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The ability of Flexibacter columnaris to attach to fish cells was studied. Four of the five bacterial strains tested were able to adhere to fish cells derived from four different fish species. The attachment of these strains in vitro required magnesium but was not affected by increasing the incubation temperature from 16 to 22°C. Cells attached in higher numbers when they were at the end of the log phase of the growth cycle. The fifth strain, K4m, had an atypical colony morphology and was unable to attach to the four fish cell lines used under any of the assay conditions tested.

The four strains that were able to adhere to fish cells in vitro were also able to kill fish when infected by waterborne exposure. Strain K4m was unable to kill fish

infected by waterborne exposure, but did cause infection when fish were injected intraperitoneally with a culture of this strain. This means that the ability to infect and kill fish by natural or waterborne infection is closely related to the ability of the bacterial cells to attach to fish cells.

Electron microscopy of thin sections failed to show any discernible differences between the outer surface structures of the stain that did not adhere to fish cells (K4m) and the strains that did.

Attachment of the Pathogen *Flexibacter columnaris* to Fish Cells

by

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I. INTRODUCTION

The ability of bacterial cells to interact with host tissues in a specific manner is thought to be the primary event in which most indigenous and pathogenic microorganisms initiate the colonization of a host. That is, bacterial adherence to host tissue is a prerequisite for pathogenicity. The association of bacteria with specific tissues in vivo is a complex phenomena and dependent on a number of factors related to both the host and the bacteria. In aquatic microorganisms, the environment is a third factor that has a strong influence on the relationship between the pathogen and its host. Interference with one or more of these factors may lead to an attenuation of the strain concerned (Smith, 1977).

Flexibacter columnaris is an aquatic microorganism that infects fresh water fish. It is the etiological agent of columnaris, a disease that occurs among warm and cold water fish. It attacks fish primarily through the gills or abraded epidermal areas, but it is also capable of invading uninjured gill filaments. Gill lesions normally appear as yellow-orange necrotic areas that may involve all of the

gill tissue. Examination of gill arches reveals piles of F. columnaris at various locations on the gill filament.

When F. columnaris cells are inoculated unto sterile gill tissue, they form columnar masses that project above the tissue surface. Typical colonies tend to adhere to the surface of the agar medium. In liquid cultures, cells tend to form aggregates, particularly after prolonged incubation. These in vitro observations indicate that the bacterium has an adhesive ability allowing it to establish cell to cell contact and to attach to the agar medium. This adhesive ability would enable the bacterium to infect fish and initiate disease.

A strain of F. columnaris that had an atypical colony morphology was isolated. This strain did not adhere to the surface of the agar when grown on solid medium and did not form noticeable aggregates in liquid culture medium. It seemed that its adhesive ability was defective.

The purpose of this study was to determine the relationship between adhesive ability and virulence among strains of F. columnaris, and to study the conditions that would enhance or diminish the attachment of bacterial cells to fish cells. The isolation of a strain that was unable to attach allowed a comparison between capacity to adhere to fish cells and virulence.

II. LITERATURE REVIEW

Flexibacter columnaris is an aquatic bacterium pathogenic to salmonids and other fresh water teleosts. It is the etiological agent of columnaris disease, the cause of serious mortalities among populations of fish. Losses are greatest when the animals are injured or handled at water temperatures above 21°C (Pacha and Ordal, 1970; Holt et al., 1975). Severe economic losses have occurred as a result of epizootics among natural and hatchery populations of salmonids and other species (Becker and Fujihara, 1978). Its most important hosts among cultured species are catfish and salmon. The bacterium has a worldwide distribution in the tropical and temperate regions.

Historical background

Columnaris disease was originally described by Davis (1923) among warm water fishes from the Mississippi River. Davis was unsuccessful in isolating the etiological agent but he described the disease and the bacterium that appeared in the lesions of the infected fish. He observed that the bacteria from the lesions formed column-like masses and he named it Bacillus columnaris.

The first report among cold water fish was done by Fish and Rucker (1943) and Ordal and Rucker (1944) who described a natural outbreak among sockeye salmon (Oncorhynchus nerka)

being reared at Leavenworth, Washington. In the infected tissues they observed the same columnar masses described by Davis (1923). Ordal and Rucker (1944) were able to isolate the organism using a dilute culture medium. They proposed the name Chondrococcus columnaris, a myxobacterium, because the individual cells were elongate and flexible and because of what they described as fruiting bodies. Flexibacter columnaris was eventually found among other salmonids and other cultured fresh water fish (Pacha and Ordal, 1970; Becker and Fujihara 1978).

In the eighth edition of Bergey's Manual of Determinative Bacteriology, Leadbetter (1974), reclassified the organism and placed it in the family cytophagaceae, and genus Flexibacter, because it was determined that it does not produce microcysts. It is now an accepted member of this genus.

Characteristics of Flexibacter columnaris

The organism is a long, slender, gram-negative rod that measures 0.5 to 0.7 µm wide by 4 to 8 µm long. The cells are flexible and refractile and exhibit gliding motility on solid surfaces. On infected tissue, they gather in clusters resembling haystacks and then form columns, which accounts for the species designation, columnaris. Spherical and highly refractile spheroplast-like structures appear in

older cultures (Becker and Fujihara 1978; Pacha and Ordal 1970).

Flexibacter columnaris is not fastidious during in vitro culture. It grows readily in dilute media such as cytophaga (Anacker and Ordal 1959a), HS (Shieh 1980) and the medium described by Liewes et al. (1982). Growth occurs over a temperature range of 4 to 30°C. The organism is strictly aerobic and nonhalophilic. Growth is inhibited by 1% NaCl. It is proteolytic, produces H_2S and catalase, is unable to reduce nitrates and does not decompose tyrosine nor utilize citrate. (Pacha and Ordal 1970; Becker and Fujihara 1978; Pacha and Porter 1968). Sugars are not fermented but Leadbetter (1974) reports some strains that are able to utilize glucose as a carbon source.

On solid medium the colonies are typically yellow, spreading with a convoluted center and rhizoid edges, that adhere to the agar. This characteristic colony is used as a diagnostic tool.

Studies of the ultrastructure of F. columnaris were done to compare its structure to other gliding microorganisms (Pate and Ordal 1967a, 1967b; Pate et al., 1967). Thin sections of the organism showed that the structural composition, from the surface to the center are: (1) a material coating the surface that stains with ruthenium red dye; (2) the outer membrane, separated from the cell, and extremely irregular in profile; (3) peripheral

fibrils, associated with the inner layer of the outer unit membrane, which may disintegrate during preparation of the cells for electron microscopy; (4) a dense layer which probably corresponds to the mucopeptide layer of the cell wall; (5) plasma membrane, that sometimes invaginates into the cytoplasm; (6) a homogeneous cytoplasm; (7) mesosomes.

When the cells were broken, treated with phosphotungstic acid and viewed in the electron microscope, tubular structures or rhapidosomes were seen (Pate et al., 1967). The authors suggested that the compound membrane of the mesosomes breaks down to give rise to these structures. Rhapidosomes can only be seen in lysed cells. The authors concluded that they are formed during disintegration of the cells.

Liquid cultures of F. columnaris may become viscous due to the production of extracellular slime. Johnson and Chilton (1966) purified a polysaccharide produced when F. columnaris is grown in a defined amino acid medium or a synthetic medium containing casein hydrolysate. They determined that it was a high molecular weight homopolymer of D-galactosamine in which half of the amino groups were acetylated. As expected, this purified basic polysaccharide did not react with ruthenium red. It is not known whether this compound is layered on the cell surface along with the acid mucopolysaccharide or simply excreted into the medium.

Serological typing of F. columnaris revealed that there is a common antigen present in all the strains (Anacker and Ordal 1959a). The strains are not identical; several additional antigens are present in various combinations. The four recognized serological groups are composed of antigenically related strains (Anacker and Ordal 1959b, Pacha and Ordal 1970). These four major groups were defined as follows:

Group I. Strains with antigen 1 and 2 but not 3 and 6.

Group II. Strains with antigens 1 and 3, but not 2 or 6.

Group III. Strains with antigens 1 and 6, but not 2 or 3.

Group IV. Strains with antigen 1 but not 2, 3 or 6.

According to this grouping, antigen 1 was the common antigen while antigens 2, 3 and 6 identified individual strains.

