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RESEARCH ARTICLE

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Hydrophobicity and adhesion to fish cells and mucus of *Vibrio* strains isolated from infected fish

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Abstract The hydrophobicity of 44 *Vibrio* strains isolated from cultured, diseased gilt-head sea bream (*Sparus aurata*) was determined. Three different methods were used: (1) microbial adhesion to hydrocarbons (MATH), either with phosphate buffer or with phosphate urea magnesium sulfate (PUM) buffer, (2) aggregation in the presence of salt solutions (SAT), and (3) adhesion to nitrocellulose filters (NCF). The results show that experimental conditions exerted a significant influence on hydrophobicity. Thus, Kendall rank coefficients showed the presence of correlation only for SAT and NCF, and for SAT and the MATH assay with PUM buffer. Moreover, no relationships were observed between the bacterial hydrophobicity estimated with the methods mentioned above and the ability of the strains to adhere to fish mucus or cells. These results indicate that adhesion of pathogenic *Vibrio* strains to host surfaces is mediated mainly by specific receptor interactions, instead of by hydrophobic interactions.

Keywords *Vibrio* spp · *Sparus aurata* · Hydrophobicity · Gilt-head sea bream · Fish diseases

Introduction

Adherence of bacteria to the surface layer cells of the host enables potential pathogens to overcome the flushing mechanisms of the body secretions which cleanse mucous membranes [6, 16]. Non-specific adhesion of bacteria to host surfaces has been suggested as the mechanism by which some pathogenic bacteria, such as *Renibacterium salmoninarum* and *Yersinia enterocolitica*, establish and colonize host surfaces during initial stages of the infective process [10, 20]. However, the role of these non-specific hydrophobic interactions in bacterial colonization has not been clearly elucidated [29].

Relative hydrophobicity of bacterial cells has been determined by several methods, such as microbial adherence to hydrocarbons (MATH) [30], hydrophobic interaction chromatography (HIC) [14], aggregation in the presence of different salt solutions (SAT) [23], adhesion to nitrocellulose filters (NCF) [21], and contact angle [35]. Each method has specific advantages and shortcomings; and a wide variation in relative hydrophobicity has been reported, depending on both the method and the type of microorganism [12]. Other types of interaction, such as surface charge and bacterial structures (including extracellular polysaccharides and flagella), also influence bacterial adhesion [17, 18]. The role of each type of interaction is probably dependent on the specific strain and culture conditions.

Vibrio spp have been considered responsible for many disease outbreaks in cultured fish [1, 3, 4, 26, 32]. However, only a few studies have been carried out on the hydrophobicity and adhesive abilities of *Vibrio* strains isolated from cultured marine fish [33]. Therefore, this investigation compared three different methods for determining the hydrophobic capability of 44 *Vibrio* strains isolated from diseased gilt-head sea bream (*Sparus aurata*, L.) cultured in southwestern Spain. The ability of the strains to adhere to skin mucus and erythrocytes from gilt-head sea bream and fish cell lines was also evaluated. The relationship between hydrophobic interactions and adhesive capabilities is discussed.

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Materials and methods

Bacterial strains and growth conditions

Bacterial strains were isolated from *S. aurata* specimens affected by several diseases. They were identified by morphological and biochemical tests as belonging to the genus *Vibrio* [4]. Bacterial cultures

were stored at 80 °C in basal broth (4 g peptone/l, 1 g yeast extract, 10 g NaCl/l) containing 20% glycerol. For the different tests, bacteria were cultured in tryptone soy broth (Oxoid, Basingstoke, UK), with a final concentration of 2% NaCl at 22 °C for 20 h.

Cell-surface hydrophobicity

MATH was performed as described by Rosenberg et al. [30]. Bacteria were centrifuged at 1,000 *g* for 10 min, washed, and resuspended, either in phosphate urea magnesium sulfate (PUM) buffer [containing (per liter): 22.2 g $K_2HPO_4{\cdot}3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.02 g $MgSO_4{\cdot}7H_2O$, pH 7.1], or in phosphate-buffered saline buffer (PBS; 0.02 mol/l, pH 7.2), to an optical density at 400 nm (OD_{400})of 0.9–1.1. Cell suspension aliquots (2 ml) were then transferred to clean, round-bottom test tubes and 0.3 ml *n*-hexadecane (Sigma Chemical Co., St. Louis, Mo., USA) was added. After the mix was homogenized for 2 min, the hydrocarbon phase was allowed to separate completely and the aqueous phase was removed to determine the OD_{400}. The percentage of adhesion to hydrocarbons was calculated using the following expression:

$$\text{Percentage of adhesion} = 100 \times [OD_{400}\ (\text{initial bacterial suspension}) - OD_{400}(\text{aqueous phase})]/OD_{400}(\text{initial bacterial suspension})$$

