

A most effective method for selecting a broad range of short and medium-chain-length polyhydroxyalcanoate producing microorganisms

Dolly Margot Revelo Romo

Bioprocesses and Bioprospecting Group
Instituto de Biotecnología de la Universidad Nacional
Ciudad Universitaria, Edificio Manuel Ancizar
Bogotá, Colombia
Tel: 57 1 316 5000. Ext 16970/71
Fax: 57 1 3165415
E-mail: dmrevelor@unal.edu.co

María Victoria Grosso

Bioprocesses and Bioprospecting Group
Instituto de Biotecnología de la Universidad Nacional
Ciudad Universitaria, Edificio Manuel Ancizar
Bogotá, Colombia
Tel: 57 1 316 5000. Ext 16970/71
Fax: 57 1 316 5415
E-mail: mvgrossob@unal.edu.co

Nubia Carmenza Moreno Solano

Bioprocesses and Bioprospecting Group
Instituto de Biotecnología de la Universidad Nacional
Ciudad Universitaria, Edificio Manuel Ancizar
Bogotá, Colombia
Tel: 57 1 316 5000. Ext 16972
Fax: 57 1 3165415
E-mail: ncmorenos@unal.edu.co

Dolly Montoya Castaño*

Bioprocesses and Bioprospecting Group
Instituto de Biotecnología de la Universidad Nacional
Ciudad Universitaria, Edificio Manuel Ancizar
Bogotá, Colombia
Tel: 57 1 316 5000. Ext 16951/52/54
Fax: 57 1 3165415
E-mail: dmontoyac@unal.edu.co

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Abbreviations: 16S rDNA: 16S ribosomal gene
ATCC: American Type Culture Collection
bp: base pairs
DMSO: dimethyl sulphoxide
IBUN: Instituto de Biotecnología de la Universidad Nacional
mcl-PHA: medium-chain-length PHAs
OD: optical density
ORF: open reading frame
PCR: polymerase chain reaction
PHA: polyhydroxyalcanoate
phaC: PHA synthase gene
PhaC: PHA synthase
scl-PHA: short-chain-length PHAs

*Corresponding author

A molecular approach was used for selecting polyhydroxyalcanoate (PHA)-accumulating potential Gram-negative bacteria from different genera by colony polymerase chain reaction (PCR). Three degenerate primers were designed for amplifying a fragment from PHA synthase gene (*phaC*) (Class I), *phaC1* and *phaC2* (Class II) genes for detecting PHA-producing bacteria. Thirty-four out of 55 bacterial strains from the old collection selected using Sudan black B staining were *phaC+*. PCR was used for directly selecting 35 new collection bacterial strains; these strains were *phaC+* and their ability to produce PHA was confirmed by Sudan black B staining. Four specific primers were designed on genes of Class II PHA biosynthesis operon. These primers were used for evaluating 9 strains from the old *phaC+* collection; 6 showed Class II PHA synthase organisation. 34 from the old and new bacterial isolation were characterised by 16S ribosomal gene (16S rDNA) gene partial sequencing. The tool proposed here can be used for better directing PHA production based on PHA biosynthesis genes and bacterial genera. Class I or II *phaC* genes were detected in 9 different genera and were able to infer the type of polymer produced.

Traditional stains such as Sudan black B (Schlegel et al. 1970), Nile blue A (Ostle and Holt, 1982) and Nile red (Spiekermann et al. 1999) have been used for selecting polyhydroxyalcanoate (PHA)-accumulating bacteria. Even though the polymer can be detected in some bacteria in normal growth conditions, PHA production frequently depends on unbalanced nutritional requirements (Madison and Huisman, 1999). These traditional methods are unspecific and laborious; polymerase chain reaction-based (PCR) molecular methods (Sheu et al. 2000; Solaiman et al. 2000; Shamala et al. 2003) have thus become a highly sensitive and precise tool for detecting and amplifying the gene encoding PHA synthase.

PHA biosynthesis genes have been cloned and expressed and their genetic organization has been extensively reported (Madison and Huisman, 1999; Rhem and Steinbüchel, 1999; McCool and Cannon, 2001). PHA synthases (PhaC)

are key enzymes in PHA producing polymer granules (Rehm, 2003). These enzymes are classified into four classes according to genetic sequence-deduced primary structure, substrate specificity and subunit composition (Rehm, 2003). PHA structure (short-chain-length (scl) and medium-chain-length (mcl) PHAs) depends on the type of PHA synthase, carbon source and metabolic routes involved (Madison and Huisman, 1999; Rehm and Steinbüchel, 1999).

Polyhydroxyalcanoic acids (PHAs) have been proposed as substitutes for petroleum-derived polymers due to their biodegradable and biocompatible properties. Biosynthesis of scl- or medium-chain-length PHAs (mcl-PHAs) has been widely studied. The present study shows new primers from Class I and II PHA synthase genes which were designed and validated using environmental samples. This work was aimed at selecting native bacteria potentially producing PHA by colony polymerase chain reaction (PCR) amplification of genes encoding Class I and II PHA synthase. An old collection of 55 Gram-negative bacteria, which had been previously selected by phenotype staining methods, plus 34 new strains were evaluated by the PCR method proposed here. The results also led to detecting and differentiating PHA synthase genes, some promising strains being identified by their genetic potential as a source of genes involved in PHA biosynthesis pathways.