Pacha and Ordal (1963) assayed the virulence of over 500 strains including organisms from each of the four serological groups. Fish were exposed to F. columnaris by the waterborne route and the time required to kill 100% of them was recorded. Four categories of virulence were defined based on the time interval needed to produce 100% mortality. The categories were defined as follows:

1. High virulence: 100% kill in 24 h.

2. Moderate virulence: 100% kill in 48 h, but not 24 h.

3. Intermediate virulence: 100% kill in 96 h, but not 48 h.

4. Low virulence: over 96 h to 100% kill.

Low and high virulence strains were observed among each of the four serological groups. No correlation between virulence and serotypes was noted. Pacha and Ordal (1963) demonstrated that there were variations in pathogenicity within strains of F. columnaris. Cells isolated from a single strain, showed variations in virulence. Low virulence strains were isolated from cultures which exhibited high virulence and high virulence isolates appeared among low virulence strains. Therefore, the authors concluded that the relationship between serotypes and virulence was not possible unless strains showing stable virulence features were isolated.

Pathology

Flexibacter columnaris infects both warm and cold water fish, scaled and scaleless, wild and cultured. In adult fish, lesions may occur on the gills, skin, and musculature. The organisms attacks fish primarily through the gills or abraded epidermal areas. Gill lesions appear as yellow-orange areas of erosion that may involve part or eventually all the gill tissue (Becker and Fujihara 1978; Pacha and Ordal 1967).

Lesions usually begin at the periphery of the gills and may extend to the base. Histopathology of the gills revealed that the blood vessels were congested and the surface epithelium of the lamellae was dissociated from the capillary bed. Scattered areas of hemorrhage were also noted (Fish and Rucker, 1943; Pacha and Ordal, 1967; Wobeser and Atton 1973). Bacterial cells resembling F. columnaris are found in masses throughout the gill tissue (Snieszko 1966). Prior to death, the respiratory rate of infected fish was much greater than that of uninfected fish. This observation, together with the histopathological findings, suggest that fish die of respiratory failure (Pacha and Ordal 1967).

Body lesions begin as small, circular, greyish-white eroded areas. Initial lesions frequently go unnoticed since they occur under the scales. The erosion area expands rapidly to form large circular necrotic patches and may reach 3 to 4 cm in diameter. Frequently, the skin of the fish is completely eroded away, exposing the underlying muscle tissue (Pacha and Ordal 1967).

Histopathological studies revealed necrosis of the muscle fibers. Large numbers of bacterial cells could be found under the scales, though very few were seen in the necrotic muscle (Pacha and Ordal 1967). Large numbers of bacterial cells were seen in the advancing edges of the lesions (Pacha and Ordal 1970, Corpe 1980).

A condition described as "saddleback" disease was reported among Atlantic salmon (Salmo salar) (Cone et al., 1980; Morrison et al. 1981). The organism causing the lesion was isolated and identified as F. columnaris. The saddle lesion is a kind of pale discoloration at the base of the dorsal fin. Fin deterioration then occurred starting at the base of the fin. Usually, fish died within 48 hours of the appearance of skin discoloration and lesion formation. Histopathology of the lesions revealed that a mat of bacteria replaced the epidermis around the dorsal fin. There was extensive tissue damage in the inner layer of the epidermis as well as in the muscle. Internal petechial hemorrhages may be observed in the muscle. Lymphocyte infiltration into regions of necrosis is minimal and limited to areas involving small blood vessels. Destruction of these vessels can account for the internal petechial hemorrhages. No gill damages was observed and no internal organs seemed involved.

Virulence of Flexibacter columnaris

The necrosis noted in muscle tissue underlying the skin lesions suggests that a diffusible toxin or proteolytic enzyme may be involved in the production of these lesions. Pacha and Ordal (1967) suggested that a diffusable, necrotizing substance produced during growth of the bacterial cells on the skin might account for the extensive

necrosis observed in the muscle where few bacteria were seem. Flexibacter columnaris is known to be actively proteolytic in vitro but toxin production has not been evaluated in vivo and no specific toxin has been isolated.

The gross pathology in fish infected with highly virulent strains of F. columnaris was usually very limited. There was little or no tissue damage observable at the time of death. In such cases, the gills were the major site of damage (Pacha and Ordal 1967). Experimental exposure of fish revealed that highly virulent strains produced disease more readily by the contact method than by injection of bacterial cells. Low virulence strains were transmitted more effectively by intramuscular or intraperitoneal injection. This suggests that one difference between high and low virulence strains may be their ability to attack susceptible tissues (Pacha and Ordal 1970). The contact method tests the ability of the pathogen to initiate infection as well as to produce disease, whereas, the injection method tests only the killing capacity of the strain (Becker and Fujihara 1978). The difference between low and high virulence strains could be related to the ability of the cells to adhere to host tissues and initiate infection.

The role of bacterial adhesion in pathogenesis has been extensively analyzed (Beachey 1981; Cheng et al., 1981; Costerton et al., 1981; Smith 1977). In other systems

bacteria have been demonstrated to adhere to host tissues by specific molecules, proteins or carbohydrates (Heckels 1982; Beachey et al., 1982; Kasper et al., 1982). Specific cell substructures, namely pili, fimbriae and capsules have been reported to be required for host-pathogen interaction (Swanson 1977, Frost et al., 1977, Lindley 1980). The presence of fiber-like structures in ruthenium red-stained thin sections of one strain of F. columnaris has been reported (Pate and Ordal 1967b). The presence of an acid polysaccharide on the cell surface has also been reported and its presence associated to the ability of cells to adhere to host tissue (Pate and Ordal 1967b). No demonstration of the absence of these structures in non-adherent or low virulence strains has been reported.

There is a direct relationship between water temperature and mortality in fish exposed to F. columnaris. In 1943 Fish and Rucker showed that the degree of severity of the infection increased when experimentally infected fish were held at 21.1°C. Fish held at 12.8°C showed little signs of infection. Pacha and Ordal (1970) studied the epizootiology of columnaris disease among sockeye salmon in the Columbia River Basin over a period of several years and established that incidence increased with increasing water temperatures. Fujihara et al., (1971) reported that the susceptibility of juvenile chinook salmon (Oncorhynchus tshawytscha) and rainbow trout (Salmo gairdneri) to

F. columnaris appeared to be influenced by the water temperature as well as crowding and age of fish. Mortality of fish exposed to F. columnaris by contact with Columbia River water was higher when the water was maintained 2.2°C higher than the natural water. The effect of water temperature on the disease producing capacity of F. columnaris of different grades of virulence was examined by Pacha and Ordal (1970). Their results indicated that strains of higher virulence were better able to infect fish and produce disease at low temperatures than the strains exhibiting lower virulence. Only when the temperature was increased to 20°C were strains of low virulence able to infect fish.

The effect of water temperature on the mortality of experimentally infected juvenile chinook salmon, steelhead trout (Salmo gairdneri), and coho salmon (Oncorhynchus kisutch) was reported by Holt et al. (1975). Fish were infected by the water borne method of exposure at eight temperatures increasing from 3.9 to 23.3°C. No deaths resulting from columnaris occurred at temperatures of 9.4°C or below. At temperatures above 9.4°C, mortality increased progressively with increasing temperatures to 100% in steelhead trout and coho salmon and 70% in chinook salmon.

Fish infected with a high virulence strain at temperatures over 18°C were killed in less than two days (Pacha and Ordal 1970). Little or no gross pathology was

seen in these fish and damage was usually restricted to the gills. The same strains produce extensive body lesions on the experimental fish held at lower water temperatures. Low virulence strains produced a slow-progressive chronic infection at 20°C, while other strains were only able to produce disease if the fish were scarified or injured.

Mortality of fish by columnaris disease is related to other factors beside temperature. Fujihara et al. (1971) reported that crowding of fish plays a major role in increasing the incidence of the disease. They also suggested that increasing age rather than size may be a factor in resistance to the pathogen.

It seems clear that environmental factors affecting both host and pathogen are important in the development of columnaris disease. There seems to be little host specificity, the disease having been reported among several species of cultured fresh water fish, including salmon and trout (Anacker and Ordal 1959a; Pacha and Ordal 1963; Johnson and Brice 1952), atlantic salmon, white suckers (Catostomus commersonii) (Wobeser and Atton 1973), black bullhead (Ictalurus melas), (Bowser 1973) eels (Wakabayashi et al 1970), carp (Cyprinus carpio) (Bootsma and Clerx, 1976) among others. Mortality rates vary among these fish, but all of them are susceptible.

