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Leonardo Di Meglio[a], Juan Pablo Busalmen[b], Juan Ignacio Pastore[c], Virginia Laura Ballarin[c] & Débora Nercessian[a]

[a] Facultad de Ciencias Exactas y Naturales, Instituto de Investigaciones Biológicas, CONICET-UNMDP, Mar del Plata, Argentina

[b] Laboratorio de Bioelectroquímica, Área Electroquímica y Corrosión, INTEMA-CONICET, Mar del Plata, Argentina

[c] Facultad de Ingeniería, Grupo de Procesamiento Digital de Imágenes, UNMdP, Mar del Plata, Argentina

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Leonardo Di Meglio[a], Juan Pablo Busalmen[b], Juan Ignacio Pastore[c], Virginia Laura Ballarín[c] and Débora Nercessian[a]*

[a]*Facultad de Ciencias Exactas y Naturales, Instituto de Investigaciones Biológicas, CONICET–UNMDP, Mar del Plata, Argentina;* [b]*Laboratorio de Bioelectroquímica, Área Electroquímica y Corrosión, INTEMA–CONICET, Mar del Plata, Argentina;* [c]*Facultad de Ingeniería, Grupo de Procesamiento Digital de Imágenes, UNMdP, Mar del Plata, Argentina*

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Biofilms by the hyperhalophilic archaea *Halorubrum* sp. and *Halobacterium* sp. were analyzed, and for the first time the progression of structural features and the developmental parameters of these sessile populations are described. Optical slicing and digital analysis of sequential micrographs showed that their three dimensional structure was microorganism dependent. Biofilms of *Halobacterium* sp. developed in clusters that covered about 30% of the supporting surface at the interface level and expanded over about 86 ± 4 μm in thickness, while *Halorubrum* sp. biofilms covered less than 20% of the surface and reached a thickness of 41 ± 1 μm. The kinetics of growth was lower in biofilms, with generation times of 27 ± 1 and 36 ± 2 h for *Halobacterium* sp. and *Halorubrum* sp., respectively, as compared to 8.4 ± 0.3 and 14 ± 1 h in planktonic cultures. Differences between microorganisms were also observed at the cell morphology level. The interaction between the two microorganisms was also evaluated, showing that *Halobacterium* sp. can outcompete already established *Halorubrum* sp. biofilms by a mechanism that might include the combined action of tunnelling swimmers and antimicrobial compounds.

Keywords: antagonistic interactions; archaea; biofilm; halophiles

Introduction

Biofilms are multi-specific communities with internal functional and structural organization that can grow at virtually any interface. As the most frequent bacterial way of life in nature (Gilbert & Allison 1993; Stoodley et al. 2002; Hall-Stoodley et al. 2004), biofilms are the focus of intensive research all over the world. Indeed, the impact that these structures can have on health, industry, and the environment promotes basic and applied studies. Biofilms are ancient in nature as revealed by fossil records of up to 3.25 billion years ago (Hall-Stoodley et al. 2004) and can be found until present days in various environments, including extreme ones. Nevertheless, in spite of the ubiquity of biofilms, most studies deal with bacterial biofilms and present knowledge of archaeal biofilms is scarce. Some reports describe the presence of representatives of the domain Archaea in biofilms from different environments (Lauwers et al. 1990; Couradeau et al. 2012; Ionescu et al. 2012; Justice et al. 2012) as recently reviewed (Fröls 2013), but works focused on studying the development of archaeal biofilms in pure culture remain scarce. Among these works are those describing euryarchaeal biofilms developed by the hyperthermophilic *Thermococcus litoralis* onto hydrophilic surfaces (Rinker & Kelly 1996), by *Archaeoglobus fulgidus* whose biofilms are induced by different stress situations (Lapaglia & Hartzell 1997), and by *Pyrococcus furiosus* that uses flagella to adhere to solid surfaces (Näther et al. 2006), resembling the strategy used by the methanogenic archaeon *Methanobacter thermoautotrophicus* that uses fimbriae as adhesins (Lauwers et al. 1990; Thoma et al. 2008). In the case of the acidophilic *Ferroplasma acidarmanus,* a proteomic study comparing planktonic and biofilm cells showed the up-regulation of several proteins related to anaerobic growth in biofilm cells (Baker-Austin et al. 2010). Also dual-species biofilms have been studied, showing cells of *Methanopyrus kandleri* adhering to a surface with cells of *P. furiosus* overlaying *M. kandleri* cells (Schopf et al. 2008). Within the crenarchaeal kingdom of the archaeal domain, Koerdt et al. (2010) describe biofilm formation by three *Sulfolobus* species under different environmental conditions.