MATERIALS AND METHODS

Bacterial strains and growth conditions

PHA-positive bacterial strains were used: *Ralstonia eutropha* American Type Culture Collection (ATCC) 17699, *Azospirillum brasilense* ATCC 29710, *A. amazonense* Instituto de Biotecnología, Universidad Nacional de Colombia (IBUN) 87, *Azotobacter chroococcum* IBUN 88, *Aeromonas hydrophyla* IBUN 89 and *Acinetobacter baumannii* IBUN 90 from Class I and *Pseudomonas fluorescens* IBUN 91, *P. putida* IBUN 92, *P. aeruginosa* ATCC 27853 and *P. aeruginosa* IBUN 1040 from Class II. The *E. coli* XL-1 blue PHA negative strain

Table 1. Characteristics of the environmental samples which were the source of new bacterial isolates.

Sample No	Type of sample	Department	Rural area	Geographical coordinates ^a	Height A ^b (m.a s.l.)	pH ^c	% H ^d
1	Soil used for adult sugarcane crop. Organic agriculture.	Cundinamarca (Villeta)	Payandé (La Luisa)	5° 00' LN	850	6.73	34.62
2				74° 30' LW		7.50	32.07
3			Alto de Torres (El Paraíso)	4° 98' LN 74° 29' LW	1200	6.44	31.70
4	Marine sludge (1 m, 20 cm deep)	Bolivar (Cartagena)	Playa El Laguito	10° 23' LN 75° 34' LW	0	7.68	ND

a and b: Data obtained from the Instituto Geográfico Agustín Codazzi (IGAC).

c and d: pH value and gravimetric water content (%H) were determined according to protocols described by Alef and Nannipieri (1995).

ND: Not determined.

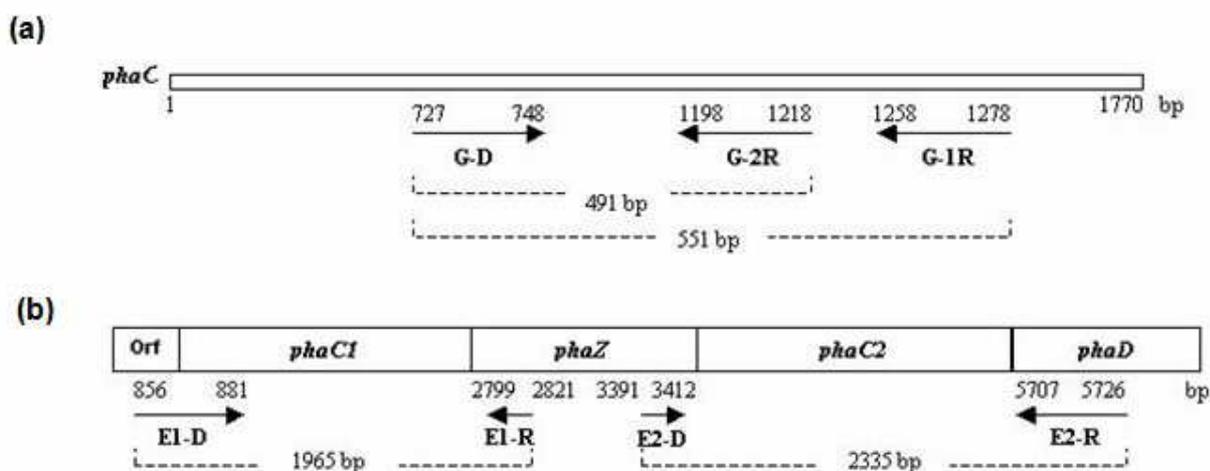


Figure 1. Location of general and specific primers on *phaC* genes.

(a) **General primers on *R. Eutropha phaC* gene.** The numbers indicate the positions of the primers on the *R. Eutropha phaC* gene (GenBank Accession No. J05003) (3'-5'). Dotted lines indicate expected fragment size in colony PCR (G-D and G-1R primers) and semi-nested PCR (G-D and G-2R primers).

(b) **Specific primers on *P. aeruginosa* Class II PHA synthase biosynthesis genes.** 3'-5': ORF, unnamed protein; *phaC1* encoding PHA synthase 1; *phaC2* encoding PHA synthase 2; *phaZ*, encoding PHA depolymerase; *phaD* encoding PhaD (protein having a function in mcl-PHA biosynthesis). The numbers indicate the positions of the primers in the *P. aeruginosa* class II synthase PHA operon sequence (GenBank Accession No. AE004919). Dotted lines indicate the expected fragment size for each pair of primers.

was used as negative control. All control strains were provided by the IBUN. An old collection of 55 bacterial strains was evaluated; this collection was previously selected on phenotype characteristics regarding presumptive polymer accumulation. 49 bacterial strains from different environmental samples (Table 1) were also isolated according to Gomez et al. (1996) and selected using the molecular tool described here. Nutritive agar medium (DIFCO) was used for maintaining the bacteria and incubated for 24 hrs at 30°C and 37°C in the case of *E. coli* XL-1 blue.

