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Portal of entry for pathogenic *Vibrio alginolyticus* into large yellow croaker *Pseudosciaena crocea*, and characteristics of bacterial adhesion to mucus

Qiang Chen[1], Qingpi Yan[1,*], Kejuan Wang[2], Zhixia Zhuang[3], Xiaoru Wang[3]

[1]Fisheries College, Jimei University, Xiamen, Fujian 316021, PR China
[2]State Key Laboratory of Marine Environmental Science and [3]College of Chemistry & Chemical Engineering, Xiamen University, Xiamen, Fujian 316005, PR China

ABSTRACT: The portal of entry for pathogenic *Vibrio alginolyticus* into large yellow croaker *Pseudosciaena crocea* is via the intestinal tract rather than gill or skin according to the kinetics of the bacterial adhesion to different mucus. The different effects on adhesion caused by proteolytic enzymes and heat treatment might be due to the different chemical compositions of mucus. Adhesion of *V. alginolyticus* to mucus depends on concerted action of bacterial surface structures such as cell-surface proteins, somatic antigens, flagella, etc. In addition, starvation and monosaccharides, especially fructose, inhibit the bacterial adhesion to the mucus. Knowledge of these adhesive characteristics should be very useful for designing more efficacious prophylactic strategies.

KEY WORDS: *Vibrio alginolyticus* · Adhesion · *Pseudosciaena crocea* · Mucus

INTRODUCTION

Large yellow croaker *Pseudosciaena crocea* is one of the main maricultured fish in southern China. Several pathogenic microorganisms such as *Shewanella putrefaciens* (Lin et al. 1999), *Vibrio harveyi* (Lin et al. 1999), *V. parahaemolyticus* (Li & Chen 2001) and especially *V. alginolyticus* (Wang et al. 2001, Yan et al. 2001) have been associated with epizootics in cultured large yellow croaker and have caused considerable losses.

Bacterial adhesion to fish tissue surfaces is an important step in the initial stage of infection (Thune et al. 1993). Therefore, the inhibition of adhesion of bacteria to the host may be useful therapeutically (Acord et al. 2005). The adhesive properties of pathogenic bacteria have been studied *in vitro*, including bacterial adhesion to tissue culture cells (Chen & Hanna 1992), red blood cells (Santos et al. 1991), and mucus (Balebona et al. 1995, Bordas et al. 1996, Yan et al. 2007).

A mucous layer covering the epithelium is found on primary and secondary gill lamellae, on the skin and along the gastrointestinal tract. Mucus is the first physical barrier to the entry of pathogenic organisms from the environment into the fish. There are specific immunoglobulins, agglutins, lectins, lysins and lysozyme in the mucus (Shephard 1994), which can kill or inhibit the invading organisms.

However, there have been few studies on the characteristics of mucus associated with different tissues and on the adhesion of pathogenic bacteria to different mucus. In this study, we attempted to uncover the portal of entry for pathogenic *Vibrio alginolyticus* into large yellow croaker by probing the characteristics of bacterial adhesion to the mucus on the skin, gill lamellae and intestinal tract of large yellow croaker.

MATERIALS AND METHODS

Bacterial strain and growth conditions. Pathogenic *Vibrio alginolyticus* (ND-01) was previously isolated and identified from naturally infected large yellow croaker *Pseudosciaena crocea* by our team and confirmed as pathogenic by artificial infection (Yan et al.

*Corresponding author. Email: yanqp@jmu.edu.cn

2001). The strain was stored at –80°C in physiological saline with 10% glycerol. Bacteria were cultured in tryptic soy broth (TSB) supplemented with 2% NaCl at 25°C for 12 h. For the adhesion assay, [methyl-^{3}H]-thymidine (30 Ci mmol^{-1}, obtained from the Shanghai Institute of Applied Physics, Chinese Academy of Sciences) was added to the medium at a final concentration of 10 µl ml^{-1} to radiolabel the bacterial cells.

Mucus preparations. Healthy large yellow croakers were obtained from marine cultured cages (Xiamen). Blood was completely drained from the caudal vessel after anesthetizing the fish with eugenol. Skin mucus was carefully scraped from the dorsal body using a plastic spatula and put into autoclaved tubes containing a small amount of sterilized seawater (filtered by a 0.22 µm filter) (Bordas et al. 1996). In order to prevent contamination by intestinal contents and sperm, ventral skin mucus was not collected. Gill mucus was prepared by cutting out the gill arches, then scraping off the mucus and putting it into autoclaved tubes containing sterilized seawater (Larsen et al. 2001). Intestinal mucus was prepared using a method modified from the one described by Olsson et al. (1996). The intestines were removed and divided into foreguts and hindguts. Both foreguts and hindguts were transferred to autoclaved petri dishes and washed by autoclaved phosphate buffered saline (PBS, 0.01 mol l^{-1} pH 7.2). These guts were then split open with a scalpel. The foregut and hindgut mucus were harvested by scraping off the inner surface of the intestines with a plastic spatula to remove the mucous gel layer covering the intestinal epithelium. Finally, this mucus was homogenized in PBS.

