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Evaluation of Morphological Changes of *Aeromonas caviae* Sch3 Biofilm Formation under Optimal Conditions*

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ABSTRACT

Aeromonas is a Gram-negative bacterium that lives in aquatic habitats. It can be infective in humans. One of its remarkable attributes is the ability of biofilm formation. Many factors are involved in the construction of biofilms as has been described for *Pseudomonas*, *Klebsiella*, and *Vibrio*, among others. The aim of this work was to study the bacterial morphology during the establishment of biofilm through scanning electron microscopy (SEM) and transmission electron microscopy (TEM) with a modified microtiter plate assay and to determine the best conditions for the establishment of *Aeromonas caviae* Sch3 biolfilm *in vitro*. We observed several phenotypic changes, including surface appearance, size, presence of extracellular vesicles from 100 to 250 nm in diameter, and flagella. The best conditions for biofilm formation were to grow cultures at 28˚C at pH 6, as determined by the crystal violet assay. This is, to the best of our knowledge, the first study that describes the cell's biological events involved in the establishment of biofilm formation of *Aeromonas caviae* Sch3 *in vitro*.

Keywords: Biofilm; *Aeromonas caviae*; Scanning Electron Microscopy; Transmission Electron Microscopy; Physical and Chemical Factors

1. Introduction

The genus *Aeromonas* is constituted by waterborne Gram-negative bacteria that live in aquatic environments, including groundwater and chlorinated drinking water [1]. This genus contains a number of different taxa, where *A. hydrophila*, *A. caviae*, *A. salmonicida*, and *A. veronii* biovar veronii are the most studied [2]. These species are considered pathogenic to humans because they cause intra- and extra-intestinal infections, and other severe illnesses, such as septicemia, wounds infection, and respiratory tract disease [2]. The *Aeromonas* mechanism of pathogenicity is not yet completely understood, although several virulence factors involved in the establishment of infection have been identified. Some of these factors are proteases, lipases, bacterial structures like flagella and pili, S layer, aerolysin, and siderophores [3,4]. In the last few years, the biofilm structure has been revealed as an important bacterial association with a significant role in exacerbating human infection, because it provides bacteria some properties that make antibiotics treatment difficult [5].

Biofilms, also known as sessile communities, are tight associations of microorganisms growing on surfaces and embedded in a matrix of extracellular polymeric substance (EPS). They have been described in Gram-positive (e.g., *Staphylococcus* spp. and *Streptococcus* spp.) and Gram-negative (e.g., *Pseudomonas* spp., *Vibrio* spp., *Klebsiella* spp., and *Escherichia coli*) bacteria. Microorganisms in a biofilm are more resistant to antimicrobial agents and innate immunity host defense than planktonic cells [6]. Biofilms constitute an intricate interplay between physical and chemical factors, and have physiological and genetic properties such as gene transfer and gene activation through bacterial communication known as quorum sensing. As a result of this complexity,

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biofilm-forming bacteria might express more virulent phenotypes [7].

Two of the most studied biofilm models are those generated by *P. aeruginosa* and *V. cholerae*. Depending on the experimental conditions used, biofilms develop as flat, mushroom-shaped, or loose protruding structures through a series of distinct steps where regulation of cellular migration and adhesiveness play important roles [8,9]. Several studies have allowed the identification of a number of relevant factors involved in biofilm development. These factors include molecules necessary for bacterial attachment and spreading, such as outer membrane proteins, polar and lateral flagella, polysaccharides, cell-to-cell interconnecting components, environmental clues such as pH, temperature, composition of culture media, oxygen availability, and some genetic elements (plasmids) [10-12].

Biofilms are surface-associated, multicellular communities of bacteria that form through a developmental process and they are the most common mode of bacterial growth in natural environments [6].

Several studies have now demonstrated that cells in biofilm state have phenotypic characteristics distinct from those of their planktonic counterparts with signifycant changes in the patterns of gene expression [6,13].

