African Journal of Microbiology Research Vol. 5(31), pp. 5644-5655, 23 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.803

Full Length Research Paper

Adhesive properties of *Aeromonas hydrophila* strains isolated from Tunisian aquatic biotopes

Nourhène SAIDI[1,2], Mejdi SNOUSSI[1,3], Donatella USAI[2], Stefania ZANETTI[2] and Amina BAKHROUF[1]

[1]Laboratoire d'Analyse, Traitement et Valorisation des Polluants de l'Environnement et des Produits, Department of Microbiology, Faculty of Pharmacy, Rue Avicenne, Monastir, Tunisia.
[2]Dipartimento di Scienze Biomediche, Sezione di Microbiologia Sperimentale e Clinica, Universita di Sassari, Sassari, Italy.
[3]Laboratoire de Traitement et de Recyclage des Eaux. Centre de Recherches et des Technologies des eaux, Technopôle de Borj-Cédria, BP 901, 2050 Hammam-Lif, Tunisie.

Accepted 31 October, 2011

The ability of *Aeromonas hydrophila* strains isolated from aquatic environment and ornamental fishes to adhere to both biotic and abiotic surfaces was evaluated. The majority of strains were able to adhere to fish skin mucus, while the fish mucus preparation exhibited a high level of anti-bacterial effect. Adhesive properties were observed between 75 and 80% of the analyzed *A. hydrophila* to cells (Hep-2 and Caco-2). In addition, 40% of the tested strains were invasive on the two cell lines. On Congo Red Agar, only 65% of the strains produced slime, 65% of the strains were able to form biofilm on glass tube with Crystal violet and 85% with Safranin. Most *A. hydrophila* strains (95%) were adhesive to polystyrene with high optical density values. These proprieties may allow the persistence to *Aeromonas* strains in the aquatic biotope in a free-living planctonic state or attached to biotic and abiotic surfaces.

Keywords: Adhesive capacities, *Aeromonas hydrophila*, biofilm, Caco-2, Hep-2 cell lines and slime production.

INTRODUCTION

Mesophilic *Aeromonas* spp strains are ubiquitous water-borne bacteria and pathogens of reptiles, amphibians, and fish (Austin and Adams, 1996). They can be isolated as a part of the fecal flora of a wide variety of other animals, including some used for human consumption, such as pigs, cows, sheep, and poultry. In humans, *Aeromonas hydrophila* have been associated with gastrointestinal and extraintestinal diseases, such as wound infections, and less commonly with septicemia of immunocompromised patients (Janda and Abbott, 1998). The swimming motility of all mesophilic aeromonads has been linked to a single polar unsheathed flagellum, expressed constitutively, which is required for adherence to and invasion of human and fish cell lines (Thornley et al., 1996; Merino et al., 1997; Rabaan et al., 2001 and Gryllos et al., 2001).

In fact, flagellar motility represents an important advantage for bacteria in moving toward favorable conditions or in avoiding detrimental environments and it allows flagellated bacteria to successfully compete with other microorganisms (Fenchel, 2002). In addition, motility and flagella play a crucial role in adhesion, biofilm formation, and colonization of several pathogenic bacteria, such as *Pseudomonas aeruginosa* (Stanley, 1983), *Salmonella enterica* (Ciacci-Woolwine et al., 1998), *Escherichia coli* (Pratt and Kotler, 1998), *Helicobacter pylori* (Eaton et al., 1996), *Vibrio cholerae* (Gardel and Mekalanos, 1996), and *A. hydrophila* (Merino et al., 1997; Rabaan et al., 2001).

Moreover, 50 to 60% of mesophilic aeromonads are able to produce many unsheathed peritrichous lateral flagella when grown in viscous environments or over surfaces (Shimada et al., 1985), which increase bacterial adherence and are required for swarming motility and biofilm formation (Gavı´n et al., 2002).

The objective of this study was to identify the adherence ability of environmental *A. hydrophila* strains to biomaterials and biotic surfaces.

MATERIALS AND METHODS

Bacterial identification and enzymatic characterization

This study includes 20 *A. hydrophila* strains: eight strains were isolated from treated wastewater (Ksour essef Station ONAS, Tunisia), six strains were isolated from the river near the seacoast of Monastir, two strains were isolated from health ornamental fishes, three strains from the bay of Khenis (Monastir) and one reference strain *A. hydrophila* ATCC 7966^T (American Type Collection Culture (Manassas, Va.)).

Bacterial strains were identified by the procedures described in Bergey's Manual of Systematic Bacteriology (Popoff, 1984). Gram staining method, cell morphology, the oxidase, catalase, motility (Mannitol-Motility agar, Pronadisa, Madrid, Spain), susceptibility to the vibriostatic compound O/129 (10 and 150 µg/disc) and ampicillin antibiotic (10 µg), growth at 30 and 37°C and growth on Rimler Shotts Agar (mRS) were the first tests employed to identify the organisms belonging to *Aeromonas* genus. Commercial miniaturized strips 20 NE Api (Non Enterobacteriacae, bioMerieux, France) were also used.

The production of lipase (Tween 80), haemolysin (Sheep blood agar, Pronadisa, Madrid, Spain) and DNA hydrolysis (DNAse Agar, Sharlau Microbiology, Barcelona, 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 caseinase activity was tested according the protocol described by Zanetti et al. (2000). *A. hydrophila* strains were cultured on Nutrient Agar containing 5% skim milk. After incubation up to 72 h at 37°C, the formation of a clear zone caused by casein degradation was considered a positive test.

Adhesion Assay to human epithelial cells Hep-2 and Caco-2

All strains were grown in Lauria Broth Base (Invitrogen), supplemented with 1% NaCl (BioBasic) in 25 ml flasks at 37°C for 12 to 18 h with shaking at 150 rpm.

Caco-2 and Hep-2 cells monolayers were grown in RPMI (GIBCO, USA) supplemented with 10% fetal bovine serum, 100 IU/ml of both penicillin and streptomycin, and maintained in a humidified atmosphere of 5% CO_2 at 37°C.

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. Twenty four well tissue trays (Falcon) were seeded with the two cells lines (10^3cells/well). Plates were incubated for 18 h at 37°C in a humidified atmosphere with 5% of CO_2. The semiconfluent monolayers were washed with PBS (pH 7.4). Adhesion of bacteria to Hep-2 and Caco-2 cells was tested as described previously by (Zanetti et al., 2000 and Snoussi et al., 2008a).

For the assay, 100 µl of 10^7cells/ml were added to Hep-2 and Caco-2 cells. The contact between bacteria and cells were promoted by centrifugation (at 1500 g) for 10 min and then the trays were incubated at 37°C in 5% CO_2 for 90 min.

