

Enhanced polyhydroxyalkanoates accumulation by *Halomonas* spp. in artificial biofilms of alginate beads

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Summary. Microbial mats are complex but stable, multi-layered and multi-functional biofilms, which are the most frequent bacterial formations in nature. The functional strategies and physiological versatility of the bacterial populations growing in microbial mats allow bacteria to resist changing conditions within their environment. One of these strategies is the accumulation of carbon- and energy-rich polymers that permit the recovery of metabolic activities when favorable conditions are restored. In the present study, we systematically screened microbial mats for bacteria able to accumulate large amounts of the ester carbon polymers polyhydroxyalkanoates (PHA). Several of these strains were isolated from Ebro Delta microbial mats and their ability to accumulate PHA up to 40–60 % of their dry weight was confirmed. According to two identification approaches (16S rRNA and *ropD* genes), these strains were identified as *Halomonas alkalicola* (MAT-7, -13, -16), *H. neptunia* (MAT-17), and *H. venusta* (MAT-28). To determine the mode of growth yielding maximum PHA accumulation, these three different species were cultured in an artificial biofilm in which the cells were immobilized on alginate beads. PHA accumulation by cells that had detached from the biofilm was compared with that of their planktonic counterparts. Experiments in different culture media showed that PHA accumulation, measured as the relative fluorescence intensity after 48 h of incubation at 30 °C, was higher in immobilized than in planktonic cells, with the exception of cells growing in 5 % NaCl, in which PHA accumulation was drastically lower in both. Therefore, for obtaining high PHA concentrations, the use of immobilized cells may be a good alternative to the PHA accumulation by bacteria growing in the classical, planktonic mode. From the ecological point of view, increased PHA accumulation in detached cells from biofilms would be a natural strategy to improve bacterial dispersion capacity and, consequently, to increase survival in stressed environments. [Int Microbiol 2012; 15(4):191-199]

Keywords: *Halomonas* spp. · polyhydroxyalkanoates (PHA) · immobilized cells · alginate beads · artificial biofilms

Introduction

In natural, clinical, and industrial environments, most bacterial populations develop communities that adhere to various

surfaces to form biofilms, while planktonic, free-swimming cells seem to be only a transitory growth mode [21]. Among the various types of biofilms, microbial mats are highly structured, comprising different functional groups in microspatial proximity and enclosed within a matrix of extracellular polymeric substances. Microbial mat environments are characterized by seasonal fluctuations of flooding and desiccation, and by diel fluctuations of temperature, light, pH, oxygen, sulfide, and nutrients.

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The intracellular storage of polymers such as the ester carbon polymers poly-hydroxyalkanoates (PHA) is a strategy that increases cell survival in changing environments [3,35,37,41]. These polymers are carbon- and energy-rich reserves but they also act as electron sinks involved in maintaining the redox balance [11]. PHA accumulation contributes to the establishment of an environment suitable for bacteria, one that contains high concentrations of organic carbon sources [40]. Microbial mats, as highly diverse and productive systems, accumulate high quantities of PHA under natural conditions [23,30]. PHA accumulation in marine microbial mats has been studied in the community as a whole [30,39] and in isolated strains under laboratory cultivation [2,23,40].

In previous works on Ebro Delta microbial mats, most of the PHA-producing strains isolated belonged to the genus *Halomonas* [2,40]. According to 16S rRNA gene sequence analysis, the family *Halomonadaceae* forms a separate phylogenetic lineage within the gamma-proteobacteria. Of its nine genera, the most common is *Halomonas*, which contains 55 species distributed into two groups, group 1 and group 2 [9]. Members of the *Halomonadaceae* are gram-negative, chemorganotrophic, aerobic or facultative anaerobic, moderately halophilic, haloalkaliphilic, halotolerant or non-halophilic.

Microbial cells in biofilms are naturally immobilized and display a variety of physiological changes compared to their planktonic counterparts [20,27]. To study the production of polyhydroxybutyrate (PHB, one of the most common PHA), Zhang et al. [44] compared the growth of *Alcaligenes eutrophus* in batch cultures (with different salts concentrations) and in biofilms formed in packed-bed reactors (using different microcarriers and ionic strengths). They observed that although biofilm formation in the packed-bed reactor was limited, the volumetric PHB yield of cells in the void volume was comparable to that of the batch culture.

In this work, we studied PHA accumulation in several *Halomonas* strains isolated from microbial mats grown in artificial biofilms in which the cells were immobilized on alginate beads. In contrast to natural or laboratory biofilms (obtained by adhesion to microcarrier surfaces), cells immobilized by encapsulation on alginate beads do not carry out an adhesion step such that the changes in gene expression that normally follow adhesion are absent [27,42].