To date, little is known of the physiological changes that occur in biofim formation in the case of *Aeromonas* species. However, we known that exopolysaccharides and flagella play key roles in biofilm formation [13]. Flagella are essential for invasion and adherence to fish and human cell lines [13]. It has been shown that polar and lateral flagella heighten biofilm formation [4]. The polar flagellum is expressed constitutively and allows bacteria to move in liquid environments, whereas lateral flagella help to move on semisolid media and are responsible of swarming motility [13]. In some studies lateral flagella have been shown to be essential for cell adherence and biofilm formation [4], while in others both kinds of flagella are required for these processes [14]. *A. caviae* strain Sch3 was originally isolated in the United Kingdom in 1991 from the diarrheal feces of a child of less than 1 year old with gastroenteritis (nausea, vomiting, and abdominal pain). It has two distinct flagellar systems, namely a polar flagellum for swimming in liquid and multiple lateral flagella for swarming over surfaces and it can adherence to (and possibly invasion) the epithelial cell line HEp-2 [14]. However, the knowledge of the morphological changes in *Aeromonas* cells during biofilm formation is not yet clearly defined nor do we how certain grown conditions can improve biofilm formation by *Aeromonas* strains. The aim of this study was to observe the morphological variants in *Aeromonas caviae* Sch3 biofilm by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) and determine the effect of some physical and chemical factors, such as temperature, pH, and incubation time on its formation.

2. Materials and Methods

2.1. Bacterial Strain

We used the *A. caviae* Sch3 strain isolated from diarrheal feces of a 5-year-old child patient. It was kindly provided by Dr. Jonathan Shaw from the University of Sheffield Medical School (United Kingdom). Genetical and biochemical characterization of *A. caviae* Sch3 was performed by Dr. Castro Escarpulli from the Escuela Nacional de Ciencias Biólogicas del Instituto Politécnico Nacional (Mexico).

2.2. Culture Conditions

A. caviae Sch3 strain was grown on 1.5% trypticase soy agar medium (TSA) (Bioxon, Mexico) at 37˚C for 16 or 18 h. Short-term storage of isolates was done in minimal maintenance medium [1%] [v/v] casein peptone, 0.3% [v/v] yeast extract, 1% [v/v] bacteriological agar and 0.85% [v/v] NaCl) at room temperature (RT). Long-term storage was done in Todd Hewitt broth (Oxoid, Mexico) containg 40% (v/v) glycerol at –70˚C.

2.3. Conditions of Biofilm Formation

Quantitative biofilm formation experiments were performed in a microtiter plate, as previously described by Gavín [4] with some modifications. Briefly, several colonies from an overnight culture were gently resuspended in 10 mL of trypticase soy broth (TSB) (Bioxon, Mexico) and adjusted to an optical density of 0.8 at 600 nm. Then, aliquots of bacterial suspension (5 mL) were placed in each well of a polystyrene microtiter plate (Costar) and incubated for up to 48 h at 37˚C without shaking at different values of pH (5.0, 5.5, 6.0, 6.5, 7.0, and 8.0). To determine the effect of temperature, cultures were grown at 8, 28, 37, and 42˚C for 48 h. The effects of incubation times were analyzed at 24, 48, 72, and 96 h at 28˚C (this temperature was previously determined as optimal in this study). After the establishment of the best conditions for biofilm formation, cells attached were carefully washed with phosphate-buffered saline (PBS), and fixed with 2.5% (v/v) glutaraldehyde for 10 min at RT and stained with 0.4% (w/v) crystal violet for 20 min at RT. We used the medium as control. The biofilm-bound crystal violet was solubilized with 2 mL of ethanol-acetone (80/20, v/v) and the absorbance for each well (4 mL) was measured at 570 nm in a spectrophotometer Optimus 10,000 xs (Spectronic 20D Genesis). The cut-off OD was defined as the mean OD negative control. Each test was performed in triplicate.

Statistical analysis. Biofilm results were statistically

evaluated by Student's *t* test (test of variances) for related samples with a confidence interval of 95%, differences with a P value < 0.05 were considered statistically significant and one-way ANOVA analysis using the SPSS Predictive Analytics software version 18.0.