Colony PCR

Designing general primers. General primers were designed by multiple sequence alignment, amplifying a fragment of the gene encoding Class I and II PHA synthase. *phaC* (Class I) and *phaC1* and *phaC2* (Class II) gene sequences deposited in GenBank/EMBL/DDBJ databases were analysed using ClustalW software (Thompson et al. 1994). The 28 sequences used belonged to the following species of Gram-negative bacteria, their GenBank accession number being shown in brackets: *Acinetobacter* sp. (L37761), *Aeromonas caviae* (D88825), *A. hydrophyla* (AY093685), *R. eutropha* (J05003), *Alcaligenes latus* (AF078795), *A. latus* (U47026), *Alcaligenes* sp. (U78047), *Azotobacter* sp. (J3119748), *A. vinelandii* (AF267243), *Bradirhodobium japonicum* (AY077580), *Chromobacterium violaceum* (AF061446), *Methylobacterium extorquens* (L07893), *Paracoccus*

denitrificans (D43764), *Pseudomonas aeruginosa* (AE004919) (*phaC1* and *phaC2*), *P. oleovorans* (M58445) (*phaC1* and *phaC2*), *P. putida* (AB085816), *P. putida* (AY113181) (*phaC1* and *phaC2*), *Pseudomonas* sp. (AB014757), *Pseudomonas* sp. (AB014758) (*phaC1* and *phaC2*), *P. stutzeri* (AY278219) (*phaC1* and *phaC2*), *Rhizobium etli* (U30612), *R. meliloti* (U17227) and *Zooglea ramigera* (U66242). Primers designed and selected for this work were:

G-D (5'GTGCCGCC(GC)(CT)(AG)(GC)ATCAACAAGT3'), G-1R (5'GTTCCAG(AT)ACAG(GC)A(GT)(AG)TCGAA3') and G-2R (5'GTAGTTCCA(GC)A(CT)CAGGTCGTT3') (Figure 1). They were synthesised by MWG-Biotech Inc., Mendenhall, USA.

Optimising PCR conditions. PCR with general primers was optimised using the following components: 1X buffer, 0.2 mM dNTPs, 0.1 µM of each primer, 2.5 mM MgCl₂, 0.05 U/µl of Taq DNA polymerase (Promega) and water to complete 25 µl reaction. 3% dimethyl sulphoxide (DMSO) (Sigma) and 20 mM ammonium sulphate [(NH₄)₂ SO₄] were used as reaction additives. Amplification reactions were carried out in an thermocycler (MJ Research, Inc.) using the following programme: 1 cycle of 94°C for 10 min, 60°C for 2 min and 72°C for 2 min followed by 40 cycles at 94°C for 20, 55.5°C for 45 sec and 72°C for 1 min and a final cycle at 72°C for 5 min. DNA template was obtained as follows: colonies isolated from bacteria grown on solid medium on plates for 12 hrs were suspended in 100 µl

sterilised water until reaching 0.7 OD₆₀₀. The cell suspension was heated at 95°C for 10 min and 2.5 µl were used in the reaction.

Designing specific primers

Specific primers were designed for amplifying and differentiating Class II *phaC1* and *phaC2* genes by aligning and analysing Class II PHA biosynthesis operon sequences belonging to the following bacterial species: *Pseudomonas* sp. (AC AB014758), *P. aureofaciens* (AB049413), *P. aeruginosa* (AE004919), *P. resinovorans* (AF129396), *P. nitroreducens* (AF336849), *Burkholderia caryophylli* (AF394660), *P. pseudoalcaligenes* (AY043314), *P. putida* (AY113181), *P. stutzeri* (AY278219), *P. putida* (AY286491), *P. oleovorans* (M58445) and *P. aeruginosa* (X66592). Primers designed for amplifying the *phaC1* gene were E1-D (5'GGAGCGTCGTAGATGAGTAACAAGAA3') and E1-R (5'AGGTTGGCGCCGATGCCGTTGAA3'). The primers designed for amplifying the *phaC2* gene were E2-D (5'TGCTGGCCTGGCGCATTCCCAA3') and E2-R (5'AAGTGGTAGTAGAGGTTGCC3') (Figure 1). These were synthesised by MWG-Biotech Inc., Mendenhall, USA.

Optimising PCR conditions. PCR using with specific primers was optimised as follows: 1X buffer (complemented with 20 mM ammonium sulphate), 0.2 mM dNTPs, 0.4 µM of each primer, 3.0 mM MgCl₂, 0.05 U/µl of Taq DNA polymerase (Fermentas) and water to complete up to 25 µl of reaction. 2.5 µl bacterial lysate were used as DNA template. Reactions were carried out in a thermocycler (MJ Research, Inc.) using the TouchdownPCR strategy (Don et al. 1991). The *phaC1* gene was amplified as follows: 1 cycle at 94°C for 5 min, followed by 14 cycles at 94°C for 30 sec, 65°C for 30 sec (reduced by 1°C for each 2 cycles) and 72°C for 1 min, followed by 20 cycles at 94°C for 30 sec, 59°C for 30 sec and 72°C for 1 min and then 72°C for 5 min. The *phaC2* gene was amplified as follows: 1 cycle at 94°C for 5 min, followed by 12 cycles at 94°C for 30 sec, 65°C for 30 sec (reduced by 1.5°C for each 2 cycles) and 72°C for 1 min, followed by 22 cycles at 94°C for 30 sec, 57.5°C for 30 sec and 72°C for 1 min and then at 72°C for 5 min.

Detecting PCR products

Electrophoresis on 1% (w/v) agarose gels (1X TAE) was used for detecting PCR amplification products. λ DNA/*Hind* III (0.2 µg/µl) was used as DNA size marker. Run conditions were 6.5 volts/cm for 2 and half hrs. The gel was stained with a 0.5 µg/ml ethidium bromide solution and amplified DNA fragments were visualised under UV light and recorded with a GelDoc image digitaliser (Bio-Rad).