To remove non adherent bacteria, the coverslips were washed gently two times with PBS (Phosphate Buffer Saline, pH 7.4). Finally, bacteria bound to cells were fixed with methanol for 30 min, stained with May Grünwald's reagent (Carlo Erba Reagenti, Italy) and Giemsa Stain (Riedel-de Haën, Germany); 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 by using optic microscope (GX100). All organisms were tested three times.

The adhesion index was assayed as described previously (Zanetti et al., 2000 and Snoussi et al., 2008a) and were classified as non adhesive (NA: 0-10 bacteria/cell), weak adhesion (W: 10-20 bacteria/cell), medium adhesion (M: 20-50 bacteria/cell) and strong adhesion (S: 50-100 bacteria/cell).

Internalization assay to Caco-2 and Hep-2

Internalization of bacteria in cells was analysed with Gentamicin exclusion assay as described by Fleiszig et al. (1994). In brief, 100 µl (10^6 CFU/mL) of each bacterium was added to confluent monolayers of cultured cells (CaCo-2 and Hep-2). Infected cells were placed for 3 h at 37°C in 5% CO_2. After incubation, the cells were rinsed with PBS, and RPMI supplemented with 300 µg/mL of Gentamicin was added to kill extracellular bacteria. After 1 h, the cells were washed again and lysed with PBS (0.5% Triton X-100) for 20 min. One hundred microlitres (µl) of pellet was plated in LB agar and incubated at 37°C for 24 h for counting of bacterial colonies. The assay was performed in duplicate.

A control was performed to ensure that sufficient antibiotic had been added to kill all potential extracellular bacteria. Cells were first lysed with Triton X-100, and Gentamicin was added, followed 1 h later by plating of centrifuged bacterial pellet.

Qualitative biofilm formation on glass surface

Wolfe test

The ability of *A. hydrophila* strains to adhere to abiotic surfaces (glass) was tested on 10 ml glass tube (0.5 cm of diameter) according to the protocol described by Wolfe et al. (2004). All tested bacteria were grown on Subwoofer wireless transmitter (SWT) broth prepared by mixing 5 g of Bactotryptone, 3 g of Yeast Extract and 3 ml of glycerol in 700 ml of filtered seawater plus 300 ml distilled water. Glass tubes were incubated overnight at 37°C. A 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% (w/v) Crystal Violet for 15 min and then washed with distilled water. Bacteria which form a purple pellicule on the cultures air surface were considered as glass-biofilm positives (Snoussi et al., 2008b).

Christensen test

This method consisted of inoculating 9ml of Tryptone Soy Broth with a loopful of microorganisms from a blood plate culture and incubating the broth culture tube overnight (18 h) at 37°C. The culture tubes were emptied of their contents and stained with 0.1% Safranin (Merck).

Slime production was visible through a film that occurred on tube walls. We estimated the amount of slime production as absent (score 0), weak (score 1), moderate (score 2), strong (score 3) or very strong (score 4) (Christensen et al., 1983).

Slime production on Congo Red Agar

Congo Red Agar plate test was prepared by adding 0.8 g/L Congo red (Bio Basic INC) and 36 g of Saccharose (Merck), both of which had been previously autoclaved separately, to 1 L of Brain Heart Infusion Agar (Scharlau Microbiology, Pronadisa, Madrid, Spain). Plates were incubated for 24 h at 37°C and subsequently overnight at room temperature (Freeman et al., 1989; Ziebuhr et al., 1997, Sechi et al., 2002 and Chaieb et al., 2007). Slime-producing *Aeromonas* strains grew as black colonies, while non slime-producing strains grew as red colonies. The original test was optimized by using a colorimetric scale with six tonalities: very black, black and almost black were considered as positive results, while burundy, red and very red were considered as negative results (Subashkumar et al., 2006). *Staphylococcus epidermidis* CIP 106510 was used as positive control for slime production and *Staphylococcus aureus* ATCC 25923 was used as negative control.

Qualitative and quantitative estimation of biofilm formation on polystyrene surface

Quantitative determination was carried out by the micromethod proposed by Pfaller et al. (1988) using tissue culture plates of 96 flat-bottomed wells. Each well was filled with 0.2 ml of 10^5 CFU/ml of a bacterial suspension in tensile strength at break (TSB). After 48 h of incubation in aerobiosis at 35ºC, the contents were aspirated and the plates were washed twice with PBS (pH 7.2). The wells were stained with 0.25 % safranin for 30 s. The plates were read in an enzyme- linked immunosorbent assay (ELISA) reader (Benchmark Biorad) at 490 nm. Sterile TSB was used as negative control.

A three-grade scale was used to evaluate the strains slime producing ability: negative, ODs < 0.500; (+) ODs 0.500-1.500; (++) ODs >1.500. *S*. *epidermidis* CIP 106510 was used as positive control for slime production and *S*. *aureus* ATCC 25923 was used as negative control.

Bio-assay with fish mucus

Collection of fish skin mucus

Raw mucus was prepared by rubbing body surface of healthy *Carassius auratus*, double centrifugation at 20 000 X g for 30 min at 4°C, and filtration of the final supernatant was done through 0.45 and 0.2-µm pore-size filters. The fish skin mucus was stored in sterile glass tubes in deep freezer at -20°C until use as described by Fouz et al. (2000).

Enzymatic assays

The enzymes secreted by *C*. *auratus* were evaluated using the semi-quantitative micro method Api Zym system (Bio Mérieux, Marcy l'Etoile, 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 (under a powerful light source (1000 W bulb)) for about 10 seconds, negative reactions become colourless.

Adhesive ability to fish skin mucus

Thirteen microliter of mucus suspension was applied to glass slides, and the adhesion test was assayed following the methodology described previously by Krovacek et al. (1987). Bacterial suspensions in saline solution were placed in petri dishes containing saline solution and mucus-coated glass slides, incubated at 20°C for 1 h with continuous gentle shaking and washed thoroughly several times in saline solution (Krovacek et al., 1987). After air drying, the slides were fixed with absolute methyl alcohol (20 min at 20°C)) stained with crystal violet and observed under a light microscope. The number of bacteria attached to 1 mm^2 of the mucus-coated glass slides was determined in the different assay conditions by counting 20 microscopic fields. Slides without mucus were used as controls to verify the non-adhesion of bacteria to the glass.

Anti-bacterial 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 30°C, the appearance of a growth inhibition halo around the discs indicated the antibacterial activities among *A*. *hydrophila* strains.

