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PCR Cloning of Polyhydroxybutyrate Synthase Gene (phbC) From *Aeromonas hydrophila*

استنساخ جين السينسيز المسئول عن تخليق مركب البولى هيدروكسى بيوتيرات من بكتريا *Aeromonas hydrophila* باستخدام التفاعل التسلسلى للبوليمراز

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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 high production cost. A polymerase Chain Reaction (PCR) protocol was developed for the specific detection and isolation of full-length gene coding for polyhydroxybutyrate (PHB). (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. The cloned fragment was named ($phbC_{Ah}$). The nucleotide sequence of ($phbC_{Ah}$) gene exhibits similarity to (PHB) synthase genes of *Alcaligenes latus* and *Pseudomonas oleovorans* (97%), *Alcaligenes* sp. (81%), and *Comamonas acidovorans* (84%).

Keywords: Cloning Polymerase Chain Reaction (PCR), Poly-hydroxybutyrate, *Aeromonas hydrophila,* Bacteria, Biodegradation, Plastics.

المستخلص: 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 بالبيئة، حتى الآن فان استخدام مثل هذه المادة مازال محدود وذلك لارتفاع تكلفة الإنتاج. فلأول مرة من بكتريا *Aeromonas hydrophila* تم استخدام التفاعل التسلسلى للبوليمراز (PCR) وذلك لتحديد وعزل الطول الكامل لجين السينسيز PHB 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 جين السنسيز phbCAh مع جين السنسيز المعزول من بكتريا *Alcaligenes latus* ومن بكتريا *oleovorans Pseudomonas* واظهرت نسبة التشابه حوالي 97٪. بينما كانت نسبة التشابة مع جين السنسيز المعزول من بكتريا *Comamonas acidovorans* حوالي 84٪.

كلمات مدخلية: بكتريا، *Aeromonas hydrophila*، المخلفات البلاستيكية، استنساخ، تحلل، بيئة.

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 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. They demonstrated that a mutant of *Sphaerotilus natans,* defective in filamentation, exhibited a higher capability of (PHB) production than the parent strain.

Recombinant *Escherchi 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). Furthermore, 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 alignment of the cloned synthase gene with other (PHB) synthase genes was reported.

Material and Methods

(**1**) Bacterial strains and growth conditions

Pseudomonas aeruginosa, Pseudomonas putida, Aeromonas caviae and *Aeromonas hydrophila* were isolated from wastewater 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 μg/ml) was added to the medium to maintain the stability of plasmids.

(**2**) 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 endonucleases, and transformation of *(E. coli)* were carried out by standard procedures (Sambrook *et. al.,* 1989).

(**3**) Polymerase Chain Reaction (PCR)

To amplify a partial fragment of (PHB) synthase gene from genomic DNA of *Aeromonas hydrophila,* we performed (PCR) with two specific oligonucleotide primers

P1: 5'- CC (C/G) CC (C/G) TGGATCAA (T/C) AAGT (T/A) (T/C) TA (T/C) ATC-3'

M1: 5'- (G/C) AGCCA (G/C) GC (G/C) GTCCA (A/G) TC (G/C) GGCCAACCA-3'.

(PCR) conditions as follows: (35 cycles) of denaturation at (95 °C) for (30 seconds) annealing at (62 °C) for (30 seconds) and elongation at (72 °C) for 1 minute. To amplify full-length sequence of (PHB) synthase gene, (PCR) was performed in the presence of two specific primers named:

(**I**) FLP1: 5'-ATGAGCCAACCATCTTATGG - 3' and

(**II**) FLM1: 5'-CTTCCAGGGATTGTGCG-3'

under (35 cycles) of denaturation at (95 °C) for (30 seconds) annealing at (58 °C for 30 seconds) and elongation at (72 °C for 2 minutes). The (PCR) products were analyzed by agarose gel electrophoresis in (1X TBE) (Tris-Borate-EDTA) buffer and gel was stained with 0.5 μgml ethidium bromide.

