NEW MICROBIOLOGICA, **31**, 489-500, 2008

Adhesive properties of environmental *Vibrio alginolyticus* strains to biotic and abiotic surfaces

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SUMMARY

The ability of *Vibrio alginolyticus* strains isolated from a bathing and fishing area (Khenis, Centre of Tunisia) to adhere to both biotic and abiotic surfaces was evaluated in the present work. The biochemical, physiological and enzymatic activities of all strains was also investigated. Three morphotypes of *V. alginolyticus* were obtained on Congo red agar and only 14 strains produced black colonies. The majority of strains were able to degrade the skin mucus of both *Sparus aurata* and *Dicentrarchus labrax* fishes while the fish mucus preparation of these two specimens exhibits a high level of anti-*V. alginolyticus* strains. Adhesive properties were observed in 37.5% of the analyzed *V. alginolyticus* strains to Hep-2 cells and 50% to Caco-2 cells. All strains were able to form a purple pellicule on glass tube when they were stained with Crystal violet. Fifteen percent of *V. alginolyticus* strains (16/32) were strongly adhesive to polystyrene with a values ranging from 3.04 to 18.25 at 595 nm and only four strains were weak biofilm forming. *V. alginolyticus* bacterium possess a strong adhesive power to both biotic and inertes surfaces. These proprieties may allow to these strains to persist in this biotope in planctonic state or attached to both biotic and abiotic surfaces.

KEY WORDS: Vibrio alginolyticus, Biofilm, Slime production, Glass surface, Caco-2, Hep-2 cell lines

Received March 10, 2008 *Accepted* May 05, 2008

INTRODUCTION

Vibrio alginolyticus is present in estuarine and marine environments and is frequently isolated from bathing areas as free-living bacteria and associated to different surfaces (Tamplin *et al.*, 1990; Barbieri *et al.*, 1999; Baffone *et al.*, 2000). The attached forms are able to survive in the seawater longer than the free-living *Vibrio* spp. strains. Larger numbers of *Vibrio* spp. strains were frequently isolated from zooplacton, especially from copepods (Carli *et al.*, 1993; Mauguri *et al.*, 2004; Baffone *et al.*, 2006). The ability to form biofilm on biotic and abiotic surfaces and to activate a survival state called "Viable but not cultivable" allow to *V. alginolyticus* strains to persist in seawater under environmental stress conditions (Ben Kahla-Nakbi *et al.*, 2007).

The transmission of *V. alginolyticus* strains from the aquatic environment, fishes, shellfishes and humans has been well documented. This bacterium is frequently associated with mass mortality of *Sparus aurata* and *Dicentrarchus labrax* larvae and older fish in many Tunisian hatcheries installed along the Mediterranean seacoasts (Bakhrouf *et al.*, 1995; Ben Kahla-Nakbi *et al.*, 2006; Snoussi *et*

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al., 2006; 2008a, b). Adhesion ability is an important factor in bacterial pathogenicity since it precedes penetration of the microorganisms in the host tissues promoted by the production of toxins. It is well known that *V. alginolyticus* produces many proteases involved in the interaction between the bacterium and the hosts (human and animals) and plays an important role in human infection (ear, wound and eyes infections) and fish pathology (Hare *et al.*, 1983; Lee, 1995).

The mechanism of pathogenicity induced by *Vibrio* infections is still complex and related to several factors including cytotoxins, enterotoxins and lytic enzymes (Baffone *et al.*, 2001; Zanetti *et al.*, 2000). Adhesion ability to human epithelial cell lines (Hep-2 and Caco-2) and fish mucus (Balebona *et al.*, 1995) seem to be diffused among *Vibrio alginolyticus* strains and may represent a potential infection risk for aquatic stressed animals (Zorrilla *et al.*, 2003; Baffone *et al.*, 2006).

The present study was undertaken to explore the ability of environmental *V. alginolyticus* strains to adhere to both biotic and abiotic surfaces including *S. aurata* and *D. labrax* fish mucus, Hep-2 and Caco-2 cell lines, glass surface and polystyrene microtiter plates.

MATERIALS AND METHODS

Bacterial identification and enzymatic characterization

This study includes 32 *Vibrio alginolyticus* strains: 28 were isolated from a bathing and fishing area near a fish rearing farm installed along the Mediterranean seacoasts, two strains (I_{17} and I_{23}) were isolated from the port of Alghero (Sardegna, Italy) and two *Vibrio alginolyticus* ATCC 17749 and 33787 (American Type Collection Culture) were kindly provided by Prof. Stefania Zanetti (I_{17} and I_{23}).

