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Kinetics of Adhesion of Selected Fish-Pathogenic *Vibrio* Strains to Skin Mucus of Gilt-Head Sea Bream (*Sparus aurata* L.)

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The kinetics of adhesion of *Vibrio* strains isolated from diseased fish to skin mucus of gilt-head sea bream was studied. A modified Langmuir adsorption isotherm was calculated, and the results obtained indicate that the strains tested (*Vibrio alginolyticus* DP1HE4 and *Vibrio anguillarum*-like DC12R8 and DC12R9) showed a saturation kinetics except for *V. alginolyticus* (CAN), which showed a proportional adsorption kinetics. The adhesive capability for skin mucus does not seem to be an essential virulence factor of pathogenic strains of *Vibrio*, since this specific interaction depended on several environmental factors, temperature and salinity being the most important. However, the absence of an inhibitory effect of mucus on the pathogenic microorganisms, and the capability of the *Vibrio* strains to utilize mucus as a carbon source, could favor their settlement on the skin with a potential for infection of cultured, stressed fish.

The most significant factor that affects fish cultures is the incidence of microbial pathologies, mainly bacterial in origin. There are different marine fish species, such as gilt-head sea bream (*Sparus aurata* L.), which are being extensively cultured in the aquaculture industry, especially in Southern Europe. Several pathogenic microorganisms which are involved in epizootic outbreaks in gilt-head sea bream (*S. aurata* L.) cultures, such as *Pseudomonas* spp. (11), *Pasteurella piscicida* (46), *Aeromonas salmonicida* (39), and several species of *Vibrio* (7, 47), have been described elsewhere. Among all the *Vibrio* species isolated from diseased *S. aurata*, *Vibrio alginolyticus* (4) and *Vibrio anguillarum*-like (7) are the most important species involved in outbreak mortalities.

The bacterial attachment to external surfaces and tissues is an essential initial step in the colonization of host tissues and subsequent occurrence of infection in pathogenic systems (34), involving different structures named adhesins (18, 21).

The external body surface of the host is the first defense barrier. In the case of fish, this external surface is covered by a mucous layer which is secreted by specialized globet cells located in the epidermal layer (36), similar in chemical composition to that of the mucus found in other animals (23, 42). In fish, the mucous layer is related to several important activities, such as osmoregulation, locomotion, mechanical protective function, and antibacterial activities against different fish pathogens (1, 14, 42, 48). Westerdahl et al. (48) reported that the mucous layer is involved in the prevention of microbial colonization of the pathogens by a mechanism of competition with the microbiota present on the mucus. However, the role played by fish mucus as the first layer involved in microbial adhesion or as a defense mechanism against pathogenic invasion has not been completely elucidated yet (4).

It is well documented that the occurrence of a fish disease depends on the balance among three factors, environment, pathogenic agent, and host (7). Temperature and salinity are two important environmental factors that affect the infection rates and the adhesion process of fish pathogenic *Vibrio* species (2, 10, 29). However, there are only a few studies of the effect of environmental factors on the adhesion of fish pathogenic *Vibrio* strains to fish surfaces (2, 8, 28).

The scope of this study is to evaluate the process of adhesion of fish pathogenic strains of *Vibrio* to skin mucus of gilt-head sea bream, as well as the influence of temperature and salinity on this process.

MATERIALS AND METHODS

Microorganisms. Thirty marine *Vibrio* strains isolated from diseased gilt-head sea bream over 2 years were used to study the adhesion to superficial fish mucus. Bacteria were cultured in tryptic soy broth (Difco Laboratories, Detroit, Mich.) with 1.5% NaCl (TSBS) and incubated at 22°C for 24 h. They were then centrifuged at 4°C (1,000 × *g* for 30 min), and bacterial pellets were washed twice and resuspended in saline solution (0.145 M NaCl, pH 7.2) to obtain a suspension with approximately 10^9 bacterial cells per ml.