Antibody production

Survivors of F. columnaris infection develop antibodies, and their production can be monitored by agglutination reactions. Yearling rainbow trout that survived columnaris infection as juveniles in the Columbia River, were not killed by re-exposure via incoming river water the following year (Becker and Fujihara 1978). The relationship between immunity and antibody production was studied by parental vaccination of yearling rainbow trout with heat-killed suspensions of virulent isolates. The magnitude of antibody development increased rapidly particularly in fish injected with heat-killed cells plus adjuvant. Injection of virulent cells killed all nonimmumized fish in three weeks. Among immunized fish, subcutaneous challenge caused 20% mortality, but intraperitoneal challenge killed all fish in two weeks. Intraperitoneally vaccinated rainbow trout were exposed during one year to virulent F. columnaris cells shed from infected fish. There was no mortality among vaccinated fish, and antibody titer values where higher than controls. Antibody titers among vaccinated fish ranged from 1:1000 to 1:650, while the titers among control fish ranged from 1:20 to 1:400 during exposure to columnaris.

When oral immunization was studied, coho salmon fed heat-killed cells developed resistance, and mortality was 8% compared to 48% in the control group. Rainbow trout fed

with sonically disrupted cells developed no resistance (Becker and Fujihara 1978).

No vaccine against columnaris disease is available at present. The main factor for prevention of columnaris disease is avoidance of conditions that increase the infection and mortality rates such as high water temperatures, and crowding and handling of fish which result in injuries and abrasions that facilitate the onset and development of the disease.

Treatment of columnaris disease

Columnaris disease can be controlled by systemic and external antibacterials. Terramycin (oxytetracycline) is effective against columnaris (Amend 1970). It can be administered therapeutically in the feed at 50 to 75 mg/kg/day for 10 days. This treatment clears the fish internally, as well as externally, of bacteria. Terramycin baths at 1 ppm active ingredient can also be used (Becker and Fujihara 1978).

Diquat can also be used to control mortalities. Four daily 1 h baths of Diquat at 2 to 4 ppm of active Diquat cation are effective if treatment is started early in the epizootic. The disease recurrs after treatment is stopped if the water temperature is maintained at 20°C. Apparently when the infection becomes systemic, treatments are without effect (Amend 1970).

Furanace (Ni-furpyrinol) can also control columnaris infections. It is readily absorbed by the fish and is eliminated from tissues within hours and shows a low level of toxicity (Amend and Ross, 1970). Control is achieved by exposure of infected fish to 1 ppm of Furanace in the water for 1 h or to 0.5 ppm for two 1-h treatments 24 h apart.

III. MATERIALS AND METHODS

Bacterial cultures

Five isolates of Flexibacter columnaris were used in this study (Table 1). Four of them were isolated from infected fish. DD3 and IC8 had been grown on Cytophoga agar (Anacker and Ordal 1959a) lyophilized and stored at -20°C for approximately 15 years. DW and K4 had been recently isolated from infected fish. For storage of these two strains, one colony showing typical morphology was selected and grown on HS medium, (Shieh 1980), pelleted, resuspended in sterile skim milk and lyophilized. The fifth strain K4m appeared among K4 colonies as a colony showing atypical morphology. It was selected for this study because it did not adhere to the surface of the agar as the typical colonies do. The three recent isolates, DW, K4 and K4m were identified as F. columnaris by biochemical and slide agglutination tests.

Before using them for the in vitro or in vivo experiments, lyophilized cultures were resuspended in 1 ml sterile water inoculated into HS medium and incubated on a shaker at 22°C. Cultures were examined for purity and for the absence of spheroplasts by Gram stain.

Table 1. Isolates of *Flexibacter columnaris* used in this study

Isolate	Source	Host	Year Isolated	Isolated by
DD3	Willamette River Oregon	spring chinook salmon (*Oncorhyhchus tshawytscha*)	1969	J. Sanders
IC8	Blue Lake Ranch Idaho	catfish (*Ictalurus punctatus*)	1969	J. Sanders
DW	Dexter Ponds Oregon	spring chinook salmon (*Oncorhyhchus tshawytscha*)	1983	T. Amandi
K4	Rogue River	sucker (*Catostomus* sp)	1983	T. Amandi

K4m: isolated among colonies of K4 and chosen for its atypical colony morphology and its lack of adherence to the agar medium

Culture media and growth conditions

Flexibacter columnaris was grown in HS medium (Shieh, 1980) composed of (in g/100 ml): peptone 0.5, sodium pyruvate, 0.01, citric acid, 0.001; yeast extract, 0.05; K_2HPO_4, 0.01; KH_2PO_4, 0.05; $MgSO_4 \cdot 7H20$, 0.03; NaHCO3, 0.005; $CaCl_2$, 0.001.

To test the effect of glucose in the culture medium as a factor affecting the adherence of the bacteria to fish cells, in vitro, HS medium was supplemented with 0.1g glucose per 100 ml.

Attachment of Flexibacter columnaris to fish cell lines

Quantification of the attachment of F. columnaris strains to fish cells involved an in vitro system using cell lines derived from fish tissue. Four fish cell lines were used (Table 2). These cell lines were grown in Eagle's minimal essential medium, MEM (Flow laboratories) supplemented with 5% ($^{v}/v$) fetal calf serum, MEM-5 (Hy-Clone) and the pH adjusted to 7.6 with 7.5% ($^{w}/v$) sodium bicarbonate. Cells were grown in plastic culture flasks (Corning Glass Works) and incubated at 15°C.

For the attachment assay, cells were grown on glass coverslips, placed in the bottom of 6-well tissue culture plates (Falcon). One milliliter of cell suspension was placed on each coverslip, so that it was confined within the limits of the glass by surface tension. After a period of

Table 2. Fish cell lines used to determine and compare adhesive properties of *Flexibacter columnaris*

Cell line	Origin	Cell type	Reference
CHSE -214 (ATCC CRL 1681	chinook salmon embryo (*Oncorhynchus tshawytscha*)	epithelial	Lannan et al, 1984
EPC	carp epithelioma (*Cyprinus carpio*)	epithelial	Tomasec and Fijan, 1971
BB	brown bullhead, caudal trunk (*Ictalurus nebulosus*)	epithelial	Wolf and Quimby, 1969
CCO	channel catfish ovary (*Ictalurus punctatus*)	fibroblast	Bowser, 1976

1.5 h, to allow for cell attachment, 1 ml of MEM-5 was added. The plates were covered with adhesive coversheets (Linbro), and incubated at 15°C until 90% cell confluence was obtained. Cells were then washed three times with Hank's balanced salts solution (HBSS) to remove serum and spent medium and were then quickly washed three times with phosphate buffered saline (PBS) 0.01 M pH 7.2. Unless otherwise stated, the PBS was supplemented with 5×10^{-3} M MgSO4 and 5×10^{-3} M $CaCl_2$.

The attachment assay was done as follows: 1 ml of bacterial suspension was added to each well containing one of the fish cell cultures and incubated at 15-16°C for 10 min. Each sample was done in triplicate. After incubation an additional 3 ml of PBS was added to remove the bacterial cells that had not attached to the tissue cells. The resulting suspension was aspirated off. The fish cells with the attached bacterial cells were quickly washed three times with PBS, then fixed in absolute methanol for 10 min at room temperature. The alcohol was removed and the fixed cells were stained using Giemsa stain for 15 min and washed with distilled water.

The attached bacterial cells were counted using 400x magnification in a light microscope. Five fields were counted for each preparation. Results represent the mean number of bacterial cells per field $\pm$ the standard error.

To determine non-specific attachment, or the adherence of the bacterial cells to surfaces other than fish cells, suspensions of F. columnaris were incubated on clean cover slips without cells. Bacteria that remained attached after washing were fixed, stained, and counted as described.

Selected factors affecting the adherence of Flexibacter columnaris to fish cells in vitro.

The ability of F. columnaris strains to adhere to fish cells in vitro under different experimental conditions was studied. These included: 1) culture age, 2) presence of glucose, 3) presence of divalent cations.

