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Isolation and characterization of biosurfactant-producing *Alcanivorax* strains: hydrocarbon accession strategies and alkane hydroxylase gene analysis

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Abstract

Biosurfactant-producing bacteria belonging to the genera *Alcanivorax*, *Cobetia* and *Halomonas* were isolated from marine sediments with a history of hydrocarbon exposure (Aristizábal and Gravina Peninsulas, Argentina). Two *Alcanivorax* isolates were found to form naturally occurring consortia with strains closely related to *Pseudomonas putida* and *Microbacterium esteraromaticum*. Alkane hydroxylase gene analysis in these two *Alcanivorax* strains resulted in the identification of two novel *alkB* genes, showing 86% and 60% deduced amino acid sequence identity with those of *Alcanivorax* sp. A-11-3 and *Alcanivorax dieselolei* P40, respectively. In addition, a gene homologous to *alkB2* from *Alcanivorax borkumensis* was present in one of the strains. The consortium formed by this strain, *Alcanivorax* sp. PA2 (98.9% 16S rRNA gene sequence identity with *A. borkumensis* SK2^T) and *P. putida* PA1 was characterized in detail. These strains form cell aggregates when growing as mixed culture, though only PA2 was responsible for biosurfactant activity. During exponential growth phase of PA2, cells showed high hydrophobicity and adherence to hydrocarbon droplets. Biosurfactant production was only detectable at late growth and stationary phases, suggesting that it is not involved in initiating oil degradation and that direct interfacial adhesion is the main hydrocarbon accession mode of PA2. This strain could be useful for biotechnological applications due to its biosurfactant production, catabolic and aggregation properties.

Keywords: *Alcanivorax*; Biosurfactant; Hydrocarbon accession mode; *alkB*

1. Introduction

Petroleum-derived hydrocarbons include a complex mixture of compounds of natural origin that, especially during the last century, have been released into the environment because of accidental or systematic anthropogenic actions. Among the main traits of hydrocarbon-degrading microorganisms, there exist different mechanisms for uptake of these molecules and a diversity of catabolic pathways to degrade them. Microbial strategies have been developed to overcome the low aqueous solubility of hydrocarbons, and thus, their low availability for microorganisms. The different hydrocarbon accession mechanisms include direct cellular contact with these compounds in solid or liquid state, contact with pseudo-solubilized hydrocarbons by biosurfactant excretion and oil–water emulsions [11,25,27].

Biosurfactants are amphipathic molecules with several roles identified [31]. In the degradation of lipophilic compounds such as hydrocarbons, they may enhance their bioavailability by either increasing apparent hydrocarbon

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solubility in the aqueous phase or by expanding the contact surface area due to emulsification [18,20]. Cell-surface hydrophobicity and biosurfactant production also take part in regulation of adhesion and detachment of microorganisms to interfaces [21,25].

A variety of microorganisms that degrade hydrocarbons have been described in marine environments. Some of them, such as members of the *Alcanivorax* and *Cycloclasticus* genera, are highly specialized hydrocarbon degraders [12]. *Alcanivorax borkumensis* uses aliphatic hydrocarbons as its main carbon source for growth and produces an anionic glucose lipid biosurfactant [1,32]. There is also evidence that *Alcanivorax* strains play a major role during bioremediation of hydrocarbon pollution in marine habitats [14].

Several areas along the Patagonian coast (Argentina) are exposed to hydrocarbon pollution mainly associated with petroleum exploitation and port activities. Field surveys showed that the highest concentrations of aromatic and aliphatic hydrocarbons in this region were present at the Aristizábal Peninsula [5]. The aim of this study was to isolate and characterize promising biosurfactant-producing microorganisms from this site and to analyze the hydrocarbon accession mode of a naturally occurring consortium formed by one of these strains, a hydrocarbonoclastic bacterium belonging to the genus *Alcanivorax*.

2. Materials and methods

2.1. Sampling and isolation of biosurfactant-producing strains

Superficial intertidal sediments (0–3 cm) and old asphaltic residues were collected at four stations in Aristizábal and Gravina Peninsulas (45° 13′ S 66° 32′ W and 45° 09′ S 66° 28′ W, respectively), Argentina, in February 2006. Suspensions of the samples were serially diluted in sterile natural seawater and plated on three different media containing, per liter of seawater: 5 ml crude oil (CO); 1 g NH_4NO_3, 4 ml of a phosphate solution (25 g/l $Na_2HPO_4.12H_2O$ and 3.6 g/l NaH_2PO_4), 0.2 g yeast extract and 5 ml crude oil (CONP); the same as the previous medium but, instead of crude oil, phenanthrene (0.05 g/l) was added (PhNP). All media contained 12 g/l of agar–agar. The plates were incubated at 25 °C for 15 days. Individual colonies were selected and purified by repeated streaking on the same media.