Commercial alginates are produced mainly by the brown algae *Laminaria hyperborea*, *Macrocystis pyrifera*, and *Ascophyllum nodosum*. Alginate is a polymer of 1,4-linked β -D-mannuronic acid and α -L-guluronic acid residues in varying proportions, sequence, and molecular weight. Alginate forms a gel when multivalent cations (usually Ca^{2+}) inter-

act ionically with the blocks of guluronic residues between two different chains, resulting in a 3-D network [24]. From this alginate mass, beads can be produced, as described in Materials and methods. Calcium ions are not uniformly distributed throughout the bead; rather, they are strongly bound in the surface and subsurface of the beads, but only weakly bound in the center [28]. The final strength of the gel depends on the overall fraction of guluronic acid residues, the molecular weight of the polymer, and the Ca^{2+} concentration at the time of gelation. Optimal concentrations for the gellification of the Na-alginate complex range from 1 % to 2 % (w/v) [34]. Depending on the characteristics of the alginate beads, bacteria growing on their surfaces are able to form microcolony-like cellular aggregates that can be easily detached and released into the surrounding medium [15,17,19,22,28]. Cells immobilized on alginate beads have been used in the degradation or biotransformation of pollutants [1,10], the production of enzymes [45], and the preservation of cell viability [4].

The main objective of the present work was to investigate the influence of different culture media and growth modes (batch culture of planktonic cells and artificial biofilms made of alginate beads) on PHA accumulation by several strains of *Halomonas* isolated from microbial mats. Accordingly, PHA accumulation in cells detached from alginate beads was compared with that of their planktonic counterparts to determine whether bacterial immobilization enhanced PHA production. We also considered whether PHA accumulation in newly released cells could be one of the strategies used by the microbial communities of natural biofilms to cope with stressful environmental conditions.

Materials and methods

Phylogenetic analysis and strain identification. DNA extraction and PCR amplification of the 16S rRNA gene for five strains isolated from Ebro Delta microbial mats [2] were performed using previously described methods [25]. The *rpoD* gene of strains MAT-7, MAT-13, MAT-16, MAT-17, and MAT-28 was PCR-amplified as described by de la Haba et al. [9], except that the temperatures for annealing and extension were 43 °C and 72 °C, respectively. Gene *rpoD* of strain MAT-28 could not be amplified under these conditions. Two primers were designed in this study on the basis of the complete *rpoD* sequences derived from the whole-genome sequences of *H. elongata* DSM 2581T (GenBank number FN869568), *H. boliviensis* LC1T (JH393258), *Halomonas* sp. GFAJ-1 (AHBC01000043), *Halomonas* sp. HAL1 (AGIB01000009), and *Halomonas* sp. TD01 (AFQW01000002). The sequences were aligned using MegAlign (Lasergene, DNASTAR, Madison, WI, USA). The following primers were designed using Primer3 [31]: 119F (5'-CGGATCAGGTGAAGACATC-3') and 1357R (5'-ATCATRTGCACGGAATACG-3'). The PCR (50 μ l) contained 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 0.25 mM of each dNTP, 1 μ M of primers 119F

and 1357R (Isogen Life Science, De Meern, the Netherlands), 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems-Life Technologies, Carlsbad, CA, USA), and 250 ng of DNA template. The reaction was done in a 2720 thermal cycler (Applied Biosystems) as follows: initial denaturation at 95 °C for 5 min, 35 cycles of 94 °C for 60 s, 56 °C for 60 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min.

PCR products were purified using the Purelink PCR purification kit (Invitrogen-Life Technologies, Carlsbad, CA, USA) and measured in a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) to assess their optimal concentrations and purity. The BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used for sequencing reactions. The nucleotide sequences were determined by the Scientific and Technological Center of the University of Barcelona (CCiTUB), using an ABI PRISM 3730 DNA analyzer (Applied Biosystems).

Sequence alignment, pairwise distance and phylogenetic analyses (neighbor-joining method with the Jukes-Cantor model and the pairwise deletion option) were conducted using MEGA5 software [36]. The topological robustness of the phylogenetic trees was evaluated by a bootstrap analysis through 1000 replicates. Isolates were identified using the EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>) [18] and pairwise distance values [26] on the basis of 16S rRNA and rpoD sequence data, respectively.

