

## ADHERENCE ASSAYS AND SLIME PRODUCTION OF *VIBRIO ALGINOLYTICUS* AND *VIBRIO PARAHAEMOLYTICUS*

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### ABSTRACT

In this study we investigated the phenotypic slime production of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains, food-borne pathogens, using a Congo red agar plate assay. Furthermore, we studied their ability to adhere to abiotic surfaces and Vero cells line. Our results showed that only *V. alginolyticus* ATCC 17749 was a slime-producer developing almost black colonies on Congo red agar plate. Adherence to glass tube showed that all *V. alginolyticus* strains were more adherent than *V. parahaemolyticus*. Only *V. alginolyticus* ATCC 17749 was found to be able to form biofilm on polystyrene microplate wells ( $OD_{570} = 0.532$ ). Adherence to Vero cells showed that all tested strains were non adherent after 30 min, however after 60 min all the studied strains become adherent. The percentage of adherence ranged from 1.23% to 4.66%.

**Key words:** *Vibrio*, slime production, adherence, abiotic surface, Vero cells

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### INTRODUCTION

Bacterial biofilms are complex communities of microorganisms embedded in a self-produced matrix and adhering to inert or living surfaces (7). Biofilms have been observed on a variety of surfaces and were considered to be the prevailing microbial lifestyle in most environments (26). The formation of biofilms on food and food-processing surfaces, and in water distribution systems, constitutes an increased risk for product contamination with spoilage or pathogenic microflora (9). The development of biofilms can be seen as a five-stage process (24): (i) initial reversible adsorption of cells to the solid surface, (ii) production of extracellular polymeric matrix substances resulting in an irreversible attachment, (iii) early development of biofilm architecture, (iv) maturation, and (v) dispersion of single cells from the biofilm.

Biofilm formation on abiotic surfaces is extensively examined and represents very important issues in food sanitation. It has been shown that bacterial cells trapped in the biofilm matrix created by the exopolysaccharide glycocalyx are more resistant

to sanitizers and other environmental stresses than free cells (29). Pathogenic bacteria released from the biofilm definitely lead to food hygiene problems (8).

Biofilm formation of marine vibrios has been reported elsewhere for *V. alginolyticus* (14), *V. cholerae* (12), *V. harveyi* (13). Lopez-Cortes *et al.* (15) reported the adhesion property of pathogenic vibrios to seafood. Comprehensive examination of the adherence of pathogenic vibrios to biotic surfaces could elucidate the pathogenesis of these bacteria in the host.

The aim of this study was to investigate the slime production and the ability of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains to adhere to polystyrene microplate, glass tube and Vero cells line.

### MATERIALS AND METHODS

#### Bacterial strains

Nine *Vibrio* strains were used in this study including two *V. alginolyticus* strains (S3 and S4) isolated respectively from the internal organs of aquacultured diseased gilthead sea bream

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(*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) according to method previously described by Ben Kahla *et al.* (2) and three *V. alginolyticus* strains designed respectively S5, S6, and S7, isolated from Tunisian seawater were also used. All the isolated strains were identified biochemically using Api 20NE system (Bio-Merieux).

In addition, *V. parahaemolyticus* strain isolated from the Calich estuary (Alghero, Italy), and three reference strains: *Vibrio alginolyticus* ATCC 33787, *V. alginolyticus* ATCC 17749 and *V. parahaemolyticus* ATCC 17802 were included in this study (Table 1). All these strains were provided gratefully by Professor S. Zanetti (Dipartimento di Scienze Biomediche, Sezione di Microbiologia Sperimentale e Clinica, Università degli studi di Sassari, Sassari, Italy).

### Phenotypic characterization of slime-producing bacteria

Qualitative detection of biofilm formation was studied by culturing the strains on Congo red agar (CRA) plates as described previously (10). *Vibrio* strains were inoculated into the surface of CRA plates, prepared by mixing 0.8 g Congo red with 36 g saccharose (Sigma) in 1 L of brain heart infusion agar, and were incubated for 24 h at 30°C under aerobic conditions and followed overnight at room temperature (4) Slime producing bacteria appeared as black colonies, whereas non-slime producers remained non pigmented (25).

### Biofilm formation assays by *Vibrio* strains

#### Biofilm formation in glass test tubes

For the biofilm formation assay, each *Vibrio* strain, was cultured in SWT medium containing (per liter): 5 g of Bacto-Tryptone (Difco), 3 g of yeast extract (Difco), 3 ml of glycerol, 700 ml of filtered seawater, and 300 ml of distilled water, at 28°C with shaking and then transferred to glass test tubes. The cells were incubated without shaking for 10h at 28°C, then stained with 1% crystal violet solution to visualise cells attached to the test tube (28). After incubation for 15min, the tubes were rinsed with sterile distilled water. Biofilms formed at the air liquid interface were stained purple. All the strains were tested in triplicate.