Isolates were screened on blood agar plates containing 10% (v/v) of sterile sheep blood, incubated at 25 °C for 5 days. Hemolysis of red blood cells around the colonies indicated potential biosurfactant production [4]. All strains were subcultured in the corresponding isolation medium to assess biosurfactant production. For each isolate, three 125 ml flasks containing 20 ml of medium were inoculated with a 24 h culture at 1% (v/v) ratio and incubated at 25 °C on an orbital shaker at 150 rpm. After 15 days of incubation, an aliquot of the aqueous phase was carefully withdrawn and used to measure surface tension (ST) at 25 °C with a DuNouy ring tensiometer (CSC Scientific, Fairfax, VA) to evaluate biosurfactant production. The ability to form stable emulsions after mechanical agitation of a culture sample with diesel–oil was used as an indication of bioemulsifier production [7].

2.2. Hydrocarbon utilization test

Selected isolates were analyzed for their ability to grow on *n*-hexadecane, phenanthrene and ship bilge waste hydrocarbons (SBWH; [23]). Inoculum preparation and culture conditions were described in Nievas et al. [22]. Briefly, three 5 ml replicates of liquid mineral medium were inoculated with 1% (v/v) of bacterial suspension and 0.5% (v/v) of liquid carbon sources. Two mg of phenanthrene were distributed in sterile tubes dissolved in acetone, the solvent was allowed to evaporate aseptically, and 5 ml of the mineral medium and inocula were added. After an incubation for 15 days at 25 °C and 150 rpm, growth was assessed by CFU enumeration on agar plates, where a filter paper saturated with SBWH was placed inside the plate lid to supply hydrocarbon vapors [8]. Controls without carbon sources were also prepared for each isolate. Differences in the means of microbial growth measurements between control and culture treatments were tested by *T*-test for independent samples using InfoStat software [http://www.infostat.com.ar].

2.3. Molecular and phylogenetic analyses

DNA was extracted using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI), quantified with the dye Hoechst 33258 (Amersham Biosciences, Piscataway, NJ) on a Hoefer DyNA Quant 200 Fluorometer (Hoefer Scientific Instruments, San Francisco, CA), and used as template for PCR amplifications of 16S rRNA and *alkB* genes. The 16S rRNA gene was amplified as in Olivera et al. [24]. For detection of *alkB* genes, three different primer sets were used: Mon F401–Mon R820 [15], TS2S 403–Deg1RE 959 [28] and AlkB484F (5′ GGKCAYTTCTWCRTYGARCA 3′)–AlkB824R (5′ CCGTAGTGYTCRABRTARTT 3′) (this study). PCR reactions were carried out on an Eppendorf Personal Mastercycler® (Eppendorf, Hamburg, Germany), or a PTC-0200 DNA Engine thermal cycler (Bio-Rad, Hercules, CA) in 25 µl reactions containing 50 mM KCl, 10 mM Tris–HCl pH 9.0, 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$ (AlkB484F–AlkB824R) or 2.5 mM $MgCl_2$ (Mon F401–Mon R820 and TS2S 403–Deg1RE 959), 0.2 µM of each dNTP, 0.5 µM of each primer, 1 U of T-PLUS DNA polymerase (Inbio-Highway, Tandil, Argentina) and 1, 5, 10 or 20 ng genomic DNA. The amplification program consisted of 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 45 °C (AlkB484F–AlkB824R and Mon F401–Mon R820) or 50 °C (TS2S 403–Deg1RE 959) and 30 s at 72 °C, followed by 15 min at 72 °C. When necessary due to low yields, PCR products were cloned into the pCR®4.0 vector (TA Cloning kit for sequencing, Invitrogen, Carlsbad, CA), following manufacturer's instructions. A QIAprep™ Spin Miniprep kit (Qiagen, Valencia, CA) was used for plasmid purifications.

Table 1
Screening for biosurfactant-producing microorganisms.

Media	N° isolates	N° hemolytic isolates	N° emulsifying isolates	N° isolates reducing ST
CO	20	9	0	0
CONP	33	9	5	7
PhNP	43	8	3	10

Sequencing was performed on both strands by the commercial services of Macrogen Inc. (Seoul, Korea). Sequences were analyzed phylogenetically using the MEGA4 program [29]. Phylogenetic trees were constructed using the neighbor-joining (NJ) algorithm, from a distance matrix calculated following Kimura's two-parameter model for 16S rRNA gene and Dayhoff's PAM matrix for *alkB*-deduced amino acid sequences. Stability among the clades was assessed with 1000-replication bootstrap analysis. Sequences were deposited at the GenBank database under accession numbers EU647558–64 (16S rRNA genes) and EU688983, FJ173004-5 (*alkB* genes).