Infectivity trials were carried out to determine the degree of virulence (50% lethal dose [LD_{50}]) of the strains for *S. aurata* (10 g in average body weight). Five fish for each dose were intraperitoneally injected (0.1 ml) with bacterial doses ranging from 10^4 to 10^8 cells per fish. Inoculated fish were maintained in 50-liter aquaria at 18°C and 35-ppt salinity with aeration. Control fish were inoculated with the same volume of sterile saline solution.

The cell surface hydrophobicity of the strains was examined by the salt aggregation test described by Lindahl et al. (31). The degree of hydrophobicity of the strains was evaluated according to the criteria described by Santos (41): high, 0 to 0.5 M; moderate, 0.5 to 1 M; weak, 1 to 2 M; and nonhydrophobic, higher than 2 M.

Collection and characterization of the mucus. Raw skin mucus was prepared by the technique described by Balebona et al. (8): the skin mucus was rubbed from gilt-head sea bream body surface and purified by a double centrifugation process at 20,000 × *g* for 30 min at 4°C, followed by filtration of the final supernatant through 0.45- and 0.2-μm-pore-size filters.

The detection of enzymatic activities from skin mucus of gilt-head sea bream was performed by using the API Zym System (BioMérieux Ibérica, Madrid, Spain), composed of 19 enzymatic substrates. The detection of caseinase, gelatinase, phospholipase, amylase, and lipase activities was carried out in plates by a radial diffusion method using a basal agar (8 g per liter of saline phosphate buffer [0.1 M and pH 7.2]) containing the appropriate substrate: 1% gelatin (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), 4% skimmed milk, egg yolk emulsion (Oxoid) (1:1 in saline solution), 0.4% starch, and 1% Tween 80 (Sigma Chemical Co., St. Louis, Mo.). Determination of elastase activity was conducted on elastine medium according to the techniques described by Hsu et al. (22) and Williams et al. (49).

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Bactericidal and bacteriostatic effects of the mucus were evaluated by both a nephelometric technique and a dish diffusion method (14). Briefly, each strain was cultured in TSBS for 16 to 20 h at 22°C. After this incubation period, the A_{600} was adjusted with tryptic soy broth at 1 and 1.5 ml of bacterial suspension was mixed with 0.5 ml of the mucus suspension (control test, 1.5 ml of bacterial culture mixed with 0.5 ml of sterile seawater). This mixture was incubated at 20°C for 16 h, and then the A_{600} was determined. In the dish diffusion method, a bacterial suspension (about 10^5 CFU/ml) was streaked on tryptone soya agar (Oxoid) supplemented with 1.5% of NaCl. Then, 20 μl of mucus was placed on the surface of the streaked agar and the plates were incubated at 20 to 22°C for 24 h (14). The inhibitory effect of the mucus corresponded with a clear zone surrounding the mucus deposit. The presence of lysozyme in the mucus was evaluated according to the method described by Ellis (13).

The ability of the strains assayed to use the skin mucus as sole carbon source was performed by addition of 1% agarose to phosphate-buffered saline (PBS) (0.2 M; pH 7.2), supplemented with 2 ml of a skin mucus suspension (1 mg/ml) and poured in 90-mm-diameter plates. The strains were streaked on this medium and incubated at 22°C for 20 h. Then, the plates were examined to observe the bacterial growth. Plates containing 0.2 M PBS (pH 7.2) supplemented with 1% agarose (FMC BioProducts, Rockland, Maine) were used as controls.

