RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2014) 17:205-212
doi:10.2436/20.1501.01.223. ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

Biofilm formation on polystyrene in detached vs. planktonic cells of polyhydroxyalkanoate-accumulating *Halomonas venusta*

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Received 15 September 2014 · Accepted 20 October 2014

Summary. Biofilm development is characterized by distinct stages of initial attachment, microcolony formation and maturation (sessile cells), and final detachment (dispersal of new, planktonic cells). In this work we examined the influence of polyhydroxyalkanoate (PHA) accumulation on bacterial surface properties and biofilm formation on polystyrene in detached vs. planktonic cells of an environmental strain isolated from microbial mats, *Halomonas venusta* MAT28. This strain was cultured either in an artificial biofilm in which the cells were immobilized on alginate beads (sessile) or as free-swimming (planktonic) cells. For the two modes of growth, conditions allowing or preventing PHA accumulation were established. Cells detached from alginate beads and their planktonic counterparts were used to study cell surface properties and cellular adhesion on polystyrene. Detached cells showed a slightly higher affinity than planktonic cells for chloroform (Lewis-acid) and a greater hydrophobicity (affinity for hexadecane and hexane). Those surface characteristics of the detached cells may explain their better adhesion on polystyrene compared to planktonic cells. Adhesion to polystyrene was not significantly different between *H. venusta* cells that had accumulated PHA vs. those that did not. These observations suggest that the surface properties of detached cells clearly differ from those of planktonic cells and that for at least the first 48 h after detachment from alginate beads *H. venusta* retained the capacity of sessile cells to adhere to polystyrene and to form a biofilm. [**Int Microbiol** 2014; 17(4):205-212]

Keywords: *Halomonas venusta* MAT-28 · PHA · alginate beads · cell surface physicochemical characteristics · adhesion on polystyrene

Introduction

Our perception of bacteria as planktonic life forms is deeply rooted in the axenic ("pure") culture paradigm. Growth in liquid culture has been exploited to study many bacterial activities. However, in nature, bacteria rarely grow as axenic planktonic cultures; rather, the normal mode of bacterial growth involves attachment to a surface, followed by the development of a microbial community and biofilm formation [1,21,22,34]. Microorganisms in biofilms are coordinated functional communities that are much more efficient than mixed populations of floating planktonic organisms. In fact, biofilms resemble the tissues formed by eukaryotic cells with respect to their physiological cooperation and the extent to which they are protected from variations in bulk-phase condi-

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tions by a primitive homeostasis afforded by the biofilm matrix of exopolysaccharides [13].

Biofilm development is characterized by distinct stages of attachment, growth, and detachment (the dispersal of planktonic cells) [2,28,30]. Naturally occurring immobilized microbial cells, such as those in biofilms, differ physiologically from planktonic (free swimming) cells in a variety of ways. These modifications occur at the beginning of biofilm formation, following cell adhesion, when the cells develop surface-sensing responses [18]. The immobilization of cells in alginate beads, which serve as artificial biofilms, bypasses the adhesion step. However, the similar physiological responses of artificially and naturally immobilized microorganisms, as evaluated based on protein expression patterns, support the existence of a specific metabolic behavior by "sessile" cells [18,20,39].

The functional strategies and physiological versatility of bacterial populations growing in biofilms allow the cells to resist changing conditions within their environment [16]. In fact, prokaryotes have evolved numerous mechanisms of resistance to stress conditions. For example, many microorganisms have an inherent ability to form resting stages (e.g., cysts and spores) [25]; others, such as the spirochete *Spirosymplokos deltaiberi,* become swollen and form refractile resistant bodies on exposure to air [26]. The accumulation of intracellular storage polymers such polyhydroxyalkanoates (PHA) increases cell survival in changing environments [2,5,19] by enhancing bacterial environmental fitness, especially under environmental stress conditions such as UV irradiation and osmotic, thermal, and oxidative stress [33,35,38]. PHAs are energy- and carbon-rich storage compounds that accumulate as intracellular granules, but they can be mobilized and used under unfavorable conditions. Under stress conditions, bacterial cells with a higher PHA content survive longer. than those with a lower PHA content.