Bacterial strains were identified by the procedures described in Bergey's Manual of Systematic Bacteriology (Holt, 1994). Gram nonstaining method (KOH method: Fluharty *et al.*, 1967), cell morphology, the oxidase test, O-F test, motility (Mannitol-Motility agar, Pronadisa, Madrid, Spain), susceptibility to the vibriostatic compound O/129 (10 and 150 µg/disc), swarming, growth in 0, 6, 8 and 10% NaCl, growth at 4, 15 and 37°C and growth on thiosulfate citrate bile sucrose modified agar (TCBS modified agar, Scharlau Microbiogy, Spain) were the first tests employed to identify the organisms belonging to *Vibrio* genus. Commercial miniaturized strips RapID^{TM} NF plus (REMEL Inc., USA) were also used. A species level was genetically confirmed using the collagenase gene which amplifies a 737-pb fragment as described by Di Pinto *et al.*, 2006. The production of lipase (Tween 80), haemolysin (Human blood agar, Bio-rad, France) and DNA hydrolysis (DNase Agar, Scharlau Microbiogy, Spain) were tested as described previously by Snoussi *et al.*, 2006. The enzymes amylase and lecithinase were detected on media prepared with Phosphate Buffer Saline (PBS) supplemented respectively with 0.5% starch and 5% egg yolk emulsion. The protease activity was tested according the protocol described by Zanetti *et al.*, 2000. *Vibrio alginolyticus* strains were cultured on Nutrient Agar containing 5% skim milk. After incubation up to 72h at 37°C, the formation of a clear zone caused by casein degradation is considered a positive test.

Qualitative detection of biofilm formation by *Vibrio alginolyticus* strains

Detection of slime production by Congo Red Agar (CRA) method

The ability of producing-slime by *Vibrio alginolyticus* strains was tested according to the protocol described by Freeman *et al.*, 1989. All tested bacteria were cultered on Congo red agar plates preapared by adding 0.8 g of Congo red (Sigma) and 36 g of saccharose (Labosi, France) to 1 L of brain heart infusion agar (Bio-rad). The Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121°C for 15 min and was added when the agar had cooled to 55°C. Plates were incubated at room temperature at 37° C for 24 h under aerobic conditions and followed overnight at room temperature. After incubation, pigmented colonies (generally black colour) were considered as slime positive, whereas unpigmented bacteria (formed pinkish red, smooth colonies with a darkening at the centre) were interpreted as slime-negative strains (Sechi *et al.*, 2002).

Bio-assay with fish mucus

Collection of fish skin mucus

Surface mucus was collected by scraping the skin of five healthy specimens of *S. aurata* and *D. labrax*

older fish (Weight 200-300 g) harvested from the fish farm installed near the site of study with sterile glass slides as described by Fouz *et al.* (1990, 1993) and Amaro *et al.* (1995). The mucus was subsequently filtered using 0,45 and 0,2 µm pore-size membranes (Millipore, Sartorius Minisart CE 0297, Germany), and stored in sterile glass tubes in deep freezer at -20°C until use as described by Fouz *et al.* (2000). The enzymes secreted by both *S. aurata* and *D. labrax* mucus were evaluated using the semi-quantitative micromethod Api Zym system (Bio Mérieux, Marcy l'Étoile, France). For the experiment, 100 µl of the purified skin mucous was used to inoculate each cupule. After 4 h of incubation at 37°C, Zym A and Zym B reagents were added in each cupule. After light exposure (5 s), negative reactions were colorless.

Use of skin mucus as a sole source of carbone
Utilization of sea bream and sea bass skin mucus as the sole source of carbon was tested according to the method described by Bordas *et al.*, (1996). The skin mucus was diluted in sterile seawater and purified (twice) by ultra-centrifugation at 20,000 rpm for 30 min at 4°C, then filtrated through 0,45 and 0,2 microns- pore-size filters (Millipore, Sartorius Minisart CE 0297, Germany). Two millilitres of the skin mucus suspension were added to 0,2 M PBS (pH 7,2) mixed with 1% agarose. All identified *V. alginolyticus* strains were streaked onto this medium and plates were incubated at 22°C for 24 to 72 h. Petri dishes without skin mucus were used as a negative control.

Anti-Vibrio alginolyticus activity of fish mucus
The antibacterial effect of skin mucus was evaluated using the disc diffusion method on agar plates according to the protocol previously described by Fouz *et al.* (1990). For the experiment, sterilized 6 mm diameter discs (Whatman paper N° 3) impregnated with 20 µl of the mucus solution were applied to freshly seeded bacterial lawns containing about 1×10^5 cfu ml^{-1}. After 18 to 24 h of incubation at 25°C, the appearance of a growth inhibition halo around the discs indicated the antibacterial activities among *V. alginolyticus* strains (Fouz *et al.*, 2000).

Biofilm formation on glass surface
The ability of *V. alginolyticus* strains to adhere to abiotic surfaces (Glass) was tested on ten millilitres glass tube (0.5 cm of diameter) according to the protocol described by Wolfe *et al.*, 2004. All tested bacteria were grown on SWT broth preapared by mixing 5 g of bactotryptone, 3 g of yeast extract, 3 ml of glycerol in 1 L (700 ml of filtered seawater + 300 ml distilled water). Glass tubes were incubated overnight at 37°C with shaking. 100 µl of this pre-enriched culture was added to a new glass tube containing the same medium and incubated at 37°C for 10 h without shaking. All glass tubes were stained with 1% Crystal violet (Merck, France) for 15 mn and then washed with distilled water. Bacteria which form purple pellicule on the air-surface tubes were considered as glass-biofilm positives.

