Screening of polyhydroxyalkanoate-producing bacteria and PhaC-encoding genes in two hypersaline microbial mats from Guerrero Negro, Baja California Sur, Mexico

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ABSTRACT

Hypersaline microbial mats develop through seasonal and diel fluctuations, as well as under several physicochemical variables. Hence, resident microorganisms commonly employ strategies such as the synthesis of polyhydroxyalkanoates (PHAs) in order to resist changing and stressful conditions. However, the knowledge of bacterial PHA production in hypersaline microbial mats is limited to date, particularly in regards to medium-chain length PHAs (mcl-PHAs), which have biotechnological applications due to their plastic properties. The aim of this study was to obtain evidence for PHA production in two hypersaline microbial mats of Guerrero Negro, Mexico by searching for PHA granules and PHA synthase genes in isolated bacterial strains and environmental samples. Six PHA-producing strains were identified by 16S rRNA gene sequencing; three of them corresponded to a *Halomonas* sp.. In addition, *Paracoccus* sp., *Planomicrobium* sp. and *Staphylococcus* sp. were also identified as PHA producers. Presumptive PHA granules and PHA synthase genes were detected in both sampling sites. Moreover, phylogenetic analysis showed that most of the phylotypes were distantly related to putative PhaC synthases class I sequences belonging to members of the classes Alphaproteobacteria and Gammaproteobacteria distributed within eight families, with higher abundances corresponding mainly to Rhodobacteraceae and Rhodospirillaceae. This analysis also showed that PhaC synthase class II sequences were closely related to those of *Pseudomonas putida*, suggesting the presence of this group, which is probably involved in the production of mcl-PHA in the mats. According to our state of knowledge, this study reports for the first time the occurrence of *phaC* and *phaC1* sequences in hypersaline microbial mats, suggesting that these ecosystems may be a novel source for the isolation of short- and medium-chain length PHA producers.

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INTRODUCTION

Microbial mats are highly diverse ecosystems characterized by both seasonal and diel fluctuations under several physicochemical variables, hence resident microorganisms must adapt to changing conditions of their environment [4]. Several functional strategies as well as their physiological versatility allow them to resist these conditions. For instance, carbon and energy-rich polymers such as poly-3-hydroxyalkanoates (PHAs) are accumulated as discrete granules to maintain the metabolic activities under unfavorable conditions and nutrient imbalance [4, 8].

Currently, microbial mats are considered productive systems that accumulate high quantities of PHA under natural conditions, and bioprospecting of PHA-producing bacteria in marine and hypersaline microbial mats has been done mostly using culture-dependent approaches [4, 23, 33, 40]. Consequently, these ecosystems have been proposed as excellent sources for isolating new PHA-producing strains with industrial applications, since PHAs show similar material properties to those of some common plastics such as polypropylene [4, 24].

PHAs are synthesized by many Gram-positive and Gram-negative bacteria [25]. PHA synthases encoded by *phaC* genes are the key enzymes that polymerize PHA monomers [29]. The composition of PHA is clearly affected by the choice of the microorganisms and the carbon source [15, 25]. Four major classes of PHA synthases (class I to IV) can be distinguished based on their primary structures, as well as the number of subunits and substrate specificity [29], allowing the use up to 150 chemically different monomers [30]. The most studied classes are PHA synthases I and II, comprising enzymes that consist of only one type of subunit (PhaC). PHA synthase class I polymerizes short-chain length PHAs (scl-PHA), while class II polymerizes medium-chain length PHAs (mcl-PHA), both with different rheological properties desirable in biotechnological developments [36, 39, 42].

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Distinctive diel patterns of *in situ* PHA accumulation, the molar percent ratio of hydroxyvalerate (HV): hydroxybutyrate (HB) repeating units [4, 33, 40], and the composition of taxa, have been recognized in different stratified-marine [4, 23, 40] and hypersaline microbial mats [4, 5, 28, 40]. Nonetheless, the analysis of PHA synthase gene sequences from environmental samples and isolated strains has not been performed for hypersaline marine microbial mats, except for a metatrancriptomic analysis of Elkhorn Slough mats [5].