The interest in the study of bacterial biofilms has increased over time, because of the improvement they can introduce in the efficiency of industrial and biotechnological processes (Rosche et al. 2009; Halan et al. 2012). In the same direction, halophilic microorganisms are also useful and have an enormous potential for biodegradation of contaminant organic matter in industrial application, eg in the food and tannery industries as well as in saline wastewater treatment (Margesin & Schinner

*Corresponding author. Email: dnercess@mdp.edu.ar

2001; Kargi 2002; Ruiz & De Castro 2007; Oren 2010; Ruiz et al. 2010). In addition, microorganisms produce several secondary metabolites and enzymes that have potential biotechnological applications, such as osmolites, surfactants, proteases, and other hydrolytic exo-enzymes (Oren 2010). Taking this into account, exploring the capacity of halophiles to grow to form biofilms acquires a special interest, since learning about how they are structured and determining the conditions that favor their development could improve biotechnological applications. Of particular interest in industrial applications is the ability to produce antimicrobial compounds (bacteriocins), ie metabolites that inhibit the growth of other microorganisms (Mills et al. 2011). Depending on the producing strains and the produced compound, the specificity on target strains may vary, affecting from only one to several species of competitor organisms. Halophilic archaea also produce and release antimicrobial compounds named halocins (Meseguer et al. 1986; Torreblanca et al. 1994; O'Connor & Shand 2002; Shand & Leyva 2007) that can be of interest for human activities. Many of these compounds are stable at high salt concentrations, but the stability and effect of others is not salt-dependent (Platas et al. 2002; Sun et al. 2005), making them potential candidates to control halophilic and non-halophilic microbial contaminants in industry and public health (Wellman et al. 1996; Lequerica et al. 2006; Mills et al. 2011). Not only halocins but other compounds such as quorum-sensing inhibitors released by archaeal cells are involved in antimicrobial actions, thus allowing these inhibitors to be utilized as antifouling agents in halophilic environments (Abed et al. 2013). Notably, and in spite of the possibilities mentioned, it was not until recently that the first report showing halophilic archaeal biofilms appeared (Fröls et al. 2012). In that work the authors interrogated 20 haloarchaeal species for adhesion ability under static cultivation conditions and determined that 13 of them were able to adhere to glass slides, which ultimately led to biofilm formation; however, fundamental information about hyperhalophilic biofilms is still lacking.

In the present work, the capacity of two hyperhalophilic archaea, *Halorubrum* sp. and *Halobacterium* sp, for biofilm development under permanent flow was tested and proved. The microorganisms selected for this study are able to grow at salt concentrations around 20%, but tolerate saturating NaCl concentrations (35%) and are widely distributed in salterns around the world (Oren 2002; Burns et al. 2004; Birbir et al. 2007). Due to their ecological significance, these organisms have been selected as models for exploring the biofilm developmental process in hyperhalophilic archaea for the first time.

Parameters including biofilm thickness, growth kinetics, volumetric fraction, and surface coverage were measured *in situ* and *in vivo*. Complimentarily, taking into account that selected microorganisms were found to compose an antagonistic-target couple, the direct interaction between them was explored in the biofilm system.

Materials and methods

Microorganisms, culture conditions, and biofilm assays

Halobacterium sp. and *Halorubrum* sp. were isolated from La Colorada Grande saltern, located in La Pampa province, Argentina (38°17′S; 63°43′W) and identified by sequencing a fragment of the gene encoding for the 16S rRNA subunit after PCR amplification.

Planktonic cultures of both microorganisms were grown in saline water (SW) medium, containing (g l^{-1}) NaBr 0.65; $NaHCO_3$ 0.17; KCl 5.0; $CaCl_2$ 0.72; $MgSO_4.7H_2O$ 49.5; $MgCl_2.6H_2O$ 34.5; NaCl 195.0; yeast extract (YE) 2.0 (Mutlu et al. 2008) at 30 °C and under constant stirring at 150 rpm. In planktonic cultures, growth was determined by measuring the optical density at 600 nm (OD_{600}) of samples taken at increasing times of incubation. The OD_{600} values were plotted against time to calculate the generation time (μ) from the linear portion of the graph, corresponding to exponential growth.

Biofilms were developed at 30 °C in a flow cell consisting of a transparent polymethylmethacrylate (PMMA) plate of 45 × 45 × 2 mm with a central hole 10 mm in diameter that functions as the growth chamber. It was sealed at bottom and top with two glass coverslips using silicone grease. To serve as a substratum for cell adhesion, glass surfaces were chemically cleaned by immersion in 0.02 M $KMnO_2$ in 1 M H_2SO_4 for 24 h, washed with 10% H_2O_2 – 1 M H_2SO_4, and washed again in distilled water to eliminate residual acid before use. The cell including the PMMA core and the two coverslips was secured between two stainless steel plates using silicone O-rings and screws (see Figure S1 [Supplementary material is available *via* a multimedia link on the online article webpage]). Direct microscopic observation was performed through the coverslips.

Flow was controlled by a peristaltic pump (Longerpump BT100-2J DG10) at 0.06 ml min^{-1} throughout the experiments, giving a residence time of 2.6 min, much shorter than measured duplication times. The flow cell was connected through silicon tubes to reservoirs containing fresh and wasted medium, and the system was operated without recirculation.

Bubbles were prevented from reaching the growth chamber by interposing a bubble trap at the liquid entrance. This trap also served to prevent cell migration from the main chamber to the medium reservoir.

The assembled flow system, composed of tubings and reservoirs with the corresponding culture medium, was sterilized in an autoclave before connecting the

chamber that was filled with 70% ethanol for 30 min and washed with sterile culture medium lacking YE before inoculation.