2.4. Analysis of a Pseudomonas–Alcanivorax hydrocarbon-degrading consortium

To analyze the role of individual strains of the hydrocarbon-degrading consortium in biosurfactant production, three replicates of mixed and axenic cultures were grown into 125 ml flasks containing 20 ml of CONP medium with 0.5% (v/v) SBWH instead of crude oil. Cultures were incubated 7 days at 25 °C on an orbital shaker (150 rpm). Growth was monitored by CFU enumeration as described above. Colonies on the plates were counted after 3 days of incubation at 25 °C. The *Alcanivorax* isolate formed transparent colonies on agar plates and could thus be differentiated from the creamy-white *Pseudomonas* colonies. ST was measured as described previously. Cell-surface hydrophobicity was examined using the microbial adhesion to hydrocarbons (MATH) assay [26]. Live cultures were examined by light microscopy (Axiolab re, Carl Zeiss, Germany) to determine the location of bacteria, i.e. free vs. oil-associated arrangement. For analyzing bacterial aggregation, samples were fixed with glutaraldehyde (2.5% v/v), prepared according to Lozano [17], and examined using a scanning electronic microscope (SEM) (JEOL Model 35CF, Tokyo, Japan).

3. Results

3.1. Isolation and characterization of biosurfactant-producing bacteria

After incubation of sediment or asphaltic residue suspensions in three culture media (CO, CONP, PhNP), 96 morphologically different bacterial strains were isolated. Hemolytic and bioemulsifying activities as well as ST reduction were measured for all isolates (Table 1). Hemolytic activity was detected in 26 of these strains, isolated in all three media. However, none of them reduced ST, indicating that they were not biosurfactant producers. These results indicate that, even though the hemolytic activity test has been and can be used as a screening tool for searching biosurfactant-producing microorganisms, it needs to be supported by ST measurements.

Bioemulsifying activity and ST-reducing capability were detected in 8 and 17 strains, respectively. All these strains were isolated from CONP and PhNP media (Table 1). As a microorganism can be considered a promising biosurfactant producer when it is able to reduce the ST below 40 mN/m [6], only those isolates lowering culture medium ST below this limit were characterized (PA2, PA3, PA5, PA6, and PA7). Except for PA2, none of them showed hemolytic activity. In addition, only PA6 presented the ability to form stable emulsions (E24: 42.3 ± 2.1%). To identify these strains, phylogenetic analysis based on their 16S rRNA gene sequences (1395 bp) was performed (Fig. 1). The highest sequence

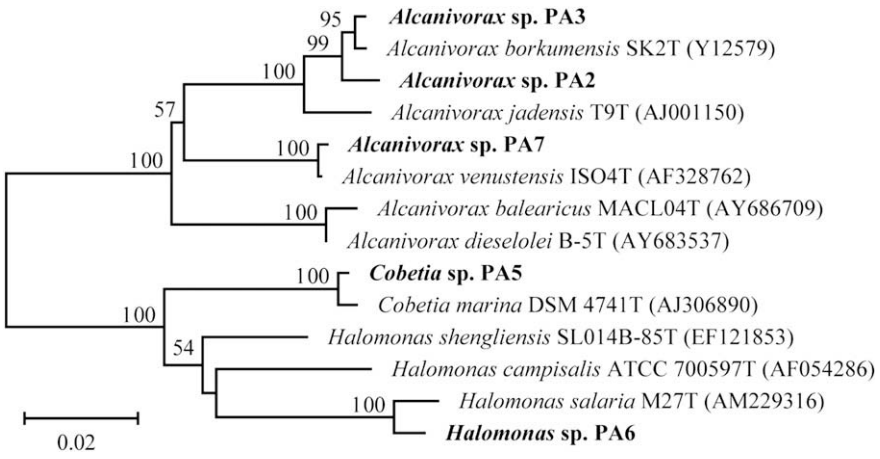

Fig. 1. NJ phylogenetic tree based on nearly full-length 16S rRNA gene sequence of biosurfactant-producing strains from this work (in bold) and related sequences. GenBank accession numbers are given in parentheses. Only bootstrap values higher than 50% out of 1000 replications are shown. Bar represents 0.02 nucleotide substitutions per site. Outgroup: 16S rRNA gene from *E. coli* K12 (AE000474).

Table 2
Hydrocarbon utilization of strains and consortium characterized in this study.