The aim of the study was to enhance our understanding of PHA producers and obtain evidence of PHA production through the recovery of both classes (I and II) of PHA synthases sequences from two hypersaline microbial mats, by constructing clone libraries and isolating bacterial strains with the ability to grow and store PHA within the first 72 hours of culture. Therefore, the recovered putative PHA synthases allowed us to elucidate the potential short- and medium-chain length PHA producers in hypersaline microbial mats from Guerrero Negro, Mexico.

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MATERIALS AND METHODS

Sample collection

Microbial mat samples were collected from concentration ponds of Area 1 (ESSA A1; 27°36.01´N 113°53.46´W) and Area 4 (ESSA A4; 27°41.41´N 113°55.19´W) at Exportadora de Sal S.A (ESSA), in Guerrero Negro, Baja California Sur, Mexico, during February, 2016.

The salinity of the studied sites was measured *in situ* (HI 931100 Hanna Instruments, Italy). Samples were taken in duplicate and preserved in RNA later® for further molecular analysis. For culture-dependent assays, mat samples were dried at environmental temperature.

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Screening, isolation and molecular characterization of polyhydroxyalkanoate-producing bacteria

The first approach to detect PHA producers in environmental samples was Nile Red staining and examination using a Nikon Eclipse 80i epifluorescence microscope under green excitation at 540 nm (Nikon, Japan).

The primary isolation of heterotrophic bacteria was done as follows: 0.1 g of mat from the photic zone was homogenized in 900 µl of half-concentration synthetic seawater (1/2X SSW) composed of (in g L^{-1}): NaCl 11.675, KCl 0.75, $MgSO_4 \cdot 7H_2O$ 12.35, $CaCl_2 \cdot 2H_2O$ 1.45, Tris–HCl buffer 1.0 M pH 7.5 [3]. Aliquots of 200 µl of serial dilutions from 10^{-1} to 10^{-9} were plated in four different culture media: (1) marine agar 2216 (Difco®), (2) *Pseudomonas* agar F (Difco®) dissolved in 1/2 X SSW, (3) YEA glucose 1% (w/v) composed of (g L^{-1}): NH_4Cl 0.5, $K_2HPO_4 \cdot 3H_2O$ 0.076, yeast extract 0.2, $FeSO_4 \cdot 7H_2O$ 0.028, glucose 10, and agar 14, dissolved in 1/2 X SSW and (4) YEA acetate with the same composition as YEA glucose, but using acetate at 1% (w/v) as the carbon source. All inoculated media were incubated at 30ºC under aerobic conditions for 72 hours.

Colonies developed on solid media plates at 72 hours were screened to select PHA producers using phase-contrast microscopy to detect RCIs and only those positive ones were subsequently assessed with two lipophilic stains: Sudan Black and Nile Red [23] under bright-field and epifluorescence microscopy, respectively (Eclipse 80i Nikon, Japan). Serial dilutions from 10^{-1} to 10^{-8} were performed using the respective isolation media to authenticate the axenic nature of the strains. Only strains that showed uniformity of colonial and cellular morphology were employed for DNA extraction.

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For taxonomic assignment and *phaC* gene detection, genomic DNA was extracted from pure cultures following the manufacturer's instructions of the DNeasy Blood and Tissue kit (QIAGEN, USA). DNA integrity and concentration were assessed by standard agarose gel electrophoresis and spectrophotometric reads using a NanoDrop Lite spectrophotometer (NanoDrop Technologies, Wilmington, DE). PCR amplifications of the 16S rRNA and *phaC* genes were performed using the GoTaq Master Mix system (Promega M7122, Madison, WI, USA), containing: 6.5 µl sterile water, 2.5 µl of each primer solution [10 µM], 12.5 µl of GoTaq Master Mix and 1 µl [10 ng µl-1] of DNA. The 16S rRNA amplifications were done using the universal primers BAC-8F and BAC-1492R [38], with the following thermocycling conditions : one cycle at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min; 58 °C for 1 min; 72 °C for 1 min; and a final step at 72 °C for 5 min. Amplifications of partial *phaC* gene were obtained employing PHACGNF and PHACGNR primer set [24] (Table 1), in one cycle at 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min; 54 °C for 1 min; 72 °C for 1 min, and a final step at 72 °C for 10 min. All the PCR assays were carried out in a Thermocycler T-100 (Bio-Rad, Berkeley, CA, USA) and analyzed by standard gel electrophoresis. All the resulting fragments were commercially sequenced by Genewiz (NJ, USA).