Biofilm optical analysis

Considering the generation time of microorganisms measured in planktonic growth, pre-cultures used as the inoculum for the biofilm system were synchronized at the late exponential growth phase (2×10^8 cells ml^{-1}) in order to inoculate all biofilm growing chambers at the same time. At this stage, cells were harvested by centrifugation and re-suspended in SW culture medium lacking YE, to prevent modification of the glass surface properties by the adsorption of organic molecules during inoculation (Busscher & Weerkamp 1987). The chamber was filled with the archaeal suspension and kept static for 30 min before starting the medium circulation to facilitate cell adhesion. The whole system was kept at 30 ± 2 °C during the experiments.

In vivo observation of biofilm development was performed by phase contrast in a Nikon TiU inverted microscope with motorized *Z*-axis, equipped with a refrigerated camera Nikon DS-Qi1Mc U2 connected to a PC. The analysis of the three-dimensional (3D) structure was performed by optical sectioning and digital analysis of stacked images (Lawrence et al. 1991). Biofilm thickness, spatial distribution, surface coverage, and the volume occupied by cells (Vc) (Busalmen & de Sanchez 2003) were determined. This last parameter was calculated as $Vc = \Sigma\, Ai\, d$, where Ai corresponds to the area covered by cells in every image, with i varying from 1 to n focal planes; and d is the distance between each focal plane (2 μm in this case). Biofilm thickness was calculated as the number of focal planes between the surface (0 μm) and the biofilm–liquid interface multiplied by 2 (Bakke & Olsson 1986). Data are reported as the mean ± standard deviation (SD) of at least three biological replicates.

To properly determine the spatial distribution and the volume occupied by cells, digital images were background subtracted using the 2D rolling ball tool of the public domain Image J software (available at http://rsbweb.nih.gov/ij/index.html), manually thresholded to select only in-focus bacteria and converted to binary format to determine the fraction of black pixels (representing cell bodies). Only focal planes with coverage of at least 1% were considered in the calculations. The generation time (μ) of biofilms was calculated as the slope of the Vc linear variation in exponential phase and data are reported as the mean ± the SD of at least three biological replicates.

3D biofilm reconstruction

Aiming to gain information about the 3D structure of biofilms, a reconstruction method was developed. Binarized images were composed in an isosurface, which is a 3D surface representation of points with equal values in a 3D data distribution. This is a level set of a continuous function whose domain is 3D space. The method used for constructing the isosurface from the volume data was the marching cubes algorithm (Newman & Yi 2006). The algorithm was implemented in MATLAB® R2008a, using the general functions of this program.

Scanning electron microscopy (SEM)

Biofilms were fixed by carefully filling the growing chamber with 3 M NaCl containing 2.5% glutaraldehyde. After incubating for 10 min at room temperature, biofilms were dehydrated by exposure to an alcohol series (15, 30, 60, 80 and 100% ethanol). The coverslip supporting the biofilm was carefully removed from the cell, air dried, and sputtered with a 100 Å gold film for SEM observation in a Joel JSM 6460 LV scanning electron microscope.

Antagonism assay in biofilms

Sets of three biofilms of *Halorubrum* sp. (target microorganism) and one of *Halobacterium* sp. (antagonistic microorganism) were simultaneously grown in independent flow systems controlled in the same 4-channel peristaltic pump. Once they were fully developed (after about 96 h), cells were interconnected in pairs, in a way that the outgoing flow from the cell containing the *Halobacterium* sp. biofilm flowed into the cell containing the *Halorubrum* sp. biofilm. Using this configuration, free swimming cells and extracellular compounds released by the antagonistic microorganism were expected to modify the biofilm structure of the target microorganism. The other two cells containing *Halorubrum* biofilms were interconnected in the same way as a control experiment for the antagonistic effect. Also, cells containing *Halorubrum* sp. biofilms were connected to *Halobacterium* sp. biofilms as a negative control. The evolution of biofilm structure after connection was analyzed by optical sectioning and digital analysis as described above.

Results

Development and structure of hyperhalophilic biofilms

As shown by results in Figures 1 and S2–S3, selected microorganisms can grow forming biofilms on a glass surface under continuous flow. Upon inoculation cells started to adhere to the glass surface. After a variable lag phase (24–72 h), cells were found to be grouped into microcolonies (Figure 2), a point defined as the starting time (day 1) of biofilm growth. At this stage, filamentous structures typically produced by dehydration of