(**4**) Cloning of (phbCAh) gene

The (PCR) amplified fragment of the ($phbC_{Ah}$) was separated by electrophoresis on 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 (LB) medium containing (100 μgml) amplicillin, (20 μgml X-gal) (5-bromo-4-chloro-3-indolyl-b-D galactopyranoside), and (120 μgml IPTG) (isopropyl-B-D-thioglucopyranoside). Plasmid (DNA) was purified from transformant with the wizard miniprep system (Promega).

(**5**) Nucleotide Sequence

The (PHB) synthase clone 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 BLASTN database search program (Stephen *et. al.*, 1997).

Results and Discussion

The major objective of this work was the cloning of (phbC) gene from a local isolate *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.

(**I**) (PCR) for amplification of (PHB) synthase gene

The Polymerase Chain Reaction (PCR) method was used to amplify 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 correspond to the *A. eutrophus* (phb) synthase (Steinbuchel *et. al.*,1992). (PCR) reaction resulted in 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 Fig. 1 & 2, respectively. Due to the presence of non-specific (PCR) product in addition to 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 Fig. 3 & 4. The size of (PCR) products agree with the length of the *($phbC_{Ah}$)* gene flanked by the (P1 & M1) primers for partial fragment and (FLP1) and (FLM1) primers for 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).

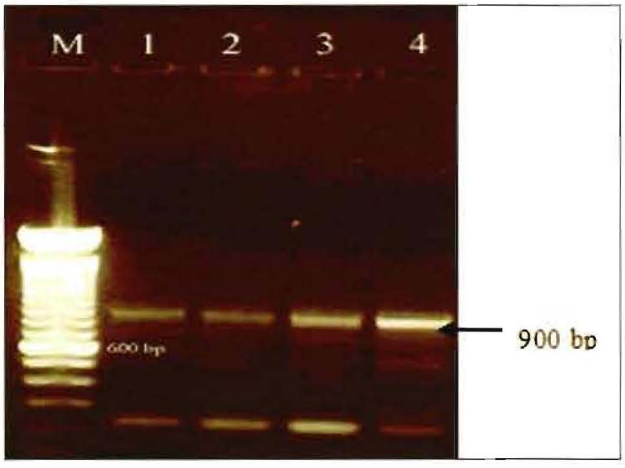

Fig. 1. Agarose gel electrophoresis of (PCR) products of $phbC_{Ah}$ fragments.
Lane M : 1 Kb DNA ladder
Lane 1: (PCR) product from *P. putida*
Lane 2: (PCR) products from *P. aeruginosa*
Lane 3: (PCR) products of *A. Caviae*
Lane 4 : (PCR) product of *A. hydrophila*

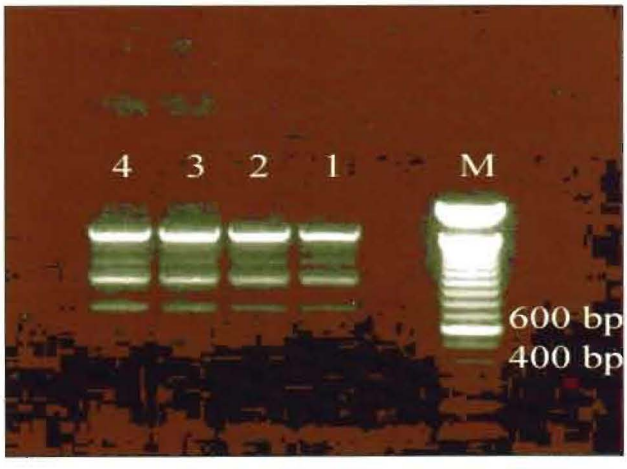

Fig 2. Detection of full-length of (PCR) products of phbCAh amplified from genomic DNA of bacterial isolates
Lane 1: (PCR) products of *P. putida*
Lane 2: (PCR) products of *P. aeruginosa*
Lane 3: (PCR) products of *Aeromonas Caviae*
Lane 4: (PCR) product of *A. hydrophila*
Lane M. 1 kb DNA ladder

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) primers, 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.

