PCR cloning of polyhydroxybutyrate synthase gene (*phbC*) from *Aeromonas hydrophila*

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ABSTRACT

Plastic wastes are considered to be severe environmental contaminants causing waste disposal problems. Widespread use of biodegradable plastics is one of the solutions, but it is limited by a high production cost. A Polymerase Chain Reaction (PCR) protocol was developed for the specific detection and isolation of full-length gene coding for polyhydroxybutyrate. PCR strategy using PHB primers resulted in the amplification of DNA fragments with the expected size from all isolated bacteria.

PHB synthase gene was cloned directly from Aeromonas hydrophila genome for the first time from this bacteria. The cloned fragment was named $phbC_{Ah}$. This fragment was partially sequenced and open reading frame was found representing the PHB synthase gene. The nucleotide sequence of $phbC_{Ah}$ gene exhibits similarity to Caulobacter crescentus phbC (40.3%), Pseudomonas oleovorans phbC (34.3%), and Rhodobacter sphaeroides phbC (40.6 %).

Keywords: *PCR, Polyhydroxybutyrate, Cloning, Aeromonas hydrophila, Biodegradable thermoplastic.*

INTRODUCTION

Poly(3-hydroxybutyrate) (PHB) is a carbon and energy reserve accumulated by several kinds of bacteria under conditions of nutrient stress, e.g. when an external carbon source is available but the concentration of nutrients such as nitrogen, phosphorus, or oxygen are limiting the growth (Senior and Dawes, 1973). Poly (3-hydroxybutyrate) biosynthesis genes are phbA (for 3-ketothiolase), phbB (NADPH-dependent acetoacetyl-coA reductase), and phbC (PHB synthase); these genes have been cloned recently and expressed in *E. coli* (Slater *et al.*, 1988).

Most *Pseudomonas* strains are able to accumulate polyhydroxyalkanoic acids (PHA) as carbon energy storage compound, consisting of saturated and unsaturated 3-hydroxy fatty acids with carbon chain lengths ranging from six to fourteen carbon atoms (Anderson and Dawes, 1990). *Alcaligenes eutrophus* is now used for commercial PHA production, but many other microorganisms accumulate PHB and can grow on more or different carbon sources than *A. eutrophus* (Page, 1992).

Takeda *et al.* (1995) reported that Poly-3-hydroxybutyrate (PHB) has potential applications for biodegradable and safe thermophilic plastics with much less environmental impact than many other

biologically stable artificial plastics. He demonstrated that a mutant of *Sphaerotilus natans*, defective in filamentation, exhibited a higher capability of PHB production than the parent strain

Recombinant *E. coli* strains harboring the *Ralstonia eutropha* PHA biosynthesis genes have been reported to have several advantages as PHA producers compared with wild type PHA producing bacteria (Choi *et al.*, 1998)

There are several methods for identifying PHA-producing organisms (Takagi and Yamane, 1997). The majority of these methods, employ lipophilic dyes to stain the polymers or cause them to fluoresce. Although highly sensitive, these reagents also react with other lipid inclusion bodies and thus are not specific for PHA. Furtheremore, the production of PHA is often dependent on specific growth conditions. If such conditions are not met and the polymer is not produced, then the dye-based screening would fail to identify the microorganism as having PHA-producing capability (Spiekermann *et al.*, 1999).

The three genes that are traditionally considered to be necessary in the PHA biosynthesis pathway were cloned from *Rhodobacter capsulatus* (Kranz *et al.*, 1997). Two types of polyhydroxyalkanoate (PHA) biosynthesis gene loci (phb and pha) of *Pseudomonas sp.* strain 61-3, were cloned and analyzed at the molecular level (Matsuaki *et al.* 1998). The cloning of PHA biosynthesis genes from two *Pseudomonads* was performed using a PCR-based cloning strategy designed on the short highly conserved stretches of PHA biosynthesis gene locus (Zhang *et al.*, 2001).

In this paper, we report a rapid and sensitive Polymerase Chain Reaction (PCR) procedure for detection of Phb synthase gene. This synthase gene ($phbC_{Ah}$) was cloned directly from the *Aeromonas hydrophila* genome for the first time. The structural analysis of the cloned synthase gene was reported.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Pseudomonas aeruginosa, P. putida , *Aeromonas caviae and Aeromonas hydrophila* were isolated from waste water and identified at Urology and Nephrology Center, Mansoura University, Mansoura, Egypt, by automatic bacterial identification (AP80/automatic 18 hr ID, Sensitiser, USA).