Adhesive assay. The adhesive capability for the skin mucus of the strains tested was evaluated with a total bacterial concentration of 5×10^5 cells per well, to avoid the problems derived from the saturation of the receptors by the nonadhered bacteria. A volume of 500 μl of mucus suspension (800 to 1,000 μg of protein per ml) was placed in a 24-well polystyrene titer plate (Nunc A/S, Kamstrup, Roskilde, Denmark) and incubated at 4°C for 18 h by the technique described previously (30). Then, each well was washed twice with saline solution to remove the nonadhered mucus excess. Afterwards, 500 μl of bacterial suspension (ca. 5×10^5 cells) was added and the plate was incubated with a gentle shaking for 1 h at 20°C. The wells were washed twice with 2 ml of saline solution to recover the nonadhered bacteria. Enumeration of adhered bacteria was carried out by the technique described by Sanchez et al. (40). Briefly, 2 ml of deadsorption buffer [zwitterionic detergent; Calbiochem; 10^{-5} M; peptone, 0.01%; ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,N′-tetraacetic acid (EGTA), 10^{-3} M; Tris buffer, 10^{-2} M; pH 7] was added to each well. All wells were homogenized with an Ultra-Turrax at 24,000 rpm for 50 to 60 s; during this step, the titer plate was kept in ice. The enumeration of the detached bacteria was performed by filtration of the sample stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) (38). The data were adjusted to give the adhesion percentage by the following: (counts of deadsorbed bacterial cells/counts of bacterial cells placed in well at time zero) × 100.

Adhesive kinetics. The evaluation of the adhesive kinetics of the strains selected was conducted spectrophotometrically with the initial bacterial concentration (10^9 bacterial cells per ml) as control at 22°C, pH 7.2, and 0.85% sodium chloride. The experimental absorbance results obtained were adjusted to a modification of the model I adsorption isotherms of Langmuir (27). The affinity index ($K_a \times n$) (3) was calculated from data represented in Fig. 1, in which K_a is the affinity constant and n is the maximal number of linkage places in the total adhesive area.

Influence of temperature and salinity on adsorption of *Vibrio* strains. The effect of different values of temperature and salinity on the adhesion of pathogenic *Vibrio* strains to skin mucus was evaluated. For this, the most virulent *Vibrio* strains (measured by their LD_{50}) and those with different mucus adhesion rates (see Table 2) were selected. The selected strains included two strains of *V. alginolyticus* with adhesion percentages between 30 and 45 (CAN and DP1HE4) and two *V. anguillarum*-like strains (DC12R8 and DC12R9), with 100 and 90% adhesion, respectively.

Bacterial cultures were prepared as described above, to obtain a suspension with approximately 10^8 bacterial cells per ml. Then 1 ml of this bacterial suspension was incubated for 8 h in seawater at different conditions of temperature (4, 22, 15, and 27°C) and salinity (10, 17, and 35‰). The different salinity values were achieved by dilution of filtered raw seawater (salinity of 35‰) with filtered deionized water. After this incubation period, the viable cell counts were carried out. The adhesion test was performed according to the method described by Krovacek et al. (26). Briefly, the bacterial suspensions were placed in petri dishes containing mucus-coated glass slides; incubated at 4, 22, and 27°C and 10, 17, and 35‰ salinity for 1 h with continuous gentle shaking, and washed thoroughly several times in saline solution (26). Then the slides were air dried and fixed with absolute methyl alcohol (20 min, at 20°C), stained with crystal violet, and observed under a light microscope. The number of bacteria attached to 1 mm^2 of the mucus-coated glass slide was determined in the different assay conditions by counting 20 microscopic yields. Slides without mucus were used as controls to verify the nonadhesion of bacteria to the glass.

A statistical analysis by applying an analysis of variance test was performed to compare the results of the different experiments of adhesion to skin mucus.

Microscopy. The arrangement of flagella was examined by the technique described by Heimbrook et al. (20).

Procedures for fixation, dehydration, and plating of specimens for scanning electron microscopy examination were conducted (7). Briefly, the dried slides were fixed with 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, for 12 to 24 h at 4°C. Then the slides were dehydrated in a graded ethanol series (50, 70, 80, 96, and 96%) for 10 min each and in two steps with absolute ethanol for 15 min each. The dehydrated samples were critical point dried and then sputter coated with gold for examination with a JEOL (JSM-840; JEOL, Tokyo, Japan) scanning electron microscope at 15 to 20 kV of accelerating potential. The micrographs were made with Ilford HP4 film.

