intimate contact with the substrate.

The difference in adhesion capacities between the high- (AJS 1) and low-virulence (AJS 4) strains found in these studies confirms the authors' previous findings with an *in vivo* model. When using the immunoperoxidase technique to study early pathogenesis of columnaris disease, it was found that strain AJS 1 was much more capable of adhering to the gills than strain AJS 4. The results of the present experiment combined with those of the previous *in vivo* findings prove that there is a close relation between virulence of *F. columnare* and its ability to adhere to gill tissue.

In order to quantify the extent of bacterial adhesion, a combination of three techniques was

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used: macroscopic observation, bacteriological titration, histological and/or SEM examination.

Although macroscopic observation may be subjective, it can offer valuable information since the formation of threads is quite characteristic for columnaris disease.

Reasonably good estimates of adhesion can be obtained from viable cell counts of organisms adherent to macerated tissues (Ofek & Doyle 1994b). However, viable counts are usually based on a logarithmic scale and only gross differences can be appreciated by this method of enumeration.

Microscopic estimates of adhesion reveal the presence of both dead and viable bacteria. Furthermore, the distribution of the pathogens can be observed. However, this method is tedious, rather subjective and not very sensitive. Indeed, only a very limited part of the gill tissue is tested. If adhesion is localized or if the gills are only lightly colonized, the bacteria may not be visible with routine histology as noted for *Flavobacterium branchiophilum* (Speare, Ferguson, Beamish, Yager & Yamashiro 1991). In the present studies, adhesion was only detected by light microscope examination if the bacterial titre of gill tissue was higher than $\approx 5 \times 10^5$ cfu g^{-1}.

In experimental work conducted by Chowdhury & Wakabayashi (1988), infection of weatherfish with *F. columnare* was found to vary with water quality. In the above authors' studies, infection occurred in every fish in both the formulated water (distilled water with 0.03% NaCl, 0.01% KCl, 0.002% $CaCl_2 2H_2O$ and 0.004% $MgCl_2 6H_2O$) and tap water. In contrast, no infection was observed in distilled water and the percentage of infection was low in solutions of the individual salts. Therefore, it was assumed that water quality was one of the important factors influencing the occurrence of columnaris disease in fish. The results from experiment II show that adhesion of the high-virulence strain to the gill tissue is influenced by the ion composition of the water. Adhesion was enhanced in the formulated water, whereas much less adhesion occurred in distilled water and distilled water with NaCl added. The formulated water was especially rich in bivalent ions (magnesium and calcium). Zaldiver (1985) correlated the ability to infect fish following waterborne exposure to the ability of the bacterial cells to attach to fish cells. He also found that the attachment of the *F. columnare* strains to epithelial cells *in vitro* required magnesium. Ofek & Doyle (1994a) claimed that multivalent ions, such as Mg^{++} and Ca^{++}, tend to reduce the surface potential. Hence, repulsive forces are more easily overcome and possible adhesins may now interact with their receptors leading to a firm adhesion.

Toranzo & Barja (1993) claimed that outbreaks of columnaris disease often occur under poor environmental conditions (presence of pollutants). Fijan (1968) suggested that columnaris disease is less likely to occur in water with low organic material levels, although disease outbreaks have been described under such conditions (Kumar, Suresh, Dey & Mishra 1986). Hanson & Grizzle (1985) claimed that nitrite at 5 mg L^{-1} induced predisposed channel catfish to columnaris disease. The results of the present studies indicate that high levels of nitrite (5 mg L^{-1}) and the presence of organic matter enhance adhesion of the high-virulence *F. columnare* strain AJS1. It certainly is worth investigating why the bacterium appears to be more capable of adhering to the gill tissue when the water quality of the organ bath is inadequate. Does the high level of nitrite and/or organic matter alter the bacterium in such a way that its adhesive capacity increases? Alternatively, does the structure of the gill tissue itself change resulting in an improved target for bacterial adhesion? By adding organic matter to the organ bath, the debris may get trapped within the mucous coat of the gills. This may promote bacterial infection of the gill and growth of established colonies of bacteria (Speare *et al.* 1991). Ferguson, Morrison, Ostland, Lumsden & Byrne (1992) claimed that gills exposed to high levels of ammonia had an altered mucous layer on top of the gills. Consequently, an enhanced susceptibility to pathogens occurred.

Adhesion of low virulence strain AJS 4 was not enhanced by the presence of organic matter or nitrite, again demonstrating the low capacity of this strain to adhere to gill epithelium.

It is generally accepted that the occurrence of columnaris disease is favoured by high environmental temperatures (Holt, Sanders, Zinn, Fryer & Pilcher 1975). The higher capacity of *F. columnare* to attach to gill epithelium at higher temperatures suggested in the present studies might play a role in this phenomenon.

The antigens or substances involved in attachment of *F. columnare* to gill epithelium are not known. Possible candidates are the capsule, fimbriae or other appendages of the outer membrane (Ofek & Doyle 1994a). Further studies are necessary to characterize the adhesins of *F.*

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columnare. These include electron microscopic examination of high- and low-virulence strains, and adhesion inhibition tests. The model developed in the present studies might prove to be very useful to further characterize the association of *F. columnare* with gill tissue. The present authors propose that the gill perfusion model is used in the future as a simple and feasible means of studying early pathogenesis of bacteria in general and evaluating bacterial virulence.

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