2.4. Scanning Electron Microscopy

Biofilms were obtained on glass coverslips (13 mm in diameter), previously treated with poly-L-lysine at 0, 24, 48, 72, and 96 h at 28˚C without shaking. After incubation, unattached cells were removed by pipetting and cells on coverslips were fixed with 2.5% glutaraldehyde and 1% OsO4 in PBS, ethanol dehydrated, critical point dried in a CO_2 atmosphere in a Samdry-780A apparatus (Tousimis Research, USA), and gold coated in a Denton Vacuum Desk II (INXS, Inc., FL. USA). Coverslips containing the biofilms were attached to aluminum holders and analyzed using a SEM JEOL 65LV (JEOL, LTD. Japan). Digital images were recorded, and photocompositions were processed with Adobe Photoshop software.

2.5. Transmission Electron Microscopy

Several colonies of *A. caviae* Sch3 were resuspended in TSB (pH 6.0), and adjusted to a cell density of 3×10^9 cells/mL. Then, cultures were incubated at 28˚C for 48 h without shaking. Next, the bacterial suspension was placed on Formvar coated grids and negatively stained with 2% (w/v) uranyl acetate (pH 4.1) for 5 min at RT. Grids were observed under a JEOL 1400 transmission electron microscope at 80 keV (JEOL LTD, Japan). Digital images were obtained and processed with Adobe Photoshop software (USA).

3. Results

Biofilm structure varies with environmental conditions; indeed, different forms of biofilms exist (O'Toole *et al.* 2000) [15]. Therefore, we studied the influence of some of these factors involved in biofilm formation of *A. caviae* Sch3 *in vitro*, and then we studied the morphological changes in a mature biofilm.

3.1. Influence of Temperature on *Aeromonas caviae* Sch3 Biofilm Formation

Cultures of *A. caviae* Sch3 were grown at 8˚C, 28˚C, 37˚C, and 42˚C for 48 h as described in Materials and methods. We considered the growth obtained at 28˚C as 100%, because this bacterium grows close to this temperature in nature [1]. Therefore, our results were normalized to this value. When cells were cultured at 8˚C, 37˚C, and 42˚C, biofilm formation dramatically decreased in 72%, 81%, and 80%, respectively, in relation to 28˚C (**Figure 1(a)**). We had guessed that the optimal temperature would be 37˚C, the human temperature. However, it is unknown if *A. caviae* Sch3 is able to form biofilms in humans.

3.2. Effect of pH on *Aeromonas caviae* Sch3 Biofilm Formation

Aeromonas has the capacity of growing at pH values between 6 to 8 [16]. Therefore, we tested the capability of this bacterium to form a biofilm at pH values of 5.0, 5.5, 6.0, 6.5, 7.0, and 8.0 at 28˚C for 48 h. We considered the culture obtained at pH 7.0 as 100%, and consequently, our results were normalized to this value. When cells were cultured at pH 5.0 and 5.5, we observed a reduction in biofilm formation of 53% and 28%, respectively. In contrast, at pH values of 6.0 and 6.5, the formation of biofilm increased 134% and 105%, respectively. Besides, when cells were cultured at pH 8.0, we observed an increase of only 31%. All these data indicate that the best pH value for an optimal formation of biofilm *in vitro* is 6.0 (**Figure 1(b)**).

3.3. Optimal Incubation Time for the Formation of *Aeromonas caviae* Sch3 Biofilm

Cultures of *A. caviae* were grown at pH 6.0 and 28˚C (the optimal conditions found in this work) for 24, 48, 72, and 96 h. We considered the incubation time of 48 h as 100% of biofilm production. When bacteria were cultured for 24 h, the formation of biofilm was 81% of the reference value. At longer incubation times, such as 72 h and 96 h, we observed a slight increase of 27% and 23%, respectively, in the formation of biofilm (**Figure 1(c)**).