Among the many bacterial species that are able to accumulate PHAs are members of the genus *Halomonas*, which belongs to the family Halomonadaceae, a Gammaproteobacteria. Members of the Halomonadaceae are gram-negative, chemoorganotrophic, aerobic or facultative anaerobic, and moderately halophilic, haloalkaliphilic, halotolerant, or non-halophilic. In this work, we used the PHA-accumulating strain *H. venusta* MAT-28, isolated from a microbial mat (a complex biofilm) from the Ebro Delta [4].

Halomonas venusta MAT-28 was cultured in an artificial biofilm in which the cells were immobilized on alginate beads and as free-swimming (planktonic) cells. Cells detached from alginate beads and their planktonic counterparts were used to: (a) investigate adhesion to polystyrene and (b) determine whether cells that accumulate PHA are better able to adhere to a new surface than those that do not.

Material and methods

Cell immobilization by alginate beads. The moderately halophilic bacterium used in this study was *Halomonas venusta* MAT28, which was isolated from microbial mats in the Ebro Delta, Spain. Sodium salt alginic acid from *Macrocystis pyrifera* (61% mannuronic acid and 39% guluronic acid; Sigma-Aldrich, St. Louis, MO, USA) was prepared as previously described [6]. The conditions used for bead preparation favored the leakage of entrapped bacteria while maintaining the integrity of the beads (Fig. 1A, B).

Growth mode: alginate beads and planktonic cells. Three different growth media were used in the assays: tryptic soy broth (TSB; Oxoid, Barcelona, Spain) + 3% NaCl, TSB-30 + 3% NaCl, and minimal medium + glucose + 3% NaCl. Only the last one allows PHA accumulation; the two TSB-based media do not [6]. The three culture media are referred to in the following as TSB, TSB-30, and MM, respectively. Two modes of bacterial growth were investigated. Thus, the cells were immobilized on alginate-beads as an artificial biofilm and cultured as free-swimming (planktonic) cells. Two Erlenmeyer flasks each containing 50 ml of one of the three culture media were prepared: one for cells immobilized on alginate beads and the other for planktonic cells. The flasks were incubated at 30ºC for 24 h (for MM, the incubation time was 48 h to allow PHA accumulation). Alginate bead cultures contained approximately 8 beads/ml. Planktonic cultures were prepared from a 1:1000 ml dilution of an overnight culture. After 24 h (or 48 h for PHA accumulation) of incubation, the affinity of detached and planktonic cells for different solvents and their adhesion properties were assayed.

Microbial affinity for solvents (MATS). The method employed was described by Giaouris et al. [15]. Detached and planktonic cells were harvested by centrifugation (7500 rpm, 10 min), washed twice with phosphate-buffered saline (PBS), pH 7.0, and resuspended in the same solution at a final optical density (OD_{600}) of 0.8. Each bacterial suspension (2.4 ml) was mixed for 60 s at maximum intensity on a vortex-type agitator with 0.4 ml each of chloroform, hexadecane, diethyl ether, and hexane (Panreac, Barcelona, Spain). The samples were allowed to stand for 60 min to ensure complete separation of the two phases. One ml was carefully removed from the aqueous phase of each sample and the optical density was measured at 600 nm. The microbial affinity for each solvent was calculated using the formula:

$$\% \text{ affinity} = (OD_0 - OD_f / OD_0) \times 100$$

where OD_0 is the optical density of the bacterial suspension before mixing with the solvent and OD_f is the absorbance after mixing and phase separation. Each measurement was performed in duplicate and the experiment was repeated three times with independent bacterial cultures. The following pairs of solvents were assayed: (a) chloroform (an acidic solvent) with hexadecane (an apolar solvent); (b) diethyl ether (a strong basic solvent) with hexane (an apolar solvent). The monopolar solvents used had similar Lifshitz–van der Waals surface tension components (Table 1) [3].