All *Pseudomonas* and *Aeromonas* strains were cultivated at 30 °C on Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). *E. coli* strain was grown at 37°C on LB medium. Amplicillin 100 ug/ml was added to the medium to maintain the stability of plsamids.

DNA manipulation

Genomic DNA of all strains (*Pseudomonas and Aeromonas*) was isolated by a Wizard Genomic DNA purification kit (Promega, Madison, Wis., USA). Isolation of plasmids, digestion of DNA with restriction endonucleses, and transformation of *E. coli* were carried out by standard procedures (Sambrook *et al.*, 1989).

Polymerase chain reaction (PCR)

To amplify a partial fragment of PHB synthase gene from genomic DNA of *Aeromonas hydrophila* , it was performed by PCR with two specific oligonucleotide primers P1:5'CC(C/G)CC(C/G)TGGATCAA(T/C)AA GT(T/A)(T/C)TA(T/C)ATC-3' and M1:5'-(G/C)AGCCA(G/C)GC(G/C)GTCCA(A/G)T C(G/C)GGCCAACCA-3'. PCR conditions were as follows: 35 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec

and elongation at 72°C for 1 min. To amplify the full-length sequence of PHB synthase gene, PCR was performed in the presence of two specific primers named FLP1:5'-ATGAGCCAACCATCTTATGG-3' and FLM1:5'CTTCCAGGGATTGTGCG-3' under 35 cycles of denaturation at 95°C for 30 sec., annealing at 58 °C for 30 sec, and elongation at 72 °C for 2 min. The PCR products were analyzed by agarose gel electrophoresis in 1X TBE (Tris-Borate-EDTA) buffer and gel was stained with 0.5 ug/ml ethidium bromide.

Cloning of $phbC_{Ah}$ gene

The PCR amplified fragment of the $phbC_{Ah}$ was separated by electrophoresis on an agarose gel in 1X TAE (Tris-Acetate-EDTA) buffer. The desired fragment was excised and eluted from the gel using the GENECLEAN II KIT (BIO 101, La jolla, Californai). The purified PCR fragment was subcloned into pGEM-T vector (Promega). The recombinant DNA was used to transform *E. coli* JM109. White transformants were selected on solid luria medium containing 100 µg/ml amplicillin, 20 ug/ml X-gal (5-bromo-4-chloro-3-indolyl-b-D galactopyranoside), and 120 µg/ml IPTG (isopropyl-B-D-thioglucopyranoside). Plasmid DNA was purified from the transformant with the Wizard miniprep system (Promega, USA).

Nucleotide sequence

The PHB synthase cloned gene was purified by wizard mini prep purification system (Promega). The nucleotide sequence was obtained by dideoxy chain termination method basically as described by Sanger *et al.* (1977), with a 310 Genetic Analyzer (Perkin Elmer). The sequencing reaction was performed in accordance with the manual supplied with the dye terminator cycle sequencing kit (Perkin Elmer). The resulting nucleotide sequence was analyzed with PC/Gene software program (IntelliGenetics).

RESULTS AND DISCUSSION

The major objective of this work was the cloning of phbC gene from a local isolate of *Aeromonas hydrophila* to be used later in the production of biodegradable plastic, hence substitute the use of petroleum polymer as a convenient method for production of non-degradable plastic. PHB synthase is a key enzyme essential for bacterial synthesis of biodegradable polyester, polyhydroxyalkanoate.

PCR for amplification of PHB synthase gene

The Polymerase Chain Reaction (PCR) method was used to amplify a partial segment and the full-length of phbC gene. A partial fragment of PhbC synthase gene was amplified from the genomic DNA in the presence of two specific primers designed from highly conserved region among known PHB synthases (244-PPWINK(Y/F)YI-252 and 547-WWPDWTAWL-55), numbering corresponds to the *Alcaligenes eutrophus* phb synthase (Steinbuchel *et al.,* 1992). PCR reaction resulted in a successful amplification of an approximately 900 bp (for partial fragment) and distinct 1.8 kb (for full-length gene) from the tested bacteria as shown in figures (1&2 respectively). Due to the presence of non-specific PCR product in addition to the expected PCR fragment, the desired PCR fragments were excised and eluted from the gel; these PCR fragments were re-amplified using the same primer pairs as shown in figures (3&4). The size of the PCR products agrees with the length of the $phbC_{Ah}$ gene flanked by the P1 & M1 primers for the partial fragment and FLP1 and FLM1 primers for the full-length fragment. Many strategies