The mucus preparations were centrifuged twice at 20 000 × *g* at 4°C for 30 min to remove particulate and cellular materials. The final supernatant was filtered through 0.45 and 0.22 µm pore size filters. The mucus samples were adjusted to 1 mg protein ml^{-1} with seawater or PBS. The protein concentration was determined using the method of Bradford (1976).

Characteristics of the mucus. Immunoglobulin (IgM) in mucus was determined using an enzyme-linked immunosorbent assay (ELISA). A 100 µl volume of 2-fold serial diluted mucus was immobilized on polystyrene microtiter plates (Corning Costar 9018) at 4°C overnight, and physiological saline was used as the blank control. After washing 3 times with PBS (0.05 mol l^{-1}, pH 7.4) supplemented with 0.05% of Tween-20 (PBST), the wells were blocked for 1 h at 37°C by 300 µl of 1% (wt/vol) bovine serum albumin (BSA) and then washed. The wells were incubated for 1 h at 37°C with rabbit polyclonal antiserum against IgM of large yellow croaker (previously prepared by our lab, diluted 1:5000 by volume) and then washed. The control samples were prepared by adding serum from non-immunized rabbits. Next, 100 µl of goat anti-rabbit IgG-horse radish peroxidase (Sino-American Biotechnology) diluted in PBST-0.5% BSA (1:1000) was added and incubated at 37°C for 1 h and then washed. After incubation in the dark at room temperature for 30 min with 50 µl of the developing solution (OPD, BioBasic), the reaction was stopped by 50 µl 2 mol l^{-1} H_2SO_4. The optical density (OD) value of each well was determined at 492 nm by a microplate reader (Thermo Multiskan MK3).

The bacteriostatic effect of the mucus was evaluated by nephelometry. Briefly, log-phase bacterial cells were collected and the density of bacterial suspension was adjusted to 10^5 CFU (colony forming units) ml^{-1} by autoclaved physiological saline. 100 µl TSB, 100 µl bacterial suspension, and 100 µl mucus were mixed in the wells of microtiter plates. The OD values at 550 nm (OD_{550}) were recorded every hour from 1 h to 12 h, and then at 15 h and 18 h. The control group was preformed by adding 100 µl of seawater or PBS rather than mucus.

Lysozyme activities of skin, gill and intestinal mucus were analyzed according to the method of Grinde et al. (1988). A standard sample was assayed as a positive control. Mucus was incubated with *Micrococcus lysodeikticus* (0.2 mg ml^{-1}, Sigma), at pH 6.4, 25°C, and the change in OD was recorded at 530 nm for 3 min.

***In vitro* adhesion assay.** The bacterial adhesion assay was conducted according to the method described by Vesterlund et al. (2005). Briefly, 150 µl of mucus was added to the wells of microtiter plates and incubated overnight at 4°C to fix the mucus. After the incubation, any unbound mucus was removed by washing the wells twice with 200 µl of sterile physiological saline. Next, 150 µl aliquots of radiolabelled bacteria were added to the wells and allowed to set at 25°C for 1.5 h. The wells were washed twice with 200 µl of sterile physiological saline to remove any non-adhering bacteria. The materials remaining in the wells were adhering bacteria which were released and lysed with 150 µl 1% SDS (sodium dodecyl sulphate)-0.1 mol l^{-1} NaOH through incubation at 60°C for 1 h. The contents of each well were transferred to 20 ml scintillation vials, which contained 10 ml scintillation fluid, and left for 12 h. Radioactivity (disintegrations per minute; DPM) was determined using a liquid scintillation counter (Packard Tri-Carb 4640). The number of adhering bacteria was assessed from the radioactivity recovered from the wells ($Y = 0.0009 \times X - 147.93$, $r^2 = 0.995$, where X is the number of adhering bacteria and Y is the radioactivity). Each adhesion assay was performed in triplicate.

Adhesive kinetics: To study the kinetics of adhesion of *Vibrio alginolyticus* to skin, gill and intestinal mucus, the bacteria were allowed to adhere to the

mucus at concentrations between 6.41×10^8 and 1.66×10^7 CFU ml^{-1}. The principle of Michaelis-Menten enzyme kinetics, described by Lee et al. (2000), was adopted here to study the adhesion of *V. alginolyticus* to mucus. The plots of the reciprocal of adhering bacterial number versus the reciprocal of the concentration of bacteria added together yielded a linear relationship, according to:

$$1/e_x = 1/e_m + k_x/e_m \times x$$

where e_x is the number of adhering bacteria, e_m is the maximal value of bacterial adhesion, k_x is the dissociation constant, and x is the concentration of the bacteria added. The values of e_m and k_x were calculated from data presented in Fig. 4.

Proteolytic enzyme treatment or heat treatment: Trypsin or Proteinase K in physiological saline (1 mg ml^{-1}) was added to the wells of microtitre plates containing immobilized mucus, and the plates were incubated at 25°C for 1.5 h. The plates were then washed to remove the proteolytic enzyme, and the adhesion assay was carried out as described above. In another test, the proteolytic enzymes were applied to bacterial suspension for 10 min at 25°C; the bacteria were then washed twice with physiological saline before the adhesion assay. The control group was prepared by treating the mucus or the bacteria with physiological saline without enzyme.

The mucus was heated at either 50, 75 or 100°C for 10 min each and then immobilized to microtitre plates for the adhesion assay. The mucus without heat treatment was used as the control.