phaC and *phaC1* gene detection from environmental DNA

Environmental DNA was extracted from 0.1 g of microbial mats' photic zone using the Power Biofilm DNA Isolation Kit (Mo Bio Laboratories, Carlsbad CA, USA). The amplification of *phaC* gene was achieved following the same procedures described for strains. However, to detect partial *phaC1* gene of *Pseudomonas* species, two sets of primers were designed after the alignment of thirteen *phaC1* sequences from different *Pseudomonas* species (Table 1; Dataset S1). The primers designed were evaluated *in silico* for secondary structure formation with

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OligoAnalyzer tool (https://www.idtdna.com/calc/analyzer), and their functionality experimentally confirmed by PCR and sequencing using DNA from *Pseudomonas putida* strain KT2440 as control (Fig. S1). Contrary to the specificity observed in control PCR reactions, PCR assays with environmental DNA from both sampling sites and primer sets, showed a low specificity (Fig. S1). Consequently, a modified nested-PCR strategy was necessary in order to obtain the expected size and enough concentration of amplicons for sequencing. For the first round, a PCR reaction with primers phaC1F2 and phaC1R1 (Table 1) was done as previously described. Thermocycling conditions consisted in one cycle at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min, with a final step at 72 °C for 10 min. Since multiple bands were observed a band of the expected size was excised and purified from agarose gel with the QIAquick Gel extraction kit (Qiagen GmbH, Hilden, Germany). Purified products were used as template in a second PCR round with phaC1F1 and phaC1R2 primers (Table 1), with the same PCR and thermocycling conditions as described in the first PCR round.

PCR products were cloned into the vector pJET1.2 (Thermo Scientific, USA). For detection of positive clones, plasmid DNA was extracted following the alkaline extraction method [34], and PCR assays with the vector primers were performed. Positive clones were also sent to Genewiz (NJ, USA) for Sanger sequencing.

Bioinformatics analysis of 16S rRNA, *phaC,* and *phaC1* genes

All the obtained 16S rRNA, *phaC* and *phaC1* sequences were analyzed with Chromas Pro v 1.5 (http://technelysium.com.au/wp/chromaspro/), and CodonCode Aligner v 4.0.4 (CodonCode Corporation, Dedham, MA, USA). Sequences were compared by BLAST v. 2.7.1+ [2], and only those showing identity with 16S rRNA, *phaC* and *phaC1* genes were selected for further analysis.

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For taxonomic assignment of strains, 16S rRNA gene sequences were phylogenetically compared with sequences obtained from GenBank using the MEGA 6 software [37], using a Maximum parsimony algorithm. Previous to the tree phylogenetic estimation, the sequences of *phaC* and *phaC1* from clones and strains were translated to amino acid sequences with the EMBOSS Transeq translation tool [11, 31].

In order to obtain the best representative sequences for the phylogenetic reconstruction, derived PhaC sequences of clones were assessed using the default parameters of CD-HIT tool with a threshold value of 97% [22]. These representative sequences, derived PhaC sequences of isolated strains, as well as several sequences retrieved from GenBank of PhaC classes I, II, III and IV, were subsequently aligned with SeaView version 4.6.2 [10, 12] under the Clustal Omega algorithm [35]. The alignment was assessed in MEGA 6 and ProtTest 3 [1, 7] to find the best amino acid substitution model, an LG model with gamma distribution was selected under the Bayes information criterion (BIC). The tree topology was estimated with PhyML 3.0 [14] using the Maximum-Likelihood method with 1000 bootstraps under the selected model assumptions.