	Isolate	Source	Isolation medium	Utilization of			Medium without substrate (CFU/ml)
				SBWH	*n*-hexadecane	Phenanthrene	
				(CFU/ml)			
Biosurfactant-producing strains	PA2	Sediments	CONP	9.0E + 07 ± 1.2E + 07*	1.9E + 07 ± 2.2E + 06*	3.7E + 04 ± 5.8E + 04	5.9E + 05 ± 3.3E + 05
	PA3	Sediments	CONP	1.1E + 08 ± 6.5E + 07*	1.3E + 08 ± 7.2E + 07*	3.9E + 03 ± 5.9E + 02	2.7E + 05 ± 1.2E + 05
	PA5	Sediments	PhNP	3.2E + 06 ± 1.6E + 06*	4.4E + 05 ± 3.0E + 05	3.5E + 05 ± 7.1E + 04*	1.5E + 05 ± 7.2E + 04
	PA6	Sediments	CONP	8.7E + 07 ± 4.1E + 07*	1.8E + 07 ± 4.6E + 06*	4.3E + 07 ± 4.7E + 07	1.6E + 06 ± 9.7E + 05
	PA7	Asphaltic residues	PhNP	4.7E + 08 ± 2.3E + 08*	1.9E + 07 ± 5.4E + 06*	3.7E + 04 ± 5.8E + 04	4.4E + 05 ± 1.2E + 05
PA2-associated strain	PA1	Sediments	CONP	2.8E + 07 ± 1.5E + 07*	1.3E + 07 ± 4.4E + 06*	6.2E + 06 ± 2.5E + 06*	1.9E + 06 ± 1.7E + 06
Consortium	PA1 + PA2			2.5E + 08 ± 1.3E + 08*	2.7E + 07 ± 1.7E + 06*	9.1E + 06 ± 4.1E + 06	1.0E + 07 ± 5.1E + 06

Note. *Significant difference between medium without substrate and cultures with hydrocarbons at $p < 0.05$.

similarities for isolates PA2 and PA3 with a strain characterized to the species level (*A. borkumensis* SK2^T) were 98.9 and 99.6%, respectively. These isolates exhibited a level of nucleotide base homology to one another of 98.9%. Strain PA7 clustered with *A. venustensis* ISO4^T (99.7% similarity). The cladogram also shows that isolates PA5 and PA6 are related to *Cobetia marina* ATCC 25374^T (99.5% similarity) and *Halomonas salaria* M27^T (98.6% similarity), respectively.

Interestingly, *Alcanivorax* sp. PA2 was found to be naturally associated with another bacterial strain. When plating the samples for the first time and during morphological screening, isolate PA1 (100% 16S rRNA gene sequence identity with *Pseudomonas putida*) was initially selected. However, only after repeated streaking could associated *Alcanivorax* sp. PA2 be purified. A second strain able to reduce ST values was also found to form a natural association. *Alcanivorax* sp. PA3 formed a consortium with isolate PA4, a strain related to *Microbacterium esteraromaticum* (99.6% similarity).

The capability of biosurfactant-producing strains, PA1 and the PA1–PA2 consortium to grow on hydrocarbons is summarized in Table 2. All of them grew on SBWH, which is an oily residue consisting of a mixture of aliphatic and aromatic hydrocarbons [23]. Most strains could grow on *n*-hexadecane, the exception being PA5 which, in turn, could use phenanthrene for growth (Table 2).

3.2. Alkane hydroxylase gene analysis of Alcanivorax *strains*

The two *A. borkumensis*-related strains (PA2 and PA3) were further characterized by screening for the presence of alkane hydroxylase genes (*alkB*). A degenerate primer set was designed using an alignment of 78 *alkB* gene sequences from pure cultures (59 of them unique), obtained from GenBank. These primers (AlkB484F and AlkB824R) cover most known groups with either zero or one mismatch each, with the exception of genes belonging to *Acinetobacter*, *Pseudomonas aeruginosa* PAO1 and related sequences (data not shown). A putative *alkB* gene fragment with low identities (60% or less at the amino acid level) with previously identified alkane hydroxylases could be retrieved from strain PA3, while no specific amplification was observed in strain PA2 using these primers. Two additional primer sets from the literature were then tested in strain PA2: TS2S 403–Deg1RE 959 [28] and Mon F401–Mon R820 [15], each one retrieving a different *alkB* gene. Primers TS2S 403–Deg1RE 959 amplified a gene fragment showing 86% identity at the protein level with a hydroxylase identified in *Alcanivorax* sp. A-11-3, isolated from surface seawater of Malaysia. Interestingly, this isolate is also reported to produce a biosurfactant (EU438898, Wu et al., unpublished). On the other hand, primers Mon F401–Mon R820 retrieved a gene fragment with high (ca. 96%) amino acid identity to AlkB2 from *A. borkumensis* SK2 and related sequences. According to phylogenetic analysis, AlkB from *Alcanivorax* sp. PA3 falls