For transmission electron microscopy examination, negative staining was carried out (7). Briefly, copper grids (400 mesh) coated with Formvar-carbon (Agar Scientific Ltd., Stansted, Essex, United Kingdom) were placed upside down for 2 min, on the surface of one drop of the samples. Subsequently, the samples were stained on one drop of a 1% uranyl acetate (Merck) solution (pH 4.5) for 3 min. After removal of the excess stain by blotting with filter paper, the grids were air dried. A JEOL 100CX electron microscope operating at 80 kV was used to examine the negative staining.

TABLE 1. Rates of adhesion of pathogenic *Vibrio* strains to skin mucus of gilt-head sea bream and evaluation of the bacteriostatic and bactericidal effects of this mucus against the *Vibrio* strains tested

Strain	Origin	% Adhesion	Optical density[a]	
			Control	TSBS
V. alginolyticus				
CAN	Asymptomatic mortality	31.75	2.28	2.35
A035	Hemorrhagic wound	38.3	2.03	2.14
DP1HE4	Hemorrhagic wound	44	2.23	2.39
V. anguillarum				
DC7R2	Exophthalmia	100	1.66	1.69
DC11R2	Distended intestine	40	1.68	1.67
DC12R7	Distended intestine	30	1.76	1.86
V. anguillarum-like				
DC12R8	Distended intestine	100	1.13	1.23
DC12R9	Distended intestine	90	1.99	2.20
V. fischeri				
P304	Exophthalmia	0	0.74	0.79
P403	Exophthalmia	0	0.74	0.87
P801	Exophthalmia	0	0.87	0.91
P802	Exophthalmia	0	0.67	0.71
P505	Exophthalmia	0	0.84	0.80
P8H1	Exophthalmia	19.7	0.94	0.91
Vibrio harveyi				
A030	Ulcers	0	0.69	0.72
DP1U1	Ulcers	0	0.89	1.03
DP1U2	Ulcers	0	1.02	1.19
DP1U3	Ulcers	0	0.88	1.08
DP1U4	Ulcers	0	0.83	0.98
DP1HE1	Hemorrhagic wound	0	0.80	0.96
DP1HE2	Hemorrhagic wound	0	1.92	1.99
DP2HE6	Hemorrhagic wound	0	1.26	1.28
DP2HE7	Hemorrhagic wound	0	0.90	1.00
Vibrio tubiashii DC10R4	Distended intestine	0	0.83	0.97
Vibrio spp.				
P3H1	Exophthalmia	0	0.69	0.72
P8H3	Exophthalmia	0	0.85	0.96
A028	Hemorrhagic wound	0	0.73	0.75
25/900	Asymptomatic mortality	0	0.78	0.76
P4R1	Exophthalmia	0	0.80	0.84
DC10R3	Distended intestine	0	0.66	0.65

[a] Values of optical density for the *Vibrio* strains obtained from control test and TSBS supplemented with skin mucus for gilt-head sea bream (A_{600}).

RESULTS AND DISCUSSION

Biological activities detected in the skin mucus. Several studies have reported the presence of different inhibitory substances which prevented the bacterial colonization by pathogenic microorganisms of the skin mucus of salmonids (6, 19) and some marine fish (14, 44). However, in the present study an inhibitory effect of the mucus of gilt-head sea bream on the pathogenic *Vibrio* strains tested has been detected neither by the nephelometric method (Table 1) nor by the dish diffusion

TABLE 2. Adhesive characteristics and LD_{50} of four *Vibrio* strains

Strain	Flagellation	% Adhesion	Agglutination in ammonium sulfate[a]	LD_{50}[b]
V. alginolyticus CAN	Mixed[c]	31.7	1	5.4×10^4
V. alginolyticus DP1HE4	Mixed	44.0	2	2.0×10^5
V. anguillarum-like DC12R8	Polar	100	2	6.8×10^4
V. anguillarum-like DC12R9	Polar	89.2	NT[d]	6.0×10^4

[a] According to the method and criteria of Santos (41).
[b] According to the criteria of virulence of Santos (41): 10^2 to 10^6 cells, high; 10^6 to 10^8 cells, moderate; $\geq 10^8$ cells, nonvirulent.
[c] Mixed, lateral and polar flagella.
[d] NT, not tested.