GenBank accession numbers

16S rRNA gene sequences from strains were deposited in GenBank with accession numbers MF804952 to MF804957. *phaC* sequences retrieved from isolated strains were deposited with the accession numbers MF939169, MF939170, MG201834, and MG201835. Environmental *phaC* and *phaC1* sequences were deposited with accession numbers MF939171 to MF939204 and MG52451.

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RESULTS

Detection of PHA granules in environmental samples and isolated strains

Salinity measurements of the brines where microbial mats were sampled were of 6.3% for ESSA A1 and 8.5% for ESSA A4. Microbial mat samples stained with Nile Red exhibited PHA granules inside long filamentous cells of various diameters (Fig. 1A and 1B). Additionally, 62 colonies were obtained from four culture media used for the isolation of PHA producers. Only six isolates achieved growth and PHA granule formation within the first 72 hours of incubation. The strains 2A, 3B and 4C were isolated in YEA-glucose medium; strains 1B and 5B were obtained in YEA-acetate and strain 6A grew in *Pseudomonas* Agar culture medium. The growth of these six strains was not evaluated in the four different culture media assayed.

The six strains showed well-defined RCIs, and PHA granules when Sudan Black was used. However, only Gram-negative strains exhibited PHA granules using Nile Red staining (Table 2). Representatives of the three techniques are shown; Nile Red of Gram-negative strains (Fig. 1C and 1D), well-defined RCIs (Fig. 1E – G) and affinity to Sudan Black of a Gram-positive strain (Fig. 1H).

Taxonomic assignment of isolated PHA-producing bacterial strains

The isolated PHA-producing strains were taxonomically assigned to four genera based on the comparison of 16S rRNA gene sequences with GenBank (Table 2). Three strains of *Halomonas* were detected in ESSA_A1, as well as strains of the genera *Paracoccus* and *Staphylococcus*, while a strain of *Planomicrobium* was the only one obtained from ESSA_A4. According to phylogenetic analysis, strains ESSAA1Ac_1B, ESSAA1Glu_3B and ESSAA1Glu_4C were closely related with *Halomonas salina* (Fig. 2). In turn, strain ESSAA1Glu_2A had high similarity with *Paracoccus chinensis*, while strain ESSAA1PAF_6A was closely related to

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The detection and quantification of *phaC2* gene in microbial mat samples from ESSA A1 and ESSA A4 were performed in a qPCR system (Rotor-Gene 6000 thermocycler, QIAGEN, Germany), with primers previously reported [41] (Table 1). A standard curve was constructed using DNA of *Pseudomonas putida* KT2440, with concentrations ranging from $8.9x10^{10}$ - $4.4x10^{8}$ *phaC2* copies μl^{-1}. qPCR reactions contained 4.25 µl of sterile water, 6.25 µl of SYBR® Green master mix (QIAGEN, Germany), 0.5 µl of each primer [10 mM], and 1 µl DNA extracted from 0.1 g of sample. Thermal cycling conditions consisted in one cycle of 94 °C for 2 min, followed by 40 cycles at 94 °C for 40 s, 53 °C for 40 s, and 72 °C for 40 s; with fluorescence detection at the end of each cycle, and a final cycle at 72 °C for 8 min. Additionally, a melting curve was run for primer dimers detection by increasing temperature from 45-95 ºC with increases of 1 ºC per cycle.¶
DNA-mass values were transformed to copy numbers with a formula described by IDT technologies [16], and statistically analyzed by R studio version 1.0.136 (Rstudio team, 2015, https://www.r-project.org/), with *a priory* Shapiro-Wilk and Fligner tests, a variance analysis (ANOVA), and *a posteriori* Tukey-HSD test.¶

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Staphylococcus saprophyticus, and ESSAA4Ac_5B with a cluster of *Planomicrobium okeanokoites* and *Planomicrobium flavidum* (Fig. 2).

