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Indigenous PAH-degrading bacteria from oil-polluted sediments in Caleta Cordova, Patagonia Argentina

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ABSTRACT

Indigenous bacteria with the capability to degrade polycyclic aromatic hydrocarbons (PAH) were isolated from polluted sediment samples recovered from Caleta Cordova by using selective enrichment cultures supplemented with phenanthrene. Bacterial communities were evaluated by denaturing gradient gel electrophoresis (DGGE) in order to detect changes along enrichment culture and relationships with the representative strains subsequently isolated. Members of these communities included marine bacteria such as *Lutibacter*, *Polaribacter*, *Arcobacter* and *Olleya*, whose degradation pathway of PAH has not been studied yet. However, isolated bacteria obtained from this enrichment comprised the genus *Pseudomonas*, *Marinobacter*, *Salinibacterium* and *Brevibacterium*. The ability of isolates to grow and degrade naphthalene, phenanthrene and pyrene was demonstrated by detection of the residual substrate by HPLC. Archetypical naphthalene and catechol dioxygenase genes were found in two isolates belonging to genus *Pseudomonas* (*Pseudomonas monteilii* P26 and *Pseudomonas xanthomarina* N12), suggesting biodegradation potential in these sediments. The successful bacterial isolation with the ability to degrade PAH in pure culture suggest the possibility to study and further consider strategies like growth stimulation *in situ*, in order to increase the intrinsic bioremediation opportunities in the polluted Caleta Cordova harbor.

1. Introduction

Caleta Cordova (Chubut, Argentinean Patagonia) is a small harbor located on the coast of the San Jorge Gulf in the Atlantic Ocean (45° 45′ S, 67° 22′ W) with an active petroleum extraction activity (5.5 million m^3 $year^{-1}$), crude oil marine terminals and loading facilities employing underwater pipelines (Commendatore and Estevez, 2007). Caleta Cordova has an arid and temperate climate with air temperatures around 6.6 °C on the coldest months and 19.6 °C on the warmest months (Servicio Meteorológico Nacional, www.smn.gov.ar). Due to daily oil transportation, the occurrence of coastal or marine pollution is a permanent risk. In the year 2007, Caleta Cordova suffered a wide crude oil spill from a tanker affecting at least 2 km of coast, and was reported later as one of the most hydrocarbon-polluted location in Patagonia Argentina (Ríos et al., 2010). Individual PAH concentrations of intertidal sediment sample CC08-1 of Caleta Cordova (sampling date: 04/08) were measured two months after the spill (Marcos et al., 2012). Total PAH was 1054 μg kg^{-1} of dry weight sediment (Marcos et al., 2012), where phenanthrene, fluoranthene and pyrene were the most abundant with concentrations of 170, 240 and 210 μg kg^{-1} respectively. These values exceed interim marine sediment quality guidelines, although below probable effect level thresholds (Canadian Environmental Quality Guidelines, http://st-ts.ccme.ca/).

Due to their chemical stability and high recalcitrance properties, the evaluation of different strategies for degradation of PAH is matter of global concern. Their prolonged persistence in environments is related to the low water solubility (Luning Prak and Pritchard, 2002), causing sorption effects in soil particles and limiting their availability to be bio-degraded by microorganisms (Cerniglia, 1992). Therefore, sediments could be considered as a pollution reservoir of PAH (Baumard et al., 1998) and decontamination of polluted sites is mandatory.

Knowledge of bacterial community structure as well as their key role in oil-contaminated environment provides a first glance of metabolic potential and physiological mechanisms that might drive hydrocarbon biodegradation (Kostka et al., 2011; Simarro et al., 2012).

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Hydrocarbon-degrading microorganisms are usually present in low abundance in marine environments; however, pollution by petroleum-derivate hydrocarbons may stimulate the growth of such organisms and lead changes on the microbial-communities profile along the contaminated area. A high diversity of bacterial strains with the ability to degrade multiple PAH can be detected by combining selective enrichment and molecular analyses, in order to follow the enrichment or consortium process and to characterize those isolates grown in pure cultures (Hilyard et al., 2008, Molina et al., 2009).

In previous studies, distinct variants of the gene encoding the Alpha subunit of the dioxygenase from PAH-biodegradation pathway were characterized in intertidal sediments of Patagonia using clone libraries targeting PAH dioxygenase genes, previously identified in Gram-negative bacteria (Lozada et al., 2008). Novel aromatic dioxygenase genes variants and those related with cultured representatives *phnA1* (*Cycloclasticus* spp.), *phnAc* (*Alcaligenes faecalis* AFK2 and other Betaproteobacteria) and *nahAc* (*Pseudomonas* spp.) were detected and quantified using qPCR assays (Marcos et al., 2012). Archetypical *nahAc* genes and the novel gene variants were detected in Caleta Cordova after the oil spill, although at concentrations below the quantification limit of assay (Marcos et al., 2012). These results suggest that PAH-degrading bacterial population carrying those genes is present in this marine environment exposed to anthropogenic contamination.

In the present work, we evaluate the bacterial population shifts during a sequential selective enrichment culture by using denaturing gradient gel electrophoresis (DGGE) combined with 16S rDNA sequence analysis for studying microbial community composition. In addition, isolated bacteria with the capability to degrade multiple PAH, were identified and their main catabolic genes characterized, in order to assess the ecological significance of these bacteria in the polluted site.