Influence of antiserum, starvation and monosaccharide on bacterial adhesion: O-antiserum and H-antiserum against *Vibrio alginolyticus*, which had previously been prepared in New Zealand rabbits in our lab, were used to evaluate their inhibition upon the adhesion of *V. alginolyticus*. Aliquots (100 µl) of O- or H-antisera were separately mixed with 900 µl bacterial suspension, and incubated at 25°C for 1 h, and the mixture was then washed with physiological saline. Bacteria treated with PBS instead of antiserum were used as the control.

Log-phase bacterial cells were harvested and suspended in physiological saline at a density of 10^8 CFU ml^{-1}. After starvation at 25°C for various time intervals (0, 1, 2, 3, 4, or 10 d) 3 aliquots of starved bacteria were sampled for the adhesion assay.

A bacterial suspension was pre-incubated at 25°C for 1 h with different kinds of monosaccharides — D(+)glucose, D(+)mannose, D(–)fructose, and D(+) galactose — which were prepared in physiological saline at a final concentration of 1 mg ml^{-1}. Finally, the mixture was washed with physiological saline. Bacterial suspensions pre-incubated with physiological saline without monosaccharide were used as the control.

Analysis of mucus by SDS-PAGE. Skin, gill and intestinal mucus were subjected to SDS-PAGE (SDS-polyacrylamide gel electrophoresis; 12%) under reducing conditions according to Laemmli (1970). The marker (ca. 14.4 to 116.0 kDa) was used to calibrate the molecular weight of the mucous proteins. The gel was stained with silver stain.

Statistical analysis. All data were expressed as means ± SD. The difference among mean values was analyzed using Student's *t*-test (SPSS 12.0 for Windows). Values of $p < 0.05$ were considered significant.

RESULTS

Biological characteristics detected in the mucus

Fig. 1 shows the IgM titers in skin, gill and intestinal mucus; the highest titer of IgM was found in skin mucus ($p < 0.05$). Of the 4 kinds of mucus, only skin mucus exhibited lysozyme activity (Table 1). The OD values at 550 nm (OD_{550}) of mucus–*Vibrio alginolyticus* mixtures increased with the incubation time. The OD values at OD_{550} of mucus groups exceeded those of control groups after incubation for 18 h (Fig. 2). The results indicated that the mucus of large yellow croaker had no bacteriostatic effect on the pathogenic *V. alginolyticus.* Fig. 3 shows the difference in chemical component among the 4 kinds of mucus. Foregut mucus and hindgut mucus exhibited a similar SDS-PAGE profile, which was quite different from those of skin mucus and gill mucus.

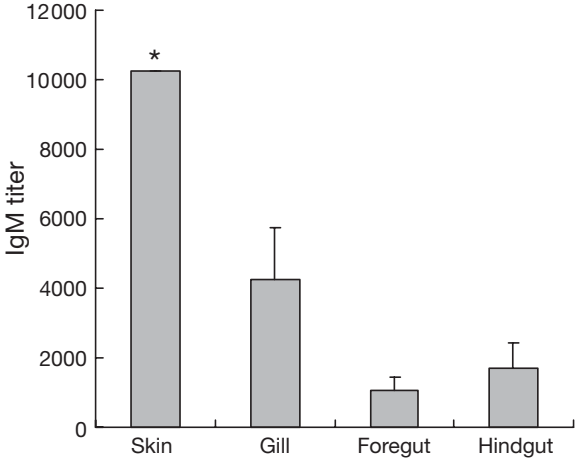

Fig. 1. Determination of immunoglobulin (IgM) present in the 4 mucus types. The highest dilution of mucus in which the ratio of optical density $OD_{492\ tested}$ to $OD_{492\ control} > 2$ was considered as the IgM titer in mucus. *Compared with other mucus groups, $p < 0.05$. Error bars represent mean + SD

Table 1. *Vibrio alginolycticus*. Mean (±SD) optical density values at 530 nm (OD_{530}) at 0 and 3 min and lysozyme activity of different mucus

	Standard	Mucus			
		Skin	Gill	Foregut	Hindgut
OD_{530nm}					
0 min	0.607 ± 0.018	0.707 ± 0.012	0.736 ± 0.015	0.746 ± 0.015	0.703 ± 0.006
3 min	0.178 ± 0.010	0.684 ± 0.016	0.740 ± 0.020	0.751 ± 0.014	0.721 ± 0.007
Lysozyme activity ($U\ ml^{-1}$)	2000	107 ± 30	–	–	–

Kinetics of adhesion of *Vibrio alginolyticus* to different mucus

Fig. 4 presents the hyperbola of the concentration of bacteria added versus the number of bacteria adhering on different mucus. A linear relationship was observed in all 4 kinds of mucus. Table 2 summarizes the values of bacterial adhesion (e_m), dissociation constant (k_x), and affinity index (e_m/k_x). Hindgut mucus exhibited the highest e_m and k_x, while the highest value for e_m/k_x was found in foregut mucus ($p < 0.05$).

