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ORIGINAL ARTICLE

Screening and Isolation of PHB-Producing Bacteria in a Polluted Marine Microbial Mat

Alejandro López-Cortés · Alberto Lanz-Landázuri · José Q. García-Maldonado

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Abstract The characteristics of microbial mats within the waste stream from a seafood cannery were compared to a microbial community at a pristine site near a sandy beach at Puerto San Carlos, Baja California Sur, Mexico. Isolation of poly-β-hydroxybutyrate (PHB)-producing bacteria, recognition of brightly refractile cytoplasmatic inclusions, lipophilic stains with Sudan Black and Nile Red, and chemical extraction of PHB were used as a culture-dependent strategy for the detection of PHB-producing bacteria. The culture-independent approach included denaturing gradient gel electrophoresis of phylotypes of 16S rRNA of microbial communities from environmental samples. Significant differences in community structure were found among the polluted and pristine sites. These differences were correlated with the physicochemical characteristics of the seawater column. At the polluted site, the seawater was rich in nutrients (ammonia, phosphates, and organic matter), compared to the pristine location. Partial sequencing of 16S rDNA of cultures of bacteria producing PHB included *Bacillus* and *Staphylococcus* at both sites; *Paracoccus* and *Micrococcus* were found only at the polluted site and *Rhodococcus* and *Methylobacterium* were found only at the pristine site. Bands of the sequences of 16S rDNA from both field samples in the denaturing gradient gel electrophoresis (DGGE) analyses affiliated closely only with bacterial sequences of cultures of *Bacillus* and *Staphylococcus*. High concentrations of organic and inorganic nutrients at the polluted site had a clear effect on the composition and diversity of the microbial community compared to the unpolluted site.

A. López-Cortés (✉) · A. Lanz-Landázuri · J. Q. García-Maldonado
Laboratorio de Ecología Microbiana Molecular,
Centro de Investigaciones Biológicas del Noroeste (CIBNOR),
Mar Bermejo 195, Colonia Playa Palo de Santa Rita,
La Paz B.C.S. 23090, Mexico
e-mail: alopez04@cibnor.mx

Introduction

Polyhydroxyalkanoates (PHAs) are a type of polyester polymer produced by many bacteria. It accumulates as discrete granules to levels as high as 90% of cell dry weight as a response to environmental stress and nutrient imbalance and plays a role as a sink for carbon and energy [8]. This polymer can be produced from different renewable carbon sources [9, 22, 32, 33]. These PHAs have properties that vary according to their monomer compositions (e.g., 3-hydroxyvalerate, 3-hydroxybutyrate, 3-hydroxyhexanoate) and chain length having commercial value as thermoplastics, elastomers, and rubbers [22, 30]. The diversity of different monomers that can be incorporated into PHAs, combined with biological polymerization system that generates high-molecular weight materials, has resulted in a situation where an enormous range of new polymers is potentially available. The advent of genetic engineering combined with modern molecular microbiology now provides us with the exceptional framework for studying plastic properties as a function of molecular biology and metabolism. Besides the typical polymeric properties described above, an important characteristic of PHAs is their biodegradability [19]. Growing concern about environmental pollution has renewed interest in the development of PHAs, which are completely biodegradable by bacteria present in most environments [15]. More important

as the biological characteristics and biodegradability of PHAs is the fact that their production is based on renewable resources. Bacterial producers of PHA have been isolated from the waste stream of several treatment facilities that often provide a mixture of substrates. Several investigations of natural isolates of *Actinobacillus*, *Azotobacter*, *Agrobacterium*, *Rhodobacter*, and *Sphaerotilius* have focused on converting organic waste to bacterial PHA [19]. First reports of PHA producer strains from marine sediments referred to the genera *Beneckea* and *Vibrio* [5, 23]. Recently, microbial mats has been considered as highly diverse and productive systems that produce high quantities of poly (3)-hydroxybutyrate (PHB) under natural conditions, and bioprospection of PHB-producing bacteria in marine microbial mats has been done for the bulk of the community [25]. PHA-producing strains from Ebro Delta microbial mats were detected and monitored under laboratory cultivation. Nevertheless, the taxonomic assignment of the strains was missing. They propose microbial mats as an excellent source for the isolation of new PHA-producing strains with industrial applications [6]. This study contributed to the understanding of the diversity of PHA-producing bacteria, with preference to PHB, in marine microbial mats associated with the waste stream from a cannery of marine products and a pristine site near a sandy beach.

Materials and Methods

Sampling

Samples of marine coastal sediment were collected from an unpolluted or pristine site, called *Curve* (24°48′19″N, 112°06′43.6″W), and a site polluted with cannery discharge, called *Cannery* (24°46′56.4″N, 112°06′21.2″W), at Puerto San Carlos, B.C.S., Mexico during October 2004. One sample and one replicate were taken from intertidal benthos at 5 and 15 m seaward from the shoreline. Samples from *Curve* (pristine site) consisted mainly of sandy sediments and those from *Cannery* (polluted site) were represented by a microbial mat. Seawater samples were also taken for physicochemical analyses.