2. Materials and methods

2.1. Sediment sampling and chemical analysis

Approximately 100 days after oil spill intertidal sediment (0–3 cm) samples were obtained from Caleta Cordova coastal (45° 45′ S, 67° 22′ W) for this study. Sampling procedure was performed according to Marcos et al. (2012). Each composite sediment sample was placed into sterile glass flasks, mixed thoroughly and stored at 4 °C until preparation of enrichment cultures, or at −20 °C for chemical analysis and DNA extraction. PAH concentrations were determined using gas chromatography coupled to mass spectrometry as previously described: phenanthrene (170 μg kg^{-1}), fluoranthene (240 μg kg^{-1}) and pyrene (210 μg kg^{-1}) were the most abundant PAH in the sediment sample reaching a total concentration of 1054 μg kg^{-1} dry wt. sediment (Marcos et al., 2012).

2.2. Culture media

Commonly used plate count agar (PCA, Merck Química Argentina) was used as microbiological growth medium to evaluate viable bacterial growth. Minimal medium (MM) (in g l^{-1}: $(NH_4)_2SO_4$, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4$, 0.01; pH 7–7.2) and JPP medium designed in our laboratory (in g l^{-1}: NaCl, 20; yeast extract, 1; peptone, 2) were used during microbiological determinations. Solid media were obtained by adding 15 g l^{-1} of plant TC agar, micropropagation grade (Phyto Technology Lab., US).

2.3. Heterotrophic bacteria count and culture conditions

Sediment samples were initially treated in order to separate cells from sediment particles. Sediment (1 g) was dispensed into 10-ml bottles with 5 ml of sterile water and 100 μl of Tween 20 (100% v/v). The bottles were homogenized in vortex during 10 min and incubated at room temperature during 30 min in orbital shaker at 180 rpm. After this treatment, sediment was allowed to settle and 100 μl of supernatant was employed for ten-fold serial dilution in sterile water. 10^{-1}–10^{-5} dilutions were plated into 15 ml of PCA plates and incubated 48 h at 20 °C. Assay was performed by triplicate and CFU ml^{-1} was determined.

2.4. Enrichment cultures and isolation procedure

In order to enrich selectively for PAH-degrading bacteria, 7 g of sediment were added into 500 ml Erlenmeyer flasks containing minimal medium (200 ml) and crystals of phenanthrene ($\sim$1 g l^{-1}). Two serial enrichments were prepared by using 10% v v^{-1} as inoculum for each subculture at 15 days intervals. All the enrichments were aerobically incubated at 20 °C in orbital shaker at 180 rpm. The initial enrichment culture was designated **A**, while the second and third subcultures were designated **B** and **C** respectively. Crystals of phenanthrene were added to each subculture to replace the lost of hydrocarbon by consumption. Positive growth was determined by an increase in the turbidity in the flasks containing phenanthrene compared to the control flasks. In order to obtain PAH-degrading bacteria, aliquots (100 μl) of the last (**C**) enrichment culture were plated on JPP-agar medium supplemented with crystals of naphthalene, phenanthrene or pyrene on the lid of Petri dishes and incubated at 20 °C during 48 h or longer when necessary. Representative colonies with different morphotypes were selected for further characterization. Naphthalene, phenanthrene and pyrene (all >99% purity) were purchased from Sigma Chem. Co. (St. Louis, USA).

2.5. PAH removal assay

In order to evaluate the capability of the isolates to degrade PAH, removal assays were performed in 25-ml flasks containing 5 ml of JPP liquid medium supplemented with 0.2 mmol l^{-1} of naphthalene, phenanthrene or pyrene (stock solutions 25 mmol l^{-1} in acetone). Acetone was permitted to evaporate at room temperature before inoculation. The flasks were inoculated with 5% v v^{-1} of an overnight culture (OD_{600} = 0.8) prepared in the same medium. Flasks were incubated in darkness at 20 °C in orbital shaker at 180 rpm. A flask with sterile medium was used to determinate the PAH loss by abiotic phenomena. Assays were performed by triplicate in all the cases. For quantification of residual PAH, cultures were sacrificed at initial, middle and final cultivation stages (0, 48 and 120 h, respectively). Residual PAH was extracted by adding 20 ml of acetone to the flasks and filtered using 0.22 μm nylon membranes (Microclar, Argentina). The samples were stored at −20 °C until analysis. PAH analysis was carried out by reverse-phase high performance liquid chromatography (RP-HPLC) using a Waters e2695 HPLC equipment with a PDA detector (Waters 2998, Waters Corporation, MA, USA) operating at 276 nm of fixed wavelength. Filtered solutions were injected into C18 μ Bondapak HPLC column (4.6 × 250 mm, 50Å pore size, 5 μ particle size). A methanol/water (9:1 v v^{-1}) solution at a flow rate of 1 ml min^{-1} (Manohar et al., 2001) was established as mobile phase during 25 min. PAH concentrations were calculated applying the external standard method.