Cell immobilization by alginate beads. Sodium salt alginic acid from *Macrocystis pyrifera* (61 % mannuronic acid and 39 % glucuronic acid) (Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving the alginate powder in warm water to a concentration of 4 % (w/v) and then autoclaving the solution at 121 °C for 20 min. Cells to be added to the alginate were grown overnight at 30 °C in tryptic soy broth (TSB) containing 3 % NaCl. The cell suspension was mixed with the alginate (1:1, v/v) at room temperature and stirred to obtain a uniform mixture. Aliquots of 1 ml were withdrawn from this mixture and transferred dropwise into a sterile solution of CaCl₂ (0.2 M), resulting in the formation of beads of ca. 2.0 mm diameter. The beads were allowed to further harden in the CaCl₂ solution for 30 min at room temperature and then washed with sterile distilled water to remove the excess Ca²⁺.

Electron microscopy. *Halomonas venusta* MAT-28 immobilized in alginate beads was grown for 48 h at 30 °C in glucose minimal medium supplemented with 3 % NaCl. After a fixation step in 2 % (v/v) glutaraldehyde for 18 h, the beads were cut, stained with osmium tetroxide and uranyl acetate, and then examined in a Leica transmission electron microscope. For scanning electron microscopy (SEM), beads at times 0 and 48 h of incubation were fixed with 2 % (v/v) glutaraldehyde for 18 h and then dehydrated in an ethanol series (20–100 %). The samples were dried, sputter-coated with gold, and observed using a Hitachi S-3400N scanning electron microscope.

Spectrofluorometric monitoring of PHA accumulation. Cellular PHA accumulation was measured as the relative fluorescence intensity of cells incubated for 48 h at 30 °C in different culture media. Two modes of growth were examined: batch culture of planktonic cells and artificial biofilms growing on alginate beads. The culture medium consisted of minimal medium (MM) containing TSB (Scharlau, Barcelona, Spain) diluted 50-fold, plus glucose or glycerol at 5 g/l, 3 or 5 % NaCl, and 0.5 µg Nile red dye (dissolved in dimethylsulfoxide)/ml. The MM was phosphate-free because phosphates retain Ca²⁺, which results in extensive disintegration of the alginate beads.

For the planktonic assays, cells were grown overnight at 30 °C in TSB containing 3 % NaCl. An aliquot (1/100) from the overnight culture was transferred to 100-ml flasks containing 25 ml MM and one of the following different combinations of carbon sources and salt: glucose + 3 % NaCl;

glucose + 5 % NaCl; glycerol + 3 % NaCl; and glycerol + 5 % NaCl. For the immobilized cells assay, alginate beads were prepared as explained above. Flasks containing 25 ml of the same media used in the planktonic assays were then inoculated with approximately 200 beads.

All flask cultures were incubated in the dark at 30 °C for 48 h with shaking (100 rpm). A 1-ml sample was then removed and centrifuged in a microcentrifuge at 10,000 rpm at room temperature. Pellets were washed and resuspended in 1 ml of phosphate-buffered saline (PBS), pH 7.0. Relative PHA accumulation was measured using an SLM Aminco 8100 spectrofluorometer. The fluorescence excitation- and emission wavelengths of the stained cells in PBS were 543 nm and 598 nm, respectively. Slits of excitation and emission were set to 10 nm at 900 V. PHA accumulation in the two growth modes, planktonic and immobilized cells, was compared. Four measurements using independent bacterial cultures were obtained for confirmation.

Results

Phylogenetic analysis and strain identification. The 16S rRNA and rpoD gene sequences from strains MAT-7, MAT-13, MAT-16, MAT-17, and MAT-28 were aligned independently with the respective gene sequences of the type strains belonging to *Halomonas* group 1 and *H. elongata* ATCC 33173T, representative of *Halomonas* group 2 [9]. Phylogenetic tree based on the 16S rRNA sequences (Fig. 1A) showed that all five isolates clustered within *Halomonas* group 1, comprising two subgroups and clearly separated from *H. elongata* (group 2). *Halomonas* group 1 was also subdivided into two subgroups in the phylogenetic tree based on rpoD sequences, but *H. elongata* clustered in one of these subgroups (Fig. 1B). The 16S rRNA and rpoD gene sequences of strains MAT-7, MAT-13 and MAT-16 were identical. Consequently, these isolates were considered as a single strain, represented by MAT-16.