Adherence assay to human epithelial cells: Hep-2 and Caco-2
Adhesion test was carried out in the Department of Biomedical Sciences, University of Sassari, Italy. Caco-2 (cells from human colon adenocarcinoma) and Hep-2 (Cells from human laryngeal carcinoma) were used in this study. The epithelial cells were grown in MEM supplemented with 10% fetal bovine serum, 1000 IU/ml of both penicillin and streptomycin. Twenty four well tissue trays (Falcon) were seeded with the two cells lines (10^3 cells/well). Plates were incubated for18h at 37°C in a humidified atmosphere with 5% of CO_2. The semiconfluent monolayers were washed with fresh MEM containing 1% foetal bovine serum. Adhesion of *V. alginolyticus* to Hep-2 and Caco-2 cells was tested as described previously by Baffone *et al.*, 2005. Bacterial strains were grown in Marine broth 2216 (MICROBIOL Diagnotici, Italy).
For the assay, 100 µl of 10^7 cells /ml was added to Hep-2 and Caco-2 cells and the 24-well plates were incubated at 37°C for 3 h in 5% CO_2. After being washed three times with phosphate-buffered saline (PBS, pH 7.4), bacteria bound to cells were fixed with methanol, stained with Giemsa stain and examined microscopically under oil immersion. Uninoculated cell lines served as negative controls. The number of bacteria adhering to each of cell lines was counted. All organisms were tested three times. The adhesion index was assayed as: NA= non adhesive (0-10 bacteria/cells); W= weak adhesion (10-20 bacteria/cells); M= medium adhesion (20-50 bacteria/cells); S= strong adhesion (50-100 bacteria/cells).

Quantitative estimation of biofilm formation by *Vibrio alginolyticus* strains on polystyrene surface

The ability of *V. alginolyticus* strains to form a biofilm on abiotic surface was quantified using the protocol described by Toledo-Arana *et al.*, 2001.

Al strains were grown overnight in Brain Infusion Broth (BHI-0.25 glucose at 37°C). The culture was diluted 1:20 in fresh BHI plus (0.25%) glucose at 37°C. 200 µl of this suspension was used to inoculate sterile 96-well-polystyrene microtiter plates (Nunc, Roskilde, Denmark). The plates were incubated aerobically at 37°C for 24 h. The cultures were removed and the microtiter wells were washed twice with phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) to remove non-adherent cells and were dried in an inverted position. Adherent bacteria were stained with 1% Crystal violet (Merck, France) for 15 mn. The wells were rinsed once more and the Crystal violet was solubilised in 200 µl of ethanol-acetone (80:20 v/v).

The optical density (OD_{595}) was measured in spectrophotometer (Amadèo Bibby, Sterlin France). The following values were assigned for biofilm determination: (-) non biofilm forming $OD_{595} \leq 1$; (+) weak biofilm forming $1 < OD_{595} \leq 2$; (++) medium biofilm forming $2 < OD_{595} \leq 3$; (+++) strong biofilm forming $OD_{595} \geq 3$. Each essay was performed in triplicate.

Statistical analysis

All data from quantitative adhesion assays were expressed as Means±Standard Deviation (S.D.). Each analysis was performed using the SPSS 13.0 statistics package for Windows. The differences in the degree of biofilm formation were examined by the Friedman test, followed by the Wilcoxon signed ranks test. P-values <0.05 were considered significant.

RESULTS

Bacterial identification

All *V. alginolyticus* strains tested produced many enzymes such as amylase (89.28%) and lecithinase (89.28%). All strains were β-haemolytic; hydrolyze the DNA, and were gelatinase and lipase positives. All strains tested amplify a 737-pb size fragment (Figure 1). Two *V. parahaemolyticus* reference strains (ATCC 17802 and 43926) produce a 271-pb region specific for this species (Di Pinto *et al.*, 2006).

Slime production on CRA plates

Phenotypic production of slime was assessed by culturing the strains on Congo red agar plates. A total of 32 strains were tested. Pigmanted colonies were considered as normal slime-producing strains, whereas unpigmented colonies were classified as non-slime-producing strains (Sechi *et al.*, 2002). Among the isolated strains, 14/32 (56.25%) were slime producing characterized by a black colonies, and the remaining 18 strains were non-slime producing characterized by pinkish-red colonies with darkning at the centre or orange colonies (Figure 2).

Assays with fish mucus

The *S. aurata* mucous was found to produce ten enzymatic activities on the Api Zym strips (Phosphatase alcaline, Esterase (C4), Esterase Lipase (C8), Leucine arylamidase, Valine arylamidase, Phosphatase acide, Naphtol-AS-BI-phosphohydrolase, β-galactosidase, N-acétyl-β-glycosaminidase and α-fucosidase) and twelve for the *D. labrax* skin mucous (Phosphatase alcaline, Esterase (C4), Esterase Lipase (C8), Leucine arylamidase, Valine arylamidase, Trypsine, Phosphatase acide, Naphtol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase, N-acétyl-β-glycosaminidase and α-fucosidase).

About (50%) of the *V. alginolyticus* strains tested in this work were able to degrade the skin mucus of *S.aurata* and 46.87% for *D. labrax* harvested from the fish farm localized near the site of study. *V. alginolyticus* bacterium gives white and small colonies on PBS medium containing agarose and supplemented with epithelial mucus of gilt head and gilt head sea bream. In bioassays performed *in vitro*, nine strains (28.12%) were resistant to the antimicrobial action of skin mucus from *S. aurata* and only seven (21.87%) for *D. labrax* specimens.