#### Quantitative adherence assay

Biofilm production by *Vibrio* strains was determined using a semi-quantitative adherence assay on 96-well tissue culture plates, as described previously (4). Strains were grown in Trypticase Soy broth supplemented with 1% (w/v) NaCl (TSB 1%, Pronadisa, Spain), Following overnight incubation at 30°C, the optical density at 600 nm ( $OD_{600}$ ) of the bacteria was measured. An overnight culture, grown in TSB 1% at 30°C, was diluted to 1:100 in TSB supplement with 2% (w/v) glucose. A total of 200  $\mu$ l of cell suspensions was transferred in a U-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark). Each strain was tested in triplicate. Wells with sterile TSB alone

were served as controls. The plates were incubated aerobically at 30°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 dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100  $\mu$ l of 1% crystal violet (Merck, France) for 5 min. The excess stain was rinsed and poured off and the wells were washed three times with 300  $\mu$ l of sterile distilled water. The water was then cleared and the microplates were air-dried. The optical density of each well was measured at 570 nm ( $OD_{570}$ ) using an automated Multiskan reader (GIO. DE VITA E C, Rome, Italy). Biofilm formation was interpreted as highly positive ( $OD_{570} \geq 1$ ), low-grade positive ( $0.1 \leq OD_{570} < 1$ ), or negative ( $OD_{570} < 0.1$ ).

### Vero cells adherence assays

Quantitative adherence assays was performed with kidney epithelial cells of the African Green Monkey (Vero) as described by Chatti *et al.* (6). Vero cells were seeded at a concentration of  $2 \times 10^5$  and grown overnight in minimal essential medium (MEM) with Earle's salts and 10% fetal bovine serum in 96-well microtiter plates at 37°C with 5%  $CO_2$ . Each *Vibrio* strain was grown overnight in brain heart infusion with 0.5% NaCl at 30°C with shaking. The bacterial cells were washed three times by centrifugation at  $6000 \times g$  for 15 min with MEM without serum and resuspended in the same medium. The number of bacteria in the suspension was adjusted to  $10^7$  CFU/ml. The monolayers of Vero cells were inoculated with  $10^7$  CFU/ml for each tested strain, and incubated at 37°C in 5%  $CO_2$  for 30 min and 60 min. Then, bacterial suspension was removed to exclude the unattached bacteria. The monolayers of Vero cells were washed 3 times with DMEM, and 1ml Triton X-100 in PBS was added for 5 min at room temperature to release the bacteria from the cells. The number of bacteria was estimated by plating serial dilutions. All experiments were performed in triplicate.

### Statistical analysis

Each analysis was performed using the S.P.S.S. 13.0 statistics package for Windows. The differences in the degree of biofilm formation (semi-quantitative adherence assay on 96-well tissue culture plates) and adherence potency to Vero cells were examined by the Friedman test, followed by the Wilcoxon signed ranks test. *P*-values of  $< 0.05$  were considered as significant. Other analysis were realised between the origin of strains, slime production and glass test tube adherence.

## RESULTS

### Determination of slime production

Phenotypic slime production was assessed by culturing the investigated strains on CRA plates. Among the 9 *Vibrio* strains tested in this study, only *V. alginolyticus* ATCC 17749 (S2) was

a slime-producer developing almost black colonies whereas the remaining 8 strains are considered as non-producers since they showed white colonies on CRA plates (Fig. 1)

**Biofilm formation in glass test tubes**

The results of adherence assay to test glass tube showed that *V. alginolyticus* strains were more adherent than *V. parahaemolyticus* which is slightly adherent (Fig. 2). Among the seven *V. alginolyticus* strains, we have observed the existence of three different phenotypes (Fig. 2). In addition, the strains S3 and S7 isolated respectively from diseased *Dicentrarchus labrax* and seawater were very adherent (Fig. 2 a). Furthermore, *V. alginolyticus* ATCC 33787 (S1) was adherent (Fig. 2b). Whereas, the strain S4 isolated from diseased *Sparus aurata*, the strains S5 and S6 isolated from seawater and *V. alginolyticus* ATCC 17749 were fairly adherent (Fig. 2c).