Biofilm visualization by atomic force microscopy

To visualize the biofilm formed on glass slides and to have an idea on the morphological changes in the cells during biofilm production, *A*. *hydrophila* ATCC 7966^T cells strongly adhesive to the glass was used as a positive control. For the experiments, the cells enriched on saline alkaline peptone water (1% (w/v) NaCl; pH 7.4) were collected, washed three times with PBS, centrifuged and the pellet was resuspended in PBS, placed on a round microscope cover slide and was examined by Atomic force Microscope (AFM, Nanoscope IIIA, Digital Instrument; Veeco) according to the method previously described (Braga and Ricci, 1998). After biofilm formation on glass slide, the piece were fixed on the round cover slide and examined by AFM.

Statistical analysis

All results are shown as the average of at least three independent experiments; variation is expressed as standard deviation.

The Pearson correlation coefficient was calculated to determine the possible relation between the adhesion to polystyrene, glass, mucus and epithelial cells and virulence of all strains. All statistics were performed using SPSS for Windows version 17.0.

RESULTS

Biochemical characterization

The *A*. *hydrophila* strains isolated in this study were biochemically heterogeneous on the basis of their biochemical activities tested on 20NE plus strops and their exoenzymes profile (Table 1). In fact, all *A*. *hydrophila* strains tested were catalase and oxidase positive. Positive reaction was observed for the mobility and for gas produced from glucose fermentation. They hydrolyzed gelatin (50%) and esculin (71.42%). Assimilation N-acetyl-glucosamine (100%), L-arabinose (69.90%), caprate (100%), malate (100%), adipate (100%) and citrate (100%). Acidification of maltose (96.15%) and mannitol (96.15%).

All *A*. *hydrophila* strains tested produced many enzymes such as amylase, lecithenase (100%), caseinase, β-haemolytic and gelatinase (50%). All strains were lipase positives, hydrolyzed DNA and resisted to ampicillin antibiotic (10 µg), and vibriostatic compound O/129 (10 and 150 µg/disc).

Table 1. Biochemical characteristics of *A. hydrophila* isolated from treated wastewater, river water, seawater (bay) and healthy fish.

		% of positive tests	
		A. hydrophila **(n = 20)**	***A. hydrophila*** **ATCC 7966**
Conventional tests			
1	Oxidase	100	+
2	Catalase	100	+
3	Motility	100	+
	API 20NE plus strops		
4	D-Glucose	92.30	+
5	Arginine dihydrolase	96.15	+
6	Urea	0	-
7	Esculin	71.42	+
8	Gelatin	50	+
9	D-Arabinose	69.90	+
10	D-Mannose	100	+
11	Mannitol	96.15	+
12	N-acetyl-glucosamine	100	+
13	Maltose	96.15	+
14	Gluconate	66.66	+
15	Caprate	100	+
16	Adipate	100	+
17	Malate	100	+
18	Citrate	100	-
	Exoenzymes production		
19	Amylase	100	+
20	Caseinase	50	+
21	Lecithenase	100	+
22	Lipase	100	+
23	Gelatinase	50	+
24	DNase	100	+
25	Beta-hemolysis on sheep blood	50	+
	Sensitivity to		
26	O/129 (10 µg)	0	-
27	O/129 (150 µg)	0	-
	Susceptibility to		
28	Ampicillin (10 µg**)**	0	-

Adhesion and invasion ability of *A. hydrophila* strains to human epithelial cells: Hep-2 and Caco-2

The tested *A. hydrophila* strains were able to adhere to the two cell lines used in this study with different degree: 80% to human colon adencarcinoma cells (Caco-2 cells) and 75% to human laryngeal carcinoma cells (Hep-2). In fact, a weak adhesion was shown from 8 strains (40%) to Caco-2 and 7 strains (35%) to Hep-2 cells.

Medium adhesion was detected in 7 isolates for Hep-2 and Caco-2 (35%). Only one strain was able to adhere strongly to both Caco-2 and Hep-2 cell lines (Figure 1). However, large differences of invasion frequencies between the 20 *A. hydrophila* strains were noted to Caco-2 and Hep-2 cells. The percentage of the invasive *Aeromonas* for Caco-2 and Hep-2 cells was the same (40%), it was just one strongly invasive strain to Caco-2 ($1\ 10^5$ CFU/ml) and Hep-2 ($82\ 10^4$ CFU/ml). Fourteen percent of *A. hydrophila* strains tested was adhesive and invasive to Caco-2 and Hep-2 (Table 2).