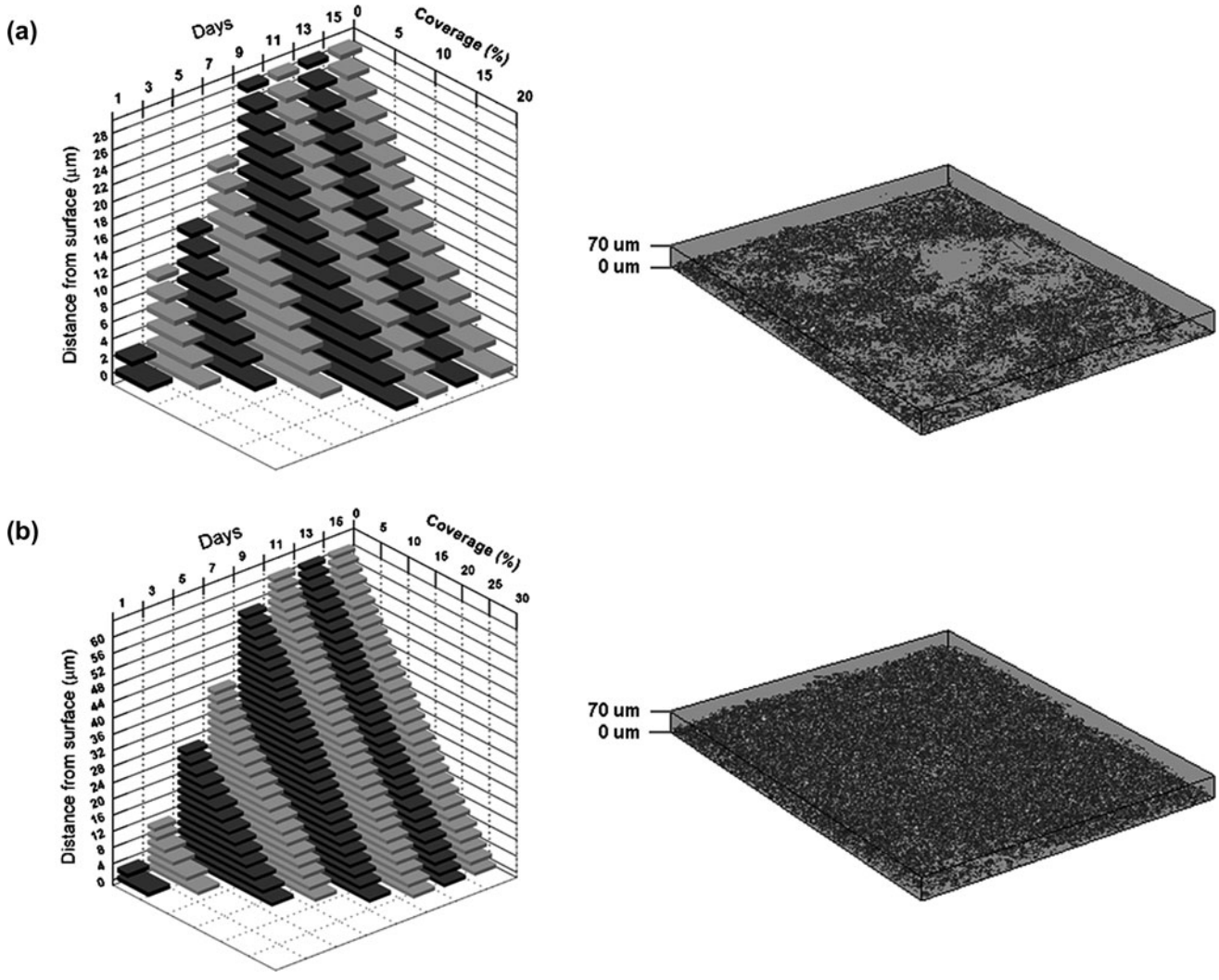

Figure 1. Left panels: development of the surface coverage in biofilm focal planes at increasing distances from the glass surface, during biofilm growth. Right panels: 3D representation of mature biofilm structure at the end of the experiment (see "Materials and methods"). Upper panels (a): *Halorubrum* sp.; lower panels (b): *Halobacterium* sp.

exopolysaccharides were observed in between neighboring cells, and in between cells and the glass surface (Figure S4), as described in *Sulfolobus* (Koerdt et al. 2010) and *Halobacterium salinarum* DSM 3754^T (Fröls et al. 2012).

After that, an exponential growth phase led to the formation of clusters, separated by channels and voids (Figure 2). The volume and height of the clusters increased with the time of growth, progressively covering the glass surface and protruding to the solution. According to the evolution of biofilm thickness (Figure S2), biofilms of *Halorubrum* sp. grew faster over about 8–9 days until reaching the stationary phase of growth, presenting a final and stable thickness of about 41 ± 1 µm at this stage. Biofilms of *Halobacterium* sp. presented a period of slow growth once microcolonies were formed (Figure S2) and before entering exponential growth (at day 2–3). After growing exponentially over about 7–9 days *Halobacterium* sp. biofilms reached a maximum thickness of 86 ± 4 µm (Figure S2) in stationary phase. The structure remained stable for at least two weeks, in spite of the permanent flow conditions (Figures 1 and S2–S3).