within a cluster that includes alkane hydroxylases from *Alcanivorax*, *Thalassolituus*, *Oleiphilus*, *Pseudomonas* and other yet uncultured marine microorganisms (Fig. 2). Despite the fact that *Alcanivorax* sp. PA3 was closely related to *A. borkumensis* by 16S rRNA gene analysis, its AlkB sequence was excluded from the clade that includes AlkB1 from *A. borkumensis*. On the contrary, AlkB1 from *Alcanivorax* sp. PA2 falls within a cluster that includes AlkB from *Alcanivorax* sp. A-11-3 and from uncultured soil microorganisms, forming a highly supported group (81% bootstrap). As expected, AlkB2 from strain PA2 clustered with AlkB2 from A. *borkumensis* and related sequences (Fig. 2).

3.3. Characterization of the Alcanivorax sp. PA2-Pseudomonas sp. PA1 consortium

In order to gain insight into naturally occurring associations involving *Alcanivorax* strains, one of the identified consortia was chosen for detailed analysis. The *Alcanivorax* sp. PA2 and *P. putida* PA1 consortium and individual strains were analyzed for their roles in hydrophobicity and biosurfactant production, while growing on medium with SBWH (Fig. 3). After a 2-day incubation period, bacterial counts of *Alcanivorax* sp. PA2 cultures reached 2.2 E + 08 CFU/ml, a value significantly greater ($p < 0.05$) than the *P. putida* PA1 cultures (6.3 E + 07 CFU/ml). After day 2, both strains entered the stationary phase of growth (Figs. 3a,b). A similar trend was observed for each strain in mixed compared to individual cultures (Figs. 3a–c). Only *Alcanivorax* sp. PA2 presented biosurfactant activity, as no appreciable reduction of ST was observed during PA1 growth on SBWH (Figs. 3a,b). ST in consortium cultures reached significantly lower values than those found for *Alcanivorax* from day 4 to 7 ($p < 0.05$) (Figs. 3a–c). According to MATH results, *Alcanivorax* sp. PA2 exhibited high cell-surface hydrophobicity during late exponential growth (72.7%), diminishing its adherence to hexadecane in the stationary phase to values of nearly 50% (Fig. 3a). In contrast, *P. putida* PA1 cultures presented low hydrophobicity throughout the time course of the experiment (Fig. 3b).

After 24 h of incubation, hydrocarbon dispersion in the aqueous phase of *Alcanivorax* sp. PA2 cultures was evident. Light microscopic observations revealed multiple hydrocarbon