Physicochemical Analyses

The pH and temperature were measured *in situ*. Salinity, dissolved oxygen, ammonia, nitrites, nitrates, orthophosphates and total phosphorus were determined in the laboratory [29]. Significant differences were determined by ANOVA at probability (P) value <0.05 confidence, using SPSS 12.0 software. P value <0.05 means significant differences of the parameters between sites.

Culture Media and Isolation of Bacteria

Six culture media were used for retrieving bacteria by performing serial dilutions from 1 g of sediment in half concentration of 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 [4]. Inocula from the serial dilution were used for plating on solid medium. Culture media were: (1) marine agar 2216 (Difco), (2) *Pseudomonas* agar (Difco), (3) diazotrophic medium (AZ) composed of (in g L^{-1} distilled water): glucose 5, mannitol 5, $CaCl_2{\cdot}2H_2O$ 0.1, $MgSO_4{\cdot}7H_2O$ 0.1, $Na_2MoO_4{\cdot}2H_2O$ 0.005, K_2HPO_4 0.9, KH_2PO_4 0.1, $FeSO_4{\cdot}7H_2O$ 0.01, $CaCO_3$ 5, agar 15, (4) luminescent marine bacteria medium (LM) composed of (in g L^{-1} 1/2X SSW), NH_4Cl 1, K_2HPO_4 0.076, $FeSO_4{\cdot}7H_2O$ 0.028, yeast extract 5, tryptone 5, $CaCO_3$ 1, agar 15, glycerol 0.3% [3], (5) peptone–yeast medium (PY) composed of (in g L^{-1} distilled water), peptone 5, yeast extract 3, glucose 20, agar 15, pH adjusted to 7.5 with NaOH [26], (6) peptone–yeast marine medium (PYM) composed of (in g L^{-1} 1/2X SSW), peptone 5, yeast extract 3, glucose 20, agar 15. Plates were incubated at 30°C for 96 h and colony counts were made at 48 and 96 h. Colonies with different morphologies were transferred to PYM medium, which allows PHB accumulation.

PHB-producing Bacteria Detection

Three microscopic techniques were used to detect PHB-producing bacteria. Phase contrast microscopy detected brightly refractile cytoplasmic inclusions (RCI) in PHB-producing bacteria in axenic cultures. The lipophilic stains, Sudan Black and Nile Red, were used to identify PHB under bright field and epifluorescence microscopes, respectively. Nile Red was prepared as 0.25 mg Nile Red dissolved in 1 mL dimethyl sulfoxide (DMSO) and added to sterile culture medium to a final concentration of 0.5 µg Nile Red mL^{-1} medium, working as an *in vivo* stain [28]. Epifluorescence was observed exciting Nile Red at 540 nm. Sudan Black was performed on heat-fixed samples and prepared as 0.3 g Sudan Black B dissolved in 75 mL 95% ethanol, bringing it to 100 mL with distilled water. Samples were stained with Sudan Black solution for 10 min, dried with filter paper, and clarified with xylene drops, dried again with filter paper, and counterstained with 0.5% aqueous safranine for 5 s.

Chemical Identification of PHB

Chemical extraction of PHB was conducted as described earlier [18]. Each bacteria strain was grown in 1 L flask with 300 mL of PYM medium for 24 and 48 h with orbital agitation at 100 rpm. The cell pellet was resuspended in a volume of sodium hypochloride equal to the original

volume of medium. After 1 h at 37°C, the lipid granules (PHB) were centrifuged, washed with water, and then washed with acetone and ethanol. The polymer of PHB was dissolved by extraction with three small portions of boiling chloroform; the chloroform solution was filtered, and the filtrate was used. The excess of chloroform was evaporated, and 10 mL of concentrated H_2SO_4 was added and heated for 10 min at 100°C in a water bath to convert PHB to crotonic acid. PHB was determined as crotonic acid and quantified by spectrophotometry at 235 nm. A standard curve with commercial PHB (Sigma) was made to determine the PHB quantities in our samples. Simultaneously, 15 mL of culture were centrifuged and lyophilized to determine the dry weight of cells.

Phenotype Diagnostic Tests

Colony and cellular morphologies were recorded. Other tests were Gram stain, motility on soft agar (0.1% yeast extract, 0.01% K_2HPO_4, and 0.2% agar), growth in 12% NaCl nutrient agar, anaerobic growth using Brewer thioglycolate and anaerobic Brewer agar (Difco), growth on diazotrophic medium AZ, oxidase, and catalase production.