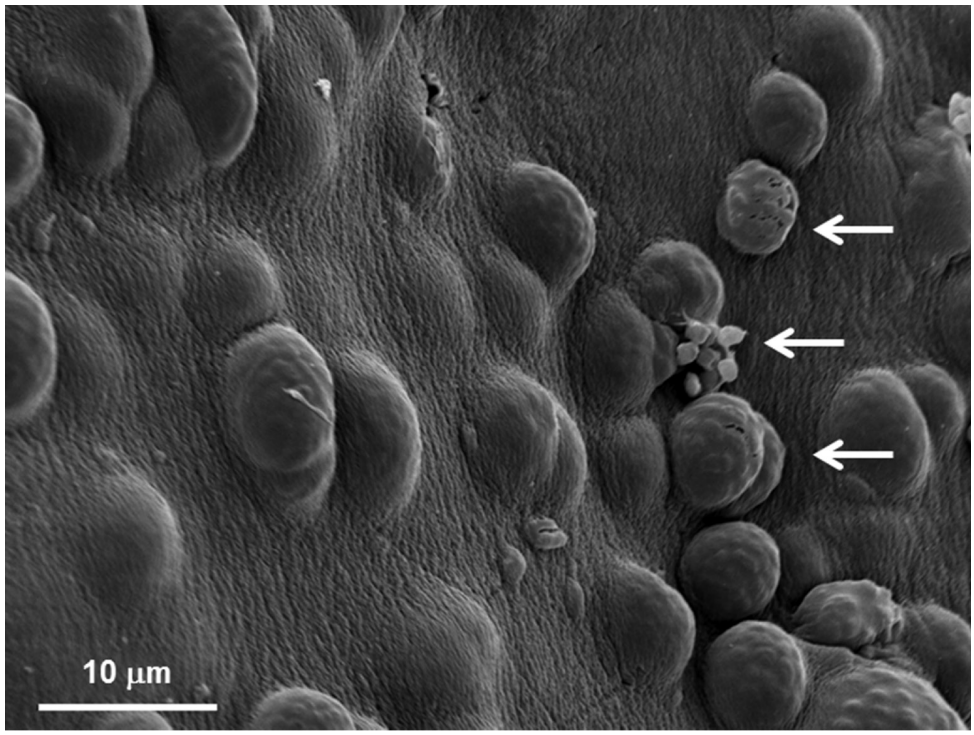

Fig. 1. Micrograph of immobilized cells of *Halomonas* in alginate beads and cultured in TSB-30 at 30ºC for 24 h. The SEM micrograph shows microcolonies formed at the surface of a bead that are about to detach.

Biofilm formation. Aliquots (150 ml) of the bacterial suspensions (1:100 dilutions of the detached and planktonic cells) were transferred to each well of a microtiter plate (plastic). The bacterial suspensions were prepared in fresh TSB, TSB-30, or MM, depending on the culture medium used previously. After a 24-h static incubation at 30ºC, the biofilm index was determined as described by O'Toole and Kolter (1998) [27].

Atomic force microscopy (AFM). AFM images were recorded using a Multimode microscope controlled with Nanoscope V electronics (Bruker AXS, Santa Barbara, CA) equipped with a 10-μm piezoelectric scanner. The bacteria were immobilized by adsorption onto a clean mica surface. A 50-μl aliquot of a bacterial suspension was placed on 1 × 1 cm of cleaved mica and incubated for 5 min at room temperature. Non-adsorbed bacteria were eliminated by gentle rinsing of the surface with PBS. The sample was dried under a nitrogen stream. All images were taken in air in contact mode with a silicon cantilever with a nominal spring constant of 40 $N \cdot m^{-1}$. The applied force was kept as low as possible to minimize damage of the sample. The images were processed using commercial Nanoscope software.

Results

Influence of the growth mode on the physicochemical properties of the bacterial surface.