PHA synthase genes from PHA-producing strains and microbial mats

Four of the six PHA-producing strains (ESSAA1Ac_1B, ESSAA1Glu_2A, ESSAA1Glu_3B, ESSAA1Glu_4C), assessed by the molecular approach to detect *phaC* gene resulted in amplicons of the expected size (~500 bp) (Table 2). Although strain ESSAA4Ac_5B showed RCIs and was positive to Sudan Black (Fig. 1H), an unexpected amplicon of ~1000 bp was obtained. However, the sequence had low quality, therefore *phaC* gene assignation was not possible In turn, ESSAA1PFA_6A strain did not present any PCR fragment amplification.

Amplicons from strains ESSAA1Glu_1B, ESSAA1Glu_2A, ESSAA1Glu_3B, and ESSAA1Glu_4C were successfully sequenced. The partial *phaC* sequence of ESSAA1Glu_2A strain showed an ORF of 528 bp encoding 176 amino acids, with an identity value of 98% with the sequence from *Paracoccus chinensis* (SDK97885). Both strains ESSAA1Ac_1B and ESSAA1Glu_3B showed an ORF of 534 bp encoding 178 amino acids. Meanwhile in ESSAA1Glu_4C, the ORF was of 528 bp encoding 176 amino acids. All showed relatively high identity values of 97, 97 and 94 %, respectively against PHA synthase class I of *Halomonas aestuarii* (WP071941987) (Table 2).

A unique *phaC* fragment of ~500 bp was also obtained from environmental DNA PCR assays using primers PHACGN (data not shown). In turn, a nested strategy to detect *phaC1* resulted in a first fragment of ~1100 bp (Fig. S), which was used as template for the second round, allowing unique fragments of ~500 bp which were subsequently used for cloning assays (Fig. S).

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After screening clone libraries, 22 confirmed *phaC* sequences from ESSA A1 (16 sequences) and ESSA A4 (6 sequences) were analyzed by BLAST (Table 3). The sequences were binned to PHA producers presumed to belong to eight families of Alphaproteobacteria and Gammaproteobacteria, and to unassigned bacteria (Fig. 3), with identity values ranging from 67 – 99 % (Table S 1). Differences in composition at the presumed family level were observed in the sites (Fig. 3). For ESSA A1 seven families were detected meanwhile for ESSA A4 only four, with Rhodobacteraceae and Rhodospirillaceae the most abundant families, respectively. It is remarkable that most of the sequences recovered from ESSA A4 (with an abundance of ~ 60 %), despite showing high quality, were assigned to hypothetical proteins and with proteins lacking an assigned taxon (unassigned taxa) (Fig. 3).

Only six partial-putative PhaC amino acid sequences had a relatively close similarity (>90 %) with sequences reported previously; two clones to *Marinobacter* sp., (99 % and 97 %), one clone to *Cobetia amphilecti*, (98%), two more to *Marivita hallyeonensis* (94 % and 96 %), and a last one to a bacterium of the Rhodobacteraceae family (91 %) (Table S 1). In turn, we obtained 13 confirmed PhaC sequences deduced from *phaC1* genes, five from ESSA A1 and eight from ESSA A4(Table 3), however all the putative amino acid sequences showed a high identity value (99 %) with PHA synthase class II of *Pseudomonas putida* (Table S 1).

Phylogeny of PHA-synthase sequences recovered from microbial mats

CD-HIT analysis resulted in 21 clusters (20 for PhaC class I and one for PhaC class II) (Table S 2), which were submitted to a phylogenetic analysis using the Maximum-Likelihood method. Phylogenetic analysis (Fig. 4) showed that the partial amino acid sequences deduced from *phaC*

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genes binned into PhaC class I of PHA producers from Alphaproteobacteria and Gammaproteobacteria.