Bacterial adhesion to NCF was determined according to the technique described by Lachica and Zink [21]. Bacterial cultures were centrifuged at 1,000 *g*, washed as described above, and resuspended in saline solution (0.85% NaCl, pH 7.2) at an $OD_{600}$of 1.0. Suspensions were passed through a 13-mm NCF filter (type CS, 8.0-μm pore size; Millipore, Belford, Mass., USA). The OD_{600} of each filtrate were measured and the percentage of adhesion was expressed as:

$$\text{Percentage of adhesion} = 100 \times [OD_{600}(\text{initial bacterial suspension}) - OD_{600}(\text{filtrate})]/OD_{600}(\text{initial suspension})$$

The SAT assay, described by Lindhal et al. [23], is based on bacterial precipitation in the presence of salts. Bacterial cultures were centrifuged at 1,000 *g*, washed, and resuspended in PBS (0.002 mol/l, pH 6.8), to a concentration of $5{\times}10^9$ cells/ml (OD_{600} of 1.1). Then, 30 μl of the bacterial suspensions were mixed with equal volumes of decreasing molarities of buffered ammonium sulfate solutions in the range 0.025–4.0 mol/l. Hydrophobicity was expressed as the lowest molarity [$(NH_4)_2SO_4$)] in a mixture which produced visual clumping. Molarity values were converted to percentages by considering 0% hydrophilicity as the aggregation at 0 mol ammonium sulfate/l and 100% hydrophilicity as the aggregation at 4 mol ammonium sulfate/l (maximum concentration). Kendall rank coefficients were calculated to determine the correlation between the different hydrophobicity tests assayed.

Adhesion to skin mucus, erythrocytes, and tissue culture cells

Adhesion of the strains to mucus was determined according to Krovacek et al. [19], as modified by Balebona et al. [2]. Raw skin mucus was scraped from the surface of gilt-head sea bream specimens and suspended in sterile seawater. Mucus suspensions were purified by double-centrifugation 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. Then, 300 μl of the mucus suspensions were placed on microscope slides and dried overnight at room temperature. After fixation with methyl alcohol for 20 min, mucus-coated glass slides were used for adhesion assays.

Adhesion to erythrocytes was performed using gilt-head sea bream erythrocyte suspensions (3% v/v) in PBS (0.01 mol/l, pH 6.8). Equal volumes of bacterial suspensions (10^{10} cells/ml) and erythrocyte suspensions were mixed on glass slides. The test was considered negative if visible agglutination did not occur within 10 min.

Two fish cell lines were used for adhesion assays, Chinook salmon embryo (CHSE) and epithelioma papullosum of carp (EPC). Cells were maintained in Eagle minimum essential medium (MEM) containing 10% fetal calf serum, and 1% antibiotics [(10,000 IU penicillin, 10 mg streptomycin)/ml]. Fish monolayers were trypsinized and resuspended in fresh MEM medium at a concentration of $2{\times}10^6$ cells/ml. Then 30 ml of the cell suspension were placed in a Petri dish containing sterile polystyrene slides and incubated for 4–5 days. At 2 h before performing the adhesion experiments, the old medium was replaced by fresh medium without antibiotics. The slides were kept in the fresh medium for 2 h before being washed with 0.145 mol NaCl/l and fixed with methyl alcohol.

To perform the adhesion assays, bacterial suspensions containing 10^8 cells/ml were placed in Petri dishes containing mucus or cell-coated glass slides and incubated at 20 °C with gentle shaking for 1 h. After being washed thoroughly with 0.145 mol NaCl/l, slides were air-dried and fixed with methyl alcohol for 20 min. Then, slides were stained with crystal violet and examined under the microscope.

Results

Different results were obtained, depending on the method used to estimate cell-surface hydrophobicity (Table 1). Most of the isolates (90.9%) aggregated in the presence of <1 mol ammonium sulfate/l. Adhesion to NCF yielded the highest values of hydrophobicity for most of the isolates, the percentages of adhesion ranging from 17.4% to 99.5%. A variable degree of adhesion to *n*-hexadecane was observed, with percentages of adhesion ranging from 0% to 41% when PUM buffer was used as aqueous phase and from 8.6% to 46.9% when PBS buffer was used.