The cell surface of *Halomonas venusta* growing in TSB had a higher Lewis-acid character, with a high affinity for chloroform and the lowest affinity for diethyl ether. Cells growing in TSB-30 and MM also exhibited Lewis-acid characteristics, both detached and planktonic cells, but their affinity for chlo-

Table 1. Properties of microbial affinity for solvents (MATS)-type solvents [3]

MATS solvent	Formula	Lifshitz-van del Waals (mJ/m^2)	Electron donor (γ^-) (mJ/m^2)	Electron acceptor (γ^+) (mJ/m^2)
Chloroform	$CHCl_3$	27.2	0	3.8
Diethyl ether	$C_4H_8O_2$	16.7	16.4	0
Hexane	C_6H_{14}	18.4	0	0
Hexadecane	$C_{16}H_{34}$	27.7	0	0

Table 2. Lewis acid-base and hydrophobicity surface characteristics

	MATS-type solvent			
Strain (growth mode)	Chloroform	Hexadecane	Diethyl ether	Hexane
[a]*Halomonas*-P	62.4 ± 5.0	24.3 ± 4.4	41.8 ± 2.9	28.9 ± 6,8
[a]*Halomonas*-DC	64.7 ± 5.6	25.9 ± 2.6	42.7 ± 6.2	30.2 ± 3.5
[b]*Halomonas*-P	54.97 ± 4.68	17.2 ± 3.06	47.71 ± 5.63	18.41 ± 3.34
[b]*Halomonas*-DC	56.90 ± 3.97	26.2 ± 4.66	49.75 ± 3.75	26.31 ± 1.35
[c]*Halomonas*-P (PHA)	52.3 ± 4.4	12.6 ± 2.4	44.3 ± 3.6	17.1 ± 4.6
[c]*Halomonas*-DC (PHA)	55.5 ± 3.5	24.1 ± 4.4	48.8 ± 6.7	25.3 ± 3.5

P, planktonic cells; DC, detached-cells. PHA, polyhydroxyalkanoate accumulation.
[a]TSB growth medium.
[b]TSB-30 growth medium.
[c]Minimal medium allowing PHA accumulation.

roform was lower in either of these media than in TSB medium. In general, *H. venusta* was hydrophilic, as indicated by its low affinity for apolar solvents such as hexadecane and hexane. However, detached cells displayed a slowly increasing hydrophobicity compared to planktonic cells (Table 2). Similar results have been previously reported [30].

Influence of growth mode on surface adhesion. Static biofilms from *H .venusta* were allowed to develop on polystyrene microplates. Detached cells were better able to adhere to plastic than cells growing planktonically (Fig. 2). Detached cells of PHA-accumulating *H. venusta*-PHA were less adherent to plastic than detached *H. venusta* cells that did not accumulate PHA, perhaps because of the slight differences in surface hydrophobicity (Table 2). Similar results were obtained in *Pseudomonas* [14]. Cells that accumulated PHA were more hydrophilic (lower affinity for apolar solvents) than those that did not. This result can be explained by a redirection of the carbon flux to fatty acid biosynthesis in cells that did not accumulate PHA. According to Chang et al. [10], a higher fatty acid accumulation is related to enhanced cell-surface hydrophobicity. In TSB medium, detached and