method (data not shown). Similarly, Magariños et al. (32) have not detected either inhibitory effect of the mucus of sea bass and gilt-head sea bream on *Photobacterium damsela* subsp. *piscicida* (formerly *Pasteurella piscicida*), *Flexibacter maritimus*, *V. anguillarum*, and *Vibrio damsela.*

Different enzymatic activities of the *S. aurata* mucus were tested in the present study, but only the following 12 enzymatic activities were detected: alkaline phosphatase, esterase, esterase-lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-B1-phosphohydrolase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, and α-fucosidase. The high levels of alkaline phosphatase, arylamidase leucine, acid phosphohydrolase, and *N*-acetyl-β-glucosaminidase detected in the skin mucus of gilt-head sea bream did not show bacteriostatic or bactericidal effects on the bacterial strains assayed. In addition, lysozyme activity of this skin mucus was negative, and even all the bacterial strains tested could grow by using the mucus as sole carbon source.

Adhesive processes. Bacterial adhesion to fish tissue surfaces is an important step of the initial stage of infection (45). It has been suggested that the mucous layer covering the host epithelial surface may protect underlying cells from bacterial colonization by inhibiting bacterial attachment to the epithelial cells (9). However, pathogenic microorganisms can establish cellular interactions, and some of them can cross this barrier and penetrate the epithelium (33). The bacterial linkage to the mucus is an essential requirement for the infection by different pathogenic bacteria (43), and thus, the capability of adhesion to mucus is considered a bacterial virulence factor (9, 12).

The origin and percentages of adhesion of all the strains tested are given in Table 1. On the basis of the results, the strains can be grouped into four categories. First is high adhesive capability, strains ranging between 90 and 100% in adhesion rate. Only 10% of the strains tested were in this group, including strains belonging to *V. anguillarum* and *V. anguillarum*-like. Second is moderate adhesive capability, strains that ranged between 75 and 40% in adhesion rate. Only two strains belonging to *V. anguillarum* and *V. alginolyticus* were included in this category. Third are low-adhesive-capability strains that exhibited between 39 and 1% in adhesion rate. Four strains, belonging to *Vibrio fischeri* ($n = 1$), *V. alginolyticus* ($n = 2$), and *V. anguillarum* ($n = 1$), were included in this group. Last, more than 70% of the strains tested ($n = 21$) did not exhibit adhesion capability for the skin mucus of gilt-head sea bream.

The flagellation, adhesive characteristics, and LD_{50} of the four strains selected (ranging between 5.4×10^4 and 2.0×10^5 cells) are summarized in Table 2. As can be observed, the adhesive capability for skin mucus and the mixed or polar flagellation are not essential factors for the virulence of the strains of *Vibrio* tested, since only the *V. anguillarum*-like strains showed the highest adhesive capabilities for mucus (between 89.2 and 100%), while the *V. alginolyticus* strains presenting similar virulence showed adhesion rates lower than 45%.

Adhesive kinetics. In this study, Langmuir's model has been applied. This adsorption isothermic equation has been previously applied to the adsorption of oral streptococci and other microorganisms (3, 10), allowing comparative studies of the bacterial adsorption onto surfaces. Since bacterial concentrations this high are unlikely in natural waters, the affinity, which is not concentration dependent, was selected as the best indicator of relative bacterial attachment rates (16). The discrimination between surface saturation kinetics and proportional adsorption kinetics was artificially imposed, by using the criterion of correlation determined from Lineweaver-Burk plots (Fig. 1). Bacterial adsorption kinetics which correlated at $r \geq 0.9$ was described as surface saturation kinetics, whereas kinetics which correlated at $r < 0.9$ was termed proportional adsorption kinetics according to the criteria proposed by Belas and Colwell (10).