2.6. DNA preparation and phylogenetic identification of isolates

DNA extraction (from enrichments and pure cultures) was performed by the CTAB method (Ellis et al., 1999). The quality and quantity of DNA from the several sources were checked in a 0.8%

agarose gel electrophoresis after staining with ethidium bromide. DNA purity was assessed from the A_{260}/A_{280} and A_{260}/A_{230} extinction ratios (Johnson, 1994). In order to differentiate closely related strains, ISR-PCR (PCR amplification of Intergenic Spacer Regions between the 16S and 23S rDNA) were conducted according to Benito et al. (2004). Universal primers 8f and 1492r (corresponding to position 8–27 and 1492–1509, respectively in the 16S rDNA sequence of *Escherichia coli*) were used to amplify the 16S rDNA by PCR, as previously described (Quillaguamán et al., 2004). Due to 16S rDNA sequencing analysis limitations, the selected isolates were also subjected to partial sequencing of their *gyrB* gene (Fukushima et al., 2002) and *cpn60* gene, encoding the 60 kDa chaperonin, also called *groEL* or *hsp60* (Goh et al., 1997, 2000; Brousseau et al., 2001). The *gyrB* and *cpn60* genes were amplified by PCR according to Kazunori et al. (2003) and Hill et al. (2006) respectively.

2.7. PAH-degrading genes amplification

Alpha-subunit of multimeric naphthalene 1, 2-dioxygenase gene fragment from cultured representatives *phnA1* (*Cyclocasticus* spp.), *phnAc* (*A. faecalis* AFK2 and other Betaproteobacteria) and *nahAc* (*Pseudomonas* spp.) were amplified with primer sets Cyc372F/Cyc854R (Lozada et al., 2008), Ac114F/Ac596R (Wilson et al., 1999) and Ac164/Ac1029 (Ferrero et al., 2002), respectively. Catechol 1, 2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) genes (*Pseudomonas* spp.) were determined also on selected isolates, with primer sets C12Of/C12Or and C23Of/C23Or (Sei et al., 1999). Sequencing was performed directly on PCR amplicons using Macrogen sequencing service (Macrogen Inc., Korea).

2.8. DGGE analysis

DGGE oligonucleotide primers 341F-GC (*E. coli* 16S rDNA positions 341–357) and 518R (*E. coli* 16S rDNA positions 518–534) (Muyzer et al., 1993) were used to amplify the V3 to V5 region of eubacterial 16S rDNA from whole enrichment cultures. DGGE was performed using a D-Code system (Bio-Rad Laboratories, Inc., Hercules, CA) (Ferrero et al., 2010). About 20 μl (approximately 800 ng) of PCR-product was loaded for most of the samples and the gels were run at a constant voltage of 120 V at 60 °C for 4.5 h. The gel was stained with SYBR® Gold (Molecular Probes, Eugene, OR) and visualized with a Bio-Rad UV transilluminator. Digital images of the gels were captured with the Quantity One software (version 4.3.1; Bio-Rad Laboratories, Inc., Hercules, CA) for use in comparative image analysis. The band patterns of the samples were used to construct a binary matrix. The relationships between the bacterial communities from the enrichments, was represented using a dendrogram (UPGMA cluster analysis). Selected bands for identifying were carefully excised from the gel with a razor blade under UV illumination. Immediately, they were placed in 30 μl TE buffer and incubated overnight at −20 °C to allow elution of the DNA from the gel; 2.0 μl of eluate was used as a template for PCR amplification with the original primer set, 341F and 518R (without a GC clamp). Some PCR products obtained from the excised bands were randomly re-run in DGGE gel to confirm their relative band position. Sequencing was performed directly on PCR products with the 341F primer using Macrogen sequencing service (Macrogen Inc., Korea).

2.9. Phylogenetic analysis

The identity and similarity to the nearest neighbor of DGGE band sequences, were obtained by using the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (NCBI). The isolates were identified according to the databank of 16S rRNA sequences of the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The sequences were analyzed with Chromas software (Technelysium, Tewantin, Australia). Those sequences belonging to the same genus or closely related species, available through the public databases (GenBank and RDP II) were aligned and a similarity matrix was calculated (Maidak et al., 2000) by using MEGA 4 software. Phylogenetic trees were constructed with the aid of MEGA 4 software package by using the neighbor-joining method and the Jukes–Cantor distance correction method (Saitou and Nei, 1987). Only unambiguously aligned positions from all sequences were used, and gaps were not included in the match/mismatch count. The nucleotide sequences identified in this study were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under the accession numbers HE798515–HE798540.

3. Results

3.1. Enrichment, isolation and substrate utilization

Heterotrophic bacteria count in Caleta Cordova sediment samples yield $3.3 \cdot 10^3$ CFU ml^{-1}. PAH-degrading strains were obtained from enrichment **C** and 22 morphologically distinct colonies were selected and characterized by both physiological and molecular methods. The isolates were able to grow on JPP medium agar plates exposed to vapors of naphthalene, phenanthrene and pyrene. ISR pattern (data not shown) allowed us to group the isolates within 10 OTUs (operational taxonomic units). Only one microorganism from each representative group was chosen for further studies. Isolates were arbitrary named as: N1, P3, P4, P27, P26, P13, P14, P18, P43 and N12. Some morphological and physiological features of the isolates are shown in Table 1.