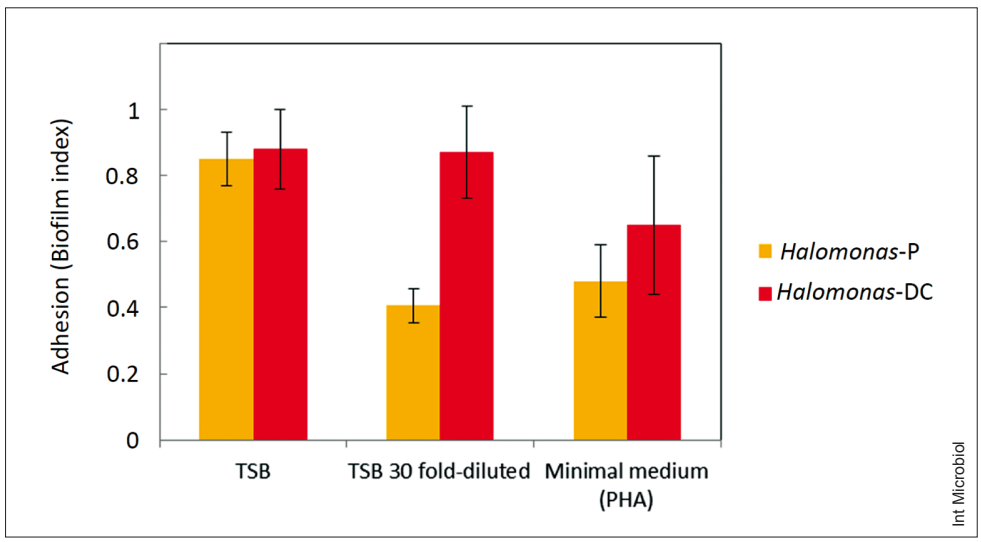

Fig. 2. Biofilm index. Adhesion to polystyrene by *Halomonas venusta* MAT28 growing planktonically (P) or on alginate beads (detached cells, DC). The height of each column is the mean of the results of three independent experiments. The standard deviations are indicated by the bars.

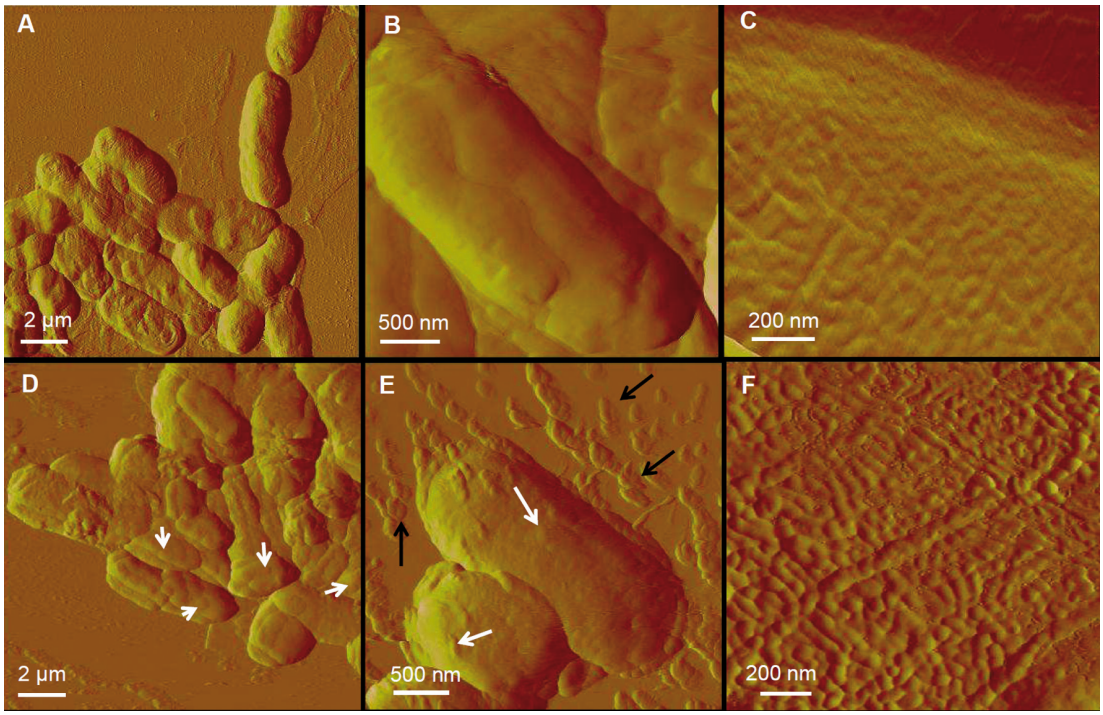

Fig. 3. Atomic force microscopy images. (**A**) *Halomonas* planktonic cells growing under conditions that did not support polyhydroyalkanoate (PHA) accumulation. (**B**) A detail of a cell from A. (**C**) A surface detail from the image in panel B. (**D**) *Halomonas* detached cells growing in a culture medium that facilitates PHA accumulation. (**E**) Detail of a cell from D. (**F**) Surface detail from panel E.

planktonic cells did not differ in their adhesion to polystyrene.