The representation of the Lineweaver-Burk plot has allowed us to differentiate two types of kinetics (Fig. 1). First is a kinetics of saturation which is adjusted to Langmuir's type I. This kind of model indicates a saturation of all the receptors present on skin mucus, although it is not possible to differentiate between the production of a specific linkage and that of an unspecific linkage to occupy all the receptors. This kinetics type is showed by two strains of *V. anguillarum*-like and one strain of *V. alginolyticus* (DP1HE4). Second, *V. alginolyticus* (CAN) showed a proportional adherent kinetics in which saturation of the receptors by linkage with bacteria is not detected. These two types of kinetics were also observed by Belas and Colwell (10) in studying the kinetics of adsorption to chitin by different laterally and polarly flagellated strains of *Vibrio*. These authors concluded that the first type of kinetics occurred when conditions favored lateral flagella production in the bacterial strains, whereas the second type of kinetics was associated with the most polarly flagellated strains and when conditions did not favor the production of lateral flagella. For this, it seems that the lateral flagella represent an important component of the bacterial structure in the adsorption of bacteria

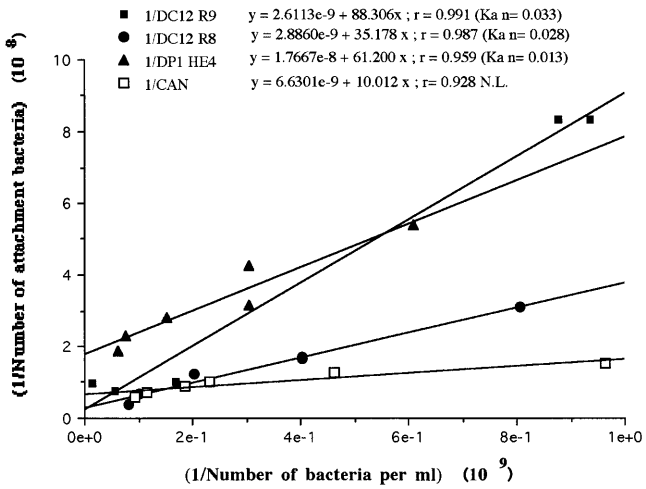

FIG. 1. Adhesive kinetics of the selected *Vibrio* strains obtained for application of Langmuir's model (representing the inverse of number of bacteria). N.L., non-Langmuir adsorption.

TABLE 3. Numbers (10^3) of bacteria adhered per square millimeter of mucus-coated glass slide incubated at different temperatures and salinities[a]

Strain	4°C			15°C			22°C			27°C		
	10‰	17‰	35‰	10‰	17‰	35‰	10‰	17‰	35‰	10‰	17‰	35‰
DC12R9	3.66	11.6	18.8	5.72	10.8	20.1	8.33	14.0	19.0	13.6	19.2	15.1
DC12R8	2.53	2.58	4.06	1.44	1.94	2.00	6.57	12.3	10.0	7.82	6.25	6.70
DP1HE4	2.99	4.80	6.50	0.86	2.18	3.31	3.52	3.44	4.08	3.97	3.46	3.27
CAN	1.76	2.13	5.45	1.72	1.29	4.97	4.45	3.20	3.54	3.13	2.63	3.25

[a] The values represent the arithmethic means of 10 experiments.

to surfaces. However, conflicting results regarding the involvement of flagella in the attachment of pathogenic bacteria such as *Campylobacter jejuni* (17, 35), *Proteus mirabilis* (50), *Pseudomonas fluorescens* (37), *Salmonella typhimurium* (25), and *Vibrio cholerae* (5).