Effect of culture age - One hundred milliliters of HS medium was inoculated with 1 ml of a 12 h culture of each bacterial strain and incubated on a shaker at 22°C, for 21, 32, and 41 h. A sample of each was taken, washed twice in PBS, adjusted to 0.3 A_{520} and assayed for ability to adhere to tissue cells.

Effect of glucose - Addition of glucose to the culture medium has been reported to enhance synthesis of the external polysaccharides of F. columnaris (Shieh 1980, Johnson and Chilton 1966). Because the presence of this external polysaccharide has been associated with the ability of F. columnaris to adhere to host cells (Pate and Ordal 1967b), the strains were grown in HS medium containing 0.1% ($^{w}/v$) glucose, and transferred daily for 3 days to fresh

medium. Cells were then washed three times in PBS and assayed for their adherent ability.

HS medium contains other carbon compounds such as citric acid, acetate and pyruvate that could lead to the synthesis of polysaccharides. The effect of their absence in the culture medium, both in the presence and absence of glucose, was also assayed.

Effect of divalent cations - The initial adhesion of microbial cells to a surface is usually related to the net charge on the cell surface as well as on the bacteria (Beachey 1981). In addition to net charge, specific divalent cations have been reported to be involved in cell attachment (Smith 1977). Divalent and trivalent cations are also required for cell aggregation of virulent strains of Aeromonas salmonicida (Udey and Fryer 1978, Udey 1977).

To test the effect of divalent cations Ca^{++} and Mg^{++}, the attachment was carried out using PBS with or without addition of Ca^{++} and Mg^{++} or both. Each strain was grown in HS medium for 30 h and washed five times in either of the following buffers:

1) 5×10^{-3} M $CaCl_2$, 5×10^{-3} M $MgSO_4$ in PBS
2) 5×10^{-3} M $CaCl_2$ in PBS to test the effect of the absence of Mg^{++}
3) 5×10^{-3} M $MgSO_4$ in PBS to test the effect of the absence of Ca^{++}

4) PBS without supplements: to test the effect of the absence of Ca^{++} and Mg^{++}

The bacterial cell suspension was adjusted to an absorbance of 0.3 at 525 nm.

Fish tissue cells were washed three times in the same buffer used to make the bacterial suspension. The attachment assay was performed as before.

Estimation of virulence of Flexibacter columnaris strains: LD_{50} determination

One way of measuring the virulence of a bacterial culture is to determine the number of bacterial cells that are capable of killing 50% of the exposed host, that is, lethal dose-50 or LD_{50}. This study was concerned with the importance of the adhesive properties on virulence, so the contact method of infection or waterborne exposure was used. It was hypothesized that failure of a strain to attach to fish tissue cells would be reflected as a reduced or lack of ability to kill exposed fish. That is, that the lack of ability to adhere as determined in vitro would also mean a lack of ability to adhere to fish cells in vivo, and thus a reduced virulence.

Groups of 15 rainbow trout from Oak Springs Fish Hatchery, averaging 9-10 cm fork length were held in fiberglass tanks containing 68 liters of pathogen free

water. Fish were held at 18°C for at least 2 days before exposure to temper them to this temperature.

Bacterial strains were grown in 1.5 l of HS medium for 30 h on a shaker at 22°C. Exposure of fish was done as follows: the flow of water in the aquaria was stopped and the volume reduced to about 20 liters. Two tanks were inoculated with 500 ml of the bacterial culture, two tanks with 50 ml and two with 5 ml to accomplish a 10-fold dilution series. After an exposure time of 10 min, the water flow was resumed. Two control lots of fish were not inoculated but the water flow was also stopped and the water in the tanks was reduced to 20 liters, to subject these fish to the same conditions as the exposed fish.

To determine the actual number of bacterial cells in the water during exposure, the culture was mixed in the water and a 3 ml sample was taken. This sample was diluted in 0.2% ($^{w}/v$) gelatin in 30% ($^{w}/v$) Earle's balanced salts solution (EBSS) to reduce clumping of the bacterium and achieve a more accurate count. Dilutions were streaked on HS agar medium and typical yellow colonies with rhizoid edges counted.

Dead fish were collected daily and the organism recovered by streaking gill and kidney tissue on HS agar medium. After seven days with no mortality, the surviving fish were terminated and their gills and kidneys examined for F. columnaris. Only those fish from which F. columnaris

colonies were recovered were considered killed by the organism and used to calculate the LD_{50}. The LD_{50} was calculated according to the method of Reed and Muench (1938), and expressed as the bacterial cell concentration required to kill 50% of the fish exposed.

To test the hypothesis that a non-adherent strain is unable to infect and kill fish because it lacks the ability to attach to fish tissue and initiate infection, fish were injected with F. columnaris cultures. Three strains were tested: DD3 and K4 which were able to kill fish by waterborne exposure, and K4m which seemed avirulent by this procedure. For each strain, five rainbow trout were injected with 0.1 ml of a suspension of 10^7 bacterial cells per ml. Fish were maintained in separate tanks at 18°C with continuous flow of pathogen free water. Dead fish were necropsied and bacterial cultures prepared from gill and kidney tissue on HS agar plates and incubated at 22°C.

Preparation of samples for Electron Microscopy

The absorption of microorganisms to surfaces has been related to the presence of certain structures in their outer surfaces (Beachey 1981; Cheng et al., 1981; Costerton et al., 1981). Pate and Ordal (1967b), by electron microscopy of thin sections, reported the presence of five peripheral layers in F. columnaris. They suggested the ruthenium red-

positive material, probably an acid mucopolysaccaride, could be involved in the adhesive properties of this species.

To investigate the presence of this external acidic mucopolysaccharide in both adherent and non-adherent cells, thin sections of the five strains were prepared. Cells were grown in HS medium for 30 h on a shaker. Fifteen milliliters of each culture were centrifuged at 6000 g for 10 min and washed three times in PBS. The pellets were transferred to two microfuge tubes. One of them was carefully mixed with 0.15 ml of F. columnaris antiserum (kindly supplied by Yen-Ling Song) and incubated for 1 h. The purpose of this step was to stabilize the extracellular carbohydrates against condensation during the dehydration procedure (Costerton 1980, Mackie et al., 1979; Bayer and Thurow, 1977). Antiserum-treated and untreated cells were then fixed by adding 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 in the presence of 0.15% ruthenium red (Toussimis). Fixation was carried out at room temperature for 1.5 h. Fixed cells were then washed five times in the same buffer in the presence of 0.05% ruthenium red. They were postfixed in osmium tetroxide for 1.5 h, dehydrated in a graded acetone series in which the 30% and 50% steps contained ruthenium red (0.05%) in the aqueous buffer component, but the 70% and 90% series were composed with distilled water. The initial steps were carried out for 30 min but the 90% step was shortened to 10 min to minimize loss of stain, and

was followed by two washes in 100% acetone. Samples were infiltrated in Spurr's medium, embedded, thin sectioned on a LKB ultramicrotome using a diamond knife and stained using lead citrate. Examination of the sections was done in a Phillips model 300 transmission electron microscope.

IV. RESULTS

Appearance of Flexibacter columnaris strain K4m

During isolation of K4 colonies on HS agar plates, the appearance of colonies showing a different morphology was observed. These colonies, typical of F. columnaris, were yellow in color and had rhizoid edges; however, they differed by not adhering to the surface of the agar and having a mucoid surface (Figure 1). Gram stains of cells from this colony type revealed long, thin gram negative rods, identical to F. columnaris. Biochemical tests showed that the strain was catalase and cytochrome oxidase positive, produced H_2S, was able to grow in 0.5% NaCl but growth was inhibited by 1% NaCl, glucose was not fermented and nitrate was not reduced. Slide agglutination tests were done and K4m agglutinated with antiserum prepared in rabbits against three different strains of F. columnaris: DD3, IC8 and BH-3. Therefore, it was concluded that K4m was F. columnaris and not a contaminant with similar colony and cell morphology.

During the course of this work, the new or second colony type was frequently observed in K4 cultures, usually when the medium contained glucose. To determine whether the

Figure 1. Colony morphology of two *Flexibacter columnaris* strains.

a) Strain K4 showing the characteristic spreading nature and rhizoid edge

b) Strain K4m showing an atypical morphology and mucoid surface.