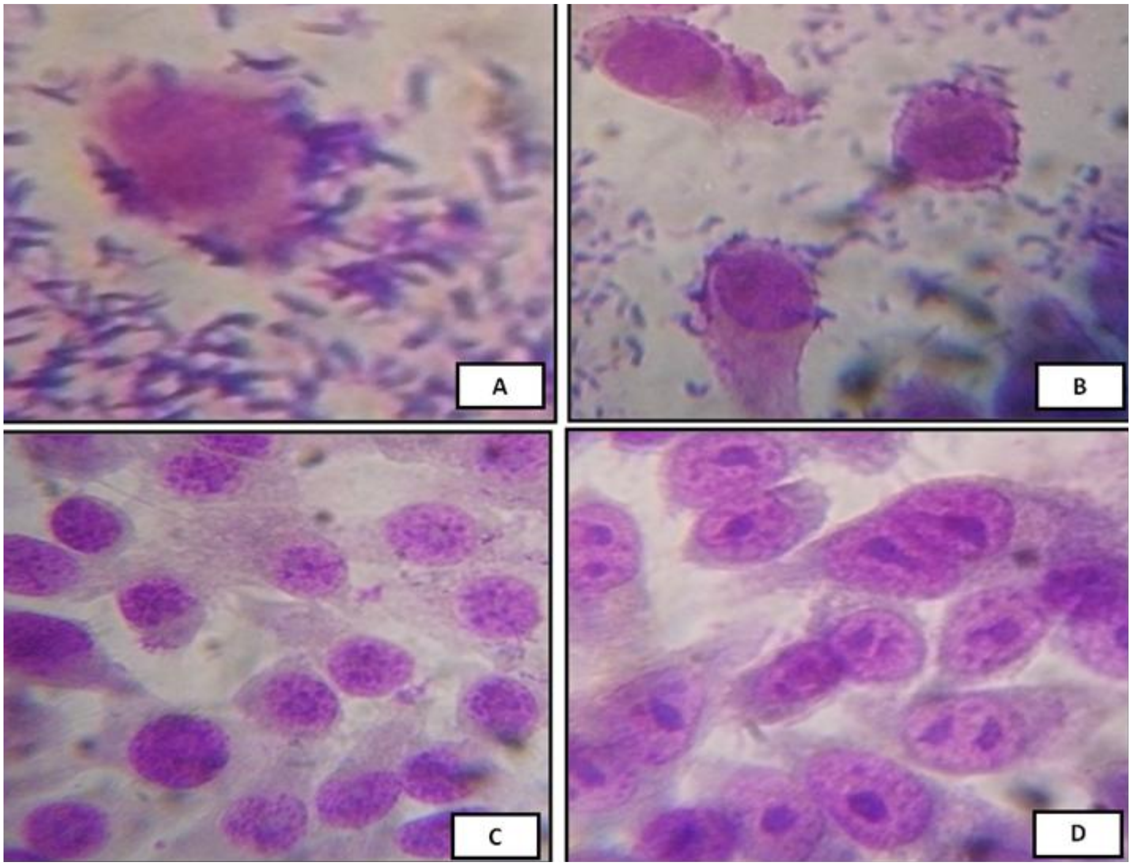

Figure 1. Optic microscopy showing the high adherence ability of *A. hydrophila* to both Caco-2 and Hep-2 monolayers. The adherence assay was performed as described in the text. Giemsa stain: magnification (x1000). (A) and (B): *A. hydrophila* strain strongly adhesive to Caco-2 and Hep-2 cells respectively. (C) and (D): Negative control for Hep-2 and Caco-2 cells.

Qualitative biofilm formation on tube methods

Biofilm formation on glass surface

Almost *A*. *hydrophila* strains were able to adhere to the glass that uses both methods; Christensen and Wolfe tests: 65 and 85%, respectively, giving a coloured pellicule (purple or red) on the air-surface of the glass tube: (Table 3). We noted that both the intensity and the width of pellicule differ from strain to strain. In fact, 5/20 (25%) give a large purple pellicule with Christensen test and 3/20 (15%) with Wolfe test (Figure 2).

Slime production

Phenotypics production of slime was assessed by culturing the strains on Congo Red Agar plates. A total of 20 strains were tested. Pigmented colonies were considered as normal slime-producing strains, whereas unpigmented colonies were classified as non-slime-producing strains. Among the isolated strains, 65% were slime producing (Table 3) characterized by very black and black colonies (Figure 3).

Qualitative and quantitative estimation of biofilm formation by *A. hydrophila* strains on polystyrene surface

Of *A*. *hydrophila* strains (95%) were able to adhere to polystyrene surface (Figure 4), 40% were strongly adhesive with a values ranging from 2.016 to 7.183 at 490nm. Only one strain was non biofilm forming with an OD_{490}<0.500 (Table 3).

Bio-assay with fish mucus

The *C. auratus* mucous was found to produce 17 enzymatic activities on the Api Zym strips (Phosphatase alcaline, Esterase (C4), Esterase Lipase (C8), Lipase (C14), Leucine arylamidase, Valine arylamidase, Cystine arylamidase, α Chymotrypsin, Phosphatase acide,

Table 2. Adhesion and invasion abilities of *A. hydrophila* strains to human epithelial cells (Hep-2 and Caco-2).

Strains	Adherence				Invasion				Adhesion/Invasion	
	Cells/Caco-2±SD	O	Cells/Hep-2±SD	O	Cells in Caco-2±SD	O	Cells in Hep-2±SD	O	Caco-2	Hep-2
ATCC 7966^T	2.3±2.5	NA	15.6±7.2	WA	0±0.0	NI	41.5±1.7 10^3	I	NA/NI	A/I
Wt1	27±3.6	MA	24.3±2.5	MA	0±0.0	NI	1.5±0.5 10^3	I	A/NI	A/I
Wt2	23.6±1.1	MA	26±1.7	MA	1±0.0 10^5	I	82±4.6 10^4	I	A/I	A/I
Wt3	23.6±1.1	MA	22.6±2.8	MA	5±0.0 10^3	I	10±0.0 10^3	I	A/I	A/I
Wt4	100±0.0	SA	100±0.0	SA	0±0.0	NI	0±0.0	NI	A/NI	A/NI
Wt5	10.3±0.5	WA	1±1.0	NA	0±0.0	NI	0±0.0	NI	A/NI	NA/NI
Wt6	15.3±5.1	WA	22.6±7.5	MA	0±0.0	NI	0±0.0	NI	A/NI	A/NI
Wt7	6±0.0	NA	16.3±4.6	WA	0±0.0	NI	0±0.0	NI	NA/NI	A/NI
Wt8	21.3±8.0	WA	12±5.5	WA	1±0.0 10^3	I	6±0.0 10^3	I	A/I	A/I
R1	17.3±2.5	WA	15.6±4.0	WA	0±0.0	NI	0±0.0	NI	A/NI	A/NI
R2	16.3±0.5	WA	16±3.6	WA	4±0.0 10^3	I	4±0.0 10^3	I	A/I	A/I
R3	25±12.2	MA	12.6±8.1	WA	0±0.0	NI	0±0.0	NI	A/NI	A/NI
R4	39±7.0	MA	39±6.5	MA	6±2.3 10^3	I	1±0.0 10^3	I	A/I	A/I
R5	13±1.0	WA	28±3.4	MA	0±0.0	NI	0±0.0	NI	A/NI	A/NI
R6	17±4.3	WA	17.3±6.6	WA	0±0.0	NI	0±0.0	NI	A/NI	A/NI
E2	4±1.7	NA	5.3±0.5	NA	0±0.0	NI	0±0.0	NI	NA/NI	NA/NI
E3	15.3±4.1	WA	2.3±0.5	NA	5±0.5 10^2	I	0±0.0	NI	A/I	NA/NI
S6	20.3±11.8	MA	23.6±5.1	MA	41.5±2.8 10^3	I	26.5±4.0 10^3	I	A/I	A/I
S7	0.0±0.0	NA	0.0±0.0	NA	0±0.0	NI	0±0.0	NI	NA/NI	NA/NI
10S	0.0±0.0	NA	0.0±0.0	NA	0±0.0	NI	0±0.0	NI	NA/NI	NA/NI
S13	32±3.4	MA	19±6.2	WA	72±8.6 10^3	I	49±4.6 10^3	I	A/I	A/I
% of positive tests		**80**		**75**		**40**		**40**	**40**	**40**

Hep-2: Cell from human laryngeal carcinoma, **Caco-2:** Cells from human colon adeno-carcinoma. Adhesion degree: NA; none adhesion; WA: weak adhesion; MA: Moderate adhesion; SA: strong adhesion. Viable intracellular bacteria in CaCo-2 and Hep-2 Cells were quantified by Gentamicin survival assays (CFU/ml). **(I)**: Invasion and **(NI)**: None invasion. Numbers represent the mean ±standard deviation. O: observation.

Naphtol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glycosaminidase, α-mannosidase and α-fucisidase).