were applied to clone PHA synthase gene (Rehm and Steinbuchel, 1999). Among them, a rapid and convenient PCR strategy for cloning type II PHA biosynthesis genes, namely medium chain length (mcl) PHA synthase genes (Zhang et al., 2001). Similarly, the PCR cloning strategy was applied for cloning of PHA biosynthesis genes from *Burkholderia caryophylli* (Hang *et al.,* 2002). This successful $PhbC_{Ah}$ gene cloning from *Aeromonas hydrophila* showed that the rapid PCR cloning strategy can be used for cloning PHA synthase genes from other microorganisms.

However, additional PCR products with smaller sizes were amplified from the tested bacteria by the PHB primer, which implies that amplification was due to non-specific binding of the primers. Therefore, re-amplification with PHB primers was carried out to further specifically identify the amplification fragments.

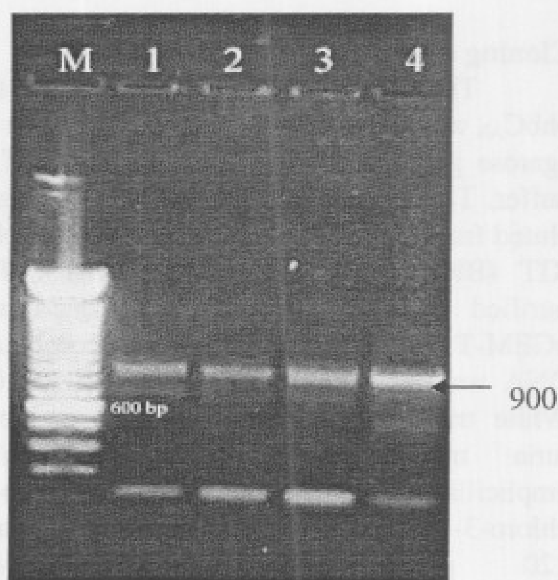

Fig. (1): Agarose gel electrophoresis of PCR products of $phbC_{Ah}$ fragments.
Lane M: 1 Kb DNA ladder.
Lane 1: PCR product from Pseudomonas putida.
Lane 2: PCR products from P. aeruginosa.
Lane 3: PCR products of Aeromonas Caviae.
Lane 4: PCR product of A. hydrophila.

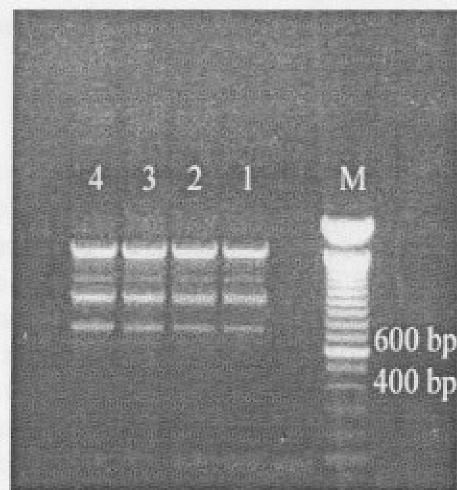

Fig. (2): Detection of full-length of PCR products of phbCAh amplified from genomic DNA of bacterial isolates.
Lanes1: PCR products of P. putida.
Lane 2: PCR products of P. aeruginosa.
Lane 3: PCR products of Aeromonas Caviae.
Lane 4: PCR product of A. hydrophila.
Lane M. 1 kb DNA ladder.

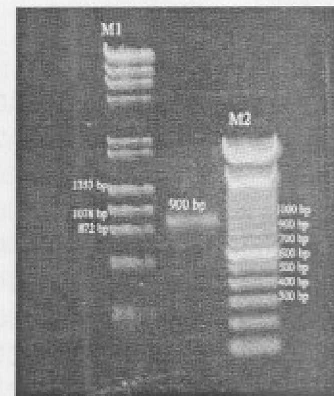

Fig. (3): Agarose gel electrophoresis of re-amplified PCR products of phbCAh fragments using P1 and M1 primers.
Lane M1: Lambda DNA Hind III and Hae III.
Lane M2: 1 kb DNA ladder.