3.4. Scanning Electron Microscopy of *Aeromonas caviae* Sch3 Biofilm Formation

Finally, we studied through SEM the characteristics of *A. caviae* Sch3 biofilm obtained on a polystyrene surface at optimal growth conditions *in vitro* at different incubation times. At 24 h, we observed few rod-shaped bacteria on the surface of the polystyrene dish (**Figure 2(a)**) with sizes varying between 1.2 and 4.6 μm in length. Some dividing cells are shown at an amplification of 10,000× (**Figure 2(b)**). When cells were observed at a larger amplification (20,000×), some cells exhibited a smooth surface while others were slightly rough. Moreover, we observed extracellular vesicular material (**Figure 2(c)**, ar row). At 48 h, bacteria number increased (varying in size) attached to the polystyrene surface (**Figure 2(d)**). In **Figure 2(e)**, most bacteria showed the smooth phenotype. At a larger magnification, some cells exhibited a rough phenotype, while others showed the smooth and semi-smooth phenotypes (**Figure 2(f)**). In addition, two cells

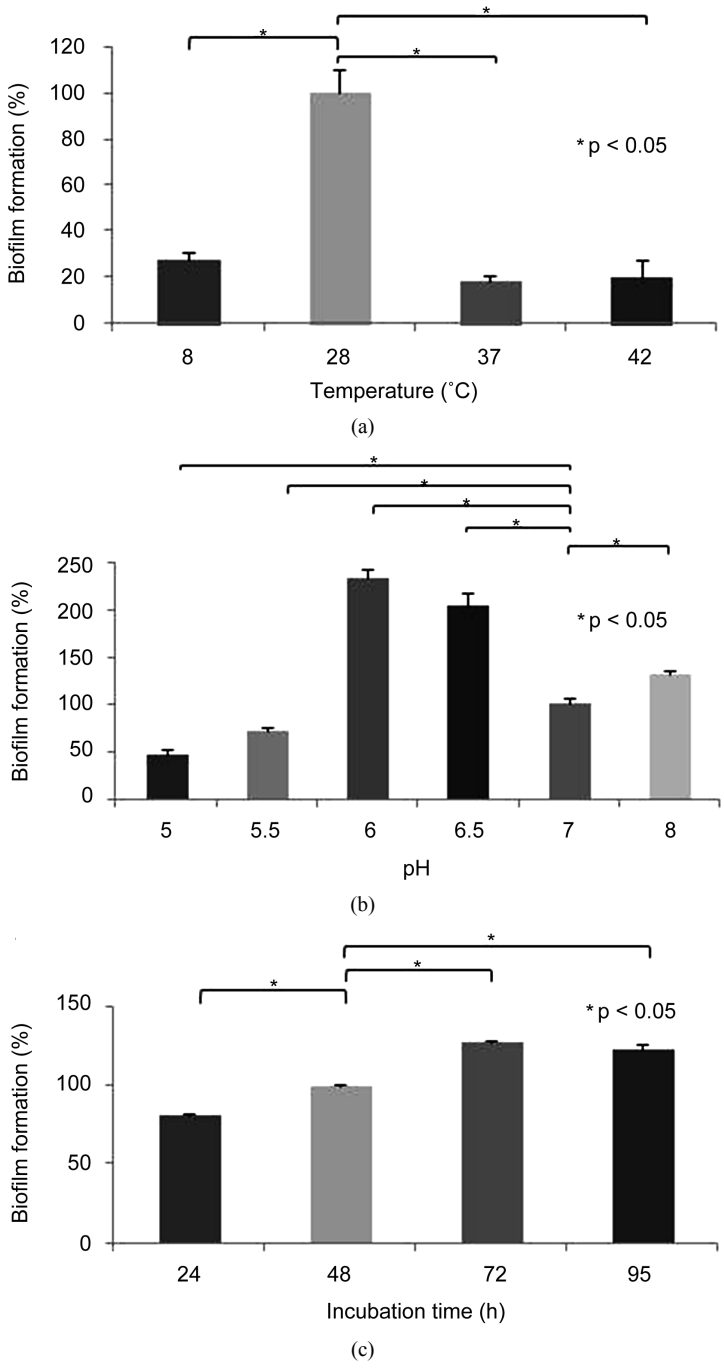


Figure 1. Biofilm formation of *Aermonas caviae* Sch3 at different conditions. Cells were grown in TSB medium without shaking. The amount of biofilm was determined by crystal violet method, as described in Materials and methods. (a) Effect of temperature; (b) Influence of pH; (c) Evaluation of incubation time in the biofilm formation. The analysis was done with Student *t*-test. Statically significance was set at P < 0.05 (*). Each bar represents the average of three replicates, and vertical lines represent standard errors.