DNA Extraction

DNA from environmental samples was extracted with FastDNA Spin Kit (Bio 101 System) for soil samples. Purification was required using Geneclean Kit (Bio 101 System). DNeasy Tissue Kit (Qiagen) was used to obtain DNA from axenic cultures.

PCR Conditions

PCR amplifications were performed with a gene cycler thermal cycler (Bio-Rad). The oligonucleotide primers 341F and 907R were applied to selectively amplify bacterial 16S rRNA gene segments from bulk DNA and DNA from bacteria cultures. These primers yields amplification products up to 465 bp in length. Partial amplification of 16S rRNA gene was done with the following reaction mixture: Each 25 μL reaction contained 14.46 μL sterile water, 5 μL 5X green reaction buffer (Promega), 1.5 μL of $MgCl_2$ solution, 1.32 μL of each deoxynucleotide triphosphate (dNTP) (G,A,T,C) at 2.5 mM, 0.26 μL of the each primer (341F+GC 145.76 nM, 907R 429.82 nM), 0.2 μL of GoTaq® Flexi DNA Polymerase (Promega), and 2 μL of templated DNA. The PCR amplification protocol starts with an initial denaturation step (5 min at 94°C). Thirty five incubation cycles followed, each consisted of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C, and a final extension of 5 min at 72°C. A 40-nucleotide, GC-rich sequence, referred to as a GC clamp, was attached to the 5′ end of primer 341F to improve the detection of sequence variations in amplified DNA fragments by subsequent denaturing gradient gel electrophoresis (DGGE) (see below). The following primers were used: 341F+GC: 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG -3′ and 907R: 5′-CCG TCA ATT CCT TTG AGT TT-3′ [21]. Products of the sequencing reactions were done commercially (Macrogen, Korea).

DGGE Analyses

PCR-amplified 16S rRNA gene alleles were separated by DGGE. About 800 to 1000 ng of PCR products from environmental samples and 100 ng of PCR products from cultures were applied to individual lanes in 1-mm thick 6% polyacrylamide gel containing a 30–70% chemical gradient of denaturants (100% denaturant agent was defined as 7 M urea and 40% deionized formamide; acrylamide/bisacrylamide=37.5:1). The gel was electrophoresed for 18 h at 50 V in Dcode universal mutation detection system (Bio-Rad), submerged in a tank containing 1X Tris/Acetic Acid/EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.3) that was maintained at a constant temperature of 60°C [21]. Subsequently, detection was done using the silver staining method [3]. DNA bands in the gel were documented with a scanner UMAX Powerlook 2100 XL.

Phylogenetic Reconstruction

Bacterial 16S rRNA gene sequences available from GenBank, obtained by using BLAST [1], and those determined in this study were aligned to the sequences in the database

Table 1 Mean values of physicochemical parameters from the *Curve* and *Cannery* sites

Site	Salinity (psu)	Temp. °C	D.O. (mL L^{-1})	pH	NH_4 (μM)	NO_2 (μM)	NO_3 (μM)	PO_4 (μM)	PO_{4Total} (μM)
Curve	32.42	29.6	4.846	8.20	1.127	<0.1	0.837	0.293	0.724
Cannery	32.60	31.0	4.183	8.46	3.763	<0.1	0.761	2.638	3.503
P value	0.93		0.52		0.02		0.86	<0.01	<0.01

P is the probability value from the statistical analyses; a value lower than 0.05 means significant differences of the parameters between sites. *Temp.*: temperature, *D.O.*: dissolved oxygen, *psu*: practical salinity units

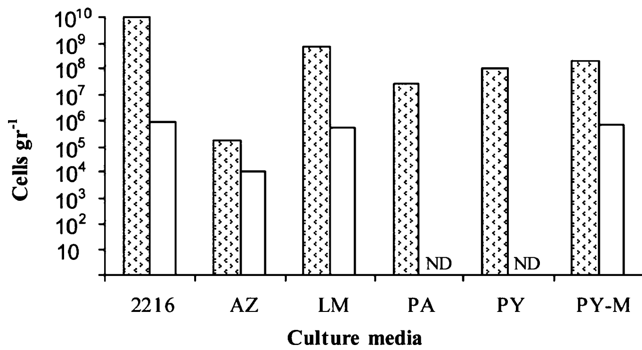

Figure 1 Cells (g^{-1}) of sediment at sites (*Cannery, hatched bars*; *Curve*, *white bars*). Estimates in six media: *2216* marine agar medium, *AZ* diazotrophic medium, *LM* luminescent bacteria medium, *PA Pseudomonas* agar medium, *PY* yeast extract–peptone medium, *PYM* yeast extract–peptone seawater medium, *ND* not determined

of the software package ClustalX v1.8. Maximum parsimony and neighbor joining were performed in the PAUP 4.0 beta 10 software for phylogenetic reconstruction.