The 16S rRNA genes of MAT-16, MAT-17, and MAT-28 showed sequence similarities higher than 98 % with those of four (*H. andesensis*, *H. hydrothermalis*, *H. venusta* and *H. alkaliphila*), six (*H. sulfidaeris*, *H. titanicae*, *H. variabilis*, *H. boliviensis*, *H. neptunia* and *H. alkaliantarctica*) and five (*H. stevensii*, *H. andesensis*, *H. hydrothermalis*, *H. alkaliphila* and *H. venusta*) type strains of the genus *Halomonas*, respectively.

In the distance matrix obtained from the rpoD sequences, pairwise distance values <3 % were as follows: 2.4 % between MAT-16 and *H. alkaliphila* DSM 16354T, 0.2 % between MAT-17 and *H. neptunia* CECT 5815T, and 0.9 % and 1.4 % between MAT-28 and *H. venusta* DSM 4743T and *H. hydrothermalis* CECT 5814T, respectively. Distance values <3 % were also obtained in com-

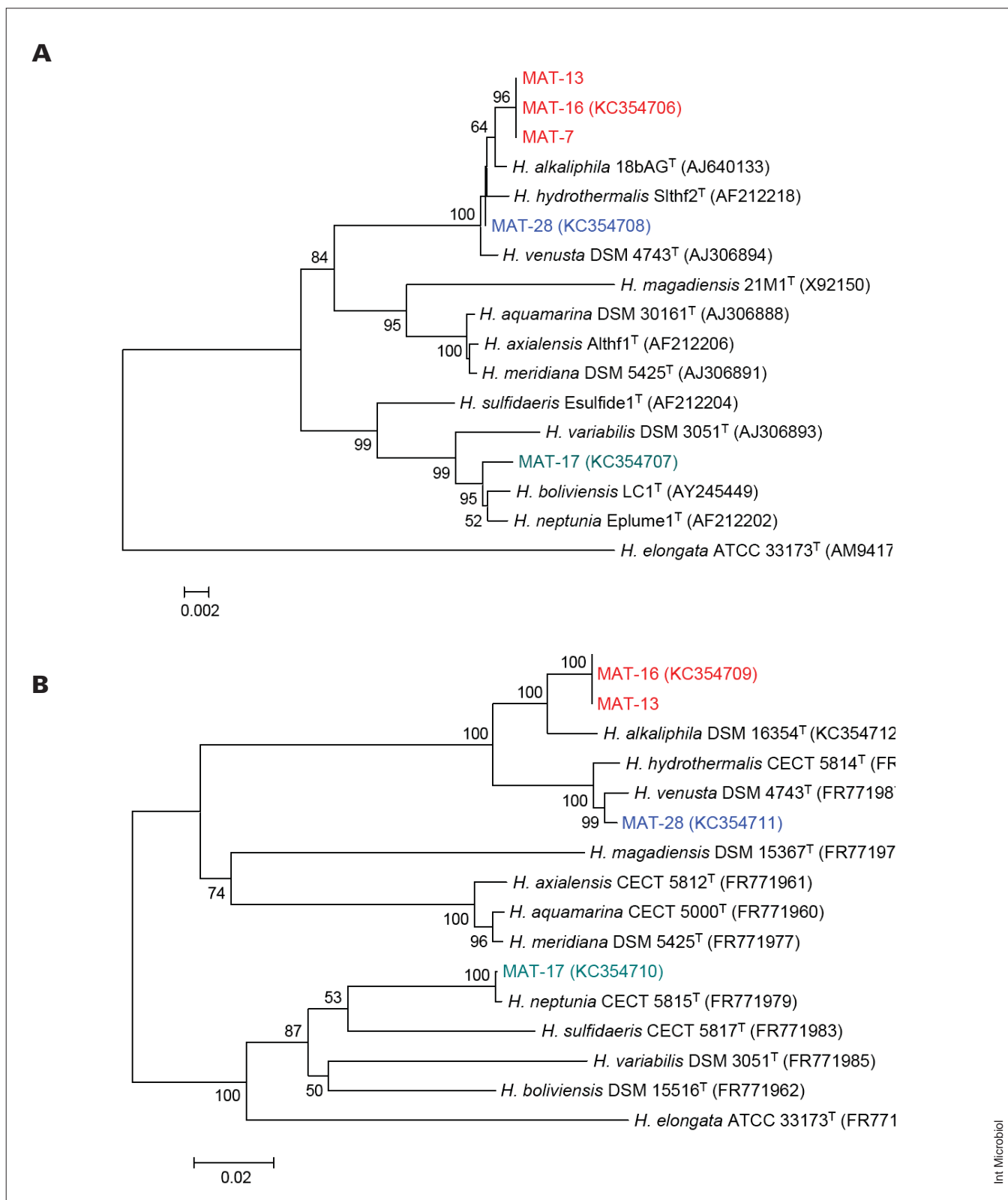


Fig. 1. (A) Neighbor-joining phylogenetic tree obtained from 16SrRNA and (B) *rpoD* gene sequences encompassing strains MAT-7, MAT-13, MAT-16, MAT-17, and MAT-28 and all type strains of *Halomonas* group 1, and with the type strain of *Halomonas* group 2, *H. elongata*. Bootstrap values (>50 %) based on 1000 replicates are shown. Bars indicate sequence distance. (Red: strains of *H. alkaliphila*; blue: strains of *H. venusta*; blue: strains of *H. neptunia*.)