To analyze the growth kinetics, Vc was determined daily. This parameter was taken as a way to estimate biomass growth in a non-invasive way. Vc evolution presented in Figure S3 closely followed the profile previously observed in thickness data for both archaea (Figure S2). Generation times (μ) were calculated from the linear range (ie exponential growth stage) of data reported in Figure S2, yielding values of 36 ± 2 and 27 ± 1 h for *Halorubrum* sp. and *Halobacterium* sp. biofilms, respectively. These values were about three times slower than those determined from planktonic cultures

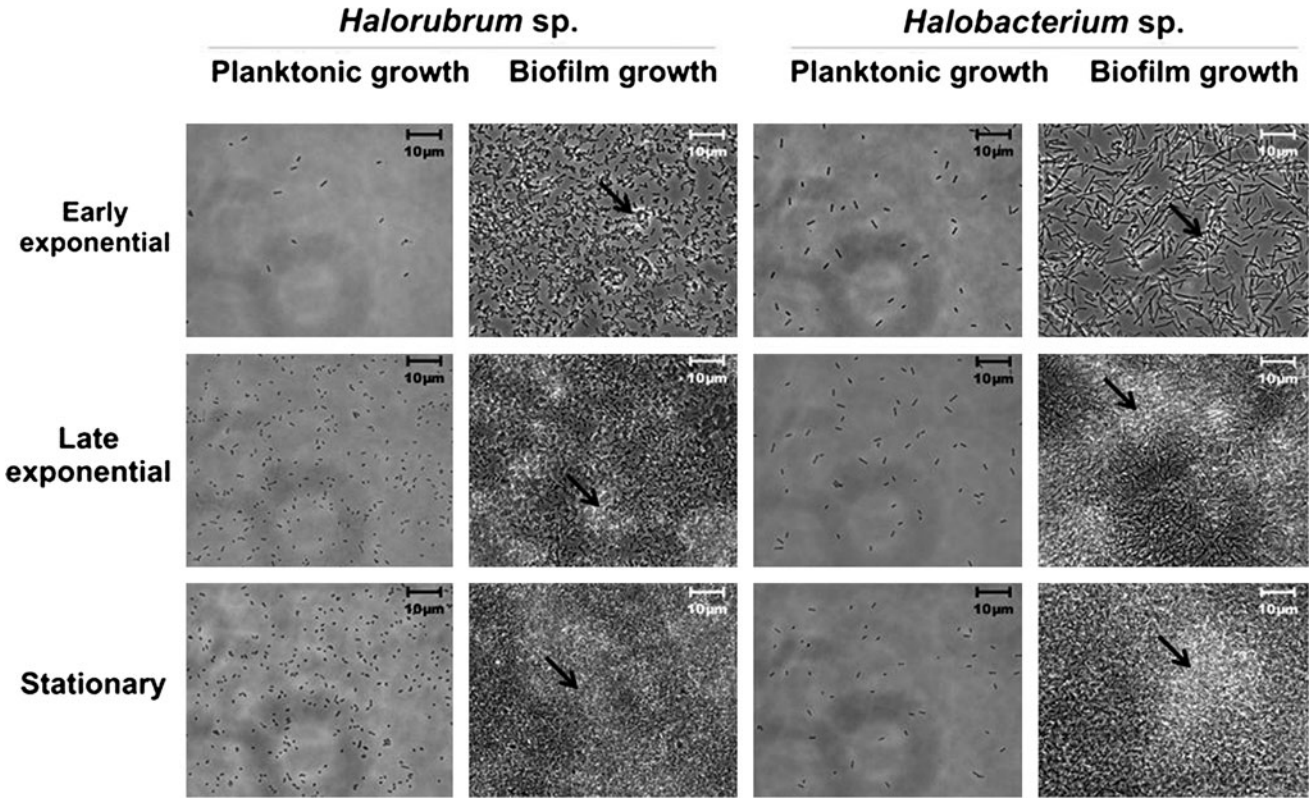

Figure 2. Micrographs showing cells grown in biofilm and planktonic culture at different growth phases. Left panels: *Halorubrum* sp. Right panels: *Halobacterium* sp. Arrows indicate microcolonies and cumuli.

developed in batch under the same conditions, measured at $\mu = 14 \pm 1$ and 8.4 ± 0.3 h for *Halorubrum* sp. and *Halobacterium* sp., respectively.

Changes in Vc can be more clearly visualized in Figure 1, in which both the area covered by cell bodies at any focal plane and the thickness of the biofilm were represented as a function of time. As can be observed, biofilms of *Halobacterium* sp. progressively spread over the glass surface until covering about 30% of the available area (Figure 1b). The maximum surface coverage for biofilms of *Halorubrum* sp. was always lower than 20% (Figure 1a). Clusters of *Halobacterium* sp. cells were thicker at any plane than those formed by *Halorubrum* sp. and protruded deeply into the solution producing 2-fold thicker biofilms. Biofilm structures are better illustrated in Figure 1 by the 3D reconstruction of optical sectioning data (Bouchet et al. 2013).

Changes in cell size for *Halorubrum* sp. were observed only during development in planktonic cultures, where cell length changed from 2 to 1.5 µm in size (Figure 3a). During biofilm growth, the cell length was almost constant at 1.5 µm for all cells in the whole population (Figure 3a), with the exception of a few extremely long rods of 3–4 cell bodies in length, found at the biofilm–glass interface. Morphological changes at the cell level were evident during growth of *Halobacterium* sp. biofilms. As can be observed in Figure 3b, during the initial stages of growth, the mean length of cells in the biofilm reached about 6.5 µm. This change was not observed during planktonic growth, where cell length was about 2 µm during the different phases (Figure 3b). Long cells were also observed in mature biofilms, intertwined in upper biofilm layers well exposed to liquid streams (data not shown).