3.2. DGGE analysis of bacterial communities

The DGGE analysis of 16S rRNA gene fragments from the enrichment and sediment samples from Caleta Cordova showed the development of different bacterial communities at different culture conditions. Between 3 and 8 sharp bands were found in each enrichment culture per sample (Fig. 1). Cluster analysis of DGGE bands lead to group **A** and **B** enrichments together. A second clustering level was observed for enrichment **C**, with the emergence of novel dominant bands (data not shown). Identities of selected bacterial members by different enrichment conditions were obtained by excision and sequencing of both representative (e.g., most frequent ones) and rare bands from the DGGE gels (Fig. 1). DGGE band sequences showed similarities with different "un-identified" or "un-cultured bacteria". However, those sequences closely related to cultured microorganisms showed high identity level (98–100%). Regarding DGGE sequence analysis, bacterial community from original Caleta Cordova sediment was mainly composed of Bacteroidetes and Proteobacteria in minor proportion. Only two dominant band sequences (2 and 3) recovered from the original sediment sample were present in first enrichment cultures (A and B). Sequences were closely related (~100%) to *Polaribacter* sp. and "un-cultured marine bacterium" species, both belonging to Bacteroidetes group. Most sequences from enrichment cultures A and B were closely related (~99%) to Proteobacteria and Bacteroidetes groups. Sequences from band 4 belonging to un-cultured Alpha-proteobacterium showed relative low similarities (93%). Alternatively, sequences from bands 5 and 7 were related to cultured members of Bacteroidetes, being *Lutibacter* sp. and *Olleya* sp. their closest related species, respectively, both with 100% of identity. In addition, sequence from band 6 showed

Table 1
Morphological characteristics, growth temperature and phylogenetic affiliation of isolated strains.

Isolate	PAH[a]	Micro/macro morphology	Growth optimum (range) °C	Closest relative (acc. number)[b]	Identity (%)	Phylogenetic group
P26	Naphthalene	Gram (−), short rods, white, circular, mucoid colonies; entire margin	30 (15–40)	*P. monteilii* CIP 104883^T (NR_024910.1)	99.6	Gammaproteobacteria
N1	Naphthalene	Gram (−), long rods; white, circular colonies, irregular margin	20 (15–30)	*P. xanthomarina* KMM 1447^T (AB176954)	99.3	Gammaproteobacteria
P3	Pyrene	Gram (−), rods, orange, circular, mucoid colonies, lobate margin	25 (15–30)	*P. sabulinigri* J64 (NR_044415.1)	99.4	Gammaproteobacteria
P4	Pyrene	Gram (−), rods, white, regular margin, dry colonies, entire margin	15 (15–20)	*P. sabulinigri* J64 (NR_044415.1)	99.6	Gammaproteobacteria
P27	Pyrene	Gram (+), rods, orange, circular, dry colonies, entire margin	20 (15–40)	*Salinibacterium amurskyense* KMM 3673^T (AF539697)	100	Actinobacteria
P13	Phenanthrene	Gram (−), long rods, colorless, dry colonies, entire margin	25 (15–40)	*Marinobacter antarcticus* ZS2-30^T (FJ196022)	98.8	Gammaproteobacteria
P14	Phenanthrene	Gram (+), cluster cocci, light pink, irregular colonies; lobate margin	20 (15–40)	*Bacillus cereus* ATCC 14579^T (AE016877)	99.8	Firmicutes
P18	Phenanthrene	Gram (+), rods, colorless, dry colonies, entire margin	25 (15–40)	*Salinibacterium amurskyense* KMM 3673^T (AF539697)	99.7	Actinobacteria
P43	Phenanthrene	Gram (+), single cocci, light pink colonies; lobate margin	20 (15–30)	*Brevibacterium epidermidis* NCDO 2286^T (X76565)	99.1	Actinobacteria
N12	Naphthalene	Gram (−), rods, light cream, circular colonies, lobate margin	25 (15–40)	*P. xanthomarina* KMM1447^T (NR_041044.1)	99.3	Gammaproteobacteria

[a] PAH used in isolation procedure.
[b] The nearest GenBank neighbors for nearly complete 16S rRNA sequences obtained from isolates and accession numbers. The sequences were aligned with related sequences retrieved from EzTaxon database.

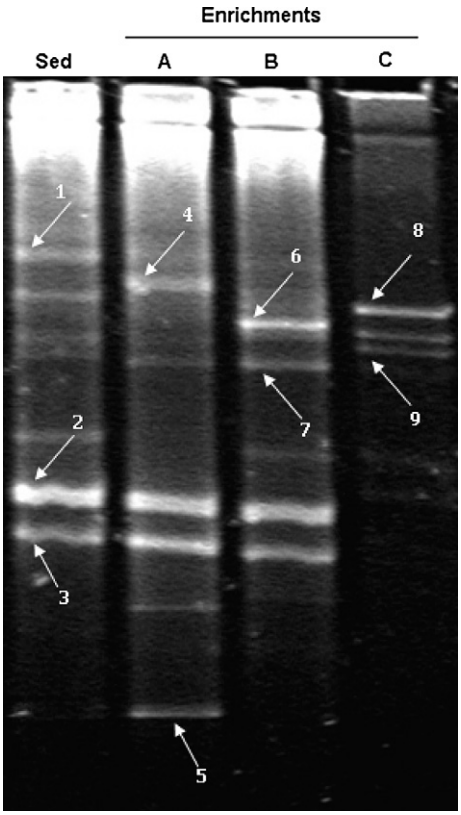

Fig. 1. Image of a denaturing gradient gel electrophoresis (DGGE) of bacterial community developed in each subculture supplemented with phenanthrene (A, B and C) and those from Caleta Córdova sediment sample (Sed.). When bands across several lanes could be identified as being the same, they all have the same number.