Moreover, it was found that the diameter of growth inhibition zone was ranging from (1 to 2.33) mm to gilt head sea bream mucous and from (1 to 5.33) mm when the gilt head sea bass mucous preparation was tested (Table 1). These

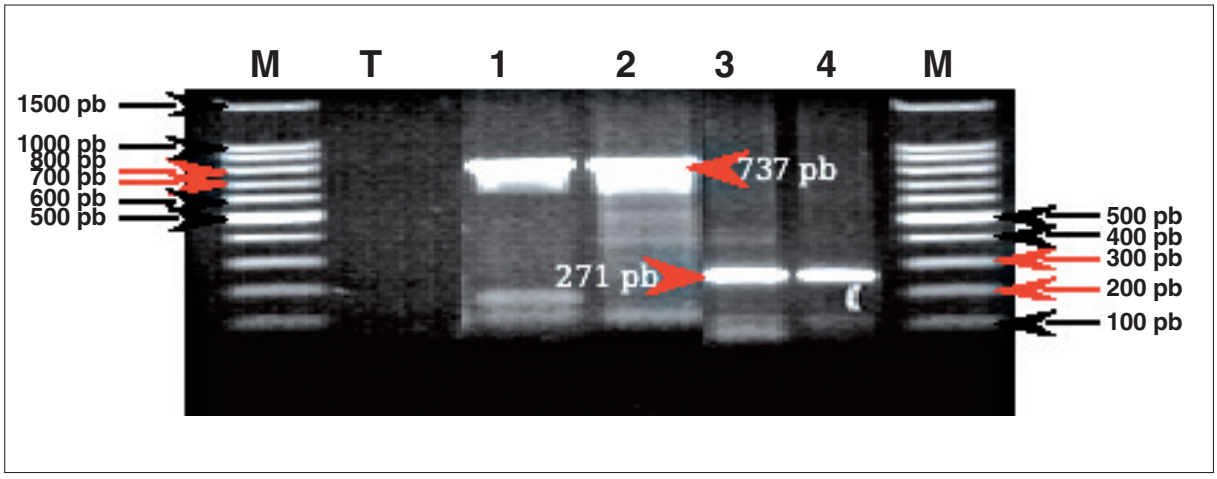

FIGURE 1 - *Agarose gel electrophoresis (1% agarose) of the amplification products obtained for collagenase genes. (M): molecular weight marker (100-pb DNA ladder, Invitrogen). Lane (1): V. alginolyticus ATCC 33787; lane (2): V. alginolyticus (strain K_5); lane 3 and 4: V. parahaemolyticus reference strains (respectively ATCC 17802 and 43926) positives for collagenase gene.*

findings demonstrate that both mucus preparations possess an anti-*V. alginolyticus* activity (Figure 3).

Adherence assay to human epithelial cells: Hep-2 and Caco-2

The tested *Vibrio alginolyticus* strains were able to adhere to the two cell lines used in this study with different degree: 50% to human colon adenocarcinoma cells (Caco-2 cells) and 37.5% to human laryngeal carcinoma cells (Hep-2). In fact, a weak adhesion was shown from 10 strains (31.25%) to Caco-2 cells and only from four strains (12.5%) to Hep-2.

A medium adhesion was detected in one isolate only for Hep-2. Six strains (18.75%) were able to adhere strongly to both Caco-2 and Hep-2 cell lines (Figure 4). All these data were summarized in table 2.

Biofilm formation on glass and polystyrene surfaces

All *Vibrio alginolyticus* strains were able to adhere to the glass giving a purple pellicule on the air-