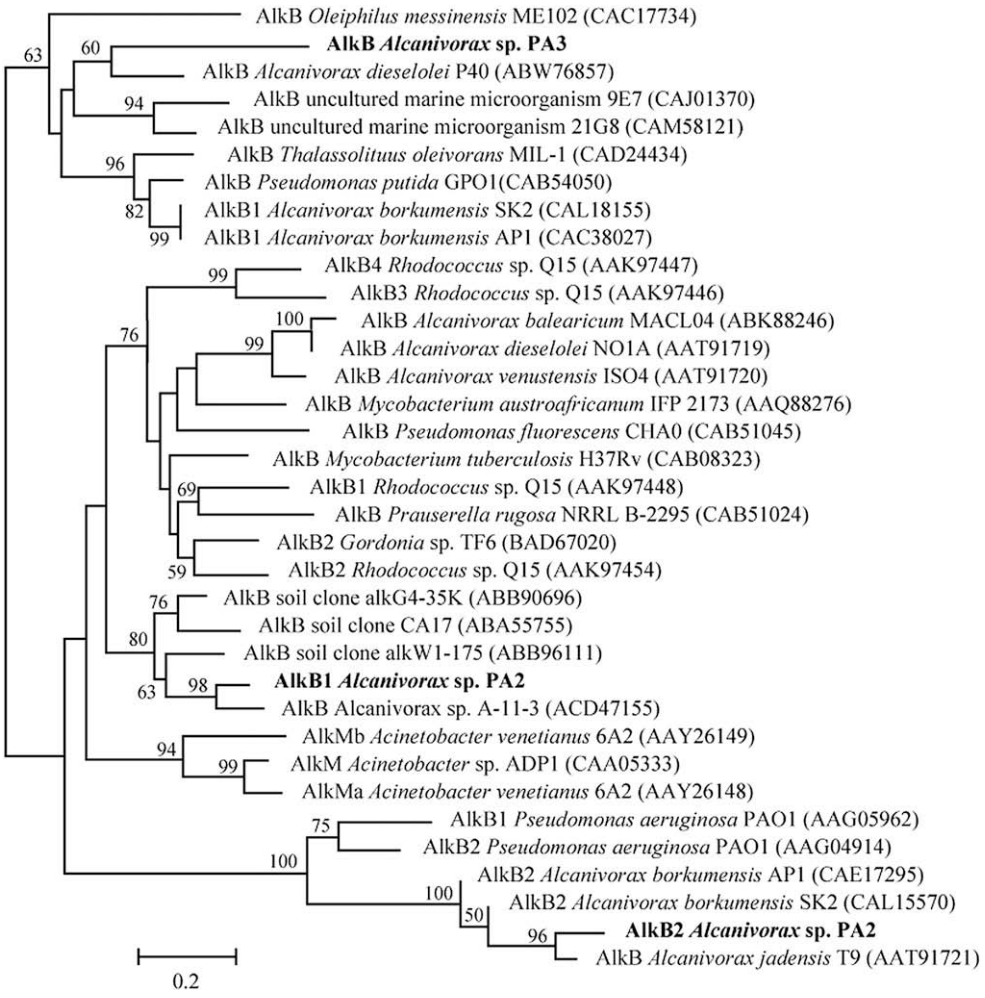

Fig. 2. NJ phylogenetic tree of AlkB partial deduced amino acid sequences obtained from *Alcanivorax* sp. PA2 and PA3 (in bold) and related alkane hydroxylase sequences. GenBank accession numbers are given in parentheses. Only bootstrap values higher than 50% out of 1000 replications are shown. Bar represents 0.2 amino acid substitutions per site. XylM from *P. putida* TOL plasmid pWWO (AAA26026) was used as outgroup.

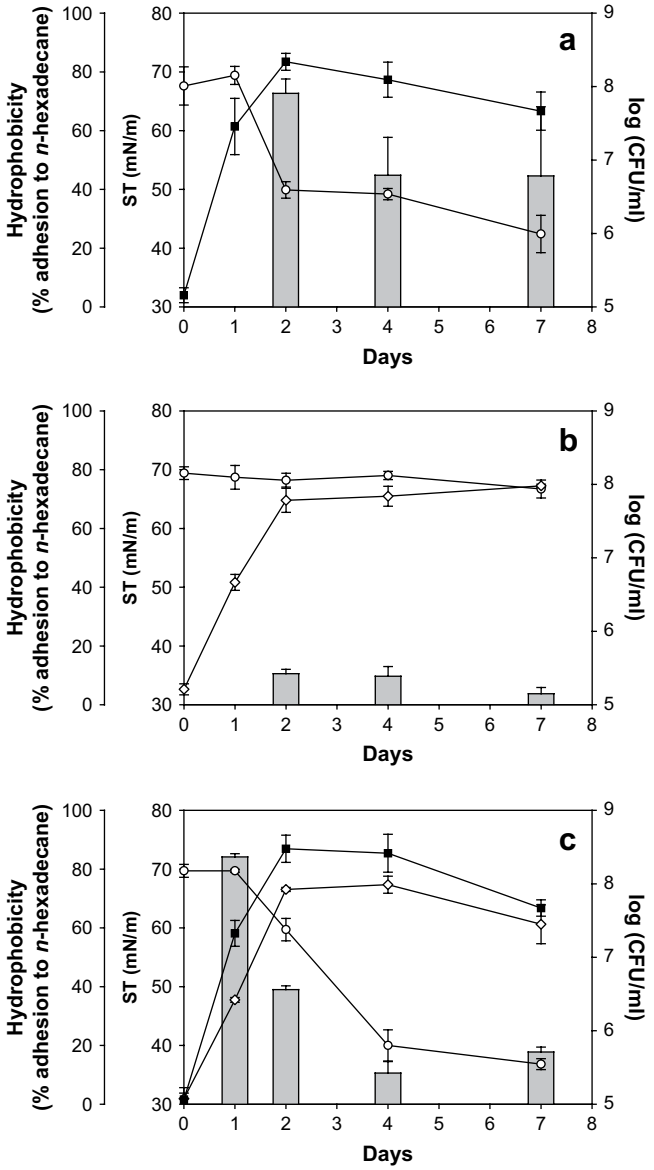

Fig. 3. Time-course experiments measuring microbial growth, ST and hydrophobicity of (a) *Alcanivorax* sp. PA2, (b) *P. putida* PA1, and (c) the consortium (*P. putida* PA1 + *Alcanivorax* sp. PA2). Hydrophobicity is indicated as % adhesion to *n*-hexadecane (bars), ST is expressed in mN/m (○) and growth is expressed as CFU/ml of *P. putida* PA1 (◇) and *Alcanivorax* sp. PA2 (■).