Validating colony PCR

PCR specificity was verified by analysing the nucleotide sequence of a DNA fragment having the expected size and

amplified with G-D and G-1R primers in native strain S0804 (chosen randomly). PCR sensitivity was determined by cell concentration limit which was also expressed as being the minimum quantity of DNA template required for amplification. *P. aureginosa* IBUN 1040 (Class II PHA biosynthesis organisation) was cultivated in nutritive broth (Mikrobiologie) up to 0.7 OD₆₀₀. The number of cells per ml at this optical density (OD) was determined by the plate-count method, in triplicate, at different times. One ml of culture was centrifuged at 2,432 x g for 6 min; the biomass was washed once with sterilised water, suspended in 100 µl water and lysed by heating at 95°C for 10 min. This bacterial lysate was used as DNA template in increasing volumes (0.2 µl to 5 µl) for PCR reactions.

Discarding false positives and false negatives. A semi-nested PCR was optimised using general primers G-D and G-2R (internal) for discarding false positives and false negatives and confirming partial PCR amplification of the *phaC* gene using G-D and G-R (external) general primers. Semi-nested PCR conditions were as follows: 2 µl DNA template from the first *phaC* gene amplified product were added to this reaction for positive products and 5 µl for negative products. Amplification conditions were the same as those optimised with the general primers.

Determining *phaC* gene presence in native bacterial strains using general primers

Fifty-five bacterial strains from a collection having presumptive PHA-accumulation ability were screened by the PCR method described here. New bacterial isolates were obtained from different environmental samples (Table 1). 10 g of soil or 10 ml sea-sludge were enriched in 90 ml of mineral salts medium (MSM), pH 7.0 (Gomez et al. 1996), containing glucose, fructose and saccharose (5 g/l of each one) as carbon source, 3 g/l ammonium sulphate as nitrogen source and 0.001 g/l crystal violet and 0.03 g/l cycloheximide as gram-positive bacteria and fungi inhibitors. 0.1 ml of serial dilutions were spread on solid MSM medium containing 5 g/l glucose, fructose or saccharose and incubated at 30°C for 24 hrs. Colonies having different macroscopic and microscopic morphology were maintained in nutritive agar (DIFCO). Colony PCR was used for directly selecting bacteria having PHA-accumulation ability. The presence of the PHA synthase gene in the bacteria was designated as being *phaC+* and its absence as *phaC-*; *phaC+* strains were then cultured on nitrogen-deficient MSM medium plates (1 g/l ammonium sulphate) for 5 days and PHA granules were visualised by Sudan black B staining (Schlegel et al. 1970).

Partial characterisation of the 16S ribosomal gene (16S rDNA)

Randomly chosen native bacteria from the old and new collections, which had been previously subjected to *phaC* gene determination, were characterised by partial

sequencing of the 16S rDNA gene. A, E (direct) and J (reverse) primers (primer J was modified by Montoya et al. 2001) were used for amplifying gene. The amplification reaction used the following mixture: 1X buffer, 0.18 mM dNTPs, 0.3 μ M of each primer, 1.5 mM MgCl₂ and 0.05 U/ μ l of Taq DNA polymerase (Promega) and water up to 25 μ l. 2.5 μ l bacterial lysate were added as DNA template. All PCR reactions were done in a PTC-100 thermocycler (MJ Research, Inc.) in the following conditions: 1 cycle at 94°C for 2 min, 25 cycles at 94°C for 15 sec, 62°C for 30 sec and 72°C for 50 sec and then one cycle at 94°C for 15 sec, 62°C for 30 sec and 72°C for 7 min. PCR products were sequenced by the Macrogen Company (Korea). The search for similarity amongst 16S rDNA gene partial sequence from native bacterial strains was done by alignments with sequences from GenBank/EMBL/DDBJ databases using BLAST software (Altschul et al. 1997). Native 16S rDNA gene partial sequences and database reference sequences were aligned using ClustalW program (Thompson et al. 1994); MEGA 3.0 software (Kumar et al. 2004) was used for making a similarity dendrogram.

Sequence accession numbers. The 16S rDNA gene partial sequences have been deposited in GenBank under accession numbers: DQ813307, DQ813308, DQ813309, DQ813310, DQ813311, DQ813312, DQ813313, DQ813314, DQ813315, DQ813316, DQ813317, DQ813318, DQ813319, DQ813320, DQ813321, DQ813322, DQ813323, DQ813324, DQ813325, DQ813326, DQ813327, DQ813328, DQ813329, DQ813330, DQ813331, DQ813332, DQ813333, DQ813334, DQ813335, DQ813336, DQ813337, DQ813338, DQ813339 and DQ813340. The *phaC* gene partial sequence had been deposited in GenBank under accession number DQ826388.

RESULTS

Amplifying the *phaC* gene fragment

Three degenerate primers (general) were designed by analysing 28 Class I and II *phaC*. The so designed G-D and G-1R general primers directly amplified a *phaC* gene 551 base pairs (bp) fragment in 8 of the bacteria used as positive control corresponding to 5 bacterial genera: *Ralstonia*, *Aeromonas*, *Azospirillum*, *Acinetobacter* and *Pseudomonas*. *A. amazonense* IBUN 87 and *A. chroococcum* IBUN 88 could not be directly detected when G-D and G-1R primers were used. However, semi-nested PCR using G-D and G-2R primers confirmed *phaC* gene presence (Figure 2). These results confirmed the efficiency of the reaction for amplifying the two types of *phaC* genes, type I (*R. eutropha*) and type II (*P. aeruginosa*), using a G-D and G-1R primers.