The criteria of hydrophobicity proposed by different authors [22, 32] were used to evaluate the hydrophobicity of the *Vibrio* strains assayed in the present study (Table 2). According to these criteria, none of the isolates was highly hydrophobic with the MATH assay, using PBS or PUM buffer. However, 11.4% of the strains showed strong hydrophobicity with SAT and 68.2% with the NCF assay (Table 2). Most of the isolates were considered to have moderate or weak hydrophobicity with SAT (88.7%) and MATH-PBS (63.6%), whereas 11.4% and 25% of the strains were included in the moderately hydrophobicity group using the NCF and MATH-PUM assays.

Kendall rank coefficients showed a significant correlation ($P<0.05$) between SAT and the MATH-PUM assay ($P=0.013$) and between SAT and the NCF assay ($P=0.050$). However, no correlation was observed among the other tests assayed ($P>0.05$). A weak correlation between MATH assays in the presence of PBS or PUM buffer was detected ($P=0.055$). These findings suggest that adhesion to *n*-hexadecane is influenced by the use of PBS or PUM buffer as an aqueous phase.

A total of 24 strains were selected on the basis of their different biochemical profiles. The results of the

assay of adhesion of these strains to mucus, erythrocytes, CHSE cells, and EPC cells showed that 33.3% of the isolates adhered to skin mucus from gilt-head sea bream. Adhesion to CHSE cells and sea bream erythrocytes was observed in 29.2% of the strains, whereas one only isolate (4.2% of the strains tested) adhered to EPC cells (Table 3). All *V. alginolyticus* and *V. anguillarum* strains assayed showed adhesion to skin

Table 1 Determination of cell-surface hydrophobicity of *Vibrio* strains using different methods. *MATH* Microbial adhesion to hydrocarbons (percentage of adherence to *n*-hexadecane), *PUM* phosphate urea magnesium sulfate buffer, *PBS* phosphate-buffered saline, *NCF* nitrocellulose filters (percentage of adherence), *SAT* salt solutions (lowest molarity of ammonium sulfate producing visible aggregation)

Bacterial strains	MATH			
	PUM	PBS	NCF	SAT
Vibrio anguillarum				
DC12R7	0.0	11.7	17.4	2.00
DC11R2	13.9	24.9	36.5	2.00
DC12R8	22.1	44.1	46.7	2.00
V. alginolyticus				
CAN	0.3	9.0	50.0	1.00
AO35	10.2	28.8	77.6	1.00
DP1HE4	0.0	36.4	78.5	2.00
110	41.0	27.3	55.3	1.00
139	8.0	23.9	47.4	1.00
21	17.7	25.4	76.5	0.02
V. harveyi				
28	15.2	37.9	94.0	2.00
DP1U3	7.9	23.5	84.5	2.00
DP2HE6	6.6	15.6	98.2	1.00
P8H5	21.1	41.5	75.4	1.00
DP1U1	7.4	26.8	99.5	1.00
DP1U2	8.3	14.2	97.3	2.00
V. fischeri				
140	18.0	39.0	92.1	2.00
P3O4	15.6	17.7	92.5	2.00
P5O5	21.1	46.9	98.9	2.00
P8H1	26.0	32.2	61.7	1.00
V. tubiashii				
DC10R4	9.7	16.4	96.5	2.00
Vibrio spp				
P3H2	25.6	21.7	42.9	0.05
127	2.4	26.7	48.9	2.00
V3	22.0	17.9	74.7	1.00
P4R1	21.0	30.4	91.4	2.00
141	4.5	17.5	92.1	0.40
P3H3	20.9	38.8	59.1	1.00
P5O3	12.4	24.0	99.0	1.00
P8R3	22.1	27.2	93.6	1.00
DC7R1	4.7	32.5	97.6	0.01
128	3.0	23.0	94.9	2.00
122	9.6	25.1	94.5	2.00
P4R2	4.6	15.0	83.5	1.00
AO28	13.5	17.5	39.2	2.00
DP2HE3	18.3	21.8	98.7	2.00
DC10R3	11.0	14.4	86.3	1.00
DC11R1	6.5	13.2	95.8	1.00
AO30	16.0	13.8	47.2	1.00
25900	18.1	17.2	38.4	1.00
143	0.0	30.3	85.8	2.00
144	15.1	30.4	93.3	2.00
104	5.6	38.3	77.8	0.50
105	10.4	20.0	77.5	2.00
25	21.5	8.6	97.5	1.00
114	16.2	17.1	82.8	1.00