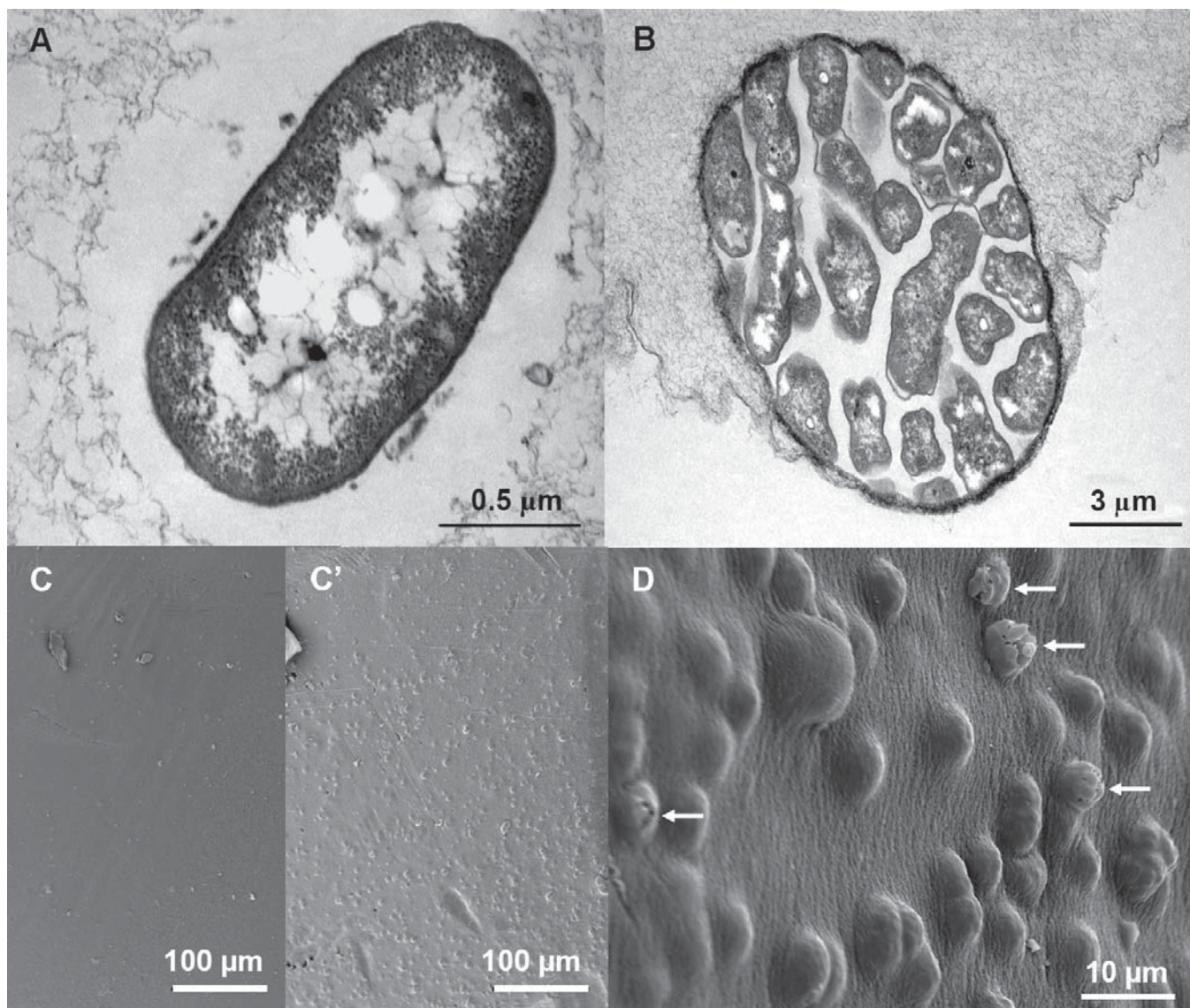


Fig. 2. Transmission electron micrograph of *Halomonas venusta* MAT-28 immobilized-cells growing in MM-glucose with 3 % NaCl after 48 h at 30 °C. (A) Individual cell near the center of an alginate bead. Note the electron-dense peripheral zone surrounding the cytoplasm. (B) Group of cells forming a microcolony near the surface of the bead, indicating an active state of growth. (C) Scanning electron micrograph of immobilized cells of *Halomonas* strain MAT-28 at time 0, and (C') after 48 h of incubation. (D) Microcolonies formed at the surface of a bead and about to detach. Note that several individual cells are protruding from the bumps produced by the presence of the microcolonies (arrows).