AFM produces a three-dimensional topographic image of the specimen surface with nanometer resolution. Figure 3A shows a three-dimensional topographic image of the surface of *H. venusta* grown in TSB-30 deposited on a mica surface. The bacteria are seen as ellipsoid structures with a mean length of 3.3 ± 0.3 µm, a mean width of 1.7 ± 0.2 µm, and a mean height of 290 ± 30 nm. These values, especially bacterial height, may be underestimations because of probable deformation of the cells after they attach to a flat surface. A thin film with a step height of 12 nm from mica surface was also observed. A representative zoom image of a cell is shown in Fig. 3B. The cell surface is flat and smooth, with a nanostructure of small protrusions of 6 ± 2 nm (Fig. 3C). Figure 3D shows cells detached from alginate-beads that had been suspended in MM medium, which facilitates PHA accumulation, and deposited on a mica surface. The rounded structures (white arrows) inside the bacteria are probably PHA granules. A representative image of bacteria with the above-described protrusions is shown in Fig. 3E (white arrows). Material of an undetermined nature is seen outside the cell adsorbed on mica (black arrows). Closer inspection of the bacterial surface (Fig. 3F) showed the nanostructures to be small round particles with a mean diameter of 58 ± 9 nm and a mean height of 3.8 ± 0.5 nm. Those nanoscale surface differences between *H. venusta* without PHA and with PHA may have resulted in a modification of the cells' surface properties that accounted for the difference in their adhesion capacity on polystyrene (see Table 2, Fig. 2).

To determine whether the growth mode (alginate beads or planktonic) of *H. venusta* resulted in differences in polystyrene adhesion, a principal components analysis (PCA), as a multivariate data analysis tool, was applied. The results obtained with the adhesion datasets are shown in Fig. 4 as a two-dimensional plot. A cloud of points is observed, with each point representing *H. venusta* growing under different conditions with respect to culture medium and growth mode (alginate beads and planktonic). Despite the large dispersion of the dataset (Fig. 4), a general grouping depending on the growth mode can be seen. The exception was detached and planktonic cells growing in TSB, as there were no difference is adhesion under these conditions (see Fig. 2). Otherwise, the characteristics of the detached cells were clearly different from those of planktonic cells.

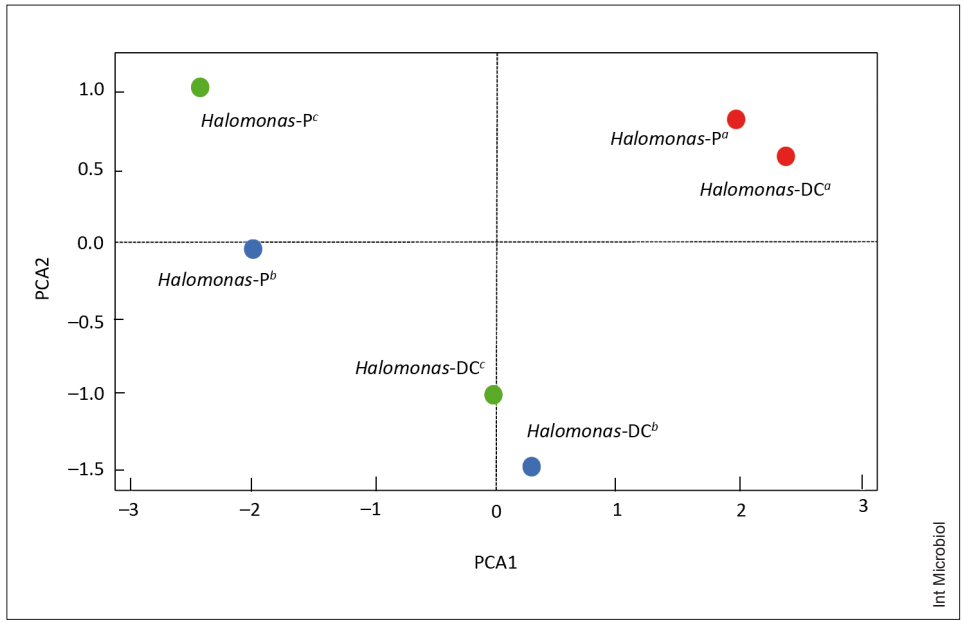

Fig. 4. Analysis of the main components of *Halomonas* detached (DC) and planktonic (P) cells, based on the physicochemical characteristics of the cell surface and cellular adhesion capability. *Halomonas* were grown in TSB[a], TSB-30[b], or minimal medium (MM)[c]. Only MM allowed PHA accumulation.