Validating colony PCR

PCR specificity was determined by analysing partial sequence *phaC* gene of native bacterial strain IBUN S0804. G-D and GD-1R primers were used for amplifying *phaC*

gene. Theoretically, this should have given 551 bp, but 514 bp were obtained. The partial *phaC* gene sequence (DQ826388) from this strain showed 95% similarity with the *P. aureofaciens phaC1* gene sequence. PCR sensitivity was defined as being the minimum of viable cells. This limit detection was around 2×10^4 viable cells, corresponding to 345 ng DNA; the best band resolution was obtained with 4 μ g DNA from $\sim 2.3 \times 10^5$ viable cells. Thus, 2.5 μ l bacterial lysate was used in all subsequent reactions.

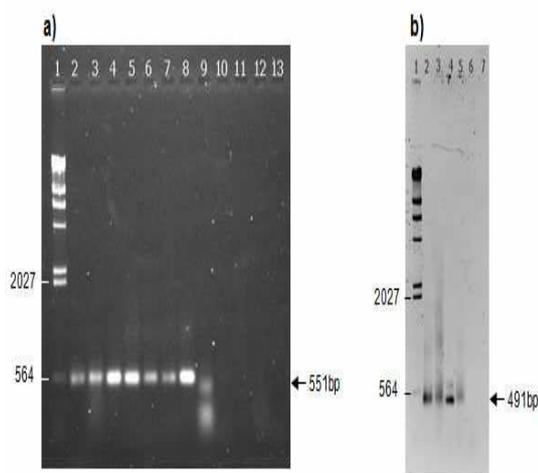


Figure 2. PCR amplification of *phaC* gene for control strains.

(a) Colony PCR amplification of a *phaC* gene fragment using G-D and G-1R primers. Lane 1, molecular weight marker (λ ADN HindIII); 2, *R. eutropha* ATCC 17699; 3, *A. hydrophyla* IBUN 89; 4, *A. brasilense* ATCC 29710; 5, *A. baumannii* IBUN 90; 6, *P. aeruginosa* IBUN 1040; 7, *P. aeruginosa* ATCC 27853; 8, *P. fluorescens* IBUN 91; 9, *P. putida* IBUN 92; 10, *A. chroococcum* IBUN 88; 11, *A. amazonense* IBUN 87; 12, *E. coli* XL-1 blue (negative control); 13, reagent control.

(b) Confirmation by semi-nested PCR using G-D and G-2R primers. Lane 1, molecular weight marker (λ ADN HindIII); 2, *P. aeruginosa* ATCC 27853 (*phaC*+); 3, *P. putida* IBUN 92 (*phaC*+); 4, *A. amazonense* IBUN 87; 5, *A. chroococcum* IBUN 88; 6, *E. coli* XL-1 blue (negative control); 7, reagent control.

Selecting native PHA-producing isolates by amplifying a *phaC* gene (Class I and II) fragment and Class II *phaC* gene differentiation

Thirty-four of the 55 bacterial isolates belonging to the old collection were *phaC*+. 16S rDNA characterisation of 18 of these positive strains showed that they belonged to the following genera: *Burkholderia*, *Rhizobium*, *Pseudomonas* and an isolate from the *Enterobacteriaceae* family. A *phaC*-isolate was included in this analysis and was very similar to *Stenotrophomonas maltophilia* which has not been reported as being a PHB-producer (Palleroni and Bradbury, 1993). The primers were also able to amplify this gene in other PHA- accumulator bacterial species which are different to the genera represented by the reference strains (*Burkholderia*, *Rhizobium* and a genus from the *Enterobacteriaceae* family).

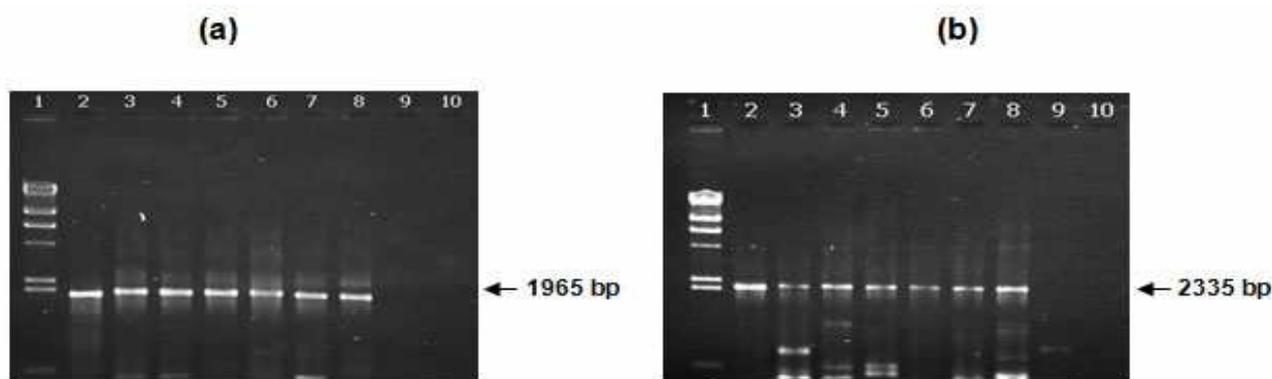


Figure 3. Colony PCR amplification of the *phaC1* and *phaC2* genes in reference bacteria and native bacteria from the old collection.

(a) Amplifying the *phaC1* gene using E1-D and E1-R primers.

(b) Amplifying the *phaC2* gene using E2-D and E2-R primers.

Lane 1, molecular weight marker (1 ADN *Hind*III); 2, *Pseudomonas fluorescens* IBUN 91; 3, *Pseudomonas* sp. IBUN C14; 4, *Pseudomonas* sp. IBUN C16; 5, *Pseudomonas* sp. IBUN S0804; 6, *Burkholderia* sp. IBUN S1602; 7, *Pseudomonas* sp. IBUN S1804; 8, *Pseudomonas* sp. IBUN 1G-3*; 9, *E. coli* XL-1 Blue (negative control); 10, reagent control.