Nucleotide Sequence Accession Numbers

The sequences of the determined bacteria in this study were deposited in the GenBank and their accession numbers are listed in Table 2.

Results

Physicochemical Parameters and Recognition of Microbial Mats

Higher concentrations of organic nutrients, phosphates, and ammonia from the water–sediment interface in front of the polluted site (Table 1) correlated with higher counts of viable bacteria cultured in five media (Fig. 1). In addition, differences in microbial composition were found when these communities were compared between them (Table 2). Conspicuous development of a microbial mat occurred in the sediment at the polluted site, but not at the pristine site. Microscopic analysis of the mat showed six cyanobacteria (*Microcoleus chthonoplastes*, *Lyngbya aestuarii*, *Leptolyngbya* sp., *Oscillatoria* sp., *Geitlerinema* sp., and *Gloeocapsa* sp.), one phototrophic, anoxygenic bacteria (*Chloroflexus* sp.), and several heterotrophic bacteria. The criteria used to identify cyanobacteria were cell shape, cell dimensions (diameter, length), number and regularity of planes of binary fission, sheath description, trichome type, motility, and color of cells [7].

Detection and Chemical Identification of PHB

We isolated 126 colonial morphotypes of aerobic heterotrophic bacteria, 72 at the polluted site and 54 at the pristine site. Phase contrast microscopy indicated that up to 25% of the morphotypes had well-defined, brightly refractile cytoplasmatic inclusions (Fig. 2a, b, c), suggesting accumulation of PHB. We selected 14 PHB-producing strains as positive lipophilic strains (Fig. 2d–i). PHB-producing strains were 80% more abundant in the microbial mat occurred in the sediments of the polluted site than in the sandy bottom of the pristine site. Production of PHB was confirmed by chemical extraction, obtaining significant amounts of crystalline, thermoplastic biopolymer. Chemical extraction was successful for nine strains as presented in Fig. 3. Results from each strain at 48 h (cell DW and percent of PHB production) are presented in Fig. 3.

Identification and Phylogeny of Bacteria

Based on the partial sequence of 16S rRNA gene and phenotypic traits, the 14 strains were assigned to belong to 6 genera. *Bacillus* and *Staphylococcus* were detected at

Table 2 List of PHB-producing strains recovered from the *Cannery* and *Curve* sites and taxonomic assignments based on 16S rRNA gene partial sequences

		Cannery			*Curve*		
Group	Gram	Genus	Strain	Accession number[a]	Genus	Strain	Accession number[a]
BLS[b]	Positive	*Staphylococcus* sp.	E4	DQ316058	*Bacillus* sp.	C18	DQ316053
		Staphylococcus sp.	E63	DQ316066	*Bacillus* sp.	C19	DQ316054
		Bacillus sp.	E13	DQ316059	*Staphylococcus* sp.	C20R	DQ316055
α Proteobacteria	Negative	*Paracoccus* sp.	E33	DQ316060	*Methylobacterium* sp.	C54	DQ316057
		Paracoccus sp.	E45	DQ316061			
		Paracoccus sp.	E46	DQ316062			
		Paracoccus sp.	E71	DQ316063			
Actinobacteria	Positive	*Micrococcus* sp.	E36	DQ316064	*Rhodococcus* sp.	C20L	DQ316056
		Micrococcus sp.	E51	DQ316065			

[a] Sequence accession number in the GenBank.
[b] *Bacillus–Lactobacillus–Streptococcus*