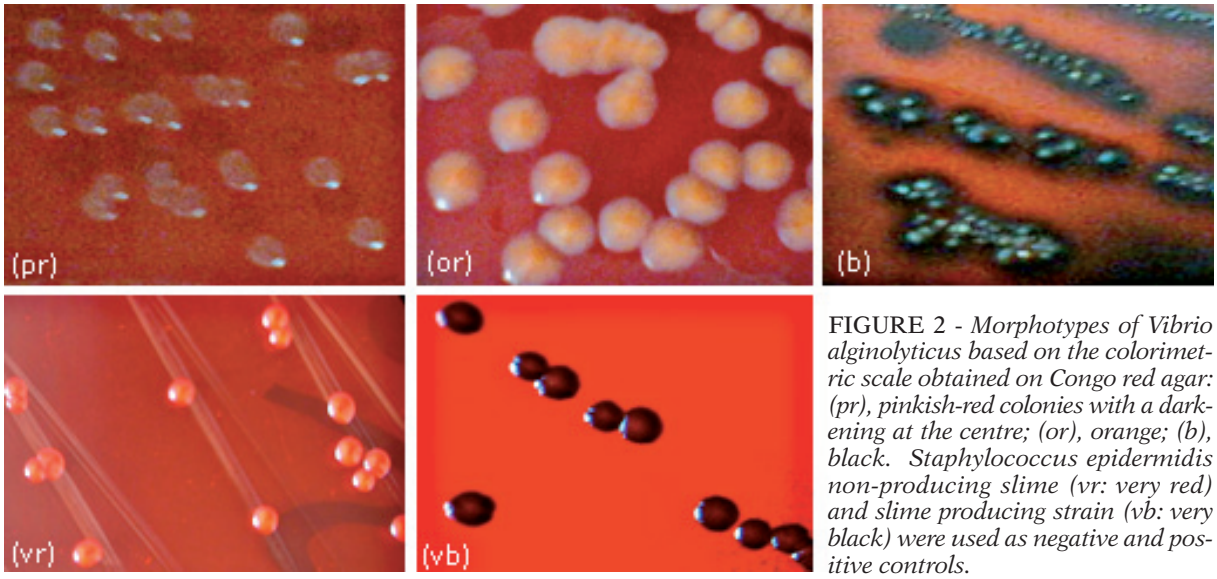

FIGURE 2 - *Morphotypes of Vibrio alginolyticus based on the colorimetric scale obtained on Congo red agar: (pr), pinkish-red colonies with a darkening at the centre; (or), orange; (b), black. Staphylococcus epidermidis non-producing slime (vr: very red) and slime producing strain (vb: very black) were used as negative and positive controls.*

surface of the glass tube. We noted that both the intensity and the width of the pellicule differe from strain to strain. In fact, 27/32 (84.4%) give a large purple pellicule (Figure 4).
50% of *V. alginolyticus* strains (16/32) were strongly adhesive to polystyrene with a values ranging from (3.04) to (18.25) at 595 nm.
Only four strains were non- biofilm forming with an OD_{595} ≤1. Of the six strains able to adhere strongly to the two epithelial cell lines, three strains were highly adhesive to polystyrene surfaces and also three strains were slime producers on CRA plates characterized by black colonies.
Three strains were non adhesive or exhibit a weak power of adhesion to epithelial cells tested (241, 234 and EM_4) including two strains slime producers. All these data were summarized in table 2.

TABLE 1 - *Bioassays on fish mucus: use of skin mucus as a source of carbone and inhibition zone in diameter (mm ± SD) around the discs impregnated with 20 µl of fish mucus (Diameter of the discs 6 mm).*

Strains	*Use of a skin mucus of*		*(mm ± SD)*	
	1	*2*	*1*	*2*
ATCC 33787	+	-	7±0	8±0
ATCC 17749	-	+	7±0	8±0
I_{17} Alghero	+	+	6±0	10±0,81
I_{23} Alghero	+	+	7±0	8±0
K_1	+	+	7±0	7±0
K_3	+	+	7±0	7±0
K_5	+	-	6±0	7±0
K_6	-	-	6±0	6±0
K_7	+	-	6±0	6±0
K_8	-	-	6±0	8±0
K_9	+	+	7±0	6±0
K_{11}	+	-	7±0	6±0
K_{12}	-	-	8±0	7±0
P_1	-	-	8,33±0,57	11,33±1,69
P_4	-	-	6±0	6±0
$P_{7'}$	-	-	7±0	7,66±0,47
P_8	-	+	7±0	7±0
213	-	+	7±0	7±0
224	-	-	7±0	7±0
225	-	-	7±0	7±0
226	-	-	7±0	7±0
227	+	+	7±0	7±0
228	-	+	7±0	7±0
233	-	-	6±0	6±0
234	+	+	7±0	10,66±1,24
241	+	-	7±0	7±0
244	+	-	7±0	8±0
OM_1	-	-	7±0	7±0
EM_1	-	+	7±0	6±0
EM_2	+	+	6±0	7±0
EM_3	+	+	7±0	7±0
EM_4	+	+	6±0	7±0
% of positive test	50	46.87	71.87	78.12

[1]*S. aurata* mucous; [2]*D. labrax* mucous. After 24 h of incubation at 25°C, the appearance of a growth inhibition halo around the discs indicated that antibacterial substances were present in the mucus preparation.

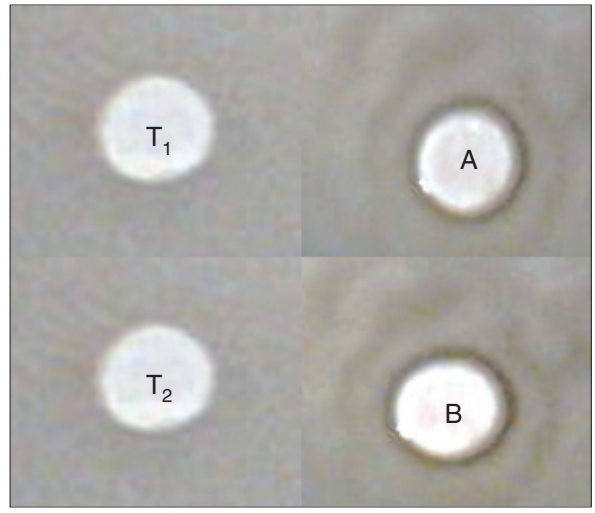

FIGURE 3 - *Effect of mucus preparation on bacterial growth. Bacterial strains were seeded on Mueller Hinton agar supplemented with 1% NaCl. Paper disks (6 mm of diameter) containing 20 µl of skin mucus were placed onto the seeded agar. (A) and (B) represent respectively the antimicrobial activity of S. aurata and D. labrax mucus on V. alginolyticus ATCC 17749. T_1 and T_2 were the negative strains for the two mucus tested.*