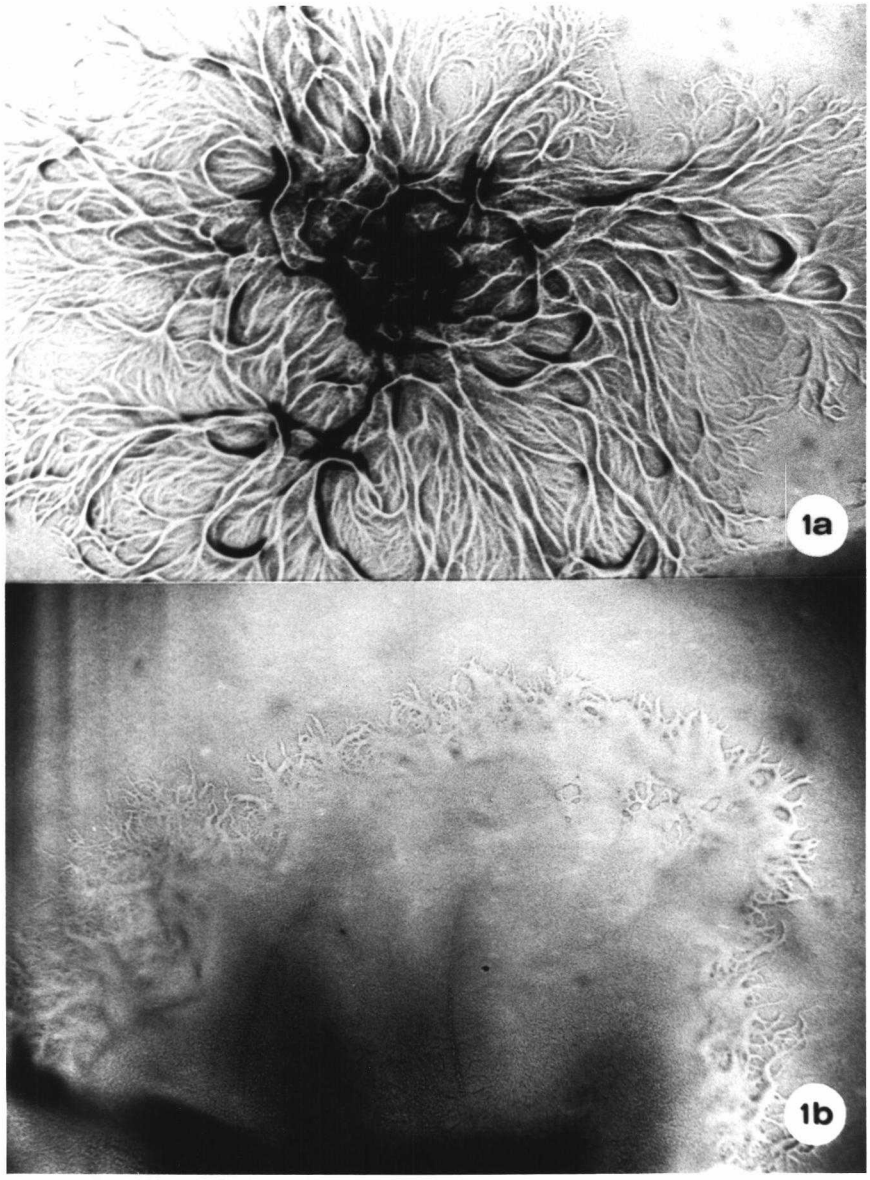


Figure 1.

colony type was a phenotypic feature determined by the glucose in the agar medium, one colony was transferred to two HS agar plates, one with and one without glucose. They were transferred daily for five days in the same medium. In all cultures, only the mucoid colony type was found. It was concluded that K4m was a spontaneous mutation and retains its colony morphology regardless of the culture medium.

Strain K4m was chosen for further experiments because it could not adhere to the agar surface which might reflect an inability to attach to fish cells in vitro and to fish tissue in vivo.

Attachment of Flexibacter columnaris to fish cell lines

To determine the optimal conditions for this in vitro assay, the effect of bacterial concentration and time of incubation of the bacterial cells with the fish cells was examined. The conditions selected were: bacterial cell concentration of 0.3 A_{520} and an incubation time of 10 min. These conditions were chosen because results were reproducible, there was minimum of bacterial clumping at this concentration, and there was a minimum of attachment to glass. A typical result using CHSE-214 cells is shown in Table 3 using a 30 h culture.

Table 3. Attachment of *Flexibacter columnaris* cells to CHSE-214 cells, after 10 min incubation at 16°C, using a bacterial concentration of absorbance 0.3 at 520 µm.

	Number of bacterial cells attached to[a]	
Bacterial strain	CHSE-214 cells	glass
DD3	124 ± 14	7 ± 4
IC8	89 ± 13	10 ± 7
Dw	133 ± 15	7 ± 5
K4	118 ± 21	5 ± 3
K4m	14 ± 5	5 ± 2

[a]Average number of bacterial cells, ± standard error, per field at 400x magnification using a light microscope. Cells in five fields were counted.

This experiment was repeated eight times. The number of cells attached to CHSE-214 varied by about 25%. Nevertheless, in each case the same trend was observed. 1.) The number of K4m cells attached to CHSE-214 cells was negligible, indistinguishable from the controls, in which no fish cells are present. 2.) In each experiment, the number of IC8 cells attached to CHSE-214 cells was low compared to DD3, DW and K4.

These results suggested that the K4m strain is deficient in its adhesive ability to CHSE-214, and that IC8 cells have less affinity for these tissue cells than the others tested. The same experiments were done to determine

the affinity of the strains to other fish cell lines, derived from different species. Identical experiments were performed using cells derived from carp, brown bullhead, and channel catfish. Strain IC8 was originally isolated from catfish, so an increased affinity of this strain to CCO cells might be expected. However, the results (Table 4) indicated no increased affinity of the IC8 strain to CCO cells, and similar to results obtained with CHSE-214 cells, the number of IC8 cells was lower than for the other three adherent strains. Therefore, the smaller number of IC8 cells attached to the four fish cell lines tested, seems to indicated that IC8 has a reduced adhesive ability for host cell membranes in vitro and that it is a characteristic of the bacterial strain and not of the fish cell line.

The same observation applies for strain K4m. The attachment of this strain is essentially the same as that of the controls without tissue cells. It can be concluded that K4m cells are not able to attach to these fish cell lines.

The lack of attachment of K4m cells to the fish cells could be an artifact of the assay. Some of the conditions were changed to further examine this possibility. If the K4m strain had slower attachment kinetics, an increase in

Table 4. Attachment of *Flexibacter columnaris* cells to different tissue cells, after 10 min incubation at 16°C using a bacterial cell concentration of absorbance 0.3 at 520 µm.

Bacterial strain	Number of bacterial cells attached to[a]			
	EPC	BB	CCO	glass
DD3	145 ± 22	131 ± 19	151 ± 21	8 ± 5
IC8	95 ± 19	87 ± 14	101 ± 15	10 ± 6
Dw	139 ± 18	125 ± 21	147 ± 17	11 ± 5
K4	131 ± 13	142 ± 22	139 ± 20	10 ± 4
K4m	17 ± 7	11 ± 6	12 ± 5	7 ± 5

[a] Average number of bacterial cells, ± standard error per field at 400 x magnification using a light microscope. Cells in five fields were counted.

incubation time would allow them to adhere and the assay would detect their presence. For this reason, the incubation time was increased from 10 to 30 min. Another factor that could reduce the adherence was temperature. The incubation temperature was increased from 16 to 22°C which is closer to the optimal growth temperature of F. columnaris. When the effect of temperature was assayed, the incubation time was maintained at 10 min to avoid damaging the tissue cells. The third factor assayed was bacterial concentration which was increased from 0.3 to 0.45 A_{520}. Each parameter was changed individually. It seems that the tissue cell type does not affect the assay, so only CHSE-214 cells were used. As positive control, bacterial strains DD3 and IC8 were chosen because both of them were able to attach to fish cells. The results (Table 5) demonstrate that increasing incubation time, or bacterial concentration does not increase the attachment of K4m to tissue cells. Under the same conditions, there is an increase in the number of both DD3 and IC8 detected. It can be concluded that under all the assay conditions tested, K4m does not attach to fish tissue cells. The results in Table 5 also show that the temperature of incubation does not affect the adhesive capacity because the number of cells attached remained the same at 16 and 22°C for both adherent and non-adherent strains.