that could be conjugating were seen (**Figure 2(f)**, arrow). At 72 h of cultivation, an early stage in the biofilm formation was observed (**Figure 2(g)**). One characteristic identified at this time was the presence of chains of bacilli as larger as 7 to 8 μm forming a nest. Most of the cells presented no septum (**Figure 2(h)**). In **Figure 2(i)**, a large amount of extracellular material containing many vesicles was detected, although some cells seemed to be damaged. After 96 h of incubation, a flat biofilm structure was found corresponding to the typical morphology of mature biofilms (**Figure 2(j)**) [9,17,18]. When the biofilm was analyzed at a magnification of 8500×, some

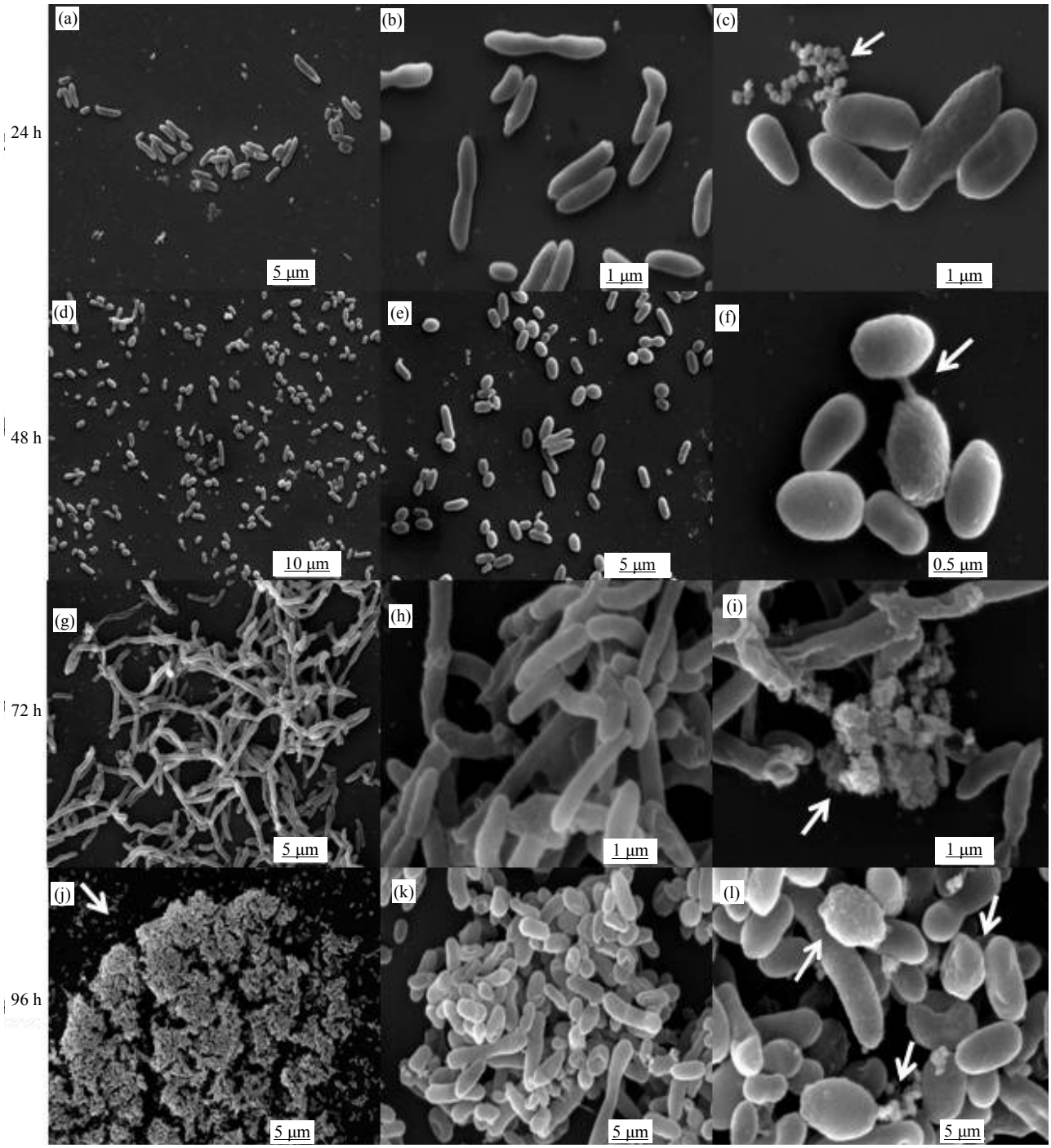


Figure 2. SEM micrographs of *Aeromonas caviae* Sch3 biofilm. Cells were grown on polyL-lysine-treateds attached glass coverslips at 28˚C in TSB and gently resuspended in PBS liquid medium without shaking after following incubation times: (a)-(c) 24 h (few bacterial cells attached to the surface); (d)-(f) 48 h (augment of number of attached cells); (g)-(i) 72 h (cell elongation, loose of bacterial septum); and (j)-(l) 96 hr (micro colonies, mature biofilm, multiple phenotypes). Arrows point out vesicles (c), (i), (l) or two putative conjugating bacteria (f) Mature biofilm (j) Different surface phenotypes (l). Scale bar = 0.5 µm - 5 µm.