droplets (about 5–25 μm diameter) with bacteria clustering around them and producing a hydrocarbon microemulsion in their periphery (Fig. 4a). During the stationary phase, hydrocarbon microemulsion increased (spheres of 2–3 μm diameter) and the bacteria desorbed from the oil droplets (data not shown). Consortium cultures presented the same general behavior. On the other hand, no relevant oil/water emulsion was observed in *P. putida* PA1 cultures. Microscopically, few hydrocarbon droplets could be detected, which were either not covered with bacteria or emulsified in their outer part (Fig. 4b). SEM studies showed that strains PA1 and PA2 form aggregates in liquid cultures containing hydrocarbons, adhering to each other through an extracellular polymeric material (Figs. 4c,d). Pure cultures of *Alcanivorax* sp. PA2 also produced these cell aggregates, which were not observed in cultures of *P. putida* PA1 (data not shown).

4. Discussion

We characterized promising culturable biosurfactant-producing bacteria from coastal environments of Patagonia which had been exposed to hydrocarbon pollution. Contamination levels in sediments of the Aristizábal Peninsula were analyzed in 1995, when concentrations as high as 1304 μg of aliphatic and 737 μg of aromatic compounds per g of dry weight sediments were found [5]. Approximately 11 years later, when we sampled the region for this study, a substantial decrease in sediment hydrocarbon levels was observed, reaching values corresponding to non-polluted sediments according to UNEP [30] for aliphatic compounds (Commendatore, personal communication), or below detection limits for PAHs [16]. However, many supra-littoral rocks at this site were still coated by an asphalt-like layer of weathered oil (data not shown), and some loose old asphaltic residues were found.

As usually happens in studies involving culture methods for obtaining microorganisms showing a certain property, the medium composition had great influence on screening results. Among the different culture media used, only those supplemented with N and P sources led to isolation of biosurfactant and bioemulsifying bacteria. The strains with the highest ability to reduce the medium ST belonged to the genera *Halomonas*, *Cobetia* and *Alcanivorax*. Our result agrees with previous work showing that *Alcanivorax* becomes dominant in petroleum-contaminated seawater when nutrients are supplied [14]. However, the medium not supplemented with N and P (CO medium) is the one that most closely resembles a natural oil spill in the marine environment and supports the need for exploring the response of biosurfactant-producing microorganisms to nutrient addition after hydrocarbon spillage.

The presence of *Alcanivorax* in environments polluted with hydrocarbons has been well documented [13,19], although these bacteria have also been isolated from presumably non-polluted environments [9]. In our study, even after a substantial decrease in hydrocarbon concentrations in the sediments, biosurfactant-producing *Alcanivorax* strains able to use different hydrocarbons for growth were present and viable. In order to characterize alkane degradation genes present in these strains, we designed a degenerate primer set able to recognize alkane hydroxylase genes from a wide range of gram-positive and gram-negative bacteria. In addition, other primers from the literature were tested. We identified three different *alkB* genes in two *Alcanivorax* strains analyzed. One gene fragment was highly similar to *alkB2* from *A. borkumensis* and related sequences, while the other two amplicons were more divergent, thus expanding our knowledge of functional gene diversity in these hydrocarbonoclastic bacteria.

Moreover, we found that two of the isolated *Alcanivorax* strains (PA2 and PA3) were naturally associated with other bacteria. To better understand the nature of the interaction between the associated strains, the consortium formed by