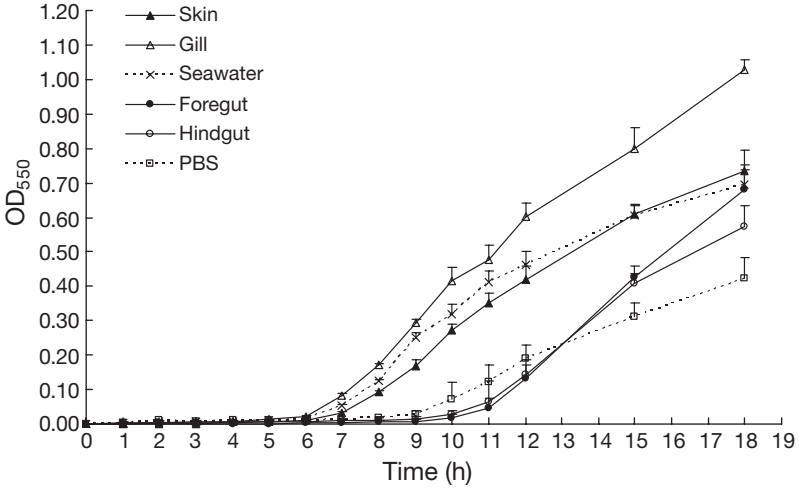

Fig. 2. *Vibrio alginolyticus*. Growth curve of incubation with different mucus. Ordinate is optical density at 550 nm (OD_{550}). Seawater: control for the skin and gill mucus groups; PBS (phospate buffered saline): control for the intestinal (foregut and hindgut) mucus groups. Error bars represent mean + SD

Adhesion of *Vibrio alginolyticus* to the mucus after different treatments

Heat treatment. Adhesion of *Vibrio alginolyticus* to the mucus after heat treatment is presented in Fig. 5. Incubating the mucus at 50°C for 10 min resulted in more bacterial adherence to skin mucus compared with the control group ($p < 0.05$), whereas incubating at 75 or 100°C for 10 min reduced the number of *V. alginolyticus* adhering to gill mucus ($p < 0.05$). No significant difference was found in the bacterial adhesion to intestinal mucus after heating.

Proteolytic enzyme treatment. Fig. 6A shows that pre-treatment of skin mucus with Proteinase K reduced the number of *Vibrio alginolyticus* adhering to the mucus ($p < 0.05$), but the enzyme treatment had no significant effect on the number of bacteria adhering to gill or intestinal mucus ($p > 0.05$). Fig. 6B shows the effect on bacterial adhesion of treating the bacteria with proteolytic enzymes. The bacterial adhesion to skin, gill or intestinal mucus was remarkably inhibited by treating the bacteria with Proteinase K ($p < 0.05$). Trypsin treatment had a significant effect on the bacterial adhesion to skin and foregut mucus ($p < 0.05$), but no significant effect on the bacterial adhesion to gill mucus and hindgut mucus.

Antiserum treatment. The results showed that O-antiserum treatment resulted in 54, 68, 56, and 47% reduction in adhesion quantities of *Vibrio alginolyticus*

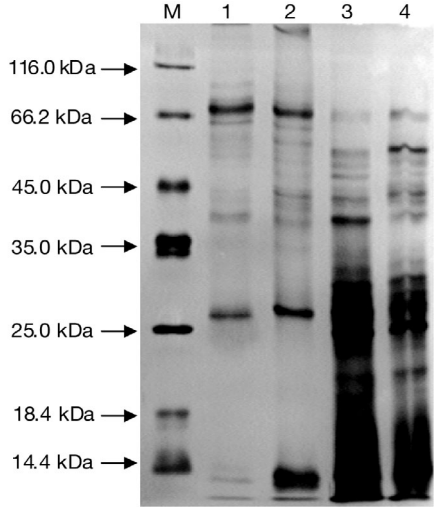

Fig. 3. Profile on SDS-PAGE. M: marker; 1: skin mucus; 2: gill mucus; 3: foregut mucus; 4: hindgut mucus