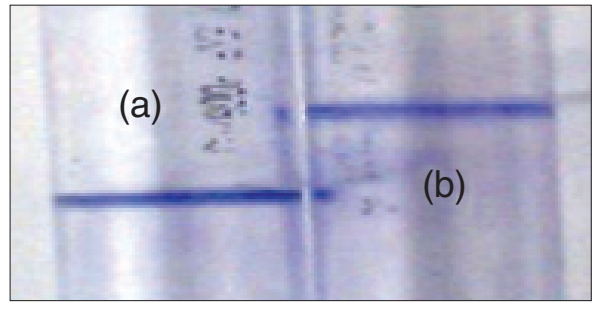

FIGURE 4 - *Pellicule formation by V. alginolyticus strains on the surface of the tested glass tube and then stained with 1% Crystal violet. (a): V. alginolyticus ATCC 17749; (b): V. alginolyticus strain K_5.*

TABLE 2 - *Qualitative and quantitative estimation of V. alginolyticus biofilm formation on CRA plates, glass tubes, epithelial cells and polystyrene microtiter plates.*

Strains	*Phenotypes on CRA*	*Slime production*	*Glass surface*	*Adherence*		*Mean OD_{595} ± SD*	**OD_{595}*
				Hep-2	*Caco-2*		
ATCC 33787	Pinkish red	NP	a	NA	NA	2.665±0.179	++
ATCC 17749	Black	P	a	NA	NA	4.218±0.057	+++
I_{17} Alghero	Black	P	a	NA	NA	6.05±0.082	+++
I_{23} Alghero	Black	P	a	NA	NA	1.708±0.095	+
K_1	Black	P	a	M	W	6.245±0.292	+++
K_3	Orange	NP	a	NA	NA	2.178±0.153	++
K_5	Black	P	a	S	S	1.638±0.132	+
K_6	Black	P	a	S	S	1.332±0.08	+
K_7	Orange	NP	a	S	S	3.258±0.179	+++
K_8	Orange	NP	a	S	NA	3.338±0.138	+++
K_9	Orange	NP	a	S	S	5.078±0.118	+++
K_{11}	Orange	NP	a	W	NA	0.048±0.008	-
K_{12}	Black	P	a	S	S	1.845±0.228	+
P_1	Orange	NP	a	NA	W	3.042±0.045	+++
P_4	Orange	NP	a	NA	NA	1.735±0.092	+
$P_{7'}$	Orange	NP	a	NA	W	4.458±0.401	+++
P_8	Orange	NP	a	NA	NA	1.668±0.118	+
213	Black	P	a	NA	NA	4.199±0.12	+++
224	Orange	NP	a	NA	NA	1.408±0.098	+
225	Orange	NP	a	S	S	6.245±0.292	+++
226	Orange	NP	a	NA	NA	1.135±0.063	+
227	Black	P	a	NA	NA	6.725±0.205	+++
228	Orange	NP	a	NA	W	1.125±0.097	+
233	Black	P	a	NA	NA	0.375±0.043	-
234	Orange	NP	a	NA	NA	6.162±0.364	+++
241	Orange	NP	a	NA	W	0.05±0.039	-
244	Orange	NP	a	W	W	18.25±0.064	+++
OM_1	Black	P	a	NA	W	6.559±0.197	+++
EM_1	Black	P	a	W	W	3.042±0.098	+++
EM_2	Black	P	a	NA	W	3.355±0.191	+++
EM_3	Orange	NP	a	NA	NA	1.235±0.066	+
EM_4	Black	P	a	W	W	0.118±0.03	-
% of positive tests		43.75	100	37.5	50		87.5

NP: slime non-producer; P: slime producer; (a): glass adhesive strain; Hep-2: Cells from human laryngeal carcinoma, Caco-2: cells from human colon adenocarcinoma. Adhesion degree: NA= none adhesive; W= weak adhesion; M= medium adhesion; S= strong adhesion. *OD595: (-) non biofilm forming OD595≤1; (+) weak biofilm forming 1<OD595≤ 2; (++) medium biofilm forming 2<OD595≤ 3; (+++) strong biofilm forming OD595≥3.

DISCUSSION

The *V. alginolyticus* strains analyzed in this study were biochemically heterogeneous on the basis of their biochemical activities tested on RapID^{TM} NF plus strips and their exoenzymes profile. All *Vibrio alginolyticus* strains were genetically confirmed at the specie level as they amplify a 737-pb fragment as described previously by Di Pinto *et al.*, 2006.
All strains tested in this study grow on Congo red agar plates and gave after 18 to 24 h of incubation at 37°C three different morphotypes on the basis of the colour of the colonies obtained. The morphotype I is characterized by a pinkish-red colonies with a darkening at the centre including only one *V. alginolyticus* strain (ATCC 33787), morphotype II with orange colonies (17/32 strains) and morphotype III which were considered as slime producers were characterized by black colonies (14/32 strains). Previous studies used this medium to study the phenotypic formation of biofilm for several bacteria including