In bioassays performed *in vitro*, 55% of *A. hydrophila* strains tested was sensitive to antimicrobial action of skin mucus from *C. auratus*. Moreover, it was found that the diameter of growth inhibition zone was ranging from 10 to 17.5 mm when the *C. auratus* mucous preparation was tested and 55% of them were very sensitive (Table 3). These findings demonstrate the adherence mucus characteristic and the anti-*A. hydrophila* activity (Figure 5).

All the strains showed positive adhesion on the mucus, in fact, the 20 tested isolates were positive for attachment to mucus-coated glass slides. From them, 15 strains adhered to mucus-coated

Table 3. Qualitative and quantitative estimation of *A. hydrophila* biofilm formation on CRA plates, polystyrene microtiter plates, glass tubes (TSB and SWT), adherence and sensitivity to fish mucus.

Strains	Slime production CRA		Biofilm formation on polystyrene		Wolfe Test			Christensen Test			Adherence and Sensitivity to fish mucus*	
	Phenotypes	Slime	Mean OD$_{490}$±SD	O	Pellicule formation in Bactotryptone broth	O	Adherence to the Glass surface	Pellicule formation in TSB	O	Adherence to the Glass surface	(cells/mm^2)±SD	mm±SD
ATCC 7966^T	Very Black	P	2.133±0.015	++	+	Strong	A	+	Strong	A	82.6±12.9	10±0.0
Wt1	Red	NP	2.136±0.07	++	+	Weak	A	+	Strong	A	45.2±30.1	17.5±8.6
Wt2	Red	NP	1.640±0.01	+	+	Weak	A	-	Absent	NA	31.8±17.2	6±0.0
Wt3	Very Black	P	1.783±0.028	+	+	Medium	A	+	Weak	A	55.2±20.8	6±0.0
Wt4	Red	NP	1.690±0.01	+	+	Strong	A	-	Absent	NA	91.2±18.5	12±0.0
Wt5	Very Black	P	1.646±0.02	+	+	Strong	A	-	Absent	NA	45.4±16.2	6±0.0
Wt6	Burundy	NP	1.513±0.015	+	+	Medium	A	+	Strong	A	37±14.3	16±0.0
Wt7	Black	P	1.523±0.015	+	+	Medium	A	-	Absent	NA	53±11.5	6±0.0
Wt8	Black	P	6.363±0.318	++	+	Weak	A	+	Weak	A	34±15.7	12±0.0
R1	Red	NP	1.473±0.02	+	+	Weak	A	+	Strong	A	99.4±0.8	10±0.0
R2	Very Black	P	1.490±0.01	+	+	Weak	A	+	Medium	A	105±14.7	6±0.0
R3	Red	NP	2.183±0.155	++	+	Very strong	A	+	Weak	A	140.8±31.7	6±0.0
R4	Very Black	P	2.706±0.066	++	+	Medium	A	-	Absent	NA	90±11.9	12±0.0
R5	Very Black	P	0.049±0.0005	-	+	Weak	A	+	Weak	A	98.8±13.5	6±0.0
R6	Burundy	NP	1.490±0.01	+	+	Medium	A	+	Strong	A	88.6±12.8	14±0.0
E2	Very Black	P	1.473±0.005	+	-	Absent	NA	-	Absent	NA	53.4±5.8	16±0.0
E3	Very Black	P	2.286±0.023	++	+	Weak	A	+	Strong	A	53±15.3	14±0.0
S6	Red	NP	1.816±0.02	+	-	Absent	NA	-	Absent	NA	62±12.5	6±0.0
S7	Very black	P	2.016±0.011	++	+	Weak	A	+	Weak	A	47±18.3	10±0.0
S10	Very black	P	5.156±0.125	++	-	Absent	NA	-	Absent	NA	66.6±14.9	6±0.0
S13	Black	P	7.183±0.263	++	+	Weak	A	+	Weak	A	53.6±1.1	10±0.0
Positive tests (%)	**65**		**95**			**85**			**65**		**100**	**55**

NP): slime non producer; (P): slime producer. Colorimetric scale with six tonalities was used: very black, black and almost black were considered as positive results, while burgundy, red and very red were considered as negative results. A three-grade scale was used to evaluate the strains slime producing ability: **(-):** ODs < 0.500; **(+)**: ODs 0.500 to 1.500; **(++)**: ODs >1.500. *S. epidermidis* ATCC 35984 was used as positive control for biofilm formation and *S. epidermidis* ATCC 12228 was used as negative control.(A): Adhesive, (NA): None Adhesive. (-): none pellicule formation and (+): pellicule formation (Wolfe et al., 2004; Christensen et al., 1983). *****: *C. auratus* mucous. After 24 h of incubation at 30°C, the appearance of a growth inhibition halo around the discs indicated that antibacterial substances were present in the mucus.

glass slides at > 50 bacteria/mm^2 and 2 strains at > 100 bacteria/mm^2. Atomic force micrograph of biofilm formed by *A*. *hydrophila* on glass surface was showed in Figure 6.

DISCUSSION

Motility and flagella play a crucial role in adhesion, biofilm formation, and colonization of several pathogenic *A. hydrophila* (Merino et al., 1997; Rabaan et al., 2001). Moreover, 50 to 60% of mesophilic aeromonads are able to produce many unsheathed peritrichous lateral flagella when grown