Antagonistic interaction in hyperhalophilic biofilms

As shown in Figure S5, *Halobacterium* sp. inhibited the growth of *Halorubrum* sp. in soft agar plates forming typical inhibition halos. Testing the same interaction in biofilms, according to Figure S6, showed that *Halorubrum* sp. biofilms fed with the outgoing flow of *Halobacterium* sp. antagonistic biofilms were rapidly and progressively colonized by invader cells. Firstly, typical long cells of *Halobacterium* sp. were found invading the spaces between *Halorubrum* sp. cumuli. Then, these cells developed microcolonies and colonized preexisting cumuli. From this stage, parameters like cell size, cell shape, and cumulus thickness gradually changed to those typically found in *Halobacterium* sp. biofilms, while the size of cumuli of the target organism slowly reduced (Figures 4 and 5). On the contrary, neither modification

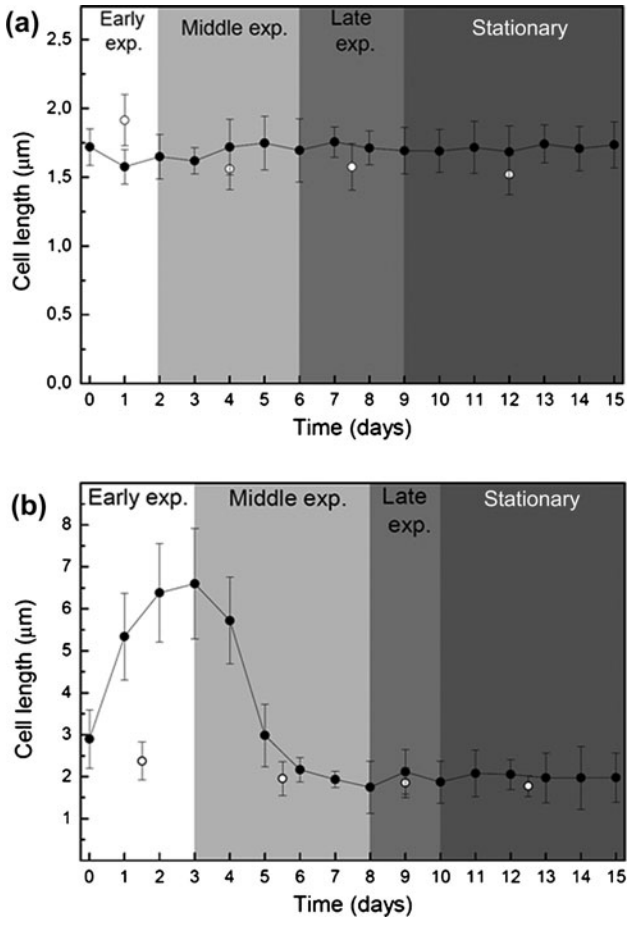

Figure 3. Variations in cellular length during the development of biofilms. Biofilm cell length (filled circles) was measured using Image J software in micrographs taken daily at the surface level (0 µm). The length of planktonic cells measured at each growth phase (open circles) is included as a reference. (a) *Halorubrum* sp. and (b) *Halobacterium* sp. Each value represents the mean ± SD of 10 measurements.

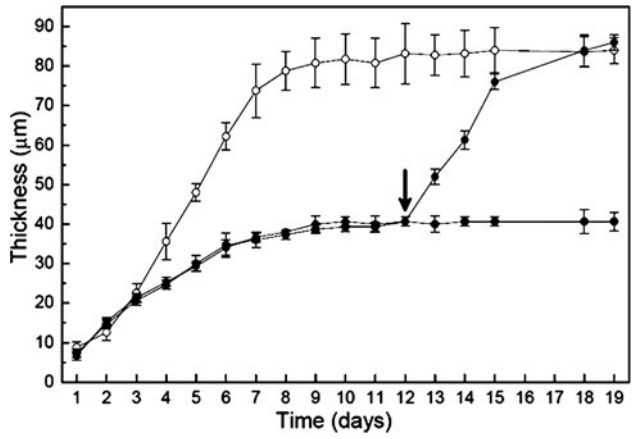

Figure 4. Visualization of the antagonistic effect of *Halobacterium* sp. onto *Halorubrum* sp. through changes in biofilm thickness. The arrow indicates the point at which the chambers containing the biofilms of the antagonist and target microorganisms were connected. Open circles: *Halobacterium* sp. biofilm. Filled circles: *Halorubrum* sp. biofilm not connected (control). Gray circles: *Halorubrum* sp. biofilm connected to the outgoing flow of a *Halobacterium* sp. biofilm. Biofilms thickness was the average of three determinations from different optical fields.

was observed in *Halobacterium* sp. exposed to the outgoing flow of *Halorubrum* sp. biofilms nor when *Halorubrum* sp. biofilms were exposed to the outgoing flow of other *Halorubrum* sp. biofilms (negative control experiments).