parison of *H. alkaliphila*–*H. axialensis*–*H. meridiana* and *H. venusta*–*H. hydrothermalis*. All remaining pairwise comparisons were >5 %.

Influence of growth mode (planktonic or immobilized cells) on PHA accumulation In previous work we observed that PHA accumulation in MAT-16, MAT-17, and MAT-28 reached steady-state concentrations after 48 h of incubation [2]. Here, *Halomonas* strains were grown in two modes, planktonically (free swimming) or

as immobilized cells in alginate beads (artificial biofilm). The conditions used for bead preparation (see Materials and methods) favored leakage of entrapped bacteria while the integrity of the beads was maintained (Fig. 2). Cells inside the alginate beads were surrounded by a transparent area, apparently without alginate polymer. These cells did not seem to accumulate PHA, but electron-dense particles were observed near the cytoplasmic membrane (Fig. 2A). On the surface of the bead, however, microcolonies formed and they were surrounded by an unspecified structure, perhaps con-

Table 1. Number of viable cells on plates of *Halomonas* strains MAT-16, MAT-17, and MAT-28 using different culture media and growth modes

Culture medium* (48 h at 30°C)	<i>Halomonas alkaliphila</i> MAT-16		<i>Halomonas neptunia</i> MAT-17		<i>Halomonas venusta</i> MAT-28	
	Planktonic mode (CFU/ml)	Immobilized (biofilm) mode (CFU/ml)	Planktonic mode (CFU/ml)	Immobilized (biofilm) mode (CFU/ml)	Planktonic mode (CFU/ml)	Immobilized (biofilm) mode (CFU/ml)
MM + glucose + 3 % NaCl	9.8×10^8	1.3×10^5	1.0×10^8	4.3×10^5	2.4×10^8	4×10^6
MM + glucose + 5 % NaCl	nd	nd	nd	nd	1.0×10^8	1.2×10^6
MM + glycerol + 3 % NaCl	3.0×10^8	1.5×10^6	1.5×10^7	1.3×10^5	3.2×10^8	2.1×10^6
MM + glycerol + 5 % NaCl	nd	nd	nd	nd	1.5×10^8	2.5×10^6

*MM: minimal medium (see text). nd: not determined.

sisting of minerals precipitated as a result of changes in the alginate polymer due to the cell metabolic activity. Outside this structure, alginate polymer strands were observed. The cells of the microcolonies in the surface lacked the electron dense particles clearly visible in the cells from the center of the beads (Fig. 2B). For *Halomonas venusta* (MAT-28), the scanning micrographs revealed the formation of bumps on the surface of the beads after 48 h of incubation at 30 °C, but not at time 0 h. Each bump was due to the presence of a growing microcolony (Fig. 2C,D).

For all three strains, the number of cells (measured as colony-forming units, CFU, per ml) that had detached from the alginate beads after 48 h and were released into the surrounding medium was two orders of magnitude lower than the CFU/ml determined for parallel cultures in planktonic growth mode (Table 1). The lower growth rate (CFU/ml) of the detached cells might be explained by low nutrient/oxygen concentration, osmotic pressure, or water activity [16]. *Halomonas* cells in alginate beads are surrounded by the gel matrix. Immediately after immobilization the cells are distributed homogeneously in the beads that entrap them. However, as substrates and waste products are carried to and from the cells by diffusion, gradients form such that the entrapped cells become heterogeneously distributed inside the bead. Consequently, cells grow and form microcolonies in the peripheral areas of the beads, while no growth occurs in cells situated in the inner parts [22]. Bacterial cells that have accumulated at the periphery were, as a consequence, easily detached from the beads and liberated into the medium.