groups of bacteria were forming microcolonies. Cells in these groups showed variation in size and shape. Most of them were from 1.9 to 2.8 µm in length, although some others measured 12.7 µm in length (**Figure 2(k)**). Finally, the mature biofilm at a magnification of 20,000× showed bacteria with the three surface phenotypes (smooth, semi-smooth, and rough) and also showed some extracellular vesicular material close to bacterial walls (**Figure 2(l)**).

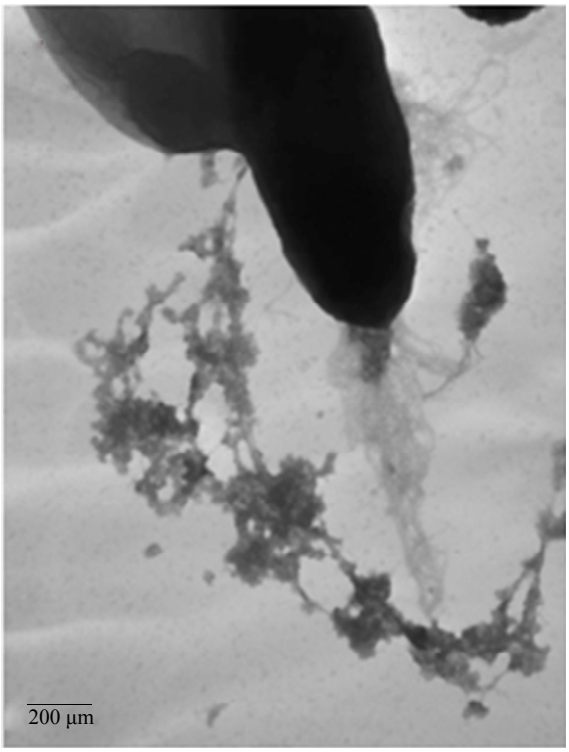
3.5. Transmission Electron Microscopy of the Biofilm Formed by *Aeromonas caviae* Sch3 under Optimal Culture Conditions

We analyzed the characteristics of *A. caviae* Sch3 biofilm formation under optimal conditions at 48 h by TEM. **Figure 3(a)** depicts one bacterial cell negatively stained by uranyl acetate. The presence of vesicular material in panel b was evident. We also corroborated the two different kinds of populations, previously observed by SEM; one of them was 4 μm in length whereas the other was only of 2 μm. Finally, panel c depicts a cell with several bacterial appendages.