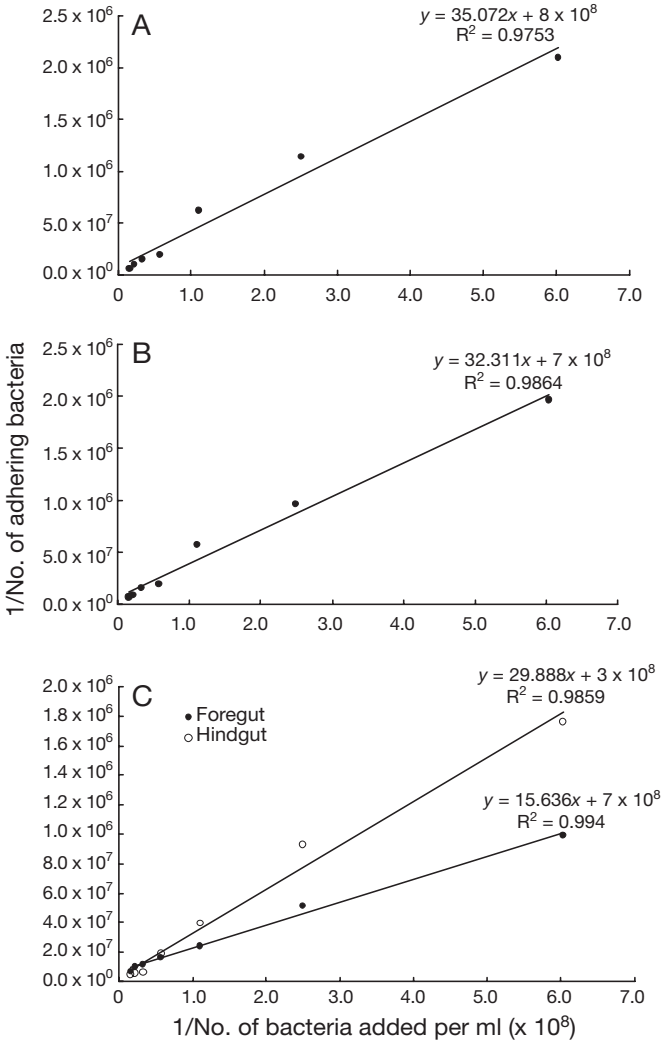

Fig. 4. *Vibrio alginolyticus*. Adhesive hyperbola to different mucus. (A) Skin mucus, (B) gill mucus, (C) intestinal (foregut and hindgut) mucus. Data are mean values calculated from 3 experiments. Note *x*-axis values are $\times 10^8$

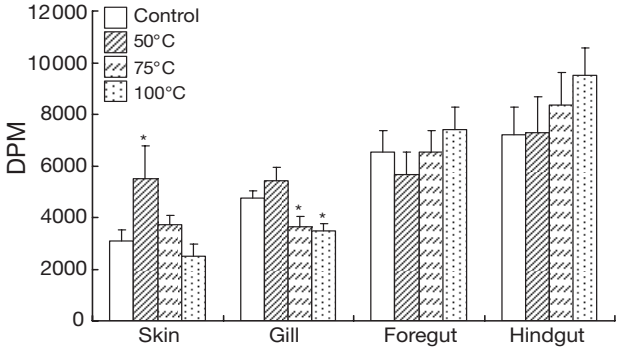

Fig. 5. *Vibrio alginolyticus*. Adhesion to mucus after heat treatment. Data are shown as disintegrations per minute (DPM) which reflected the number of adhering bacteria. *Compared with control group (without heat treatment), $p < 0.05$. Error bars represent mean + SD

Starvation treatment. Starved bacteria showed a sharp reduction in adhesion of *Vibrio alginolyticus* to skin mucus and gill mucus (Table 3). The number of bacteria adhering to skin and gill mucus decreased by 53 and 66%, respectively, after starvation for 1 d. After starvation for 4 d, the bacterial adhesion quantities were close to the blank level (radioactivity of ISO µl 1% SDS–0.1 M NaOH without labeled bacteria). Results from Yan et al. (2007) indicated that starvation of *V. alginolyticus* caused a marked reduction in the number of bacterial adhesion to the intestinal mucus.

Monosaccharide treatment. Fig. 8 shows the effect of monosaccharides on bacterial adhesion. Fructose exhibited a remarkable inhibition effect on the adhesion of *Vibrio alginolyticus* to all mucus investigated. Mannose and galactose significantly inhibited the bacterial adhesion to gill mucus. Mannose also inhibited the bacterial adhesion to hindgut mucus. Glucose showed no significant inhibition effect on the bacterial adhesion to any mucus.

to skin, gill, foregut and hindgut mucus, respectively, and H-antiserum treatment reduced the bacterial adhesion by 21, 61, 52, and 39% (Fig. 7). Both O-antiserum and H-antiserum treatment effectively inhibited the adhesion of *V. alginolyticus* to the mucus.

Table 2. *Vibrio alginolyticus*. Maximum number of adhering bacterial cells (e_m), dissociation constant (k_x) and affinity index (e_m/k_x). Values are mean ± SD
*Compared with other mucus groups, $p < 0.05$

Mucus	e_m (cells ml^{-1})	k_x (cells ml^{-1})	e_m/k_x
Skin	$1.25 \times 10^7 \pm 8.23 \times 10^6$	$4.38 \times 10^8 \pm 3.26 \times 10^7$	0.0285 ± 0.0005
Gill	$1.43 \times 10^7 \pm 9.12 \times 10^6$	$4.62 \times 10^8 \pm 4.69 \times 10^7$	0.0309 ± 0.0006
Foregut	$1.43 \times 10^7 \pm 7.56 \times 10^6$	$2.23 \times 10^8 \pm 3.12 \times 10^7$	0.0640 ± 0.0049*
Hindgut	$3.33 \times 10^7 \pm 9.78 \times 10^6$*	$9.96 \times 10^8 \pm 7.51 \times 10^7$*	0.0335 ± 0.0008

DISCUSSION

Mucus, such as mucin, glycoconjugates and glycolipids, can serve as initial sites for the attachment of invading bacteria, and these sites may retard or facilitate the ability of bacteria to attack the underlying epithelial surfaces (Forstner 1978). The results of the present study demonstrate that hindgut mucus has ca. 2 to 3 times more binding sites (e_m) than other mucus does. However, *Vibrio alginolyticus* is less likely to readily adhere to hindgut mucus due to a higher dissociation constant (k_x). Because the bacterial concentrations used in the present