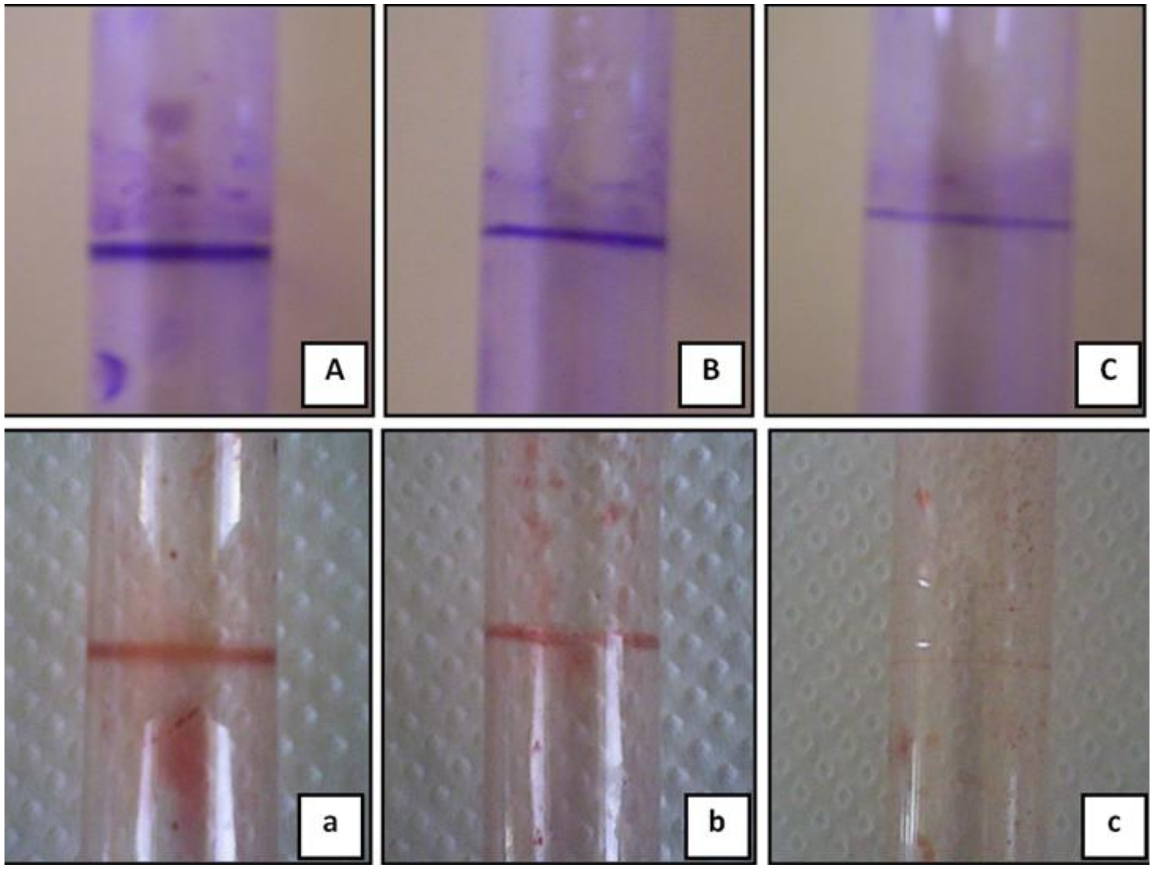

Figure 2. Pellicule formation by *A. hydrophila* strains on the surface of the tested glass tube then stained with 1% Cristal violet and 0.1% Safranin. (A,a): large pellicule formation (*A. hydrophila* ATCC7966); (B, b): medium pellicule formation, (C, c): weak pellicule formation.

in viscous environments or over surfaces (Shimada et al., 1985), which increase bacterial adherence and are required for swarming motility and biofilm formation (Gavin et al., 2002).

This study is the first carried out on environmental *A. hydrophila* strains adhesive properties isolated from coastal and internal waters and from healthy fish in Tunisia. A vital step for the bacteria to initiate infection is through adherence to host cells, allowing localisation and subsequent colonisation of the appropriate target tissues by the pathogens (Finlay and Falkow, 1997; Scoglio et al., 2001). In fact, the ability of *A. hydrophila* to invade epithelial cells has not been fully elucidated and there are few studies on this virulence attribute (Watson et al., 1985; Theodoropoulos et al., 2001).

Merino et al. (1997) demonstrated that the polar flagellum of A. *hydrophila* is essential for the invasion of fish cell lines. In addition, the ability of *Aeromonas* to invade epithelial cells has been associated with dysentery-like diarrhoea (Lawson et al., 1985; Watson et al., 1985). Theodoropoulos et al. (2001) demonstrated that Plesiomonads are able to invade human epithelial (Caco-2) cells.

In our study, the bacteria were able to adhere to the two epithelial cell lines. It seems that these isolates were adhesive on Caco-2 and Hep-2 with a degree between (75 and 80%). These data are not in accordance with the results showed by Sechi et al. (2002). In fact, these researchers were found just 33% of environmental tested strains was able to adhere on epithelial cells, when they studied the virulence factors of *A. hydrophila* strains isolated from aquatic environments.

This study proves that fourteen percent of tested *A. hydrophila* strains were invasive to epithelial cell lines. In fact, 40% of tested strains were adhesive and invasive on Caco-2 and Hep-2.

The results obtained in this study suggest the presence, in some strains, of a bacterial factor responsible for their affinity to *C. auratus* mucus. This affinity may involve specific interaction between structures (for example, pili, flagella, etc.) on the bacterial cells and receptors on the fish mucus (McSweegan and Walker, 1986; Mouricout and Julien, 1987; Metcalfe et al., 1991, Balebona et al., 1995). Thus, in its natural environment, *C. auratus* provides an optimal substrate through its mucus layer for accumulation of aquatic bacteria on its

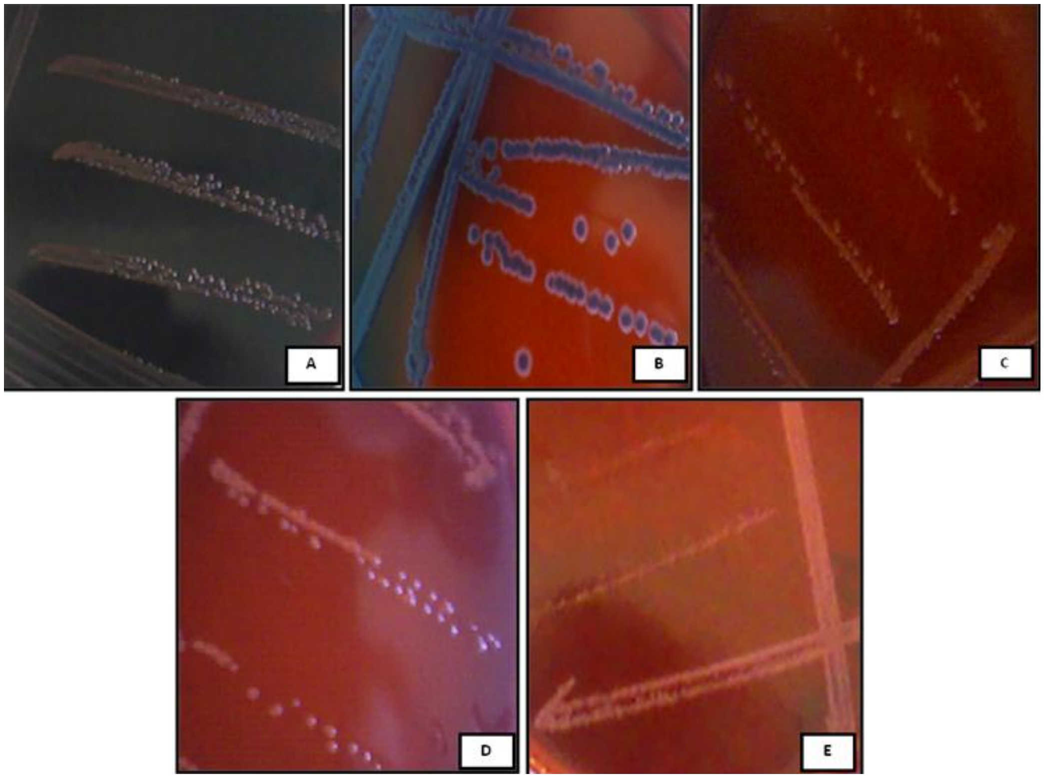

Figure 3. Morphotypes of *A. hydrophila* based on the colorimetric scale obtained on Congo Red Agar: (A) Very Black colonies (*A. hydrophila* ATCC7966^T); (B) Black colonies; (C) Pinkish-red colonies; (D) Red colonies and (E) very red colonies. *S. epidermidis* producing and non-producing slime were used as negative and positive controls.