Table 5. Effect of incubation time, bacterial concentration and incubation temperature on the attachment of *Flexibacter columnaris* to CHSE-214 cells.

Strain	incubation time (min)	concentration of bacterial cells (A520)	incubation temperature (°C)	number of bacterial cells attached[a]	control[b]
DD3	10	.30	16	135 ± 21	12
	30	.30	16	215 ± 45	38
	10	.45	16	231 ± 47	27
	10	.30	22	145 ± 34	16
IC8	10	.30	16	92 ± 19	11
	30	.30	16	168 ± 37	31
	10	.45	16	189 ± 41	24
	10	.30	22	89 ± 21	14
K4m	10	.30	16	14 ± 5	3
	30	.30	16	19 ± 4	4
	10	.45	16	21 ± 7	3
	10	.30	22	17 ± 8	5

[a] Average number of bacterial cells attached per microscope field at 400x magnification. Cells in five fields were counted.

[b] Average number of bacterial cells attached to glass coverslips.

Certain factors affecting the adherence of Flexibacter columnaris to fish tissue cells

Effect of culture age - Cells in three different phases of the growth cycle were examined for their ability to adhere to the membranes of host cells in vitro. Under the growth conditions used, the cells were in log phase after 21 h of incubation, entering the stationary phase at 32 h and were in stationary phase at 41 h. It was found that cells entering and already in stationary phase attach to tissue cells in higher numbers (Table 6A). However, after 41 h of incubation spheroplasts are present and the remaining bacterial cells tended to clump together. These conditions altered the reproducibility of the assay and increased the standard error. For this reason, 32 h was chosen as the culture age. The number of bacterial cells attached to glass remained the same at the three growth phases used (Table 6B).

Effect of glucose - It has been suggested that extracellular polysaccharides are involved in the adhesive properties of F. columnaris (Pate and Ordal, 1967b, Fletcher and Floodgate, 1973). Synthesis could also be enhanced by the addition of glucose to the culture medium (Johnson and Chilton 1966, Shieh, 1980). If so, the presence of glucose in the culture medium could be a requirement for the ability of the cells to attach. The initial experiments were done with bacterial cells grown in the presence of glucose. To

Table 6. Effect of the phase of the growth curve on the attachment of *Flexibacter columnaris* strains to CHSE-214 cells.

A Number of bacterial cells attached to CHSE-214 cells[a]

	incubation time in hours		
Strain	21	32	41
DD3	91 ± 12	138 ± 16	148 ± 38
IC8	67 ± 9	93 ± 13	104 ± 26
DW	101 ± 13	141 ± 19	157 ± 41
K4	87 ± 11	129 ± 17	138 ± 41
K4m	9 ± 5	17 ± 6	19 ± 9

B Number of bacterial cells attached to glass[a]

	incubation time in hours		
Strain	21	32	41
DD3	10 ± 6	10 ± 6	19 ± 11
IC8	7 ± 4	12 ± 6	20 ± 10
DW	8 ± 6	9 ± 5	14 ± 8
K4	5 ± 3	9 ± 4	23 ± 10
K4m	5 ± 4	4 ± 3	6 ± 3

[a]Average number of bacterial cells ± standard error, per field at 400x magnification using a light microscope. Cells in five fields were counted.

test the requirement of glucose in the adhesiveness of the bacterial cells, the five strains were grown in the absence of glucose as already described, and in the presence of 0.1% glucose. Results (Table 7) show there is no difference in attachment with either media. This could mean that, if in fact a polysaccharide is involved in attachment, it is probably synthesized using one or more of the carbon substrates of the culture media. The rest of the experiments were done in the absence of glucose.

Table 7 Effect of the presence of glucose in the culture media.

Strain	Number of bacterial cells attached [a]	
	with glucose [b]	without glucose [c]
DD3	152 ± 31	146 ± 29
IC8	111 ± 18	97 ± 15
DW	161 ± 29	149 ± 25
K4	147 ± 22	135 ± 19
K4m	18 ± 7	16 ± 7

[a] Average number of bacteria attached to CHSE-214 cells per microscope field at 400x magnification.

[b] *Flexibacter columnaris* strains grown in HS plus 1 g of glucose per liter.

[c] *Flexibacter columnaris* grown in the absence of glucose.

According to Shieh (1980) pyruvate, citric acid and acetate have a stimulatory effect on the growth of *F. columnaris*. But recently Yen-Ling Song (personal

communication) has shown there is little change in the growth curves in the presence or absence of these three compounds. Results in Table 8 show that the absence of these three carbon compounds and glucose does not significantly reduce the number of attached bacterial cells.

Table 8. Effect of pyruvate, citric acid and acetate in the adhesive properties of *Flexibacter columnaris*.

	Number of bacterial cells attached[a]			
	+ citric acid acetate pyruvate		- citric acid acetate pyruvate	
Strain	+ glucose	- glucose	+ glucose	- glucose
DD3	107 ± 17	134 ± 21	114 ± 19	109 ± 13
IC8	103 ± 9	107 ± 13	92 ± 13	90 ± 12
DW	137 ± 19	144 ± 17	121 ± 17	117 ± 19
K4	141 ± 21	137 ± 13	129 ± 14	121 ± 18
K4m	14 ± 3	17 ± 4	11 ± 4	13 ± 5

[a] Average number of bacterial cells ± standard error attached to CHSE-214 cells per microscope field at 400x magnification.

It is clear that if a polysaccharide is involved, it must be synthesized from a carbon source other than citrate, pyruvate, acetate and glucose, because their removal from the culture medium does not alter the capacity of the bacterial cells to attach. The requirement of exopolysaccharides in adherence is not ruled out. Flexibacter columnaris is a highly proteolytic organism so it could use protein as a carbon source for the synthesis of the polysaccharide.

Effect of calcium and magnesium - The importance of divalent cations in the initial step of microbial attachment has been described. In the in vitro assay used in this research, both tissue and bacterial cells are grown in culture media that contain calcium and magnesium. In addition, the buffer used during the attachment incubation period was also supplemented with them. The effect of removing one or both cations from the buffer was examined. Removal of the salts from the culture medium was not possible because they are a requirement for growth.

The results are shown in Table 9. In the absence of calcium the adhesive ability remains the same, while in the absence of magnesium the number of attached bacterial cells decreases sharply. Calcium alone cannot replace magnesium because in the presence of calcium and absence of magnesium the number of cells attached was

negligible. It seems that magnesium salts are required for initial attachment.

Table 9. Effect of calcium and magnesium on the attachment of *Flexibacter columnaris*.

	Number of bacterial cells attached[a]			
	+ Mg		- Mg	
Strain	+ Ca	- Ca	+ Ca	- Ca
DD3	107 ± 16	98 ± 17	22 ± 8	19 ± 9
IC8	91 ± 15	87 ± 14	19 ± 8	20 ± 9
DW	129 ± 22	107 ± 16	25 ± 7	31 ± 7
K	114 ± 19	109 ± 20	16 ± 6	24 ± 5
K4m	19 ± 7	17 ± 8	13 ± 6	16 ± 6

[a]Average number of bacterial cells, ± standard error, per field at 400x magnification using a light microscope. Cells in five fields were counted.

It is possible that with a longer incubation time, the bacterial cells could attach to the tissue cells even in the absence of magnesium, as this initial adhesion by electrostatic forces was reinforced by more specific adhesive mechanisms involving external bacterial components. This alternative could not be tested because tissue cells detach from the glass surface when divalent cations are removed from the buffer, making the assay impossible.

Virulence of Flexibacter columnaris

The role of bacterial cell adhesion in pathogenesis is well documented (Costerton et al., 1981; Smith 1977; Holt 1982; Gibbons, 1977; Beachey 1981). In order to examine the relationship between the affinity of some bacterial strains to fish tissue cells, or their lack of affinity as in K4m, to their ability to infect and kill fish, a series of waterborne exposures were done. Virulence of the strains was estimated as the number of bacterial cells capable of killing 50% of the exposed fish.

Tables 10 and 11 show the results of these experiments. Under the same conditions, strains DD3 and IC8 were able to kill all of the exposed fish in 2 days when the concentration of bacteria in the tank was 10^7 CFU/ml. The resulting LD_{50} was 3.2×10^6 CFU/ml. For DW, the LD_{50} is 50% higher, while for K4 it is five times higher. As expected, K4m was not able to kill fish. No mortalities were observed in the 3 weeks after exposure and no K4m-type colonies were observed when gill and kidney tissues of the fish sacrificed after termination were streaked on HS agar medium.