Four clones (59, 79, 98, and 123) were independently arranged in the tree (red branches), showing an unclear phylogenetic relation with PhaC sequences previously reported. In turn, 11 clones (blue branches in the tree) were binned to different clusters probably belonging to members of the families Rhodospirillaceae, Hyphomonadaceae, Granulosicoccaceae and Rhodobacteraceae. Only five clones (2, 4, 5, 18 and 56; green branches), were strongly clustered to known PHA producers and assigned to the family Rhodobacteraceae of the Alphaproteobacteria, and to families Alteromonadaceae and Halomonadaceae of the Gammaproteobacteria. As expected, the representative PhaC class II sequence was clustered with the well-studied mcl-PHA producer *P. putida*.

DISCUSSION

PHA-granule detection in isolated strains and environmental samples

Previous studies have suggested that the production of PHA in hypersaline microbial mats was restricted to filamentous cyanobacteria, purple sulfur bacteria [33], green non-sulfur bacteria of the phylum *Chloroflexi* [5], and heterotrophic bacteria of the genera *Sphingomonas*, *Bacillus,* and *Halomonas* [40]. Our study shows the presence of PHA granules and PHA synthases in both environmental samples as well in aerobic-heterotrophic bacterial strains isolated from the samples (Fig. 1; Table 2). Partial 16S rRNA gene sequences revealed PHA productiuon in three strains belonging to the genus *Halomonas,* which was previously found in estuarine and hypersaline mats [40]. The remaining islated strains were related to *Paracoccus*. *Planomicrobium*

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A standard curve relating CT values with *phaC2* gene copy number was constructed in order to quantify this gene in environmental mats. There was a strong linear inverse relation between CT values and $\log_{10}$ of *phaC*2 copy number ($R^2 = 0.99$). The equation describing the relationship between both parameter was CT = $-3.0702 \times \log_{10}$ (gene copy number) +46.812, with a reaction efficiency of 1.12.¶
The quantification and transformation of DNA concentration values to copy numbers of *phaC*2 gene in environmental samples resulted in an estimation of 2.1×10^8 and 5.4×10^8 copies of *phaC*2 g^{-1} of mat from ESSA A1 and ESSA A4, respectively. However, ANOVA analysis discarded the presence of statistical differences between both samples ($P > 0.05$).¶
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and *Staphylococcus*, and represent the first reported of the occurrence of these PHA-producing genera in hypersaline microbial mats (Table 2).

Most of the strains isolated in this work were closely affiliated with Gammaproteobacteria of the genus *Halomonas* (Table 2). Previous studies carried out on Ebro Delta Estuary, Spain and Camargo mats, France indicate that *Halomonas* is one of the most abundant taxa in both samples, where it appears to interact syntrophically with phototrophic partners, with direct consequences on polyhydroxyalkanoate diel dynamics in stratified systems [40]. Based on this, we suggest that PHA-producers of the *Halomonas* genus could have an important role as sink in carbon cycling [4], and PHA biosynthesis in Guerrero Negro mats.

PHA synthases from hypersaline microbial mats

Heterotrophic PHA-producing bacteria have been isolated from different environments including estuarine microbial mats [13, 40], hypersaline mats [6], and contaminated mats [23]. However, the well-studied microbial mats from Guerrero Negro have never been analyzed for PHA producers. The use of the primer set (PHACGNF and PHACGNR) targeting *phaC* of Gram negative bacteria [24] and two sets of primers (phaCF1/phaCR2; phaCF2/phaCR1) designed in this study to target *phaC1,* allowed the recovery of partial-putative PHA synthases of the classes I and II, respectively, suggesting the presence of a wide diversity of Gram negative taxa including *Pseudomonas* spp. with the potential of PHA production in the mat samples.