Table 2 Hydrophobicity degree of the strains using SAT, NCF and MATH assays, according to the criteria proposed by Santos et al. [32] and Lee and Yii [22]. *PBS* and *PUM* buffers were used as aqueous phase

Test	Values	Hydrophobicity degree	Percentage of *Vibrio* isolates
SAT	0–1.0M	Strong	11.4
	1.0–2.0M	Moderate	43.2
	2.0–4.0M	Weak	45.5
	>4.0M	Negative	0.0
NCF	>75%	Strong	68.2
	50–75%	Moderate	11.4
	<50%	Negative	20.5
MATH (PBS)	>50%	Strong	0.0
	20–50%	Moderate	63.6
	<20%	Negative	36.4
MATH (PUM)	>50%	Strong	0.0
	20–50%	Moderate	25.0
	<20%	Negative	75.0

Table 3 Adhesion of *Vibrio* strains to skin mucus and erythrocytes from gilt-head sea bream, Chinook salmon embryo cells (*CHSE*) and Epithelioma papullosum cells of carp (*EPC*)

Strain	Adhesion to			
	Mucus	CHSE	EPC	Erythrocytes
V. anguillarum				
DC12R7	+	+	+	–
DC11R2	+	+	–	+
DC12R8	+	+	–	+
V. alginolyticus				
CAN	+	+	–	–
AO35	+	+	–	–
DP1HE4	+	+	–	–
V. harveyi				
DP1U3	–	–	–	–
DP2HE6	–	–	–	–
DP1U1	–	–	–	–
DP1U2	–	+	–	–
V. fischeri				
140	+	–	–	+
P3O4	–	–	–	+
P5O5	–	–	–	–
P8H1	+	–	–	–
V. tubiashii				
DC10R4	–	–	–	–
Vibrio spp.				
P3H2	–	–	–	–
V3	–	–	–	+
P4R1	–	–	–	–
P8R3	–	–	–	–
DC7R1	–	–	–	+
128	–	–	–	–
AO28	–	–	–	–
DC10R3	–	–	–	–
25900	–	–	–	+

mucus and CHSE cells. However, only one *V. anguillarum* strain was positive for adhesion to EPC cells and two strains for adhesion to erythrocytes. None of the *V. harveyi* or *V. tubiashii* strains was positive for adhesion to mucus, EPC cells, and erythrocytes, only one *V. harveyi* strain being able to adhere to CHSE cells. In the group of *V. fischeri* strains, one isolate showed adhesion to skin mucus and erythrocytes and two strains adhered only to mucus or erythrocytes. With regard to *Vibrio* spp, 12.5% of the strains were able to adhere to sea bream erythrocytes, but none of them adhered to mucus, CHSE cells, or EPC cells. No direct relationship was observed between the adhesion and hydrophobic properties of *Vibrio* isolates (Table 3).

Discussion

The mechanisms by which bacterial cells may adhere to epithelial cells or colonize cellular secretions (mainly mucins) are often unknown, although different types of interaction have been reported to be involved [8, 15]. The *Vibrio* strains we studied showed wide differences in their hydrophobicity, and we observed no consistent patterns of hydrophobicity for the different species. These results confirm the high variability of the hydrophobicity reported by other authors for strains of *V. anguillarum*, motile *Aeromonas*, *A. salmonicida*, and *Y. ruckeri* [31]. This variability may be the result of differences in arrays of surface molecules of bacterial cells, mainly fimbriae and proteins of the outer membrane [8, 11]. Methods for measuring bacterial hydrophobicity differ somewhat in the precise properties they measure; and different types of interaction are considered when different methods are used to estimate hydrophobicity. Thus, Dickson and Koohmaraie [12] observed that the relative hydrophobicity estimated by hydrophobic interaction chromatography (HIC), microbial adhesion to hydrocarbons (MATH), and contact angle measurements for different bacterial species was dependent on the specific method tested. The results obtained in this study for *Vibrio* strains isolated from fish show that the experimental conditions imposed by the different methods used influence the observed hydrophobic interactions to some degree. Kendall rank coefficients showed that estimates of hydrophobicity with the NCF assay and MATH assays using PBS or PUM buffer are significantly different. However, a significant correlation was obtained between SAT and the NCF and MATH-PUM assays.