In the three strains assayed in this work, PHA accumulation in medium containing glucose and 3 % NaCl was higher in detached cells from alginate beads than in planktonic cells. Among all the strains, PHA accumulation was highest in MAT-28 and significantly lower in MAT-17. This result was unexpected because MAT-17 (*H. neptunia*) is related phylogenetically (by 16S rRNA analysis) to *H. boliviensis*, in which PHA yields and volumetric productivities are close to the highest amounts reported thus far [26] (Fig. 3A). In the PHA accumulation assay using glycerol as carbon source and 3 % NaCl, only strains MAT-16 and MAT-28 were tested. Accumulation was slightly higher in cells cultured in glycerol rather than glucose. Again, PHA accumulation was higher in detached cells (Fig. 3B). In the presence of 5 % NaCl, PHA accumulation by strain MAT-28 was significantly lower than in culture medium containing 3 % NaCl; this was the case in both planktonic and immobilized cells (Fig. 3C).

Discussion

Taxonomic identification and immobilized cells. Based on 16S rRNA gene sequence analysis, strains MAT-16, MAT-17, and MAT-28 were identified as belonging to the genus *Halomonas*, but they could not be identified at the species level because their similarities with several *Halomonas* species were higher than 98 % [43]. However, following *rpoD* sequence analysis, the three strains were identified as *H. alkaliphila*, *H. neptunia* and *H. venusta*,