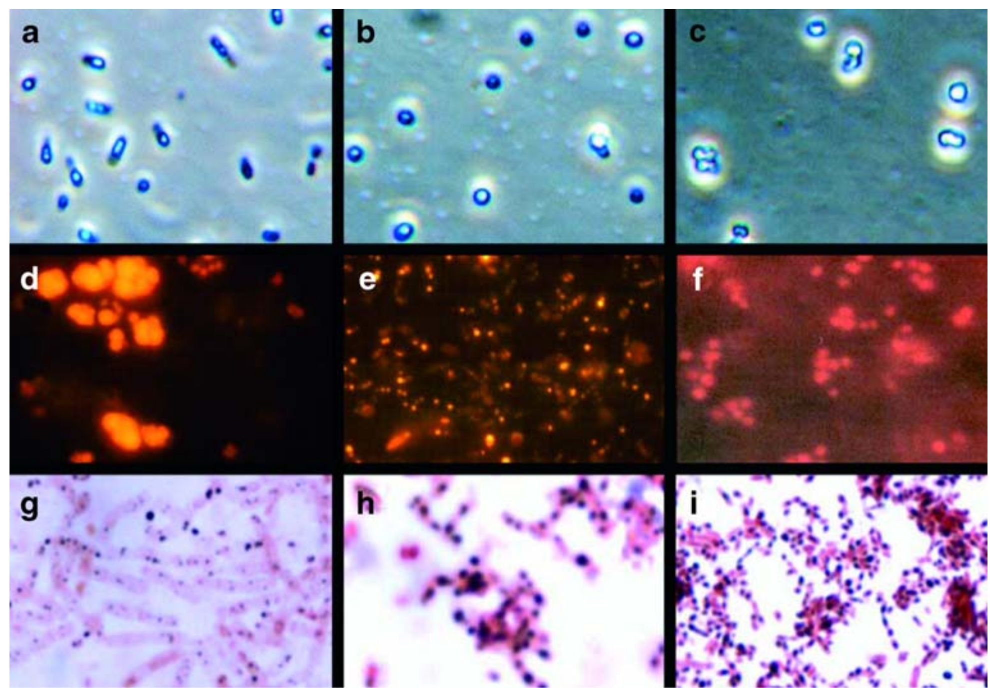

Figure 2 Micrographs of bacteria containing PHB inclusions. Brightly refractile cytoplasmic inclusions (**a**–**c**). Nile red stain (**d**–**f**). Sudan black stain (**g**–**i**). The bacteria strains are: **a** E71, **b** E13, **c** E51, **d** E13, **e** E46, **f** E4, **g** C19, **h** E33, **i** E46

both sites; *Paracoccus* and *Micrococcus* were found only at the polluted site and *Rhodococcus* and *Methylobacterium* [27] were found only at the pristine site. The GenBank accession numbers for the 16S rDNA sequences are displayed in Table 2 [1]. Sequences of C18 and E13 had 100% similarity with the sequence of *Bacillus thuringiensis* DiSz8 isolated from a polluted soil sample. The sequence of C19 had 99% similarity with the sequence of endolithic *Bacillus megaterium* WN603 isolated from weathered granite. Sequences of E33, E45, and E46 had 100% similarity with *Paracoccus homiensis* DD-R11 isolated from a sandy beach sample. The sequence of E71 had

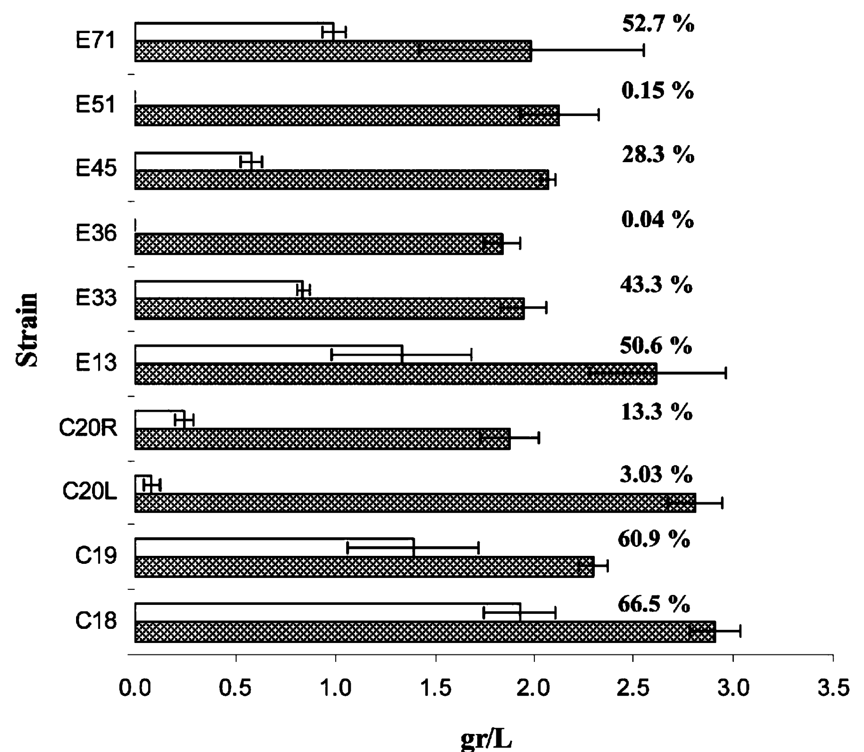

Figure 3 Mean values of dry cell biomass (*hatched bars*) and PHB production (*white bars*). *Numbers* indicate the accumulation of PHB dry cell weight in percent. *Error bars* are the SD. Cell biomass assessed after 48 h of incubation in PYM medium

100% similarity with *Paracoccus seriniphilus* MBT-A4 isolated from marine bryozoan. Sequences of C20R and E63 had 100% similarity with *Staphylococcus cohnii* GTC 728. The sequences of E4 had 100% similarity with *Staphylococcus arlettae* strain ATCC 43957. Sequences of E36 and E51 had 100% similarity with the hydrocarbon-degrading *Micrococcus luteus* AUH1. Sequences of C54 had 100% similarity with *Methylobacterium* spp. Sequences of C20L had 99% similarity with *Rhodococcus* spp (Fig. 4).