The complexity of the cell-surface mosaic resulting from hydrophobic and hydrophilic appendages and other macromolecular components might give rise to different sensitivities with different assay methods [20]. Thus, for a reliable assessment of hydrophobicity, a standard set of culture and preparation conditions should be adopted and a range of assay methods employed. Nevertheless, the correlation observed between the results obtained with SAT and the NCF and MATH assays, in conjunction with the fact that the SAT assay is easily performed, highlights the convenience of this test for estimating the cell-hydrophobicity of *Vibrio* strains.

Kendall rank coefficients showed that MATH assays using PBS and PUM buffer differ significantly from each other. Thus, percentages of adhesion to *n*-hexadecane obtained in the assays carried out using PBS as an aqueous phase were higher for most of the strains (Table 1). Lower values of hydrophobicity detected in assays carried out with PUM buffer could be attributable to the different ionic strength of this buffer [28]. The MATH assay, as described originally by Rosenberg et al. [30], used PUM buffer containing sulfate and urea to enable growth of *Acinetobacter calcoaceticus* RAG-1 when hexadecane was present. This approach facilitated the measurement of adhesion under conditions simulating growth and it showed that adhesion plays a major role in the growth of RAG-1 on hexadecane. In the case of *Vibrio* strains, although it did not result in cell lysis, magnesium sulfate and urea could interfere in the adhesion of bacterial cells to hexadecane, thus yielding lower percentages of adhesion, compared with the assays using PBS buffer.

Specific hydrophobicity assays may be useful predictors of adhesion for closely related strains of certain bacterial species, as some authors have suggested [24]. However, when working with taxonomically diverse bacteria, it may happen that a measure of surface hydrophobicity that would predict their capacity as a group to adhere to a substratum could not be identified. In the case of *Vibrio* spp isolated from diseased *S. aurata*, the results obtained in the hydrophobicity tests indicate that adhesion of these bacteria cannot be explained by hydrophobic interactions alone. Rather, adhesion is likely to be mediated by an interplay of hydrophobic and hydrophilic surface components [13, 34].

Some *Vibrio* spp showed the ability to adhere to skin mucus and fish cell lines, regardless of the degree of hydrophobicity recorded with the different methods (Table 3). This result indicates that different types of interactions are involved in the adhesion process. The importance of hydrophobic interactions in the adherence of microorganisms to cells has been indicated for many microorganisms [24, 30]. However, there is still some controversy concerning the relative importance of cell-surface hydrophobicity; and some authors relegate the overall role of hydrophobic interactions to a contributing factor in the adherence process [27], whereas others report no correlation [5]. In general, hydrophobicity was lower in the isolates which had adhesive abilities than in the isolates that did not adhere to the mucus or fish cells (Table 3). These findings indicate that adhesion of *Vibrio* strains to mucus and fish cells is not merely mediated by hydrophobic interactions, but that other forces or structures must be involved in bacterial adhesion. Several authors have pointed out the implication of specific molecules, the so-called adhesins, in the adhesion of bacteria to host cells. These molecules specifically

recognize components of the mucus or fish cells that can play a role in the colonization process. In this way, the absence of several proteins in the cell wall of *Listeria monocytogenes* strains has been related to a lack of the ability to adhere to host tissues [13]. Moreover, a proteinaceous factor has been implicated in the adhesion of *Lactobacillus acidophilus* to human enterocytes [9].

The ability of *Vibrio* strains to bind to mucus might be advantageous for the bacterium in the colonization of the mucus layer, as Paerregaard et al. [25] suggested. For such colonization to occur, the mucus-adherent bacteria must multiply at a rate exceeding that at which they are sloughed from the mucus. In previous work, we demonstrated that *V. anguillarum* and *V. alginolyticus* strains could degrade sea bream mucus and use it as source of nutrients [7]. Thus, although further studies are required, the results obtained in the present study show that these bacterial species, after adhesion by different mechanisms where hydrophobic interactions cannot be ruled out, can proliferate and colonize fish surfaces.

Pathogenic *Vibrio* strains isolated from cultured *S. aurata* show different degrees of hydrophobicity, when estimated with SAT, NCF, and MATH assays. However, no close relationship has been detected between hydrophobicity and the ability to adhere to fish mucus and cells. Thus, adhesion of *Vibrio* strains isolated from diseased gilt-head sea bream to fish surfaces must be mainly mediated by non-hydrophobic interactions.

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