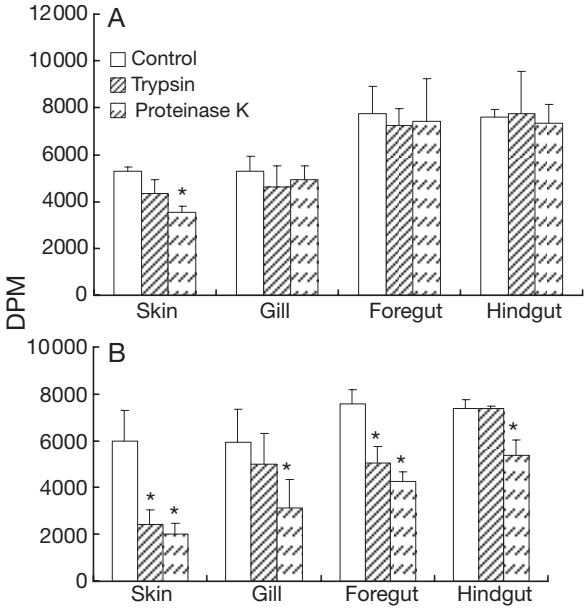

Fig. 6. *Vibrio alginolyticus*. Adhesion to mucus after proteolytic enzyme treatment. (A) Mucus and (B) bacteria treated by proteolytic enzyme prior to adhesion assay. Data are shown as disintegrations per minute (DPM) which reflected the number of adhering bacteria. *Compared with control group (without proteolytic enzyme treatment), $p < 0.05$. Error bars represent mean + SD

study were much higher than those in natural waters, the affinity index (e_m/k_x), which is independent of concentration, is the best indicator of relative bacterial attachment rates (Gordon & Millero 1984). The e_m/k_x indices of intestinal mucus (foregut and hindgut mucus) were higher than those of skin mucus and gill mucus. A similar result was presented by Chabrillón et al. (2004), who found that the adhesion e_m/k_x of *Listonella anguillarum* to intestinal mucus of gilt-head seabream was higher than those for other mucus, but they did not divide the intestine into the foregut and

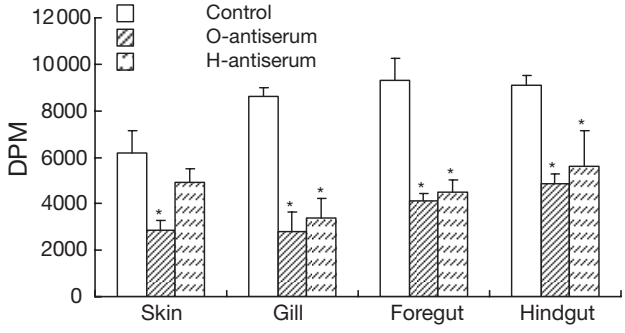

Fig. 7. *Vibrio alginolyticus*. Adhesion to mucus after antiserum treatment. Data are shown as disintegrations per minute (DPM) which reflected the number of adhering bacteria. *Compared with control group (without antiserum treatment), $p < 0.05$. Error bars represent mean + SD

Table 3. *Vibrio alginolyticus*. Adhesion to the mucus after starvation treatment. Data are shown as disintegrations per minute (DPM; mean ± SD) which reflected the number of adhering bacteria. Aliquots (150 µl) of 1% SDS–0.1 M NaOH added to 10 ml scintillation fluid were regarded as the blank control, and the DPM value of the blank control was 98.2 ± 11.7

Time (d)	Mucus	
	Skin	Gill
0	3566.5 ± 247.1	3989.2 ± 218.3
1	1686.0 ± 260.2	1339.5 ± 47.9
2	726.1 ± 44.1	551.0 ± 100.0
3	362.5 ± 21.7	272.8 ± 48.1
4	165.5 ± 27.8	121.6 ± 11.7
10	146.1 ± 60.8	123.3 ± 1.8

hindgut. However, we found that the e_m/k_x of *V. alginolyticus* to foregut mucus was 2 times higher than that of hindgut mucus. This result indicates that the foregut of large yellow croaker is the main location for the adhesion of *V. alginolyticus*. The data obtained from the characteristics of mucus show that there is more IgM and lysozyme in skin mucus than other mucus; this might be one of the reasons why the adhesion of *V. alginolyticus* to skin mucus is less than to other mucus. It has been reported that the gastrointestinal tract is the portal of entry for *V. anguillarum* into fish (Horne & Baxendale 1983, Olsson et al. 1996). The findings of the present study show that the intestinal tract rather than the gill or skin was the entry portal of *V. alginolyticus* into large yellow croaker. In addition, the number of *V. alginolyticus* adhering to intestinal mucus was always higher than the number adhering to other mucus under various conditions, which also demonstrated that the main portal of entry for the pathogenic *V. alginolyticus* is via the intestinal tract.