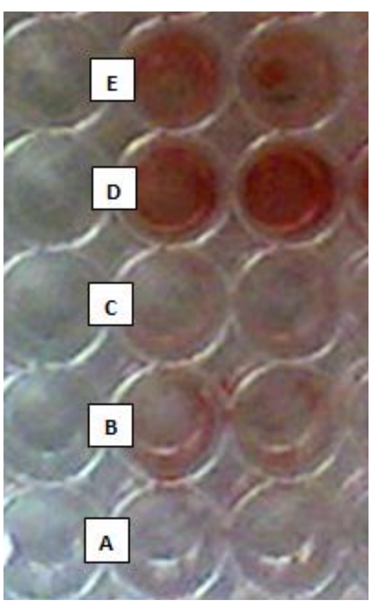

Figure 4. (A). No Biofilm formation (B, C). Weak Biofilm formation, (D, E) Strong Biofilm formation after 24 h of growth on a microtiter plate of *A. hydrophila;* Microtiter plate containing biofilms was stained with safranin (0.25%).

skin. The capability of the potential bacterial pathogens to cause disease depends on their virulence properties in relation to the mucus replacement rate (Balebona et al., 1995). In addition, the mucus layer allows a selective exchange of metabolites between the epithelial cells and the environment. Thus, cytotoxins produced by *Aeromonas* strains capable of adhesion to fish mucus may penetrate through the mucus layer and cause damage on the fish skin (Krovacek et al., 1987). Those data were accorded with our results, in fact, 100 % of the strains tested showed attachment abilities to mucus-coated glass slides. In all the cases, this capability was related to the pellicle formation in TSB and SWT. In fact, several studies carried out in mammals have shown that certain bacterial pathogens possess the phenotypic property of attaching to, and colonizing, mucus surfaces prior to infecting the host intestinal epithelial cells (Freter et al., 1981; Laux et al., 1984). Similar results were obtained by Krovacek et al. (1987). However, the process of pellicle formation related to the adhesion is still unknown. Clegg and Old (1979) observed that *E. coli* and other type of fimbriate *Enterobacteria* cultured in nutrient broth incubated statically in air or microaerophilically formed pellicles at the broth-air interface by means of aerotactic locomotion (Old and Duguid, 1970). Some of these fimbriae have been associated with adhesion

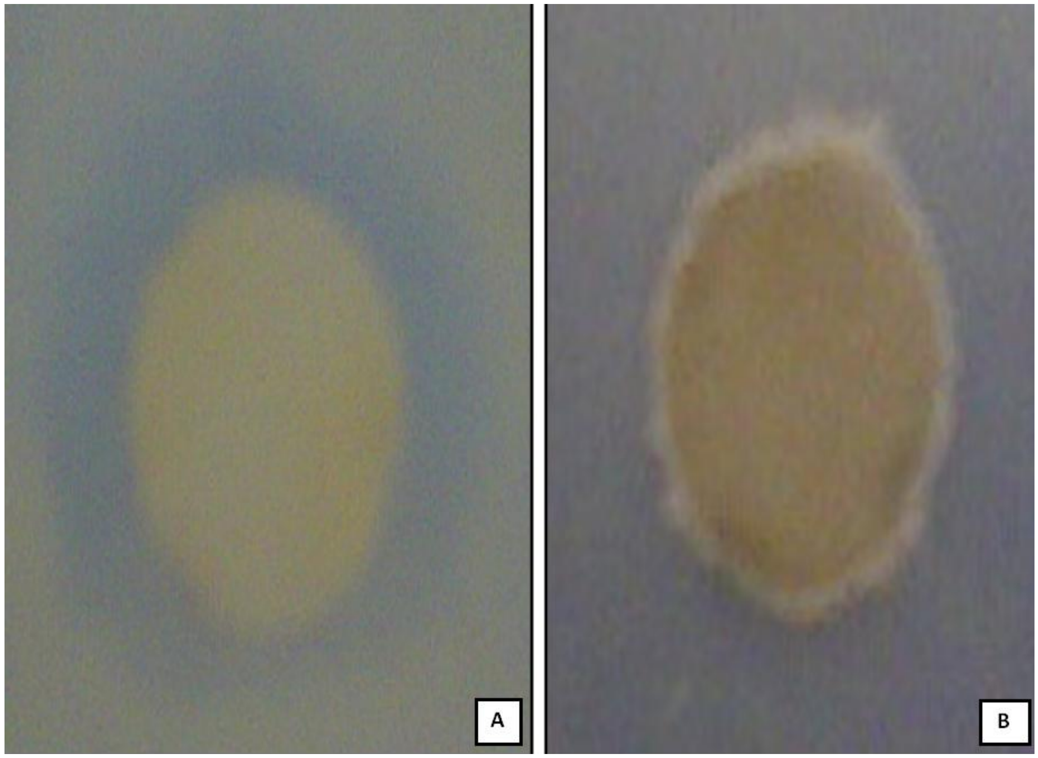

Figure 5. Effect of mucus preparation on bacterial growth. Bacterial strains were seeded on *Mueller Hinton* agar supplemented with 1% NaCl. Paper disks (6 mm of diameter) containing 20 µl of skin mucus were placed onto the seeded agar. (A) and (B) represent respectively the positive and negative antimicrobial activity of *C. auratus* mucus on *A. hydrophila* ATCC7966^{T}.