Thirty-five PHA-producing isolates from the new collection were obtained from soil and sea-sludge samples following colony PCR with G-D, G-1R and G-2R general primers. These *phaC+* isolates were then evaluated regarding their PHA-accumulating ability as described in Materials and Methods. However, polymer granules were also observed by Sudan black B staining (Schlegel et al. 1970) in some isolates strains grown on nutritive agar. Twenty-three isolates accumulated polymer on both culture mediums (9 on MSM medium and 3 on nutritive agar). Regarding the use of carbon sources in MSM medium, 13 accumulated polymer from glucose, fructose and saccharose, 3 from glucose and saccharose, one from glucose and fructose and 6, 4, and 5 from glucose, fructose and saccharose respectively. 16S rDNA characterisation of 16 of these isolates showed that they had high similarity with bacterial species from the *Pseudomonas* genus.

Differentiating Class II PHA synthase organisation

Two pairs of specific primers were designed to establish the difference between Class I and Class II PHA synthase. The *phaC1* gene was amplified by E1-D and E1-R primers and E2-D and E2-R primers amplified the *phaC2* gene. Both genes were amplified in *P. putida* IBUN 92 and *P. fluorescens* IBUN 91, in *P. aeruginosa* ATCC 27853 and *P. aeruginosa* IBUN 1040 considered to be a positive control in this study were not amplified. Nine out of 55 isolates from the old bacterial collection were evaluated with the specific primers for determining Class II PHA biosynthesis operon organisation. Native bacterial strains characterised as being *Pseudomonas* spp. (IBUN C14, IBUN C16, IBUN S0804, IBUN 1G-3*, IBUN S1804 and IBUN S1602) amplified both *phaC1* and *phaC2* genes

(Figure 3), but IBUN S1407 (*Pseudomonas* sp.) could not be amplified, nor could IBUN 2G-57 (*Burkholderia* sp.) or IBUN N3510 (*S. maltophilia*).

IBUN C14, IBUN C16 and IBUN S1407 strains (*Pseudomonas* spp.) were preliminarily characterised as having mcl-PHA polymer Class II; this was confirmed by characterising the polymer in the laboratory (results not shown). Native strain IBUN S1602 was similar to species from the *Burkholderia* genus; this type of organisation is known in the *Burkholderia caryophylli* specie (Hang et al. 2002). However, native strain IBUN 2G-57 characterised as being *Burkholderia* sp. and having a PHA preliminarily characterised by sweeping differential calorimetry as PHB (data not shown) only amplified the Class I *phaC* gene.

Partial 16S rDNA gene sequence analysis

The 16S rDNA gene partial sequence from some native bacteria was obtained by splicing the 726 bp (amplified with A and J primers) and 1420 bp fragments (amplified with E and J primers). Most of these bacterial strains (IBUN 1G-3*, IBUN C14, IBUN C16, IBUN C22, IBUN C25, IBUN C29, IBUN N2103, IBUN S1407, IBUN S1601, IBUN S1308, IBUN S1402, IBUN S1804, IBUN S1901, IBUN L41, IBUN P5, IBUN P8B and IBUN MAR1 to IBUN MAR13) were more than 96% similar to species from the *Pseudomonas* genus. IBUN N3510 was 99% similar to the *S. maltophilia* specie and IBUN S1602 had 98% and 99% similarity with *Burkholderia cepacia* ATCC 53795 and *Burkholderia* sp. CCBAU23014 respectively. IBUN 2G-57 was 98% more similar to species from the *Burkholderia* genus, IBUN B69 was 97% more similar to species from the *Rhizobium* genus and IBUN N2004 was

96% to 98% similar with genera from the *Enterobacteriaceae* family. The native bacteria included here were grouped into 5 clusters (bootstrap presented 100% confidence values for each cluster) (Figure 4). The distribution of all native pseudomonads was agreed with the classification established by Anzai et al. (2000). *Pseudomonas* spp. isolated from sea-sludge were grouped with *P. stutzeri*, a specie belonging to the *P. aeruginosa* sub-group of rRNA group I; they could be different non-fluorescent *P. stutzeri* strains, one of the most controversial bacterial species due to its extreme phenotypical diversity covering a heterogeneous set of strains (Lalucat et al. 2006).

Bacterial species from the *Enterobacteriaceae* family were shown in the second cluster where N2004 was grouped; this seemed to be distanced from *K. pneumoniae*, a bacteria described in this group as being a polymer-accumulator (Wong et al. 2002). The third cluster included *S. maltophilia* (Palleroni and Bradbury, 1993), like IBUN N3510. The fourth cluster included 2G-57, closely related to *Burkholderia* strain. The close relationship of B69 to species from the *Rhizobium* genera was observed in the fifth cluster, especially to *R. etli*, however, is necessary to consider other approaches Eardly et al. (2005).

DISCUSSION

Native PHA-accumulator Gram-negative bacteria were selected by detecting and differentiating genes encoding PHA synthase Class I and II. The great diversity of PHA-producing microorganisms could represent a multiplicity of intra-cell conditions to which PHA synthase had to adapt itself to. This could explain the low level of identity between different PHA synthase sequences (Madison and Huisman, 1999).