Structure of the Bacteria Communities

DGGE analyses of the 465 bp fragment of the 16S rDNA of the communities of bacteria from the polluted site (lanes Ca_1, Ca_2) revealed 20 bands distributed mainly along the

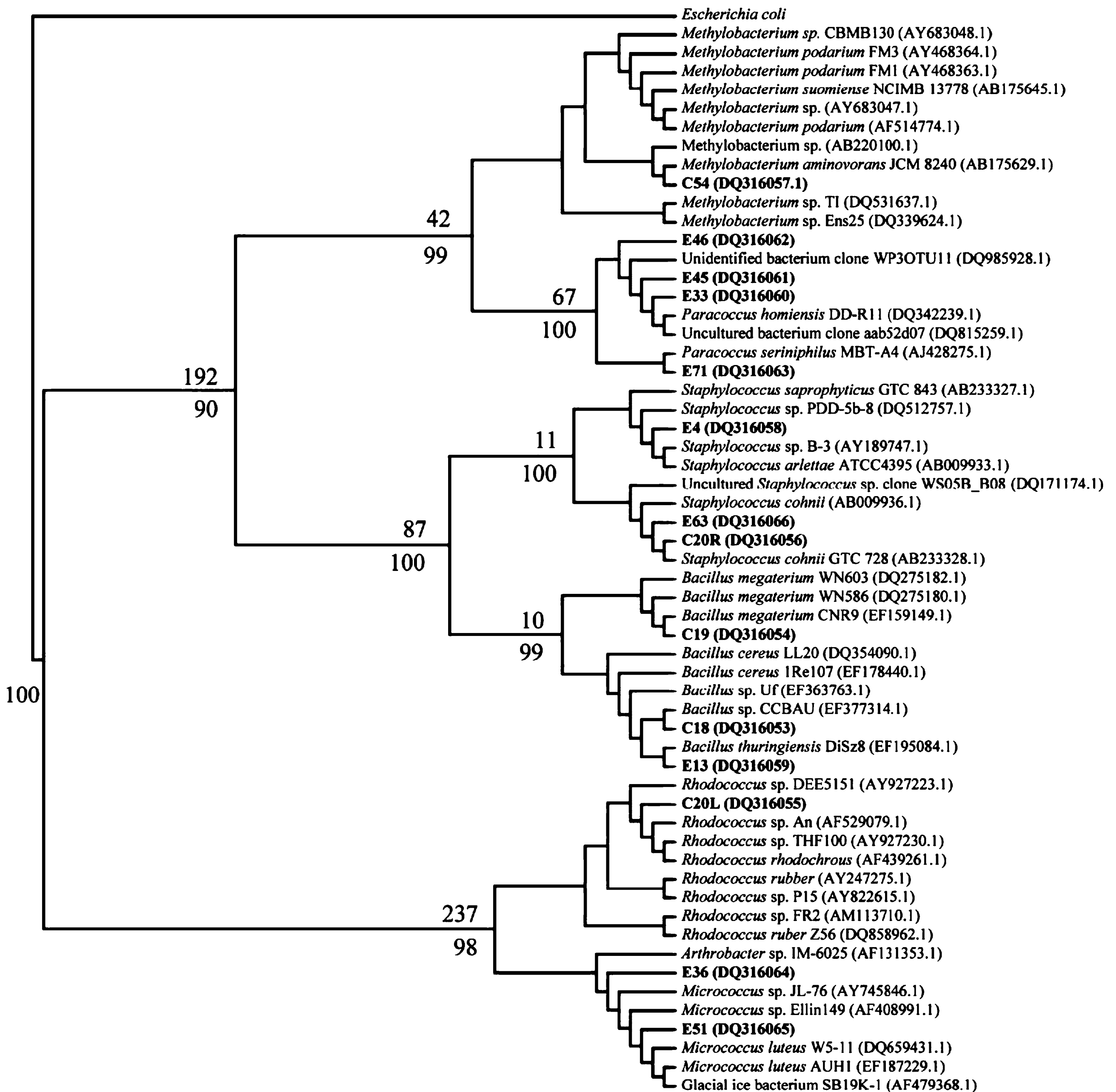

Figure 4 Phylogenetic tree of PHB-producing strains and their nearest neighbors derived from maximum parsimony analysis. Accession numbers are given in parenthesis after the taxonomic assignment. The phylogram was calculated from the divergence in partial sequences of 16S rDNA (480 to 517 base pair length). Numbers given below the branches are frequencies (expressed as percentages) with which a branch appeared in 1000 bootstrap replicates. Branch lengths are proportional to nucleotide differences as indicated by numbers on branches. Consistency index=0.878, retention index=0.980, homeoplasy index=0.122

gel, 40% to 58% of denaturant region (letters a–t) and those from the pristine site (lanes Cu_1, Cu_2) showed 10 bands concentrated in two regions of the gel, 43% to 44% and 48% to 50% of denaturant region (letters A–J). The band patterns were very similar for duplicate extracts from the same environmental sample (Fig. 5). It was noticed that the band profiles between the two sites were different.