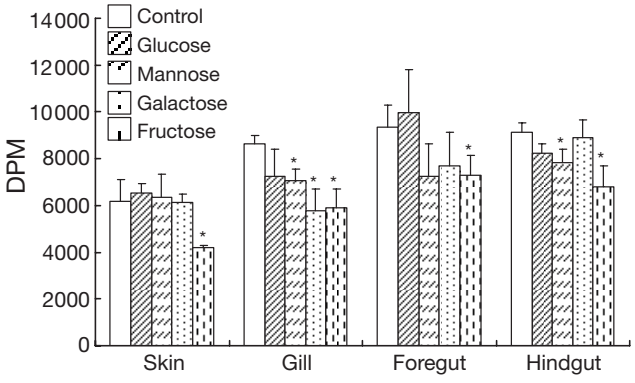

Fig. 8. *Vibrio alginolyticus*. Adhesion to mucus after monosaccharide treatment. Data are shown as disintegrations per minute (DPM) which reflected the number of adhering bacteria. *Compared with the control group (without monosaccharides treatment), $p < 0.05$. Error bars represent mean + SD

Bacterial adhesion to the mucus is mediated by macromolecules, collectively known as adhesins on the bacterial cells, and the adhesion receptors in the mucus. Because major components of fish mucus are made of glycoproteins (Creeth 1978), heat and proteolytic enzyme treatments were used to investigate characteristics of mucus. Some binding sites for *Vibrio alginolyticus* in gill mucus may have been destroyed after heat treatment (75 or 100°C for 10 min), as indicated by decreased adhesion of *V. alginolyticus* to gill mucus after treatment. However, heat treatment had no significant effect on the adhesion capabilities of skin and intestinal mucus. The data in our previous studies showed that after heat treatment (incubated at 56°C for 5 min) *V. alginolyticus* lost most of its ability to adhere to mucus (Chen et al. 2007, Yan et al. 2007). The results of the latter studies indicate that the adhesion receptors in the mucus are more thermotolerant than the adhesins on the bacterial surface.

In the present study, proteolytic enzymes, especially Proteinase K, had a great effect on *Vibrio alginolyticus* adhesion. In most cases, the adhesion has been reported to be mediated by proteins (Carnoy et al. 1994, Roos & Jonsson 2002, Vàzquez-Juárez et al. 2004). Because adhesion of *V. alginolyticus* to the mucus was reduced remarkably by Proteinase K, cell-surface proteins of *V. alginolyticus* may be important adhesins for adhesion. O-antigen lipopolysaccharide (LPS), known as the somatic antigen, was an important adhesin in *Aeromonas hydrophila* (Merino et al. 1996). Experiments with some pathogens, such as *Pseudomonas aeruginosa* (Feldman et al. 1998) and *A. hydrophila* (Merino et al. 1997), have demonstrated the important role of flagella in establishing the initial interaction with mucosal surfaces or cells. The effect of somatic antigen and flagella on the adhesion of *V. alginolyticus* was demonstrated by the effects of O-antiserum and H-antiserum on the bacterial adhesion found in the present study. Somatic antigen and flagella played important roles in the *V. alginolyticus* adhesion. In addition, starvation inhibits continuous bacterial protein synthesis, a process which is necessary for stable bacterial adherence (Finlay et al. 1989). This is likely to be one of the causes of the reduction of the adhesion of starved *V. alginolyticus*. Therefore, adhesion of *V. alginolyticus* to mucus of large yellow croaker depends on the concerted action of the surface structures of *V. alginolyticus*. The different effects on adhesion caused by heat treatment or proteolytic enzymes might be due to the different chemical compositions found in mucus as indicated by the profile of SDS-PAGE.

In many systems, adhesion is mediated by lectins presented on the surface of the infectious organism that bind to complementary carbohydrates on the surface of the host tissues (Acord et al. 2005). Soluble carbohydrates, recognized by the surface lectins of bacteria, block the adhesion of bacteria to animal cells *in vitro* (Sharon 2006). Our study demonstrates that some monosaccharides, especially fructose, might block the ligand of *Vibrio alginolyticus* and inhibit the adhesion of *V. alginolyticus* to mucus. This suggests the possibility of carbohydrates as future anti-adhesion drugs for vibriosis caused by *V. alginolyticus*.

In conclusion, our data show that there are some different chemical components among the various mucus types of large yellow croaker. These different chemical components are the basis of the characteristics of the bacterial adhesion to the mucus. The adhesive kinetics show that the intestinal tract of large yellow croaker is an important portal by which pathogenic *Vibrio alginolyticus* enters the host. Knowledge of these adhesive characteristics should be very useful for designing more effective prophylactic strategies.

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LITERATURE CITED

Acord J, Maskell J, Sefton A (2005) A rapid microplate method for quantifying inhibition of bacterial adhesion to eukaryotic cells. J Microbiol Methods 60:55–62

Balebona MC, Moriñigo MA, Faris A, Krovacek K, Mansson I, Bordas MA, Borrego JJ (1995) Influence of salinity and pH on the adhesion of pathogenic *Vibrio* strains to *Sparus aurata* skin mucus. Aquaculture 132:113–120

Bordas MA, Balebona MC, Zorrilla I, Borrego JJ, Moriñigo MA (1996) Kinetics of adhesion of selected fish-pathogenic *Vibrio* strains to skin mucus of gilt-head sea bream (*Sparus aurata* L.). Appl Environ Microbiol 62:3650–3654

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Carnoy C, Scharfman A, Van Brussel E, Lamblin G, Ramphal R, Roussel P (1994) *Pseudomonas aeruginosa* outer membrane adhesins for human respiratory mucus glycoproteins. Infect Immun 62:1896–1900