Fish were also infected by intraperitoneal injection. The purpose of this experiment was to rule out the possibility that the K4m strain was unable to kill fish due to a factor other than its lack of adhesiveness. All of the injected fish died within 48 hours. Fish injected with culture media survived.

Table 10. Comparison of virulence of *Flexibacter columnaris* strains.

Stain	Conc. in the tank (CFU/ml)[a]	Fraction that died	% of fish infected with *F. columnaris*[b]	LD50 (CFU/ml)
DD3	1.2×10^7	15/15	100	3.2×10^6
	1.4×10^6	15/15	100	
	1.2×10^6	2/15	13	
	1.0×10^5	2/15	8	
	1.2×10^5	0/15	0	
	1.2×10^5	0/15	0	
IC8	1.4×10^7	15/15	100	3.2×10^6
	1.2×10^6	14/15	100	
	1.3×10^6	3/15	13	
	1.2×10^5	2/15	13	
	1.4×10^5	0/15	0	
	1.2×10^5	0/15	0	
DW	1.7×10^7	13/15	87	4.8×10^6
	1.4×10^6	13/15	80	
	1.7×10^6	3/15	20	
	1.5×10^5	2/15	13	
	1.6×10^5	0/15	0	
	1.6×10^5	0/15	0	
K4	1.5×10^7	8/15	53	1.5×10^7
	1.5×10^6	8/15	47	
	1.5×10^6	1/15	0	
	1.4×10^6	0/15	0	
K4m	2.0×10^7	1/15	0	$> 2 \times 10^7$
	1.9×10^6	0/15	0	
	2.0×10^6	0/15	0	
	2.1×10^6	0/15	0	

[a] CFU = colony forming units.

[b] Only those from which *F. columnaris* could be isolated were considered.

Table 11. Mean time to death of rainbow trout (Salmo gairdneri) exposed to Flexibacter columnaris strains.

Strain	Bacterial concentration in tank (CFU/ml)[a]	Mean time to death (days)
DD3	1.2×10^7	2
	1.4×10^6	2
	1.2×10^6	4
	1.0×10^6	3
IC8	1.4×10^7	2
	1.2×10^6	2
	1.3×10^6	5
	1.2×10^6	4
DW	1.7×10^7	4
	1.4×10^6	5
	1.7×10^6	10
	1.5×10^6	11
K4	1.5×10^7	5
	1.5×10^6	5
	1.5×10^6	>21
	1.4×10^6	>21
K4m	2.0×10^7	>21
	1.9×10^6	>21
	2.0×10^6	>21
	2.1×10^6	>21

[a]CFU = colony forming units.

Bacterial cultures were prepared from the kidneys of dead fish and streaked on HS agar medium for recovery of the bacterial strains. All of the fish were shown to be infected, and the colony morphology was dry and adhered to the agar for the DD3 and K4 strains while K4m maintained the characteristics of being non-adherent to agar and mucoid. These results indicated that K4m is a virulent strain and that its lack of ability to infect and kill fish through waterborne exposure is due to a deficiency in its ability to adhere.

Electron microscopy

Pate and Ordal (1967b) showed that the surface of F. columnaris is coated by a ruthenium red-positive material, probably an acid mucopolysaccharide. They suggested this compound may be involved in the adhesive properties of the cells.

Thin sections of ruthenium red stained cells were examined to compare the structure of virulent and avirulent strains. In these preparations, no discernible difference was demonstrated in surface structures of virulent and avirulent cells (Figures 2 and 3). Cells that had not been stabilized with antiserum before fixation were surrounded by a loose outer layer that covered a more compact and densely stained plasma membrane and cell wall. The same structures

Figures 2-5.

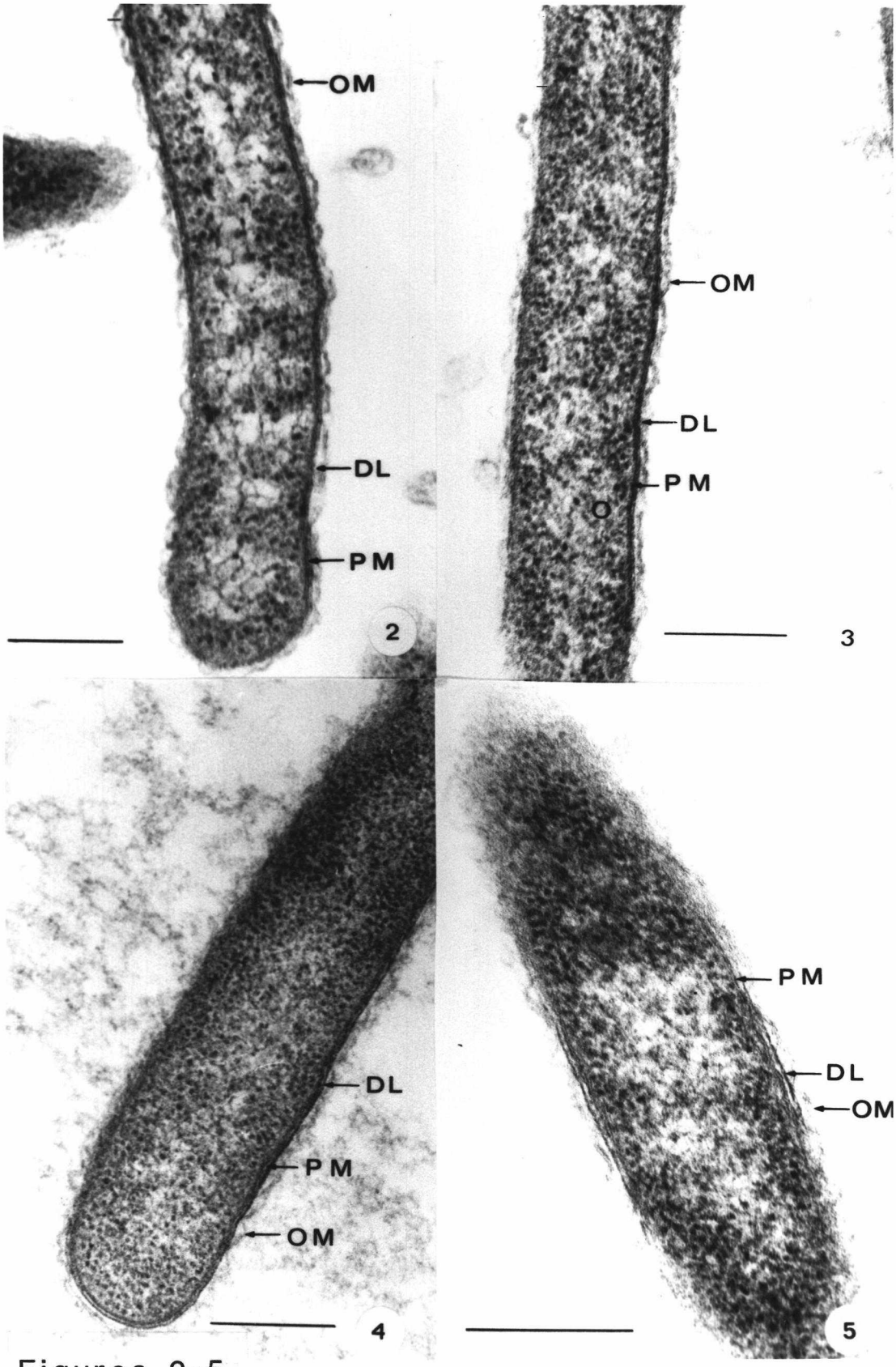
Electron micrographs of thin sections of *Flexibacter columnaris* strains. Arrows point to outer membrane (OM), dense layer (DL), and plasma membrane (PM). Bars represent 0.3 µm.

Figure 2. strain K4

Figure 3. strain K4m

Figure 4. strain DD3 treated with antiserum before fixation.

Figure 5. strain K4m treated with antiserum before fixation.



Figures 2-5.

were seen in both virulent and avirulent strains. The material described by Pate and Ordal (1967b) covering the surface, extending outward and connecting adjacent bacteria was not observed in these preparations.