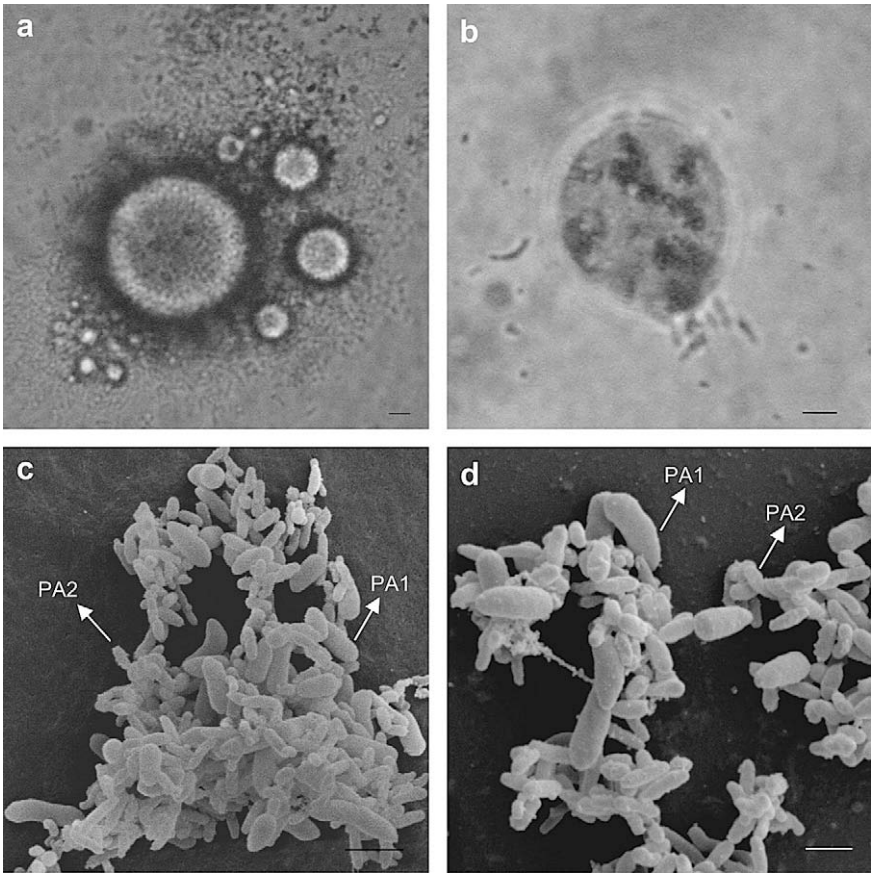

Fig. 4. Light micrographs of (a) *Alcanivorax* sp. PA2 and (b) *P. putida* PA1. SEM micrographs of (c, d) *Alcanivorax* sp. PA2-*P. putida* PA1 consortium. Bars: (a, b, c) 2 μm, (d) 1 μm.

P. putida PA1 and *Alcanivorax* sp. PA2 was analyzed with respect to their hydrocarbon accession mode. We observed that emulsification occurred only in PA2 cultures. This strain is closely related to *A. borkumensis* SK2^T, which has been described as a glucose-lipid surfactant producer [1,32]. Golyshin et al. [10] reported that cells harvested in late exponential phase produce the glucose-lipid surfactant in two forms: a glycine-containing cell-bound precursor that increases cell hydrophobicity and a glycine-lacking form that is released from the cell to the medium. Microscopic observations and hydrophobicity measurements showed that *Alcanivorax* sp. PA2 cells adhere to hydrocarbon droplets and cause the formation of a microemulsion during the exponential growth phase. Thereafter, biosurfactant production along with a reduction in cell-surface hydrophobicity occurred, suggesting that a cell-bound biosurfactant was secreted to the culture medium, causing both ST and hydrophobicity to decrease. The controlled release of biosurfactants could change hydrophobic/hydrophilic surface properties, influencing interactions with interfaces and with other bacteria [21]. The surfactant activity produced by strain PA2 was only detectable at late growth and stationary phases. Thus, our results indicate that the biosurfactant secreted to the medium was not involved in initiating oil-degradation, and suggest that direct interfacial adhesion is the main mechanism for PA2 hydrocarbon access. Instead, biosurfactant production might be more likely related to detachment of bacteria from the exhausted hydrocarbon droplets, and therefore be a consequence of *Alcanivorax* growth rather than a requirement for it. The occurrence of hydrocarbon-degrading bacteria presenting direct interfacial uptake (medium to high hydrophobicity) and biosurfactant production has already been reported for members of *Micrococcus*, *Rhodococcus*, *Corynebacterium*, *Pseudomonas*, and *Alcaligenes* genera [2,3]. Our results show that this hydrocarbon accession mode is also present in *Alcanivorax*.

Even though *P. putida* PA1 had the ability to use all tested hydrocarbons, no emulsification, adhesion to hydrocarbons or biosurfactant production were observed in this strain. In addition, no evident differences were observed in PA1 growth as an axenic culture with respect to its growth as a consortium with PA2, suggesting that PA2 does not enhance PA1 growth under the tested conditions. On the other hand, ST reduction in the consortium cultures was significantly lower than in PA2 cultures, showing that PA1–PA2 interaction had effects on the medium ST and consequently in biosurfactant production. As such reduction occurred during the late stationary phase of growth, it does not influence the exponential phase of the cultures.

Understanding the interactions between oil-degrading microorganisms is essential, not only when predicting the fate of hydrocarbons in the environment but also for the

development of successful bioremediation techniques. In this study, we characterized good biosurfactant-producing strains isolated from a region that has been exposed to hydrocarbon pollution. We particularly focused on the interactions between members of a natural consortium formed by *P. putida* and *Alcanivorax* sp. The ability of the biosurfactant-producing *Alcanivorax* sp. to form cell aggregates may play a role in this natural association. This feature could be advantageous for hydrocarbon waste treatment, if strains with complementary catabolic capabilities are selected.

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