**Quantitative Biofilm formation**

All 9 *Vibrio* strains were screened for their adherence to polystyrene microplate plates. The results showed that only *V. alginolyticus* ATCC 17749 is able to form biofilm (OD<sub>570</sub>=0,532) and was considered as low-grade positive, whereas all the other tested strains did not show any biofilm formation (Table 1).

**Vero cells adherence**

Quantitative adherence of *V. alginolyticus* and *V. parahaemolyticus* to Vero cells was assessed in two times: after 30 min of contact we have noted that all strains were non adherent, whereas after 60 min of contact we have noted that all strains were adherent but with different percentages. The adherence is ranged from 1.23% to 4.66%. Our results showed



**Figure 1.** Colourimetric scale for colony analysis of slime production by *V. alginolyticus* and *V. parahaemolyticus* using Congo Red agar assay (G8x). A: slime producing strain (almost black); B: non-producing strain (white).

that after 1h of contact, the two *V. alginolyticus* reference strains (S1 and S2) were more adherent than the other tested strains (Table 1).

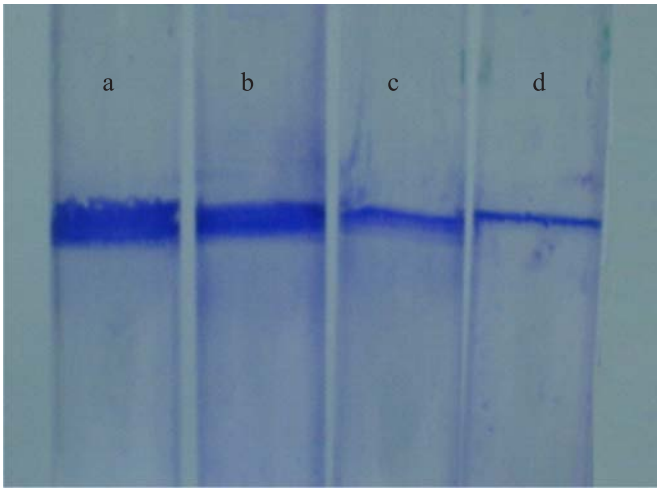
Statistical analysis revealed a significant difference between the OD<sub>570</sub> and adherence to Vero cells (*P* < 0.05). However, the statistical analysis between the origin of strains and slime production, the origin of strains and test tube adherence showed a not significant difference (*P* = 0.342 and 0.304 respectively).

**DISCUSSION**

The results, developed in this study, showed that *Vibrio*, food-borne pathogen, is able to produce biofilm on abiotic surface as well as cells. Our results showed that only *V. alginolyticus* ATCC 17749 was categorized as slime-producer

**Table 1.** Slime production and adherence to polystyrene microplate and to Vero cells of *Vibrio* strains studied.

Strains	Biotype	Sample	Phenotype of strains (CRA)	Production of slime	OD <sub>570</sub> ± SD	Vero cells adherence (%) ± SD	
						30 min	60 min
S1 : <i>V. alginolyticus</i>	7454744	ATCC 33787	White	Non producer	0.040±0.038	0	4.66±0.577
S2 : <i>V. alginolyticus</i>	7454744	ATCC 17749	Almost black	Producer	0.532±0.013	0	4.33±0.577
S3 : <i>V. alginolyticus</i>	7754444	diseased <i>Dicentrarchus labrax</i>	White	Non producer	0.024±0.008	0	3.33±0.577
S4 : <i>V. alginolyticus</i>	7456744	diseased <i>Sparus aurata</i>	White	Non producer	0.018±0.004	0	2.53±0.251
S5 : <i>V. alginolyticus</i>	7435544	seawater	White	Non producer	0.016±0.005	0	1.36±0.152
S6 : <i>V. alginolyticus</i>	7454744	seawater	White	Non producer	0.056±0.013	0	1.23±0.057
S7 : <i>V. alginolyticus</i>	7434644	seawater	White	Non producer	0.012±0.003	0	2.13±0.305
S8 : <i>V. parahaemolyticus</i>	7076744	ATCC 17802	White	Non producer	0.019±0.005	0	2.23±0.602
S9 : <i>V. parahaemolyticus</i>	7277644	seawater	White	Non producer	0.067±0.013	0	2.06±0.635



**Figure 2.** Adherence of *Vibrio* tested strains to test glaze tube: a (S3 and S7): very adherent strains; b (S1): adherent strain; c (S2, S4, S5 and S6) fairly adherent strains; d: slightly adherent strains (S8 and S9).