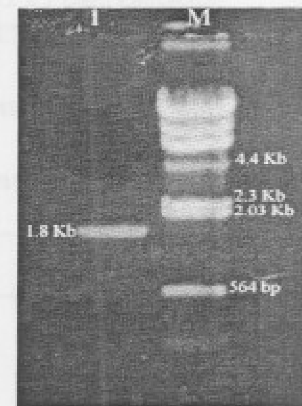

Fig. (4): Agroase gel electrophoresis of re amplified full length PCR products of phb synthase gene in the presence of FLP1 and FLM1 primers.
Lane 1: PCR products of full lenth phbC$_{Ah}$
Lane M: lambda DNA digested with Hind III.

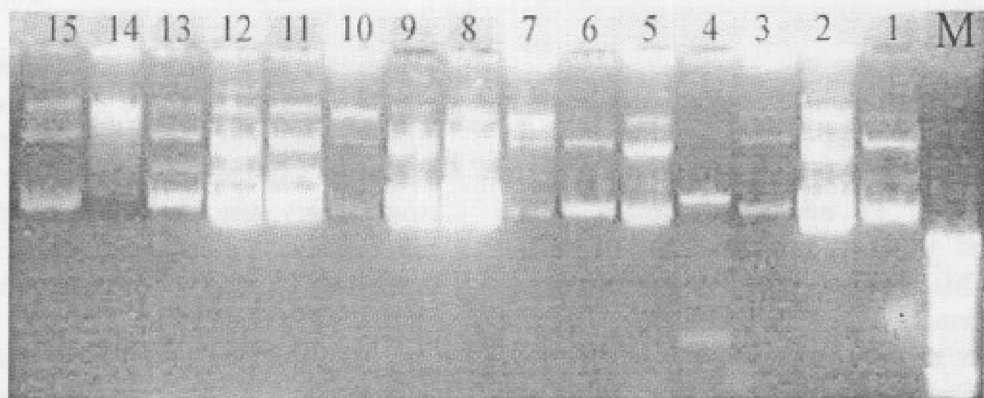

Fig. (5): Screening of putative recombinant plasmids digested with restriction enzyme, EcoR1 and separated on 1.0 % agarose gel.
Lanes from 5 to 15 and lanes 1, 2, and 3: represent non recombinant plasmids
Lane 4: represents phbC$_{Ah}$ clone
Lane M. 1kb DNA ladder

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5'AGGCCATGCAGTCGCCCATGCCTCAGGCCGCGGCTGGCTCGGCTCAGACG
GTTCAGTTCGATACCCCCAAACTGCACGGGTTGCAGCAGGAATATCTGCA
GTCCGTGCAGTCGCTCCCGGATGCCAAGCAGGTTCAGGCT CTGCTGGCC
AAGGACAATCGCTTCGCCAAGCCCGAATGGAGCTCCAATCCTGTTGCGGC
GATGGCCGGAGCCAACTATCTGCTCGGCAGCCGCATGCTGACCGGCATGG
CCGAGGCCGTGCAGGGTGATGAGAAAACGCGCAACCGCGTGCGCTTTGCG
GTCGAGCAATGGGTGGCCCTCATGGCGCCCAGCATTTTACTTGCGCTGAA
TGCCGATGCGCTCAACAAGGTCGTGGAAACCAAGGGCGAGAGCCTGGCCC
ACGGCATTGCCAATCTGCTGGCCGACATGCGCCAAGGTCATGTCTCCATG
ACCGACGAATGCCTGTTCACCGTGGGACAGAACGTGGCGACCACCGAAGG
CGCGGTGGTTTACGAGAATGAGCTGTTCCAGCTCATCGAGTACAAGCCGC
TGACAGCCAAGCTCTTCGAGAAGCCTTTACTGATGGTGCCGCCGTGCATCT3'
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Fig. (6): Partial nucleotide sequence of phbC$_{Ah}$ of A. hydrophila.

Table (1): Homology of the phbC$_{Ah}$ of Aeromonas hydrophila to phb synthase genes of other bacteria.