Chabrillón M, Bordas MA, Moriñigo MA, Balebona MC (2004) Kinetics of adhesion of *Listonella anguillarum* to the mucus of gilt-head seabream, and the implication of surface components. Aquacult Res 35:403–409

Chen D, Hanna PJ (1992) Attachment of *Vibrio* pathogens to cells of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 15:331–337

Chen Q, Yan QP, Ma S, Zhuang ZX, Wang XR (2007) Adhesion of pathogenic *Vibrio alginolyticus* to the gill mucus of *Pseudosciaene crocea.* Acta Oceanol Sin 26:101–109

Creeth JM (1978) Constituents of mucus and their separation. Br Med Bull 34:17–24

Feldman M, Bryan R, Rajan S, Scheffer L, Brunnert S, Tang H, Prince A (1998) Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. Infect Immun 66: 43–51

Finlay BB, Heffron F, Falkow S (1989) Epithelial cell surface induced *Salmonella* proteins required for bacterial adherence and invasion. Science 243:940–943

Forstner JF (1978) Intestinal mucins in health and disease. Digestion 17:234–263

Gordon AS, Millero FJ (1984) Electrolyte effects on attachment of an estuarine bacterium. Appl Environ Microbiol 47:495–499

Grinde B, Lie O, Poppe T, Salte R (1988) Species and individual variation in lysozyme activity in fish of interest in aquaculture. Aquaculture 68:299–304

Horne MT, Baxendale A (1983) The adhesion of *Vibrio anguillarum* to host tissues and its role in pathogenesis. J Fish Dis 6:461–471

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Larsen MH, Larsen JL, Olsen JE (2001) Chemotaxis of *Vibrio anguillarum* to fish mucus: role of the origin of the fish mucus, the fish species and the serogroup of the pathogen. FEMS Microbiol Ecol 38:77–80

Lee YK, Lim CY, Teng WL, Ouwehand AC, Tuomola EM, Salminen S (2000) Quantitative approach in the study of adhesion of lactic acid bacteria to intestinal cells and their competition with enterobacteria. Appl Environ Microbiol 66:3692–3697

Li QL, Chen Q (2001) Identification of bacterial pathogen and treatment after infection of *Pseudosciaena crocea* in marine cage culture. Chin J Appl Environ Biol 7:489–493 (in Chinese)

Lin KB, Zhou C, Liu JF (1999) Studies on the pathogenic bacteria of *Pseudosciaena crocea* in marine cage culture. Mark Sci 4:58–62 (in Chinese)

Merino S, Rubires X, Aguilar A, Tomás JM (1996) The O:34-antigen lipopolysaccharide as an adhesin in *Aeromonas hydrophila.* FEMS Microbiol Lett 139:97–101

Merino S, Rubires X, Aguilar A, Tomás JM (1997) The role of flagella and motility in the adherence and invasion to fish cell lines by *Aeromonas hydrophila* serogroup O:34 strains. FEMS Microbiol Lett 151:213–217

Oisson JC, Joborn A, Westerdahl A, Blomberg L, Kjelleberg S, Conway PL (1996) Is the turbot, *Scophthalmus maximus* (L.), intestine a portal of entry for the fish pathogen *Vibrio anguillarum?* J Fish Dis 19:225–234

Roos S, Jonsson H (2002) A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. Microbiology 148:433–442

Santos Y, Bandín I, Nieto TP, Barja JL, Toranzo A, Ellis AE (1991) Cell-surface-associated properties of fish pathogenic bacteria. J Aquat Anim Health 3:297–301

Sharon N (2006) Carbohydrates as future anti-adhesion drugs for infectious diseases. Biochim Biophys Acta 1760: 527–537

Shephard KL (1994) Functions for fish mucus. Rev Fish Biol Fish 4:401–429

Thune RL, Stanley LA, Cooper RK (1993) Pathogenesis of Gram-negative bacterial infections in warmwater fish. Annu Rev Fish Dis 3:37–68

Vàzquez-Juárez RC, Romero MJ, Ascencio F (2004) Adhesive properties of a LamB-like outer-membrane protein and its contribution to *Aeromonas veronii* adhesion. J Appl Microbiol 96:700–708

Vesterlund S, Paltta J, Karp M, Ouwehand AC (2005) Measurement of bacterial adhesion — in vitro evaluation of different methods. J Microbiol Methods 60:225–233

Wang J, Su YQ, Zhang ZX, Yan QP (2001) Bacterial pathogenetic biology of cultured *Pseudosciaena crocea* in southern Fujian. J Xiamen Univ (Natural Science) 40: 85–91 (in Chinese with English abstract)

Yan QP, Wang J, Su YQ, Zhang JN (2001) Studies on vibriosis in caged-cultured *Pseudosciaena crocea* (Richardson). J Jimei Univ (Natural Science) 6:191–196 (in Chinese with English abstract)

Yan QP, Chen Q, Ma S, Zhuang ZX, Wang XR (2007) Characteristics of adherence of pathogenic *Vibrio alginolyticus* to the intestinal mucus of large yellow croaker (*Pseudosciaena crocea*). Aquaculture 269:21–30

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