In the present study, the two strains with polar flagellation (*V. anguillarum*-like DC12R8 and DC12R9) showed a surface saturation kinetics. These strains saturated the skin mucus surface, yielding affinity values ($K_a \times n$) higher than those obtained for *V. alginolyticus* DP1HE4 (Fig. 1). These findings are similar to those obtained by Belas and Colwell (10), who observed that the polarly flagellated bacteria did not exhibit a surface saturation kinetics but noted a few exceptions with *V. anguillarum* and *V. cholerae*, suggesting that the adsorption kinetics and the adsorption mechanisms of polarly flagellated bacteria were different from those of laterally flagellated microorganisms, which always showed a surface saturation kinetics. We also detected a difference in adsorption behavior between the two strains of *V. alginolyticus* tested (CAN and DP1HE4), since the strain DP1HE4 showed a surface saturation kinetics, while *V. alginolyticus* CAN showed a proportional adsorption kinetics and aggregates of bacteria adsorbed to the surface of the mucus could be seen in scanning electron microscopy preparations (data not shown). These aggregates were not observed with polarly flagellated strains and *V. alginolyticus* DP1HE4. This difference in behavior between the two *V. alginolyticus* strains could be due to the higher formation of extracellular material by the strain CAN (data not shown). This material could mask other superficial components which could be involved in adherence, preventing the binding with different sites on the surface of mucus, but it also could favor the formation of aggregates among the cells of *V. alginolyticus* CAN. Jacques et al. (24) observed that the presence of capsules resulted in a decrease in binding of *Pasteurella multocida* to respiratory tract mucus and porcine tracheal rings.

Effects of temperature and salinity on the adhesion process. The importance of the temperature on the adhesion of fish pathogenic bacteria has been demonstrated by Laurençin and Germon (29) in experiments carried out with *V. anguillarum* infecting rainbow trout (*Oncorhynchus mykiss* Walbaum, formerly *Salmo gairdneri* Richardson). The minimal values of adhesion for all strains were observed at the lowest values of temperature and salinity assayed (4°C and 10‰, respectively). The numbers of bacteria attached to the mucus-coated glass slides per square millimeter in the different assay conditions are given in Table 3. The highest number of adhered cells of *V. anguillarum*-like were obtained at a salinity of 17‰ and at a temperature of 22°C (strain DC12R8) and at 35‰ and 15°C (strain DC12R9). Considering that the optimal growth temperature for *V. anguillarum* and *V. anguillarum*-like is 25°C (28), it could be assumed that at this optimal temperature the synthesis of adhesive structures such as fimbriae is favored (15). Belas and Colwell (10) reported that the synthesis of lateral flagella was maximal at temperatures and salinities ranging between 15 to 37°C and 10 to 20‰. On the other hand, Amaro et al. (2) observed that the highest mortalities achieved in assays of immersion challenges with *Vibrio vulnificus* biotype 2 occurred in experiments performed at 27 to 29°C and with a salinity of 15‰.

The highest adhesion rates for *V. alginolyticus* were achieved for both strains (CAN and DP1HE4) at 4°C and 35‰ (Table 3). Belas and Colwell (10) also demonstrated that the adsorption of *Vibrio parahaemolyticus* to chitin at 4°C correlated with a proportional kinetics and thus the number of bacteria bound to the substrate was directly proportional to the quantity added to the assay and saturation did not occur. However, these results are in contrast with those obtained in the present study for *V. anguillarum*-like and they are not consistent with those reported by Gordon and Millero (16), who suggested that a higher ionic strength of 0.1 M, such as those found in estuarine or marine waters, produced a decreased bacterial attachment on surfaces such as hydroxyapatite.

The analysis of variance test, conducted with the adhesion values obtained in conditions similar to those of the natural environment, confirmed that (i) the adhesion of *V. anguillarum*-like strains is significantly different in the same conditions of salinity and temperature, this last factor being significant only for the strain DC12R8 ($P < 0.001$), and (ii) the adhesion of *V. alginolyticus* to skin mucus is not significantly different between the strains in all conditions tested. The differences in the degree of adhesion to mucus were due to the influences of temperature and salinity and their interactions with $P < 0.001$.

In short, in this study a very important effect of environmental factors, such as temperature and salinity, on the adhesive process of pathogenic *Vibrio* strains to the skin mucus of gilthead sea bream has been observed. It has also been possible to detect a different adhesive behavior depending on the strain tested and the environmental conditions assayed. These findings, the absence of inhibitory effects of this mucus, and the capability of the pathogenic *Vibrio* strains to use this skin mucus of the fish as a nutrient could facilitate the invasion by these pathogenic microorganisms of the external fish layers. This represents an increase in infection risks, especially important in fish stressed by the manipulations they suffer in the aquaculture industry.

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