Standard sequences for bacteria from both sites were obtained using amplicons from axenic cultures. *Bacillus* strain E13 (band 1), *Staphylococcus* strain E4 (band 2), *Paracoccus* strains E33 and E71 (band 3 and band 4), respectively, and *Micrococcus* strain E36 (band 5) were used as standards for the polluted site (lane STD_{Ca}). *Bacillus* strains C18 and C19 (band I and band II), respectively. *Methylobacterium* strain C54 (band III), and *Rhodococcus* strain C20L (band IV) were used as standards for the pristine site (lane STD_{Cu}) (Fig. 5). Band profile from environmental samples of the cannery were affiliated closely with the 16S rDNA gene sequences of the standard bands of the genera *Bacillus*, *Staphylococcus*, *Rhodococcus*, *Paracoccus*, and *Micrococcus*; from the curve site, only the genera *Bacillus* and *Staphylococcus* were related.

Discussion

Effluent, rich in organic matter (0.29 mg mL^{-1} lipids, unpublished results) and inorganic nutrients from a seafood cannery supported a marine microbial mat. A level of dissolved oxygen (approximately 4.1 mg L^{-1}) at this site is a consequence of high organic material content near the surface. The high concentrations of lipids, ammonia, and phosphates are from fish residue effluent [13]. Nevertheless, the contribution of nutrients and organic matter by the cannery effluent in the polluted site, the salinity were unchanged in comparison with the pristine site (Table 1); therefore, the difference in the structure of the microbial community could not be attributed to the salinity parameter. Nitrates occur in lower concentrations at this site, probably related to absorption by the resident cyanobacteria, an important microbial mat component, which uses nitrate as its nitrogen source [12]. High concentrations of ammonia and organic matter, including lipids, produce toxic effects on bacteria communities. Synthesis of PHB has been proposed as a detoxifying mechanism of bacteria in water with high concentrations of fatty acids. Because PHA genesis is linked to lipid metabolism, PHA-producers are more competitive in these environments [16]. Thus, PHB-production in the microbial mat probably does not function only as a storage material, but also as a mechanism to cope with stressed and imbalanced nutrient environments, such as the polluted *Cannery* site.

The benthic, intertidal microbial community at the polluted site was more complex and diverse than at the pristine site. Richness of species, as indicated by the number of bands in the DGGE gel and total strains retrieved from each site were, in all cases, higher at the polluted site. Two first bands (a–b)