Since microbial mats of ESSA harbor a great diversity of microorganisms with different metabolic capabilities [21], some functional genes could show low abundance in these environments. Therefore, the detection of *phaC1* gene was difficult, requiring a modified nested-PCR strategy despite the risk of amplification biases. Nonetheless, since primers were designed

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based on 13 different *Pseudomonas* spp. (Dataset S1), it is not clear if the results obtained were due to an underestimation of diversity or that *Pseudomonas putida* is indeed the most abundant species of *Pseudomonas* in the mat.

Analysis of the approximated composition at the family level of the sequences obtained from clone libraries indicate differences in the two sites (Fig. 3), suggesting that distinctive microorganisms could be involved in the production of PHA in each mat, probably due to changes of salinity. It was also found that sequences from both sites matched with PhaC of unassigned taxa, which were interpreted as derived from uncultured bacteria and with hypothetical proteins of unknown function (Fig. 3). Similar results were found in deep-sea water samples [9], ice and cold pelagic seawater environments [26], suggesting that PHA productivity of these uncultured microorganisms have not been examined yet.

It was observed that the isolated strains were not detected in clone libraries. This could be associated with inherent biases of the methods used. In particular, we employed the detection of inner granules and growth within the first 72 h of culture as selection criteria to choose PHA producers. Staining methods are suitable for screening large numbers of strains. However, particular microorganisms will demand appropriate carbon sources as well as different incubation times to show PHA granules [23]. Hence, even when the screening of PHA producers was carried out on 62 colonies which probably had the ability of PHA production, only six fulfilled the selected criteria.

Phylogenetic relationship of PHA synthases from Guerrero Negro microbial mats

We compiled the deduced amino acid sequences of *phaC* and *phaC1* genes obtained through the culture independent approach and, by reconstructing a phylogenetic tree, compared their

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relationship with the four PhaC classes. Most of the putative amino acid sequences of PhaC directly recovered from the mats were related with PHA producers in which the percent of PHA accumulation has not been examined yet [19].

From PhaC class I sequences, four clones could not be assigned to any family (Fig. 4; red branches). These independent sequences probably derived from PhaC of unidentified Proteobacteria. In turn, five PhaC sequences (Fig. 4; green branches), were closely related to PhaCs of PHA-producers that are poorly studied: *Marinobacter* sp., (two clones), *Cobetia* sp., *Marivita* sp., and a bacterium of the Rhodobacteraceae family [9, 27, 32, 41]. Accordingly, 15 uncharacterized putative PhaC synthase fragments (<90%) from hypersaline microbial mats are reported in this work (Fig. 4; red and blue branches).

In turn, the PhaC class II representative sequence (green branch) was binned together with the PHA synthase class II of *Pseudomonas putida,* with a sequence of *Aeromonas hydrophila* and, in a separated branch, with *P. fluorescens* (Fig. 4). However, the discrepancy on the *A. hydrophila* arrangement along with *P. putida* and clone 223, could be attributed to a paralogous gene or to a HGT event, as has been previously documented [17]. Moreover, the ability of mcl-PHA production by some *Aeromonas* species has been reported [16].

This cluster of Gammaproteobacteria was closely related with another group that included clone 23 and *Rubrimonas cliftonensis*, which belongs to the Rhodobacteraceae family of the Alphaproteobacteria. Furthermore, both clusters were closely related to a branch of several Rhodobacteraceae members although as a morte distant clade (Fig. 4). This arrangement suggests that clone 23 and this particular sequence of *R. cliftonensis* could have a closer relationship to the PhaC class I of Gammaproteobacteria than to the PhaC of Alphaproteobacteria. This hypothesis is

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supported by the fact that the genome of *R. cliftonensis* has four different paralogous *phaC* genes (WGS project No. FNQM01000000), and another paralog was binned to the Alphaproteobacteria group along with the clone 13 (Fig. 4). Remarkably, the arrangement observed suggests that the origin of *phaC1* class II gene of *Pseudomonas* could be derived from *phaC* class I of Alphaproteobacteria, although a deeper analysis to confirm this hypothesis should be done. Another similar case of potential paralogs was observed in the cluster formed between clone 14 and the gammaproteobacterium *Granulosicoccus antarticus*, which was assigned into the Alphaproteobacteria group (Fig. 4).