The present work, involving designing degenerate primers from nucleotide sequences, led to amplifying the *phaC* gene (Class I and II) fragment in different bacterial species. The spectra for detecting this gene was broadened by including other sequences deposited to date in GenBank/EMBL/DDBJ databases, in addition to the 13 sequences considered by Sheu et al. (2000). Three bacterial genera were considered (*Bradyrhizobium*, *Chromobacterium* and *Azotobacter*) plus another eight species: *Azotobacter* sp., *A. vinelandii*, *Pseudomonas* sp., *P. stutzeri*, *P. putida*, *Chromobacterium violaceum*, *Bradyrhizobium japonicum* and *A. hydrophyla*. Based on the alignment, other regions were analysed which led to designing primers having a 4- to 16-fold degeneracy, thereby favouring specificity.

Analysing the G + C content of the 28 sequences used for designing the primers revealed 55% to 68% content (except

for *Azotobacter* sp. (51.82%), *Azotobacter vinelandii* (51.29%) and *Acinetobacter* sp. (41.29%)). Amplifying GC-rich regions from the *phaC* gene was optimised in this study by using 3% DMSO and 20 mM ammonium sulphate as additives (Sambrook and Russell, 2001).

Validating the PCR methodology using amplified *phaC* gene specificity, sensitivity, efficiency and confirmation parameters conferred the required reliability on the technique for amplifying the *phaC* gene in different bacterial species. Susceptible measurement detection limit corresponded to 2×10^4 viable cells; it could have been even less, in spite of the degree of primer degeneration and the low purity of the DNA used as template. The three general primers designed in this research amplified a Class I or II *phaC* gene fragment in Gram-negative bacteria belonging to 9 different genera (including control and native strains) representing PHA synthase Class I and II: *Ralstonia*, *Aeromonas*, *Azospirillum*, *Acinetobacter*, *Azotobacter*, *Pseudomonas*, *Burkholderia*, *Rhizobium* and a member of the *Enterobacteriaceae* family.

Genes adjacent to *phaC1* and *phaC2* genes (Figure 1) were considered in designing the primers for fully differentiating Class I *phaC*. Solaiman et al. (2000) designed a PCR methodology for detecting *phaC1/2* genes but it did not allow differentiating the arrangement of Class II PHA biosynthesis operon genes, since they designed primers for amplifying a fragment from the *phaC1* and *phaC2* genes. The complete open reading frames (ORF) of both genes were also amplified in this work in reference strains and some native strains. The primers designed in this work could be using in cloning strategies as shown in other studies (Zhang et al. 2001). The Touchdown PCR (Don et al. 1991) strategy allowed amplifying the *phaC1* and *phaC2* genes and this led to reducing non-specific amplifications.

Detecting and differentiating *phaC* genes in native bacteria by colony PCR, combined with the preliminary characterisation by 16S rDNA, has proved to be an instrument for the precise and directed selection of bacteria from different genera having the potential for accumulating PHAs. Native strains' genetic potential could be predicted regarding novel PHA production. *P. aeruginosa* ATCC 27853 and *P. aeruginosa* IBUN 1040 Class II PHA synthase genes could not be amplified with the specific primers; it is probable that these genes could have been different to type II (Solaiman et al. 2000).

The isolates from the old IBUN collection had been previously selected by traditional methods and were grown in the necessary conditions for detecting PHA production. The best PHA-producing strains were *phaC+*, including IBUN 2G-57 (*Burkholderia* sp.), IBUN B69 (*Rhizobium* sp.), IBUN C14 (*Pseudomonas* sp.), IBUN C16 (*Pseudomonas* sp.), IBUN S1407 (*Pseudomonas* sp.)