the highest identity value (100%) with *Pseudomonas* sp. DG1703 (EU239921), a marine bacterium isolated from oil-amended microcosms (Green, D.H. and Hart, M.C., direct submission). Also, sequence 9 was closest related (98%) to *Arcobacter* sp. sw026 (JN118550), an Epsilon-proteobacterium isolated from sea-surface water of South Pacific, China (Yu, T., direct submission) and both were relatives of *Arcobacter marinus* (Kim et al., 2010).

3.3. PAH degradation by isolated strains

All the isolates were able to grow in culture media solid and liquid, supplemented with naphthalene, phenanthrene and pyrene. However, degradation of pyrene was not detected in liquid cultures after 48 or 120 h of incubation, even when the isolates were able to grow on pyrene-containing medium. Degradation of naphthalene was observed after 48 h of incubation in the isolates P26, N12, P3, P4, P13, P14, P18 and P27 at different rates, near to 100% w v^{-1} (0.2 m mol l^{-1}) of hydrocarbon added (Fig. 2). Furthermore, P26 and N12 strains were also able to degrade 58 and 54% w v^{-1} ($\sim$0.1 m mol l^{-1}) of phenanthrene added into the culture medium, respectively. Degradation of naphthalene and phenanthrene was not observed in cultures of N1 and P43 (Fig. 2).

3.4. Identification of isolates

Sequence analysis of the 16S rRNA gene of the isolates P26, P3, P4, N1 and N12 allowed determine their relationship with to the genus *Pseudomonas* (Table 1, Fig. 3). P26 strain was closely related to *Pseudomonas monteilii* CIP 104883^T (99.6%), which was previously assigned to *Pseudomonas putida* branch by Anzai et al. (2000). N12 strain was closely related to *Pseudomonas xanthomarina* KMM1447^T (99.3%) which clustered with *Pseudomonas stutzeri* genomovar 3 branch (Romanenko et al., 2005). In the same way, P3 and P4 strains were closely related to *Pseudomonas sabulinigri* J64 (99.4 and 99.6%, respectively). The sequence similarity of 16S rDNA

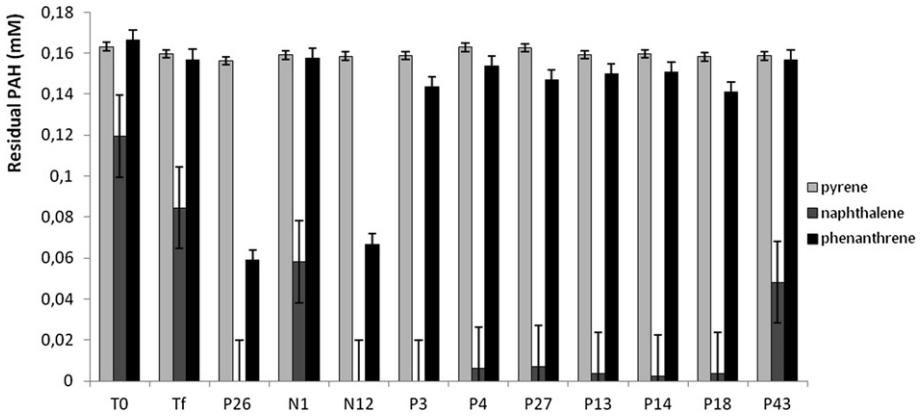

Fig. 2. Residual concentrations of individual polycyclic aromatic hydrocarbons (naphthalene, phenanthrene and pyrene) after 48 h of incubation with different strains: *Pseudomonas* sp. P26, *Pseudomonas* sp. N1, *P. xanthomarina* N12, *P. sabulinigri* P3, *P. sabulinigri* P4, *Salinibacterium* sp. P27, *Marinobacter* sp. P13, *Bacillus* sp. P14, *Salinibacterium* sp. P18, *Brevibacterium* sp. P43. Abiotic loss of each PAH is indicated as the difference of T0 (beginning of the assay) and Tf (final of the assay). Values are the average of triplicate samples.

between strains P3, P4 and *P. sabulinigri*, was more than 99%, however they were clearly different in their phenotypic profiles (Table 1). Similarly, strain P13 was closely related to *Marinobacter* sp., also belonging to Gamma-proteobacteria group (Fig. 3). On the other hand, isolates P18, P27 and P43 showed closest relationship with Actinobacteria phylum, being *Salinibacterium* and *Brevibacterium* the most related genera (99–100%). Finally, isolate P14 was closely related to *Bacillus cereus* (99.8%; Table 1).