Clones with higher identity values (Table S) as well as the sequences of the characterized strains (*Paracoccus* sp. and *Halomonas* sp.) were arranged to the expected taxonomic families and classes (Fig. 4).

The differences of variability in amino acid sequences among class I and class II PhaC in the phylogenetic tree suggests that scl-PHA are synthesized by a wide range of bacteria, while mcl-PHAs are produced primarily by some *Pseudomonas* strains [18]. In addition, a high diversity and availability of carbon sources can be found and directly synthesized to scl-PHA in the Guerrero Negro mats, i. e. organic acids produced during the fermentation process carried out by primary producers [20].

Although our results showed a bias derived from the use of primers designed only for Gram-negative bacteria, we were able to retrieve putative PhaC class I sequences organized in eight families (Fig. 4). This family distribution could be explained because the ability to synthesize PHAs is widespread in bacteria, since the PHA synthase genes can be horizontally transferred

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between different phylogenetic groups, suggesting an adaptive advantage to the microorganisms that synthesize them [16, 17].

Consistently with previous reports for marine environments [9], most of the putative sequences retrieved in this study corresponded to PHA synthase class I, which were related to the class Alphaproteobacteria. In contrast, other well-studied environments as activated sludge and soils contaminated with oil show dominant groups belonging to class I PhaCs of Betaproteobacteria [9].

Although culture-independent methods are a good tool for the detection of yet uncultured microorganisms with the potential of PHA production, cautious interpretation is needed due to the existence of paralogous genes, since some bacteria harbor more than one copy of the *phaC* gene in their genome, such as in *Cupriavidus necator* [9]. In some cases, these paralogous show low sequence similarity or even belong to different PhaC classes. Therefore, further assessments of PhaC activity either *in vitro* or *in vivo* will be required, particularly of PhaC retrieved from environmental DNA [9].

CONCLUSIONS

We report here for the first time the occurrence of PHA synthase class I and II in hypersaline microbial mats, inferred from *phaC* gene sequences and PHA granules as evidence for PHA production, which contribute to the knowledge of the PHA-bacterial producers of the classes Alphaproteobacteria and Gammaproteobacteria in hypersaline environments characterized by showing extreme metabolic diversity. Thus, hypersaline microbial mats could be considered an excellent source for the isolation of new PHA-producing strains with potential to use a wide spectra of carbon sources as sugars, organic acids, alcohols, amino acids and hydrocarbons, since

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Deleted: ***phaC2* detection in hypersaline environments**¶ Although two mcl-PHA synthase genes, *phaC1* and *phaC2*, have been identified and characterized in several species of *Pseudomonas*, *phaC2* gene has been considered as biological marker for mcl-PHA production due to a broader substrate specificity [19, 41]. Thus, potential production of mcl-PHA in hypersaline microbial mats from Guerrero Negro was assessed for a first time in this study by the qPCR quantification of *phaC2* gene (Fig 5). The abundances of this gene in both samples had not statistical differences. However, these results suggest that the analyzed samples harbor the capability of mcl-PHA production, mainly by several species of *Pseudomonas*, which has biotechnological implications due to the specific physical and mechanical properties found in these polymers in comparison with scl-PHA.¶

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the composition of PHA is clearly affected by the microorganism and the carbon source. Further studies should be directed to determine the *in situ* quantities of PHA and the monomer's types that occur in these microbial mats.

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ACKNOWLEDGMENTS

We are grateful to Exportadora de Sal, S.A de C.V. for access to the Guerrero Negro field sites. We would like to thank to Jesús Nuñez-Rojas from Laboratorio de Ecología Microbiana Molecular of the Benemérita Universidad Autónoma de Puebla, who kindly provided the strain KT2440. We also thank to Ma. Leopoldina Aguirre-Macedo for supporting the clone libraries preparation at CINVESTAV Mérida; and to Alejandra Escobar-Zepeda for the edition of Figure 3.

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