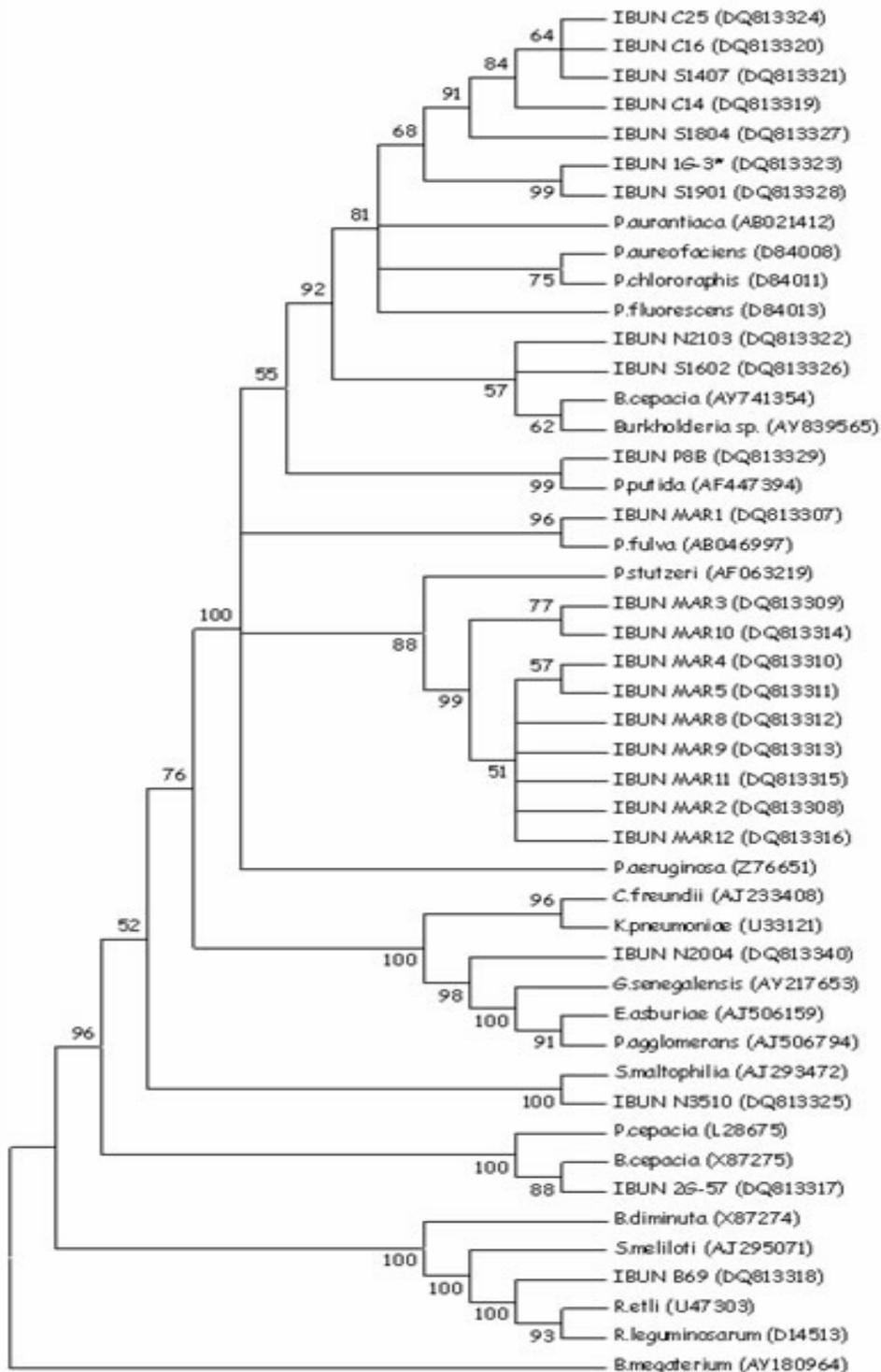


Figure 4. Dendrogram of native bacterial strain similarity based on analysing 16S ribosomal gene partial sequences.

The dendrogram was constructed from the 1,400 to 1,500 bp 16S rDNA gene sequences by the Neighbour-joining method using the P distance with MEGA 3.0 software (Kumar et al. 2004). External group, *B. megaterium*. The dendrogram shows five clusters. The first cluster corresponds to bacteria from the *Pseudomonas* genera (Anzai et al. 2000). The second cluster, formed by bacterial species from the *Enterobacteriaceae* family, belonged to IBUN N2004. IBUN N3510 was in the third cluster, similar to *S. maltophilia*. The fourth cluster contained IBUN 2G-57, closely related to *Burkholderia cepacia*. The fifth cluster was observed to be closely related to IBUN B69 with species from the genera.

and IBUN N2004 (*Enterobacteriaceae* family). Other native strains such as IBUN S1602 related to species from the strains such as IBUN S1602 related to species from the *Burkholderia* genus could represent reported Class II PHA synthase, this posed the need to confirm its identification using other taxonomic analysis strategies, due to the inference which its classification has relating to PHA biosynthesis genes. Hang et al. (2002) confirmed that species from the *Burkholderia* genus possessed at least two PHA synthetic pathways having different substrate specificity and cloned the type II system in *B. caryophylli* AS 1.2741. However, the IBUN N3510 strain (*S. maltophilia*) from the old collection was *phaC*-; this result agreed with the description of the genus (Palleroni and Bradbury, 1993) related to poly- β -hydroxybutyrate granule accumulation.

PHA synthase type II organisation was differentiated in native IBUN C14 and IBUN C16 strains, corroborating preliminary characterisation in previous work regarding the type of polymer produced by these bacteria, corresponding to mcl-PHA, a typical polymer produced by most pseudomonads, especially by species belonging to the I rRNA group (Solaiman et al. 2006). The IBUN S1407 strain also belongs to this group; however, it did not present Class IIPHA synthase organisation. A precise characterisation profile of the polymer produced by IBUN S1407, combined with a study directed towards PHA biosynthesis genes, could lead to reporting the genetic polymer-accumulation system in this bacterial strain.

Different native *Pseudomonas* spp. characterised in this study showed similarity with *Pseudomonas* from different groups established by Anzai et al. (2000): the *P. chloraphis* group, *P. fluorescens* group, *P. putida* group, *P. stutzeri* group and the *P. aureginosa* group (Figure 4). These bacteria thus represent a resource for producing different mcl-PHA from different carbon sources such as fatty acids or carbohydrates (Solaiman et al. 2006). Differentiating the Class II PHA synthase system allows native bacteria having mcl-PHA-accumulating ability to be selected and, eventually, the cloning of PHA biosynthesis genes for constructing more efficient recombinant production systems.

The new collection (containing the thirty-five new *phaC*+ potentially PHA-accumulating bacteria selected by colony PCR isolated in this study from different environmental samples) was also confirmed by PHA staining. Bacterial strains from sea-sludge constitute a biotechnological source for producing different PHAs. The *phaC* gene fragment amplified in these strains had a greater size respecting that amplified in *R. eutropha* (results not shown).

The PCR methodology for selecting different PHA-accumulator bacterial genera in this investigation represents a rapid, simple and specific tool for detecting and

differentiating type I and II PHA synthase genes, thereby eliminating some disadvantages involved in phenotypical selection using different staining, such as the inability to differentiate between scl-PHA, mcl-PHA and other lipid materials (such as triacylglycerols and waxes) and the fact that staining methods require a prior microorganism culture in conditions allowing the polymer to accumulate. Respecting other molecular methods such as hybridisation, colony PCR is a rapid and equally precise procedure which does not require pure, previously prepared DNA.

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