P26 and N12 strains were selected based on their promising results of hydrocarbon degradation for additional molecular characterization using sequence analysis of *gyrB* and *cpn*60 genes. A 1200 bp nucleotide sequence of the *gyrB* gene was obtained for P26 and N12 strains which allowed us to confirm the results obtained with 16S rDNA sequence analysis. Whereas P26 was closest related to *P. monteilii* (99.3%), the strain N12 was related to *P. xanthomarina* (99.2%). In addition, sequence analysis of *Cpn60* gene (620 bp) confirmed the assignation of both strains to major *Pseudomonas* groups: P26 belonging to *P. putida* (98.7%) group, while strain N12 belonging to *P. stutzeri* (98.9%) group.

3.5. PAH-degrading genes detection

The partial sequences of *nahAc* gene of *P. monteilii* P26 and *P. xanthomarina* N12 was compared with homologous genes

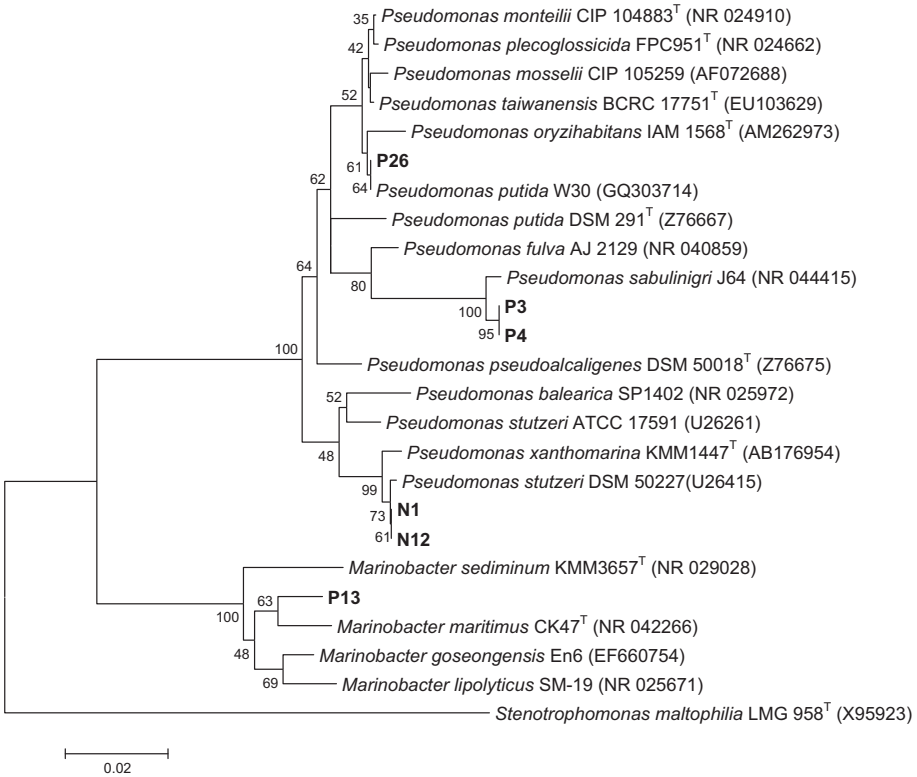

Fig. 3. Phylogenetic relationships of partial 16S rRNA sequences from the sequenced strains affiliated to Gammaproteobacteria. The tree is based on the results of a parsimony analysis that included only complete or nearly complete 16S rRNA sequences of representative bacteria. Group names indicate the most representative organism of the phylogenetic branch. The strains analyzed in this study are noted in boldface type. Bar corresponds to 0.02 substitutions per nucleotide position.

encoding isofunctional proteins from other well-known *Pseudomonas* species. Such fragments revealed high similarity (99.2 and 99.4%) with *nahAc* nucleotide sequence of *P. stutzeri*. Most *nahAc*-type sequences form two distinct and highly supported groups, one of them include sequences from *P. stutzeri* AN10, *Pseudomonas balearica* SP401, and *Pseudomonas* sp. 2N1-1 and the other clade include sequences belonging to *P. putida* G7, OUS82 and ATCC 17484 (Fig. 4). Both groups include sequences from many marine isolates and they have been described and named previously as AN10 and C18 groups, respectively (Ferrero et al., 2002). An internal fragment of *nahAc* genes of *P. monteilii* P26 and *P. xanthomarina* N12 were clustered into *P. stutzeri* AN10 *nahAc* group.

Most of aromatic compounds, including polyaromatics, are know to be metabolized to a common intermediate, catechol, which is further oxidized through the two ring-cleavage pathways, *ortho* and *meta* cleavage pathways, catalyzed by catechol 1, 2-dioxygenase (C12O) and catechol 2, 3-catechol-dioxygenase (C23O), respectively (Kivisaar et al., 1991; Carrington et al., 1994; Sei et al., 1999). In this work, C12O-like genes were not detected in all isolates while C23O-like genes were detected in both *P. monteilii* P26 and *P. xanthomarina* N12. Such genes revealed high similarity (99.0%) with C23O genes from *Pseudomonas* strains, including *P. stutzeri*, *Pseudomonas aeruginosa*, *P. putida* and *Pseudomonas pseudoalcaligenes*.