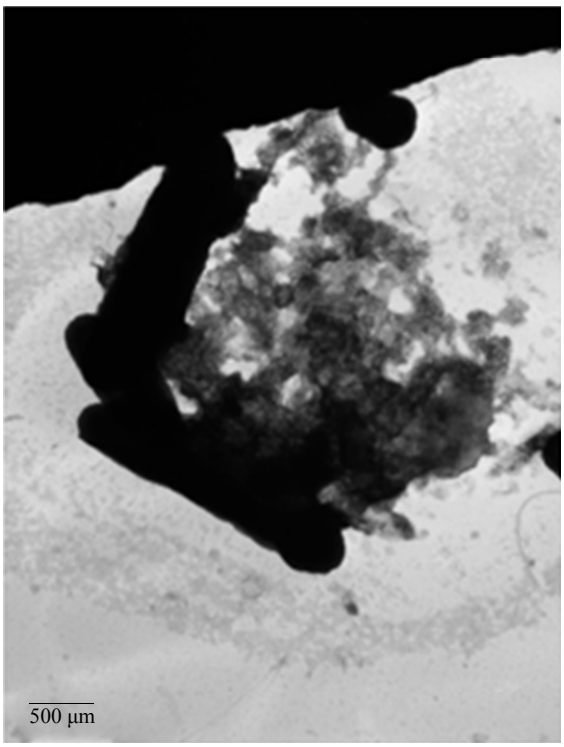
4. Discussion

Recent studies have suggested that *A. hydrophila*, *A. caviae*, and *A. veronii* (bv veronii) are responsible for approximately 85% of total infections in humans caused by bacteria from this genus [2]. One of the pathogenicity mechanisms of these bacteria is the formation of biofilms in their hosts, which contribute to an increase in the virulence of these microorganisms and in their resistance to antibiotics, consequently, in their survival [6,19].

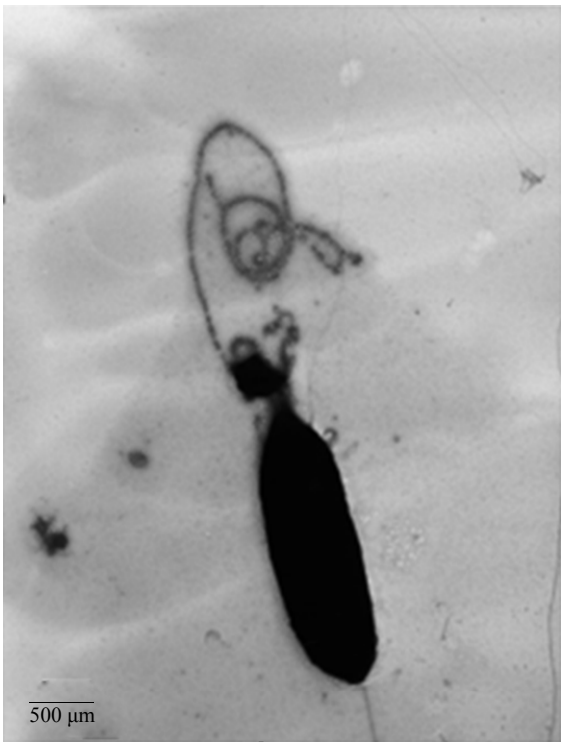
The formation of the biofilm has been thoroughly studied in *E. coli*, *P. aeruginosa*, and *V. cholerae* [20]. Several molecules have been identified to contribute to the formation of *P. aeruginosa* biofilm. The alginate, Psl and Pel exopolysaccharides are structural components of the biofilm's matrix [21-23]. Each of them is encoded in



(a)



(b)



(c)