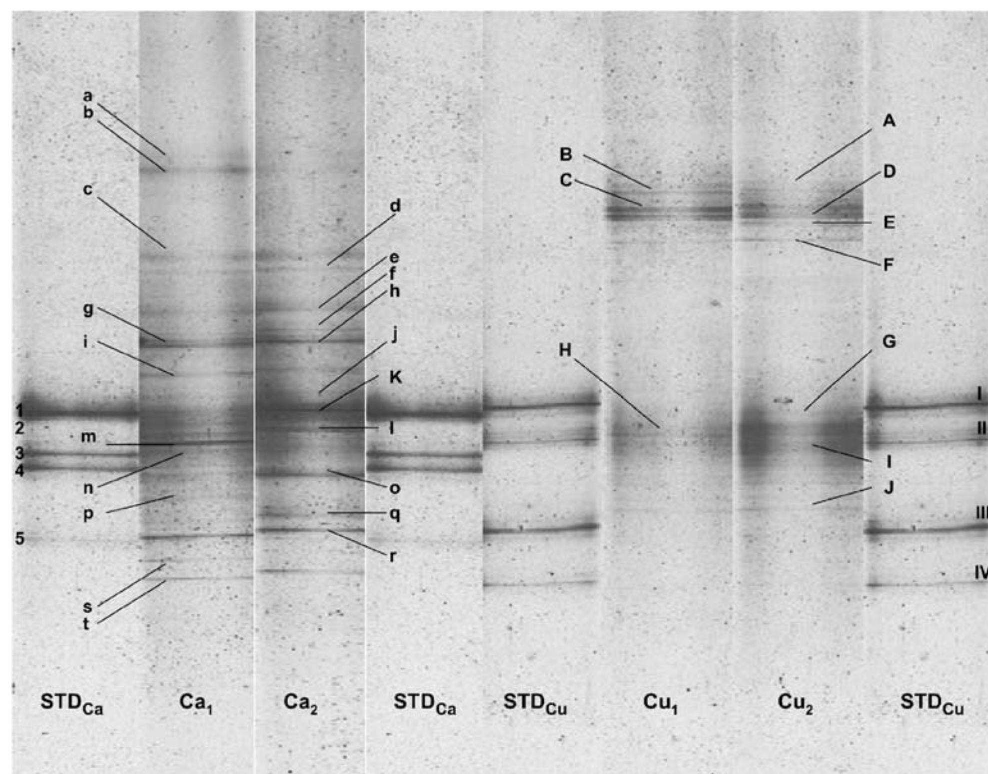

Figure 5 Image of DGGE separation patterns of 16S rRNA genes amplified from natural samples and bacterial cultures retrieved and stained with silver. Denaturing gradient was 30–70% and electrophoresis was run for 18 h at 60°C at 50 V. Lanes are: STD_{Ca} *Cannery* standards (from the top to the bottom *Bacillus* strain E13/band 1, *Staphylococcus* strain E4/band 2, *Paracoccus* strains E33/band 3 and E71/band 4, and *Micrococcus* strain E36/band 5); Ca_1 and Ca_2 amplicons from the *Cannery* site; Cu_1 and Cu_2 amplicons from the *Curve* site; STD_{Cu} *Curve* standards (from the top to the bottom *Bacillus* strains C18/band I and C19/band II, *Methylobacterium* strain C54/band III, and *Rhodococcus* strain C20L/band IV)

appear in the top of the gel of the polluted site, lanes Ca_1 and Ca_2. In lanes Cu_1 and Cu_2, bands a and b are missing, and six bands of DNA with similar sequences (A–F) were distinctive in the top of the gel and could be a fingerprint of pristine marine sediments. Seven bands were found in lanes of the polluted site (c and i); these bands were missing in lanes of the pristine site, above the standard band zone (Fig. 5). For both sites, these bands did not have cultivable counterparts. Five strong bands of the *Cannery* lanes (j–n) and two light bands (G–H) of the *Curve* lanes were affiliated closely with the *Bacillus* and *Staphylococcus* standards (1 and 2). These standard bands were overlapped in the gel of Fig. 5. However, in the construction of standards from cultures in preliminary DGGE gels, these bands were separated. Six bands from *Cannery* lanes (o–t) were recognized, three of them correlated with the *Rhodococcus*, *Paracoccus*, and *Micrococccus* standards. Two bands in the *Curve* lanes were detected (I–J), but none with cultivable counterparts. Differences in DGGE band profile are explained as a response to environmental stress and nutrient imbalance conditions in the polluted site, the cannery. The high concentrations of lipids, ammonia, and phosphates from fish residue cannery effluent promoted the biostimulation of bacterial populations, showed as bands in DGGE gel, which were not detected in the lanes of the pristine site (Fig. 5).

More diverse strains of bacteria with PHA production potential are related to unbalanced environmental nutrient conditions [9]. Microbial mats and grasslands have been considered ecosystems with higher carbon-to-biomass assimilation rates [10]. Microbial mats at the polluted site were a highly productive system, demonstrated by its capability to assimilate carbon and transform it to organic compounds, either as typical biomass or rare storage materials, such as PHB. Among microbial communities, active sludge and microbial mats had the largest concentrations of PHAs under natural conditions [25]. Conditions at the polluted site can promote the growth of PHA-producing bacteria with new chemical properties of the polymer. Three diverse clusters of bacteria may be capable of producing PHB or other copolymers (Table 2; Fig. 4). PHB production have been reported among diverse bacteria [19], including *Bacillus* [2, 14, 24, 34], which were widely studied since the discovery of PHB in a *B. megaterium* strain in 1925. PHB has also been reported in *Methylobacterium* [17, 33], *Staphylococcus* [32], *Micrococcus* [24], and *Rhodococcus* and *Paracoccus* [19, 31]. Strains of *Methylobacterium*, *Paracoccus*, and *Rhodococcus* could be candidates for producing copolymers, such as poly ([*R*]-3-hydroxybutyrate-co-hydroxyvalerate (PHB/HV), under limited nitrogen conditions using substrates such as acetate, glucose, methanol, pentathol, propionic acid, or valeric acid as a substrate, as reported for *Paracoccocus denitrificans*, *Micrococcus halodenitrificans*, and *Rhodoccocus* spp. [11, 20, 24, 31]. High yields of PHB, on a percent basis, were measured with 60–66% for *Bacillus* C19 and C18 and 43–52% for *Paracoccus* E33, *Bacillus* E13, and *Paracoccus* E71.

In conclusion, this study contributes to the comprehension of the diversity of PHA bacterial producers isolated from marine microbial mat subject to environmental stress by organic pollution of cannery of marine fish, which contribute to the imbalance of nutrients.

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