4. Discussion

The nutrient amounts surrounding marine environments, especially those based on nitrogen and phosphorus, are deficient to support some microbial growth requirements and even more after an oil spill, which is associate to an increase in the hydrocarbon level in water (Harayama et al., 2004). Microbial communities present in PAH-contaminated soils are generally enriched by the microorganisms that are able to use them as carbon and energy source (Koutny et al., 2003; Gallego et al., 2007). However, the microbial communities often include marine microorganisms surviving cell debris or intermediaries metabolism of PAHs. Heterotrophic bacteria counts ($\sim 10^3$ CFU ml^{-1}) in samples from polluted sediment of Caleta Cordova (1054 μg kg^{-1} dry wt. sediment of total PAH) were low (Marcos et al., 2012) compared, for example, to those informed ($\sim 10^7$ CFU ml^{-1}) in Almirante Storni (120 μg kg^{-1} dry wt. sediment of total PAH) which is a harbor in Patagonian coast chronically contaminated with hydrocarbon residues (Lozada et al., 2008). Similar result of heterotrophic bacteria count was found in the sandy soil chronically exposed to petroleum products, collected from a petrochemical complex in Spain (Molina et al., 2009). We relate this effect to a recent input of oil, since PAH were demonstrated to be toxic for several organisms, even in concentrations low to moderate (Margesin et al., 2000; Andreoni et al., 2004). DGGE fingerprinting is useful to analyze the population dynamics in different environments (Kao et al., 2010; HuiJie et al., 2011), because allows directly determine changes on bands profile and correlating with changes in the bacteria community (Fromin et al., 2002). These have been reported be relatively rapid methods of community analysis by comparing fingerprinting profiles (Konopka et al., 1999; Nakatsu et al., 2000; HuiJie et al., 2011). Also, the identification of key organisms playing roles in pollutant biodegradation is important in order to evaluate and develop bioremediation strategies *in situ* whose effectiveness could be evaluated by DGGE (Ríos et al., 2010; Molina et al., 2009; Harayama et al., 2004; Simarro et al., 2012). In this work, bacteria populations recovered from selective enrichment cultures supplemented with PAH and those from polluted sediment were monitored by DGGE and their members were identified by sequencing of bands. Since some sequences were recovered from enrichment cultures, but not from the sediment samples, we suggest that these bacteria were in low proportion in the original sediment and their growth were stimulated along the selective enrichment procedure. This represents an important point in this study because indigenous bacteria with catabolic capabilities to degrade PAH could be stimulated *in situ* to increase the number of cells and thus, the degradation activity into the native microbial community (Schlafer et al., 2002).

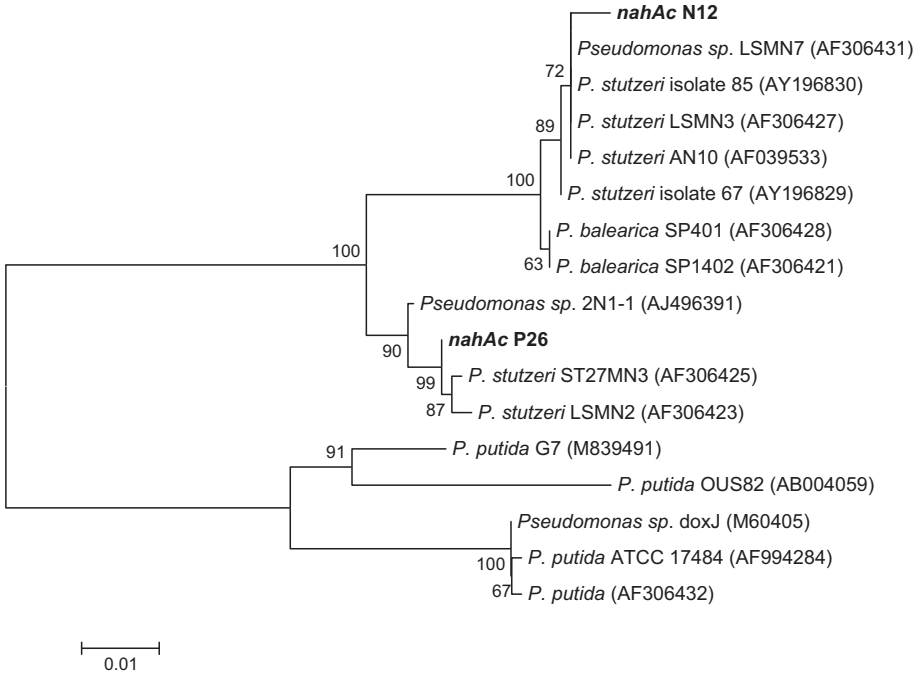

Fig. 4. Dendrogram showing the levels of homology between *nahAc* sequences from selected PAH-degrading strains and from strains retrieved from GenBank database. Bar corresponds to 0.02 substitutions per nucleotide position. The naphthalene-degrading strains analyzed in this study are noted in boldface type.

DGGE band sequences showed a dominance of representatives of Bacteroidetes, followed by Gamma-, Alpha- and Epsilonproteobacteria groups. Microbial profiles were mostly composed by marine bacteria or bacteria able to develop in high salinity environments.