on CRA plate, developing almost black colonies. Indeed, slime production play an important role in the pathogenesis of infections caused by different micro-organismes (1), and is considered to be a significant virulence factor for some staphylococci (16) as well as for *Aeromonas* spp which indicates the high-risk source contamination (23). Slimes are generally polysaccharidic materials, although other polymers may also be present. They are probably involved in the protection of microbial cells. In addition microorganisms which produce these exopolymers, such as *Vibrio*, are more resistant to desiccation, predation and toxic chemicals (20). However, these molecules are also important in the formation of biofilms on surfaces. Indeed, exopolymers have been considered to be involved in the first steps of biofilm formation (19). Pringent-Combaret *et al.* (22) found that the *E. coli* exopolysaccharide colanic acid was involved only in the ability of the cells to produce voluminous biofilm, and not in the adherence of the cells to plastic surfaces, while Gaylarde and Beech (11) demonstrated that lipopolysaccharides of the outer membrane of *Pseudomonas* spp. and sulphate-reducing bacteria were the important molecules in initial adhesion to a metal surface.

Qualitative adherence of tested *Vibrio* strains performed on glaze test tube showed that *V. alginolyticus* was very adherent contrary to *V. parahaemolyticus*. According to Wolfe *et al.* (28), this difference may be due to the presence and the expression of *rpoN* gene especially in *V. alginolyticus*.

Quantitative adherence to polystyrene microplate plates showed that among the 9 tested strains, only *V. alginolyticus* ATCC 17749 was capable to form biofilm. Indeed, biofilm

formation begins with the attachment of bacteria to abiotic surface, by means of pili, flagella or other materials, followed by the production of exopolysaccharides to form a glycocalyx (29). The attachment of bacteria, such as *Vibrio*, to glaze, polystyrene or other surfaces is affected by various physicochemical and biological factors including bacterial surface hydrophobicity (27), surface appendages, extracellular polymeric substances (5), bacterial physiological state, electrolyte concentration in the medium (17) surface charge and swimming speed. Kogure *et al.* (14) reported that the attachment of *V. alginolyticus* to glass surfaces is dependent on swimming speed.

Adherence of pathogenic bacteria, such as *Vibrio*, to host cells or tissues is a key step in virulence (31). In the present study, culture cells adherence assay performed with kidney epithelial cells of the African Green Monkey, showed the ability of two *Vibrio* species to adhere, first step, to invasion. Our results may explain the existence of some *Vibrio* species especially *V. alginolyticus*, in the internal organs of moribund cultured *Sparus aurata* and *Dicentrarchus labrax* (2). According to Wong *et al.* (29) the two *V. alginolyticus* reference strains S1 and S2 are considered more pathogens than other tested strains since have the highest adherence percentage: 4.66% for S1 and 4.33% for S2. Attachment to culture cells has been studied in some *Vibrio* species especially with Human epithelial cells (21), Human intestinal cells (30), and HeLa cells (18). Indeed, the adherence of pathogens to host surfaces is a prerequisite step in the pathogenesis of almost all infectious diseases. Bacterial adherence requires the specific interaction of bacterial molecules, termed adhesins with host cell membrane molecules or extracellular matrix proteins (3).

In conclusion, *V. alginolyticus* and *V. parahaemolyticus*, ubiquitous germs are able to adhere to abiotic and biotic surfaces. However, these two pathogens did not show a good ability to form biofilm in the polystyrene microplate contrary to glaze tube and culture cells.

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## RESUMO

### Ensaio de adesão e produção de muco por *Vibrio alginolyticus* e *Vibrio parahaemolyticus*

Neste estudo, investigou-se a produção de muco por cepas de *Vibrio alginolyticus* e *Vibrio parahaemolyticus* através do teste em placa de ágar com vermelho congo. Estudou-se também a capacidade de adesão à superfícies abióticas e células Vero.

Os resultados indicaram que somente *V. alginolyticus* ATCC 17749 produziu muco, formando colônias quase negras nas placas de ágar com vermelho congo. O teste de adesão a tubos de vidro indicou que as cepas de *V. alginolyticus* foram mais aderentes do que as de *V. parahaemolyticus*. Somente *V. alginolyticus* ATCC 17749 foi capaz de formar biofilme nos poços das microplacas de poliestireno (OD<sub>570</sub>=0,532). Testes de adesão a células Vero mostraram que nenhuma das cepas apresentou adesão em 30 min, mas todas aderiram após 60 min. A porcentagem de adesão variou de 1,23% a 4,66%.

**Palavras-chave:** *Vibrio*, produção de muco, adesão, superfície abiótica, células Vero

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