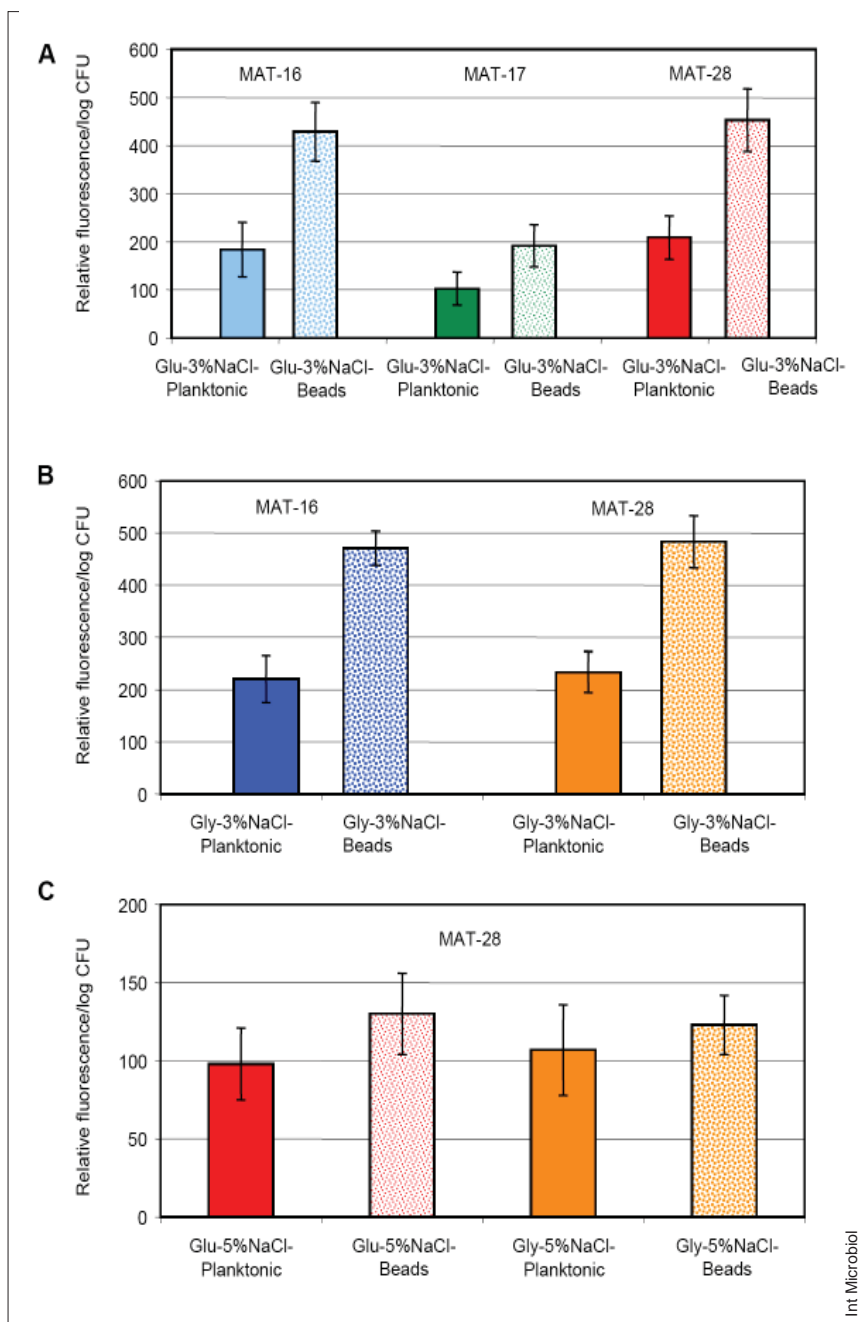


Fig. 3. PHA accumulation in *Halomonas* spp. was measured spectrofluorometrically after 48 h of incubation at 30 °C in different culture media (Glu, glucose; Gly, glycerol; and 3 % NaCl or 5 % NaCl) and in two growth modes: planktonic or artificial biofilm (alginate beads). Data shown are the average of the results of four independent experiments.

respectively, based on pairwise distance values below 3 % [26]. For the same reason, *rpoD* sequence analysis suggested that *H. alkaliphila* and *H. axialensis* are later heterotypic synonyms of *H. meridiana* and that *H. hydrothermalis* is a later heterotypic synonym of *H. venusta* [12].

Influence of growth mode on PHA accumulation.

The 4119-kb genome of *Halomonas boliviensis* contains 3863 genes, of which 160 are related to carbohydrate transport and metabolism [14]. *Halomonas* can adjust its metabolism

to optimize cell growth in response to the specific environmental conditions by engaging different combinations of metabolic pathways. Thus, carbon flow will be directed towards the synthesis of more reduced or more oxidized products according to intracellular redox conditions. PHB is synthesized from acetyl-CoA in the presence of excess NADH in the bacterial cytoplasm.

Glycerol has a lower oxidation state than glucose and its catabolism renders more reduced products in order to maintain redox balance [11]. In studies on PHB production,

with glucose as the carbon source, high aeration conditions usually favored high polymer accumulation, whereas low aeration was shown to promote the synthesis of other metabolic products derived from fermentation pathways, such as acetate. However, with glycerol as the carbon source, the highest PHB contents are obtained under conditions of relatively low aeration [7]. This was also the case in our study, in which PHA accumulation by strains MAT-16 and MAT-28 cultured in 3 % NaCl was higher with glycerol than with glucose (Fig. 3) and even higher in cells detached from immobilized alginate beads than in planktonic cells.

Alginate-immobilized cells may be subjected to higher stress than planktonic cells, e.g., due to oxygen deprivation, which could also favor polymer accumulation. In *Shewanella oneidensis* MR-1 detachment of cells from biofilms could be induced by a decrease in oxygen tension, suggesting a physiological link between oxygen sensing and detachment [38]. Other studies have shown that the yield of PHA in *Halomonas boliviensis* improves under conditions of oxygen limitation. Oxygen depletion is also known to obstruct the tricarboxylic acid cycle because the unconsumed NAD(P)H inhibits citrate synthase, which in several microorganisms results in the utilization of this cofactor for PHB synthesis [29].

In our study, 5 % NaCl, together with either glucose or glycerol resulted in significantly lower PHA accumulation by planktonic as well as immobilized cells than similarly obtained with 3 % NaCl (Fig. 3). This result may reflect the coproduction of PHA and an osmoprotector such as ectoine, in agreement with previous studies [13]. Under salt stress, there are significant variations in the expression of proteins involved in osmoregulation, stress response, energy generation, and transport [5,6,13]. At high salinity, total flux through energy-generating pathways is significantly lower and carbon sources enter in the system as citrate and are mainly diverted to osmolyte synthesis [6]. Acetyl-CoA is a common precursor for the synthesis of PHB, as noted above, and for ectoine; hence, metabolic flux to either of these products could alter production of the other. This sequence of events was proposed to explain the lower PHB production rates and yields when ectoine synthesis was promoted by increasing the salt concentration of the medium [13].

We conclude that, in artificial biofilms made by alginate beads, detached cells of *Halomonas* spp. accumulate more PHA than their counterparts growing planktonically in the same stressing culture media. In natural biofilms, it has been observed that cell detachment is favored by starvation for nutrients and/or depletion of oxygen [33,38]. As

PHA serve as an endogenous source of carbon and energy during starvation [41], under stress conditions, bacterial cells with higher contents of PHA survive longer than those with lower contents [8,32,35,37]. Detachment is a biologically controlled process [38]. Similar mechanisms might also operate in *Halomonas* spp. Thus, detached cells from immobilized alginate-beads that accumulate more PHA than planktonic cells could constitute an adaptive advantage for the dispersion in stressful environments by increasing survival in the new planktonic mode of growth.

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Competing interests. None declared.

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