Aeromonas spp. (Sechi *et al.*, 2002) and *Staphylococus* spp. (Aricola *et al.*, 2001 and 2002, Chaieb *et al.*, 2005 and Zmantar *et al.*, 2006).
Comparatively to *S. aurata* mucous preparation, the skin mucus of *D. Labrax* was less used by *V. alginolyticus* strains as a unique source of carbon and had a high range of antibacterial effect against this species. This ability may be explained by the enzymatic profile of this mucous which exhibits two enzymatic activities in addition to *S. aurata* mucous preparation. In fact, the skin mucus acts as a natural physical barrier between the external and internal environments of the fish aiming to eliminate the pathogens. The enzymatic profile obtained for gilthead sea bream mucous differ by only two enzymes (cystine arylamidase and β-glucuronidase) from the profile founded in the same fish by Bordas *et al.*, 1996. This mucus contains several proteases, lysozyme, antibodies, complement etc. (Austin and McIntosh 1988; Itami 1993).
In a previous study, Faouz *et al.*, (2000) noted that *P. damselae* subsp. *damselae* strains showed a strong ability to adhere to the fish skin mucus from eel and turbot, exhibiting a degree of adhesion similar to that previously reported for other fish pathogens (*V. vulnificus*, *V. alginolyticus*, *V. anguillarum*, *Aeromonas hydrophila, P. damselae* subsp. *piscicida* and *Flexibacter maritimus*) for the mucus of different fish species (Krovaceck *et al.*, 1987; Amaro *et al.*, 1995; Balebona *et al.*, 1995). The same strains exhibit a high power of adhesion to both glass surface and fish skin mucus fixed on glass slides. However, Magarinos *et*

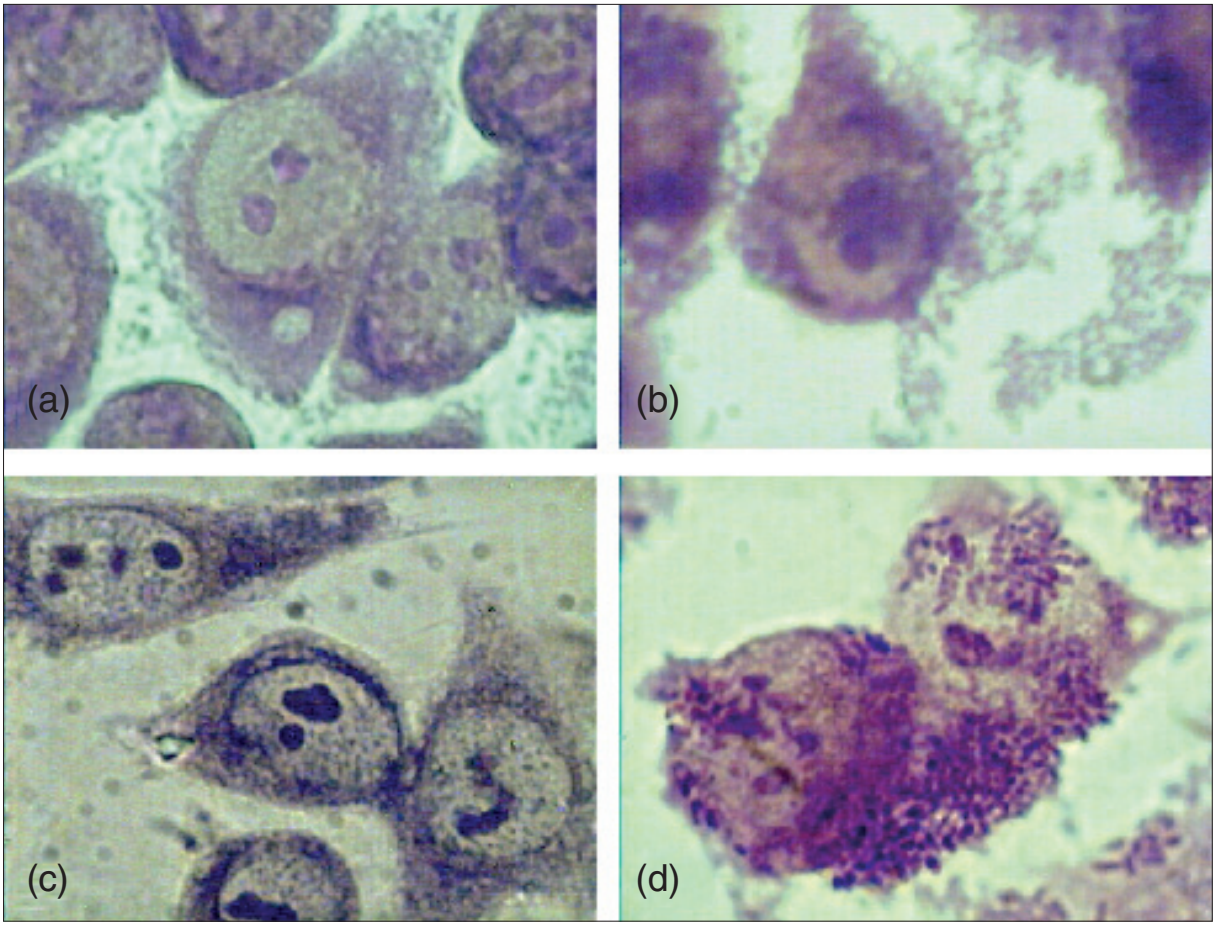

FIGURE 5 - *Optic microscopy showing the high adherence ability of Vibrio alginolyticus (strain K_5) to both Caco-2 and Hep-2 monolayers. The adherence assay was performed as described in the text. Giemsa stain: magnification (×1000). (A) and (C): Negative control for Caco-2 and Hep-2 cells. (B) and (D): Vibrio alginolyticus strain K_5 strongly adhesive to Caco-2 and Hep-2 cells respectively.*