Figure 3. TEM micrographs of *A. caviae* Sch3 biofilm. Cells were grown at 28˚C in TSB medium for 48 h without shaking and placed on grids for negative staining with 2% (w/v) uranyl acetate. (a) Cells observed at 10,000× magnification; (b) and (c) Cells observed at 25,000× magnification. Scale bar = 200 μm - 500 μm

the *algD*, *psl* and *pel* operons, respectively [22,24]. Twelve genes constitute the *algD* operon. This operon is down-regulated by the MucA polypeptide by interacting with the AlgT/U sigma factor [23]. MucA degradation or truncation results in loss of the ability to interact with AlgT/U sigma factor, allowing it to bind to its promoter and potentiate alginate production, and, ultimately, inducing a conversion to a mucoid phenotype [25].

The effect of some physical, chemical, and genetical factors in the *Aeromonas* biofilm has not been studied in detail.

The genus *Aeromonas* has a remarkable capability to tolerate high pH values, for example, alkaline peptone water culture medium (pH 8.5 - 8.8), but these bacteria can grow also at pH values of 4.6 [26]. It is well known that tolerance to acidic conditions depends on the species of *Aeromonas* [27]. In our biofilm formation model, when bacteria were grown at pH 6.0 and 6.5, we observed an increase in biofilm formation (twice higher than that obtained at pH 7.0). At pH 5.0 or 5.5, the biofilm production was equal or lower than half the value obtained in the control, whereas at pH 8.0, it presented a modest increase. This result is different from those observed with *P. aeruginosa*, *K. pneumoniae*, and *V. cholerae* biofilm models, where maximum production was achieved between 7.5 and 8.5, although they also grew at a higher level at pH of 5.5 to 6.5 than that obtained at pH 7.0 [12]. With respect to the temperature effect we found that formation of biofilm was highly efficient at 28˚C. In other models, the temperatures assayed were only 30 and 37˚C showing no significant difference in biofilm production [12,28,29].

Our SEM results of the *A. caviae* Sch3 biofilm at optimal conditions revealed the presence of vesicular material with sizes ranging between 100 nm and 250 nm (**Figure 3**), which has not been previously reported. We do not know the composition of these vesicles. In Gram negative bacteria like *P. aeruginosa*, extracellular vesicles or outer membrane vesicles (OMVs) are composed of outer membrane proteins OprD, OprE, OprF, OprG, OprH, OprI, PagL, and PcoB, as well as lipopolysaccharides, phospholipids, and DNA [30]. These elements have been implicated in cell-cell communication, attachment, aggregation and biofilm formation [31]. One advantage of their compartmentalization in vesicles is that they can exert their function far away from the place, where they were produced [31]. OMVs can nucleate and maintain cohesion of biofilms in *P. aeruginosa* and *Helicobacter pylori* [32]. However, the production of these vesicles is not exclusive of sessile cultures, but planktonic cells can produce them also in different sizes and numbers [31,33].

In this study we observed changes in the bacterial morphology. Three phenotypes were related to the bacterial surface: smooth, semi-rough, and rough. Different sizes were also detected, finding bacterial elongation as large as 7 μm, with a consequent increase in bacterial surface that could facilitate attachment to the surface or to other bacteria. These characteristics could help in the establishment or maintenance of the biofilm [34-37]; their role remains to be determined. Biofilm development involves a series of steps starting with physicochemical interactions between microbial cells and substrate, followed by cell adhesion, multiplication, and differentiation, leading to the formation of mature biofilm. Some appendages were observed through TEM. Most of them were polar flagella. Very few lateral flagella were detected. In *Aeromonas*, polar and lateral flagella have been described as essential for biofilm formation [4,38]. However, more studies are needed to reveal their role in *A. caviae* Sch3 biofilm formation.

5. Conclusion

In order to unveil the process involved in biofilm development in *Aeromonas caviae* Sch3, we established an *in vitro* model under controlled laboratory conditions. The best conditions for the formation of the biofilm were a pH value of 6.0 and a temperature of 28˚C, which allowed us to know some microscopic characteristics of this biofilm, such as different changes in bacterial morphology, presence of vesicular material of 100 to 250 nm in size, and polar flagella.

6. Acknowledgements

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