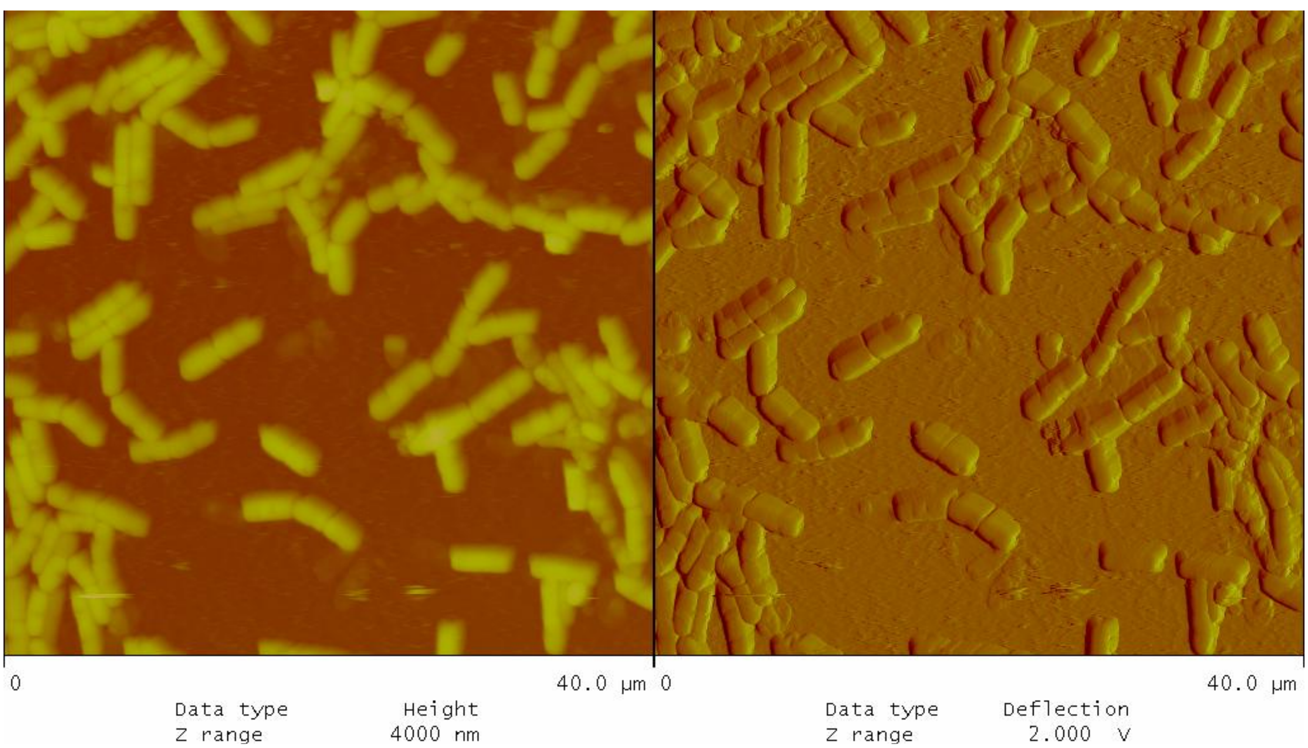

Figure 6. Atomic force micrographs of biofilm formed by *A. hydrophila* ATCC7966^{T} on glass surfaces.

(Beachey 1981). Similarly, Fletcher (1990) concluded that the bacterial attachment to surfaces is heavily influenced, not only by the species of bacteria, but also by the individual strain phenotypes and their nutrient requirements. In addition, Fouz et al. (2000) noted that *Photobacterium damselae* subsp. *damselae* strains showed a strong ability to adhere to the fish mucus of eel and turbot, with a degree of adhesion similar to that previously reported for other fish pathogens (*Vibrio alginolyticus*, *Vibrio vulnificus*, *Vibrio anguillarum*, *A. hydrophila, P*. *damselae* subsp. *piscicida* and *Flexibacter maritimus*) (Krovacekck et al., 1987; Amaro et al., 1995; Balebona et al., 1995 and Snoussi et al., 2008a).

The skin mucus of *C*. *auratus* had a high range of antibacterial effect against *A*. *hydrophila* strains. In fact, 55% of *A*. *hydrophila* strains were sensitive to *C*. *auratus* skin mucus. These results were in accordance to those reported by Beachey (1981), who founded that the mucus layer covering the host epithelial surface may protect the underlying cells from bacterial colonization by inhibiting bacterial attachment to the epithelial cells. In addition, this ability may be explained by the enzymatic profile of this mucus which exhibits the enzymatic activities. Snoussi et al. (2008b) had showed that *Sparus aurata* and *Discentracus labrax* mucous preparation had a high range of antibacterial effect against *V. alginolyticus* strains. In fact, the skin mucus acts as a natural physical barrier between the external and internal environments on the fish aiming to eliminate the pathogens.

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 morphotypes on the basis of the colour of the colonies obtained. The morphotype I is characterized by red and very red colonies including six strains, morphotype II with burundy colonies (2/20 strains) and morphotype III which were considered as slime producers were characterized by black and very black colonies (12/20 strains) including reference strain *A*. *hydrophila* (ATCC7966^{T}). Previous studies used this medium to study the phenotypic formation of biofilm for several bacteria including *Aeromonas* spp. (Sechi et al., 2002), *Staphylococcus* spp. (Aricola et al., 2001 and 2002; Chaieb et al., 2005 and Zmantar et al., 2006) and *Vibrio* spp. (Snoussi et al., 2008a, Snoussi et al., 2008b).

Our *A*. *hydrophila* strains were able to adhere to glass surface characterized by a purple and red 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 differ 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, the adhesion power developed on polystyrene material and pellicle formation on glass SWT and TSB (P-values $<$ 0.05). In fact, out of the 12 strains slime producing (black and very black colonies), 17 strains belong to the glass tube with SWT and 12 strains belong to the glass tube with TSB.

There is no correlation between virulence enzymes (lecithinase, amylase, DNase and lipase), but the protease production, β-haemolysis and adherence to epithelial cells (Hep-2 and Caco-2) had probably relation with strains isolation origin (*P*-values were lower than 0.05 for each test). Those results were accordant with those founded by Sechi et al. (2002); they had showed that skimmed milk was hydrolysed by 90% of A. *hydrophila* clinical strains and only by 55.5% of environmental strains. So, the haemolysin and protease production was found more frequently in the clinical *A*. *hydrophila* strains.

Conclusion

Our results confirmed that *A*. *hydrophila* strains shows a specific binding capability to *C*. *auratus* mucus; polystyrene and glass surfaces with varying levels of adhesion among strains. We have shown also that *A*. *hydrophila* is capable of adhering to human epithelial cells with internalization. The overall data suggest that *A*. *hydrophila* exhibits a high power of adhesion to biotic and abiotic surfaces and susceptible fish can colonized by this bacterium and that the skin can be used as portal of entry into the fish. Other studies are necessary to understand the public health significance of Aeromonads. Further investigation is needed to confirm the possible correlation found between virulence properties and environmental isolates, and to investigate if genetic exchange between these bacteria can occur in the environment.

ACKNOWLEDGEMENTS

Authors are grateful to Prof Zanetti Stefania and Donatella Usai for the help in adhesion test to epithelial cells (Dipartimento di Scienze Biomediche, Sezione di Microbiologia Sperimentale e Clinica, Universita di Sassari, Sassari, Italy) and for the correction and valuable comments on the English version of the manuscript.

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