Bacteria	Accession number	Nucleotide sequence identity (%)	Subject sequence (From-to)
Alcaligenes latus	U47026	97%	1080-1201 bp
Alcaligenes latus	AF004933	85%	1100-1221 bp
Comamonas acidovorans	AB009273	84%	1108-1451 bp
Pseudomonas oleovorans	AF422800	97%	241-839 bp
Alcaligenes sp	U78047	81%	615-737 bp

Cloning of *phbC$_{Ah}$* gene

The strategy used for molecular cloning of *phbC$_{Ah}$* gene was based on the direct cloning of PCR products. The PCR products amplified using P1 and M1 primers were purified and ligated into the tailed cloning vector pGEM-T vector. The cloned fragment was transformed into *E. coli JM109*. Clones that contain the desired *phbC$_{Ah}$* gene were selected using white/blue colony selection. Plasmid mini preparation procedure was used to screen recombinant clones, followed by digestion of plasmids by *EcoR1* restriction endonuclease. Digestion of plasmids by *EcoR1* identified only one clone containing *phbC$_{Ah}$* insert with expected molecular weight size as in figure (5).

Computer analysis and nucleotide sequence

In addition to size detection by agarose gel electrophoresis. PCR fragment of *PhbC$_{Ah}$* gene was validated by nucleotide sequence. A clone carry the recombinant plasmid was selected for nucleotide sequence. The partial sequence of *PhbC$_{Ah}$* insert was determined as shown in figure (6).

The nucleotide sequence of *PhbC$_{Ah}$* gene was compared with other phbC genes isolated from *Alcaligenes latus* (Accession number U47026), *Pseudomonas oleovorans* (Accession number AF422801), *Alcaligenes* sp (Accession number U78047), and *Comamonas acidovorans* (Accession number AB009273). The *phbC$_{Ah}$* exhibited 97% similarity with *Alcaligenes latus* and

Pseudomonas oleovorans, 84% with *Comamonas acidovorans*, and 81% with *Alcaligenes* sp. Nucleotide sequence alignment of $phbC_{Ah}$ with other synthase genes using BLASTN method is summarized in Table (1). In a previous study, the deduced amino acid sequences of phaC1 of *Pseudomonas* sp strain 61-3 (Matsusaki *et al.*, 1998) exhibited greater identity to the PHA synthase (Huismen *et al.*, 1991) of *Pseudomonas Oleovorans* (54.7%) than to the PHB synthase (Peoples and Sinskey, 1989) of *Ralstonia eutropha* (33.8 %).

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الملخص العربي

استنساخ جين السينسيز المسئول عن تخليق مركب البولي هيدروكسي بيوتيرات من بكتريا الايروموننس هيدروفيلا باستخدام التفاعل التسلسلي للبوليمراز

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تعتبر المخلفات البلاستيكية المصنعة من المواد البتروكيماوية من أهم المشاكل الخطيرة التي تلوث البيئة. وان الحل البديل هو استخدام مادة البولى هيدروكسى بيوتيرات لتصنيع بلاستيك قابل للتحلل لا يضر بالبـيئة، حتى الآن فان استخدام مثل هذه المادة مازال محدود وذلك لارتفاع تكلفة الإنتاج. لاول مرة من بكتريا *Aeromonas hydrophila* تم استخدام التفاعل التسلسلي للبوليمراز (PCR) وذلك لتحديد وعزل الطول الكامل لجين السينسيز **Synthase** فى وجود بادئات خاصة أدت إلى مضاعفة جزء خاص من هذا الجين ،تم استنساخ ناتج PCR فى البلازميد ولمضاعفة هذا البلازميد تم نقله الى بكتريا الاشرشيا كولاى وسمى البلازميد الذى يحمل جين السينسيز باسم *phbCAh* . تم دراسة تشابه الاحماض الامينية لجين السنسيز *phbCAh* مع الاحماض الامينية لجين السنسيز المعزول من بكتريا *Caulobacter crescentus* والاحماض الامينية لجين السنسيز المعزول من بكتريا *Pseudomonas oleovorans* والاحماض الامينية لجين السنسيز المعزول من بكتريا *Rhodobacter sphaeroides* واظهرت نسبة التشابه حوالي 40.3% و 34.3% و 40.6 % على التوالي .