Discussion

Development and structure of hyperhalophilic biofilms

The results presented here demonstrate for the first time that hyperhalophilic archaea can form biofilms on a solid surface when grown under continuous culture. In agreement with previous observations made on three strains of the genus *Sulfolobus* (Koerdt et al. 2010), structural features observed here differ greatly depending on the analyzed microorganism. *Halobacterium* sp. developed biofilms that were thicker and spread more widely than those of *Halorubrum* sp. (Figures 1 and S2–S3). The maximum thickness of *Halobacterium* sp. biofilms was of 86 ± 4 µm, doubling that of *Halorubrum* sp. under the same experimental conditions. It was also higher than that reported for biofilms of *H. salinarum* DSM 3754^T in a previous study (Fröls et al. 2012), although the difference may be due in this later case to the absence of flow (Lawrence & Caldwell 1987). Availability of nutrients and dissolved gases, as well as dispersion of wastes and metabolites are typically controlled by diffusion in the absence of flow and this process becomes ineffective for large distances, thus limiting growth (Stewart & Franklin 2008; Wilking et al. 2013). The effect of flow was more evident when comparing *Halorubrum* biofilms. While Fröls et al. (2012) observed loosely attached cell aggregates that took 8–9 days in presenting initial biofilm features, growth under continuous flow in the system described here was much faster, completing biofilm development after that time. All these evidences highlight the importance of implementing flow systems in obtaining biofilm structural information aimed at future applications.

The mean generation time (μ) of cells in both *Halobacterium* sp. and *Halorubrum* sp. biofilms was found to be three times higher than that determined in planktonic cultures developed in batch under the same conditions. It can be rationalized taking into account that oxygen (Xu et al. 1998) and nutrient (Stewart & Franklin 2008) depletion in the biofilm interior, typically induce physiological stratification within biofilm cells, thus lowering the mean generation time of the population. It may appear as a problem aiming at future