al. (1995) demonstrate that the sea bream skin mucus can inhibit the adhesion of *Pasteurella piscicida*, *Flexibacter maritimus*, *V. anguillarum* and *V. damsela*. Indeed, several bacterial strains tested in this work were inhibited by the mucus preparation in spite of their ability to use the skin mucus of the two specimens as a sole source of carbone and their high power to form a biofilm in glass and polystyrene biomaterials.

Our Vibrios were able to adhere to glass surface characterized by a purple pellicule on the air-surface of the glass tube, and most of them exhibit a high potential to adhere to polystyrene microplates. We noted that adhesion ability differe from strain to strain and from surface to another. Statistical analyses showed that there is no correlation between the slime producing ability on CRA plates and the adhesion power developed on polystyrene material (P-values <0.05). In fact, from the 17 strains characterized of having orange colonies on Congo red agar (slime non producers), 15 were adhesive to polystyrene microtiter plates including seven strains with high adhesion power.

Overall tested bacteria were able to adhere to the two epithelial cell lines with different degree. It seems that these isolates were more adhesive on Caco-2 cells than Hep-2 cell lines (50%). These data were in accordance with the results showed by Zanetti *et al.*, 2000 when they study the virulence factors of 31 *V. alginolyticus* strains isolated from aquatic environments. These researchers reported that the adhesion character is found in 61% of the analysed *V. alginolyticus* strains. In the same work, no association between protease activity and adhesion to Caco-2 and Hep-2 cells was shown.

In 2005, Baffone and colleagues reported that adhesion to Caco-2 monolayers was found in 69% of analyzed *Vibrio* spp. strains while 82,7% was found when Hep-2 cells were used. In this study, numerous strains exhibit adhesive properties in both epithelial cell lines and none of the environmental *V. alginolyticus* strains tested exhibit a strong "S" adhesion capacity in either cell line (Figure 4). The isolation of six strains of *V. alginolyticus* with strong ability to adhere to both Hep-2 and Caco-2 monolayers suggests a probable interaction between these strains and the human intestinal mucus and confers to these bacteria the ability to colonise the tissue of the host when they are introduced with contaminated seafood. Several studies have demonstrated that *Vibrio* spp. strains were cytotoxic respectively on CHO (Chinese hamster ovary) and HeLa cell lines (Wang, Ting & Shieh, 1992; Hassan, Rahman & Tzipori, 1994). In a recent study, the same researchers have shown that all *V. alginolyticus* strains isolated from the coastal waters of the Adriatic Sea (Italy) were adherent to Caco-2 monolayers with different degree. These strains were cytotoxic to CHO cell lines revealed by the presence of rounded and detached cells after 24h of treatment (Baffone *et al.*, 2005; Masini *et al.*, 2007).

Montanari *et al.*, (1999) demonstrated that plankton microorganisms, mainly copepods, represent a potential reservoir of *V. cholerae* and other *Vibrio* spp. strains. *V. alginolyticus*, like others bacteria, is able to enter to the so called (VBNC) state "viable but not culturable " in aquatic environment in warm conditions (Bakhrouf *et al.*, 1992; Oliver *et al.*, 1995; Baffone *et al.*, 2003; Ben Kahla Nakbi *et al.*, 2007). These VBNC forms possess the ability to resuscitate both in vitro (Weichert *et al.*, 1992) and in natural environments (Hite *et al.*, 1994) especially when the temperature increases in the biotope. This ability may explain the high amount of *Vibrionaceae* family recovered from aquatic biotopes during the summer signaled by De Paola *et al.*, (1994); Cavallo and Stabili, (2004) and Baffone *et al.*, (2006). In fact, the production of a biofilm by Gram-negative microorganisms involves the following steps:

a) the planktonic stage, during which free-swimming bacteria approach the surface;
b) the monolayer stage, during which bacteria attach to the surface;
c) when the bacteria move along the surface and associate with each other to form three-dimensional structures.

Our results confirmed that *V. alginolyticus* strains shows a specific binding capability to gilthead sea bass and gilthead sea bream mucus, polystyrene and glass surfaces with varying levels of adhesion among strains. The overall data suggest that *V. alginolyticus* exhibits a high power of adhesion to abiotic surfaces and susceptible fish can be colonized by this bacterium and that the skin can be used as a portal of entry into the fish. In conclusion to this work and considering the fact that the site of study is very frequented for both

fishing and bathing activities, a long-term programme of surveillance must be initiated to study the virulence factors of *Vibrio* spp. and their roles in human health.

ACKNOWLEDGMENTS

We are grateful to Prof. Imed Deli for the corrections and valuable comments on the English version of the manuscript. A special thanks to Prof. Ghoul-Mazgar Sonia (Laboratoire d'Histologie-Embryologie, Faculté de Médecine Dentaire, Monastir, Tunisie) for the photos of adhesion test to epithelial cells.

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