Discussion

Bacterial adhesion is a complex process that is affected by the characteristics of the bacteria (hydrophobicity, surface charge, fimbriae, production of exopolysaccharides, etc.), the surfaces properties of the material (surface charge, hydrophobicity, roughness, etc.), and environmental factors (temperature, pH, time of exposure, bacterial concentration, ionic strength, etc.) [8,11,12,24,31]. The initial adhesion of microbial cells is the product of non-covalent Lifshitz-van der Waals, electrostatic, Lewis acid-base, and hydrophobic interactions [12,24,31].

Halomonas venusta growing on TSB had the highest biofilm index on polystyrene microplates, without significant difference in adhesion between detached and planktonic cells (Fig. 2). Similar results have been reported for *Listeria* [29]. TSB is a complex mixture of casein and soy peptide hydrolyzates, along with other macromolecules that adsorb to polystyrene. The heterogeneity of the absorbed peptides in particular likely creates a variety of charged areas on the substratum that can interact with bacterial surface proteins through electrostatic as well as hydrophobic interactions. The ionic strength of the solution influences the extent of bacterial adhesion to a surface. Increasing the ionic strength decreases the thickness of the electrostatic layer surrounding a bacterium and a surface [7]. As a result, the bacterial cell can come close enough to the surface such that the strength of the van der Waals attraction may overcome the repulsive energy barrier between two negatively charged surfaces and result in bacterial adhesion [11].

The detached cells were slightly better electron acceptors (affinity for chloroform) than their planktonic counterparts, which implies an increased number of protonated groups such as NH_3 and of OH groups exposed on the bacterial surface [17]. The surfaces of the detached cells were also more hydrophobic (increase affinity for apolar solvents such as hexadecane and hexane). These differences could facilitate adhesion and biofilm formation on polystyrene, which has a negative surface charge, is hydrophobic, and is an electron donor [32].

In previous work, we showed that PHA accumulation in *H. venusta* MAT-28 reached steady-state concentrations after 48 h of incubation in MM [4] and that PHA accumulation was higher in detached (from alginate beads) than in planktonic cells [6]. From an ecological point of view, increased PHA accumulation is a natural strategy to increase survival in stressed environments [33,35]. Although detached-PHA cells were more adherent than planktonic-PHA cells, there was no difference in the ability of PHA-accumulating vs. non-accumulating cells in their adhesion to polystyrene (Fig. 2).

PCA combines two or more correlated factors into a new

variable, the principal component. Thus in PCA, the dimensionality of the dataset is reduced by replacing the original variables with a smaller number of newly formed variables that are linear combinations of the original ones and that explain the majority of the information (variability) from the experiment. PCA is an instrument of observation of multidimensional space, rather than a statistical technique [36]. As seen in Fig. 4, detached cells and planktonic cells clearly differed in their adhesion onto polystyrene, even differences in their Lewis acid-base characteristics and hydrophobicity were not substantial.

The increased biofilm index of the detached cells may reflect differences in cell-surface properties and ionic strength at the cell-substratum interface [9,12,37]. However, for bacterial cells the most important factor resulting in greater adhesion is to have derived from a biofilm, including an artificial biofilm such as alginate beads. Detached cells from a biofilm represent a transitional phenotype between the sessile and planktonic state. They maintain their biofilm-forming capacities and constitute a general response adopted by different species of bacteria [30,40]. In this study, detached *Halomonas* cells retained the ability of sessile cells to adhere to surfaces and to form biofilms for at least 48 h after detachment. Compared to permanently planktonic cells, this ability facilitates the colonization of new surfaces.

Acknowledgements. This work was supported by grant CGL2009-08922 (Spanish Ministry of Science and Technology) to RG.

Competing interests. None declared.

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