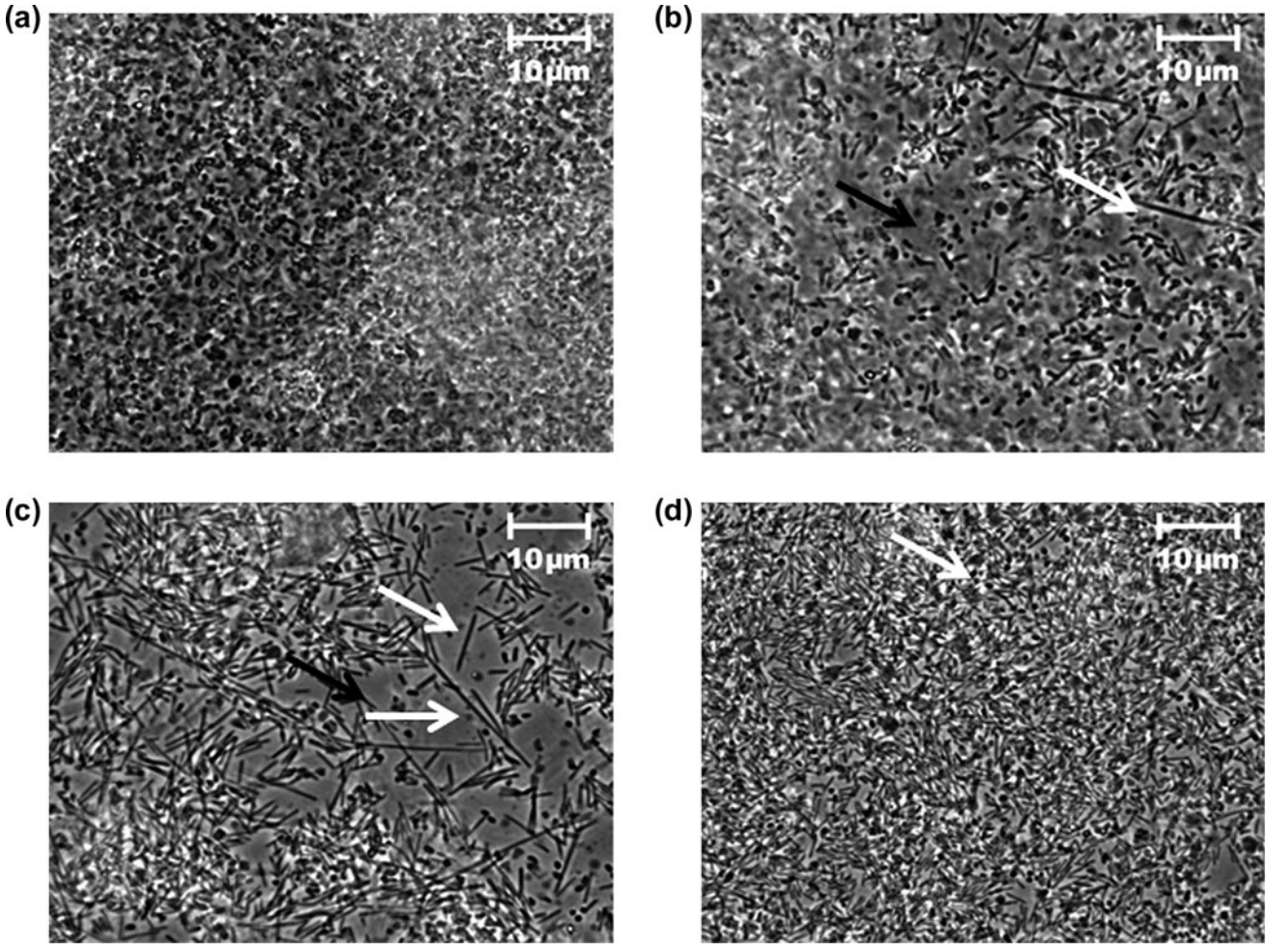

Figure 5. Antagonism assay in biofilm systems. Micrographs show the colonization of *Halorubrum* sp. biofilm by *Halobacterium* sp. cells after connection of the flow cells. Panel (a): before connection; panels (b)–(d): 24, 48, and 72 h after connection. Black arrows show empty spaces generated in *Halorubrum* biofilm after connection; white arrows show *Halobacterium* cells occupying those spaces.

applications, but the fact that hyperhalophilic archaea can be grown immobilized onto solid supports, allows the preservation of biomass during industrial applications and minimizes the impact of the larger generation time.

Besides changes at the population level, changes in cell length were clearly observed here in *Halobacterium* sp. biofilms. As shown in Figure 3b, cells enlarged up to 6.5 ± 1.2 µm upon entering into exponential growth, to later return to their typical length. Long cells were also observed intertwined in the upper layers of mature biofilms, well exposed to liquid streams (data not shown). It is well documented that the shape and size of individual cells can change in response to environmental conditions (Neidhardt et al. 1990; Koch 1996; Young 2006), eg cells have been shown to change from long to short rods for improving the surface-to-volume ratio and nutrient uptake per unit volume (Koch 1996). Nevertheless, taking into account that elongation was not observed in planktonic cultures, the observed changes are thought not to be related to a nutritional challenge, but to some surface-related factor as the need for improving cell adhesion during initial biofilm development as previously reported (Young 2006). In the upper layers, on the other hand, multiple attachments of long cells to neighboring cells can reduce detachment by shear forces (Young 2006), by providing stronger and tighter connections in biofilm streamers, which seems particularly important for biofilms of *Halobacterium* sp. because of their large thickness (Figures 1 and S2).

Antagonistic interaction in hyperhalophilic biofilms

As it is well known most, if not all, prokaryotes produce and release to the environment different molecules that act as antimicrobial compounds (Torreblanca et al. 1994; Riley & Wertz 2002). Their function is to inhibit or

prevent the development of other species that could compete with the producer for nutrients and/or space in the environment (Torreblanca et al. 1994; Jack et al. 1995; O'Connor & Shand 2002; Riley & Wertz 2002). The effect of these compounds is usually evaluated by *in vitro* assays or in agar plates, confronting the producer and the target strains. Ecological interactions are well documented in batch biofilm systems (Davey & O'Toole 2000), but no information is available on the study of antagonistic interactions in biofilm systems under continuous flow.

Cross-feeding experiments demonstrated that deleterious effects were neither observed in *Halobacterium* sp. biofilm exposed to the outgoing flow of *Halorubrum* sp. biofilms (negative control experiments), nor in *Halorubrum* sp. biofilms exposed to the outgoing flow of another biofilm of the same organism (connection control experiments). In consequence and taking into account the antagonistic effect observed in soft agar plates (Figure S5), the success of *Halobacterium* sp. cells in colonizing and outcompeting mature biofilms of *Halorubrum* sp. is thought to be due to the release of one or several antagonistic compounds, although the synergistic tunneling action of swimmers onto established biofilms described by Houry et al. (2012) may be also playing a role. In that work, planktonic bacterial swimmers were found to perturb biofilm structure and make the sessile population more susceptible to antimicrobial compounds, thus facilitating cell killing. Irrigation with planktonic cultures was not performed here, but *Halobacterium* sp. cells released from mature biofilms were detected in voids of the target biofilm (Figure 5 panel c) that could be acting as tunneling swimmers and producing the antimicrobial compounds responsible for the effect shown in Figure S5 from inside, thus promoting *Halorubrum* sp. biofilm disruption and replacement.

Conclusions

Biofilms of representative organisms of hyperhalophilic archaea were studied here in terms of their growth kinetics and tridimensional structure under permanent flow conditions. The results demonstrated that *Halorubrum* sp. and *Halobacterium* sp. formed stable biofilms with particular architectural characteristics that depended on the individual organism. Flow conditions strongly influenced structural features, illustrating the need for studying biofilms in environmentally (natural or industrial) relevant conditions. In the case of *Halobacterium* sp. biofilms, cells from different locations adopted a particular shape, suggesting a functional differentiation. Finally, the antagonistic relationship exhibited by both microorganisms was followed and analyzed by direct observation with a phase contrast microscope, showing *Halobacterium* sp. overgrowing *Halorubrum* sp. biofilms.

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