In the present study, only twenty two morphologically distinct PAH-degrading bacteria were obtained from sediment of Caleta Cordova contaminated with petroleum using a selective enrichment approach. These isolates were identified as belong to the genera *Pseudomonas*, *Bacillus*, *Marinobacter*, *Salinibacterium* and *Brevibacterium*. These bacteria can grow on PAH as the sole carbon source under aerobic conditions, indicating that those bacteria have potential to degrade PAH under those conditions. However, these bacteria were not detected between the dominant bands of DGGE. This endorses the idea that PAH produces different effects on the microbiological properties of marine sediments, inhibiting their biodegradation *in situ*. Similar results were obtained when analyzing the intrinsic bioremediation of MTBE in a soil contaminated with petroleum in Taiwan (Kao et al., 2010). In this study, Gamma-proteobacteria (especially *Pseudomonas*) showed to be an important fraction of isolated bacteria. This should not surprise, since *Pseudomonas* is usually recovered from polluted marine environments and many representatives of this genus can degrade petroleum hydrocarbons quickly and efficiently under both aerobic and anaerobic conditions (Anzai et al., 2000; Tan and Ji, 2010; Kao et al., 2010). Also, the selection for Gammaproteobacteria on standard agar plates is a well-known phenomenon that has been observed during analysis by FISH (Wintzingerode et al., 1997). Sequence analysis of the 16S rRNA gene allowed grouped the other isolates into Actinobacteria and Firmicutes groups (HuiJie et al., 2011).

Despite 16S rDNA sequencing is considered as a standard tool not only for elucidating phylogenetic relatedness but also as a mean for bacterial identification (Weisburg et al., 1991), its variation is not sufficient to differentiate between some bacterial genera and may result from slight differences between multiple gene copies within a strain (Fukushima et al., 2002). The sequence of *gyrB* gene, which encodes the subunit B of DNA gyrase, has been previously used for describing phylogenetic relationships within closely related species by Dauga (2002). In comparison with 16S rDNA, the *gyrB* gene has a greater evolutionary divergence and has been proposed as a suitable phylogenetic marker for and bacterial identification classification (Yamamoto and Harayama, 1996). Due to the 16S rDNA sequencing limitations, we have used *gyrB* gene sequencing to obtain a complementary taxonomy technique to identify our isolates. However, in this case, sequence analysis of the *gyrB* gene does not provide more information than provided by the 16S rRNA. Isolate P26 could be affiliate to *P. monteilii* using 16S rDNA (99.6%) as well as *gyrB* (99.3%) gene sequence. However, N12 strain was closely related to *P. xanthomarina* (99.3%) and to *P. stutzeri* (99.2%) using the sequences of 16S rRNA and *gyrB* gene for comparison. Also, *P. xanthomarina* and *P. stutzeri* are close related phylogenetically. Furthermore, to confirm the taxonomic position of these strains, DNA–DNA hybridization experiments between P26, N12 and type strains of phylogenetically related Pseudomonas species will be conducted, as well as the use of characteristics phenotypic and chemotaxonomic as complementary studies.

Naphthalene and phenanthrene removal by these *Pseudomonas* strains was successful considering that about 100% of naphthalene and 50% of phenanthrene were removed at 48 h. Usually, these removal values takes more time in a pure culture or when the hydrocarbon is used by the bacteria as a primary source of carbon in direct metabolism condition (Kao et al., 2010).

Microbial degradation of naphthalene was widely studied as a model of degradation of several PAH. Naphthalene dioxygenase (NahA) catalyzes the first step in the degradation of naphthalene, as well as a number of polyaromatic hydrocarbons such as phenanthrene, 2-methyl-naphthalene, toluene, ethylbenzene, biphenyl, anthracene, and benzene (Jeffrey et al., 1984). This enzyme belongs to naphthalene degradation pathways (*nahAaAbAcAdBFCED*) which involved in the conversion of naphthalene to salicylate in several *Pseudomonas* strains (Yen and Gunsalus, 1982). Archetypical *nahAc*-like genes were detected only in P26 and N12 strains. For these strains, after comparing reconstructed phylogenies of 16S rDNA gene and *nahAc* (Figs. 3 and 4), we determined that marine bacteria with different affiliations (*P. monteilii* P26 and *P. xanthomarina* N12) isolated from the same geographical location harbored nearly identical *P. stutzeri nahAc* genes (AN10 type). Archetypical *nahAc* genes were previously detected by quantitative PCR (qPCR) in intertidal sediments from Patagonia, including the same sample CC08-1 from Caleta Cordova after crude oil spill (Marcos et al., 2012). However, although *nahAc* was detected in all analyzed samples, concentrations of this gene were below the quantification limit of the assay, despite the presence of various PAH in the sediments (Marcos et al., 2012). Since polluted sediment of Caleta Cordova contained a mixture of many different PAH and this variety of compounds could support the growth of bacteria with a multiple degradation pathways, a diversity of catabolic genes could differ from the classical *nah* type. In this way, further studies are conducted to find catabolic genes in other isolates, especially belonging to Firmicutes and Actinobacteria.

5. Conclusions

Our results show that indigenous bacteria with catabolic capabilities to degrade naphthalene and phenanthrene could be enriched from marine sediments contaminated with PAH. Gammaproteobacteria (especially *Pseudomonas*) showed be an important fraction of isolated bacteria and harbored the archetypical *nahAc* and C23O genes, suggesting potential of biodegradation in these sediments. Growth of these bacteria should be stimulated *in situ* to increase the number of cells and thus, the degradation activity into the native microbial community, in order to increase the intrinsic bioremediation opportunities in the polluted Caleta Cordova harbor.

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