The use of antiserum has been described in the preparation of specimens for electron microscopy (Bayer and Thurow 1977; Mackie et al., 1979). Its role is to stabilize the outer surface components to dehydration during sample preparation. When antiserum was used, the loose outer structure seemed more diffuse and there was abundant background material (Figures 4 and 5). Because of this, any connecting fibrils would be masked by this background material. This background could represent the reaction between the antiserum with the surface antigens. Again, this material was seen in both virulent and avirulent strains.

These cells were grown under the same culture conditions as the cells for the attachment and virulence studies, yet there was no discernible difference between the outer surface of K4m and the other strains examined. This could mean that the difference in adhesive properties is not a structural difference.

V. DISCUSSION

To cause disease a pathogen must:

a) enter the host;

b) multiply in host tissues;

c) resist or not stimulate host defenses; and

d) damage the host.

Pathogens must accomplish all four of these processes to produce disease (Smith 1977). The first step in the infective process is association of one or more surface structures of the bacterial cell with receptors in the host tissues. Specificity of bacterial adherence plays an important role in colonization by pathogen. In vivo evidence indicates the association of microorganisms with host tissues is a complex process and is influenced by factors related to both pathogen and host. Pathogen factors include motility, chemotaxis and the presence of cell surface structures such as fimbriae or mucopolysaccharides. Host factors include the presence of antibacterial enzymes and antibodies.

In addition to host and pathogen, the environment plays an important role in the association between host and pathogen. With aquatic microorganisms conditions such as water temperature, electrolyte concentration, suspended organic matter and salinity can affect the attachment process. Ordal and Rucker (1944) and Holt et al. (1975)

demonstrated the effect of increasing water temperature on the severity of laboratory infections with F. columnaris in salmonids. Pacha and Ordal (1970) studied columnaris disease in salmon populations of the Columbia River and established that incidence increased with increasing water temperatures. They suggested that warm water stimulated multiplication of bacteria already residing on fish. These authors reported that some high virulence strains of F. columnaris can initiate infection at temperatures as low as 12.8°C, suggesting that disease could result from exposure of fish harboring dormant cells of F. columnaris to warm water. That is, at low temperatures F. columnaris is able to attach to fish tissues but the disease does not develop until fish are exposed to higher temperatures.

Results on the effect of two incubation temperatures on attachment of F. columnaris to fish cells in vitro agrees with the in vivo evidence (Table 5). The same number of bacterial cells attached to fish cells when incubation temperatures were increased from 15 to 22°C, indicating attachment of pathogen to host is not affected at these temperatures and that factors other than attachment are affecting the severity of the disease at higher temperatures.

Another environmental factor affecting attachment of cells is electrolyte concentration and the presence of specific ions (Gordon and Millero 1984; Smith 1977). The

effect of divalent cations on the attachment of F. columnaris to fish cells in vitro showed a requirement for magnesium ions and that calcium ions could not replace magnesium. The attachment was affected by the nature of the cation and not by ionic strength, suggesting a specific reaction between surface components and magnesium.

In natural environments, the effect of Mg^{++} on attachment could be an important factor affecting virulence. Flexibacter columnaris retains viability for prolonged periods of time in waters of higher total hardness and organic matter content, even at temperatures as high as 25°C (Becker and Fujihara 1978).

In vitro attachment of F. columnaris was also affected by the phase of the growth cycle. Results showed that there is an increase in the number of bacterial cells attached when they were in late log and stationary phases. Cell to cell association is also altered in older cultures. This is reflected in an increase of cell aggregation and the appearance of spheroplast-like cultures after prolonged periods of cell growth (Becker and Fujihara, 1978). These observations could also suggest that cells in the late phases of growth undergo changes in the outer cell structures which could increase their ability to attach to host cells.

It has been suggested that the extracellular polysaccharide present in F. columnaris is synthesized using

glucose as a substrate (Johnson and Chilton 1966; Shieh 1980). They suggested that this external polysaccharide could be responsible for the adhesive properties of the cells. Results showed that F. columnaris cells grown in the presence or absence of glucose were equally able to attach to fish cells. These results do not rule out the requirement of extracellular polysaccharides in attachment. They only indicate that glucose is not required for the synthesis of the adhesive compound. A polysaccharide could be synthesized from one of the other carbon compounds present in the culture medium.

Other cell substructures such as fimbriae and pili could be involved in attachment. Pate and Ordal (1967b) described the external structure of F. columnaris, and these did not include fimbraie or pili. These authors described the presence of a ruthenium red-positive material on the surface of the cells. In some of the thin sections, this material appeared to extend outward in filament-like structures that connect with adjacent cells. Pate and Ordal (1967b) suggested this compound could function as an adhesive, linking one cell to another. They also suggested that it could be responsible for the adhesiveness of F. columnaris to fish cells. This material would be an acid mucopolysaccharide, because the ruthenium red dye molecule has a high affinity for these compounds (Luft 1965).

These filament-like structures were not observed in ruthenium red-stained thin sections of F. columnaris (Figures 2 and 3). Antiserum has been used in the study of other bacteria, such as Escherichia coli and Streptococcus pyogenes (Corpe 1980), to stabilize external polysaccharides against dehydration; however, no differences were seen when F. columnaris cells were treated with antiserum before fixation (Figures 4 and 5).

This result was unexpected. The structures described by Pate and Ordal (1967b) are frequently cited in the literature as an example of attachment-related structures. So it was thought the strains which could attach to fish cells in vitro, such as DD3 and K4, would have these structures while strain K4m, which was unable to attach, would not. Their absence in both strain types would indicate that either they are not required for attachment or that under the conditions cultured and prepared for viewing, they were not formed. This seems unlikely because bacterial growth conditions used for the preparation of specimens for electron microscopy and for attachment and virulence studies were the same. These results suggest there could be another surface component involved in attachment.

More than one cell structure could be involved in this process. It is possible that, if the fibrils described by Pate and Ordal (1967b) participate in attachment, they are not necessarily an absolute requirement. In other

microorganisms such as Neisseria gonorrhoeae, non-fimbriated cells were still able to attach to fallopian tube mucosa but with a lower affinity (McGee at al., 1981). Thus, the ability or inability of bacteria to attach, is not always related to the presence or absence of structures visible by electron microscopy.

As expected, the strain that was unable to attach to fish cells in vitro was unable to kill fish when they were infected by waterborne exposure. However, it retained its ability to kill fish when injected intraperitoneally. That is, strain K4m is avirulent because it does not attach to fish tissues. The differences in virulence observed by Pacha and Ordal (1970) in over 500 strains is probably related to the ability of cells to attach to the host. The ability of F. columnaris to attach to host cells is an important factor in its virulence.

VI. SUMMARY AND CONCLUSIONS

1. Flexibacter columnaris strains DD3, IC8, K4 and DW were able to attach to fish cells in vitro.

2. Strain K4m, which showed an atypical colony morphology, was unable to adhere to any of the four fish cell lines tested. This strain did not attach to fish cells under any of the assay conditions tested. Changes in incubation time and temperature and the presence or absence of divalent cations did not improve its attachment.

3. Adherent strains were able to attach to the four fish cell lines tested. The fish cell lines were originally derived from four different species of cold and warm water fish. Each F. columnaris strain attached with the same affinity to the four fish cell lines.

4. Strain IC8 attached in smaller numbers to the four fish cell lines compared to DD3, K4 and DW.

5. The presence of low concentrations of magnesium is required for the attachment of strains DD3, IC8, K4 and DW to fish cells in vitro. Calcium could not substitute for magnesium.

6. The number of DD3 and K4 cells attached to fish cells did not change when the incubation temperature was raised from 16 to 22°C. In this range, temperature did not affect attachment. Strains that attached to fish cells in

vitro were also able to kill fish when they were infected by waterborne exposure.

7. Strain K4m was unable to kill fish when they were subjected to waterborne exposure. When fish were infected with a culture of this strain by intraperitoneal injection, it was able to kill fish, and the bacterium could be isolated from kidneys of dead fish. It is concluded that the inability of strain K4m to infect and kill fish is related to its defective capacity to adhere.

8. No discernible structural differences were observed in electron microscopy of thin sections of adherent and non adherent strains of F. columnaris. Peripheral fibrils previously described surrounding the cells were not detectable. The results seemed to indicate that the differences observed in the ability of the strains to attach to fish cells and to infect fish is not reflected as a structural difference at the bacterial cell surface.

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