(II) 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 Fig. 5.

(III) 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 Fig. 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 AF422800), *Alcaligenes* sp. (Accession number U78047), and *Comamonas acidovorans* (Acession Number AB009273) The *(phbC$_{Ah}$)* exhibited (97%) similarity with *Alcaligenes latus* and *Pseudomonas oleovorans,* (84%) with *Comamonas acidovorans,* and (81%) with *Alcaligens* sp. Nucleotide sequence alignment of *(phbC$_{Ah}$)* with other synthase genes using (BLASTN) method was summarized in Table 1. In 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 *P. oleovorans* (54.7%) than to the (PHB) synthase (Peoples and Sinskey, 1989) of *Ralstonia eutropha* (33.8%).

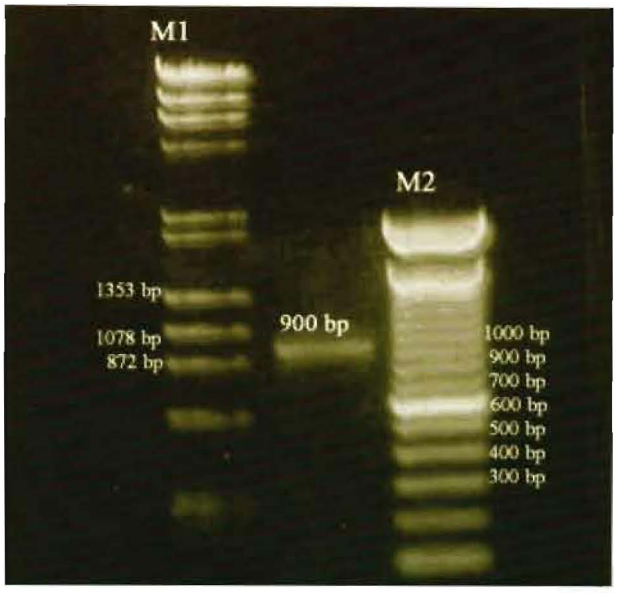

Fig. 3. Agarose gel electrophoresis of re-amplified (PCR) products of phbCAh fragments using P1 and M1 primers
Lane M1: Lambda DNA *Hind III* and *Hae III* marker
Lane M2: 1 kb DNA ladder

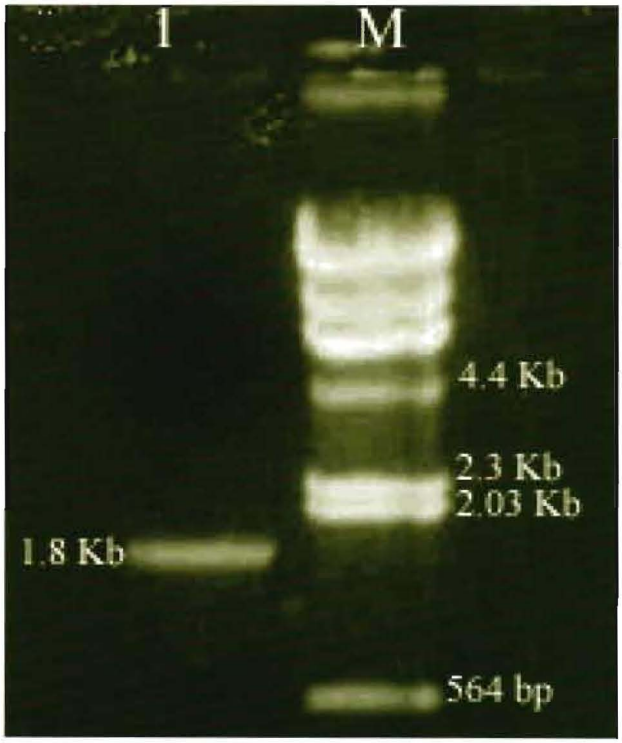

Fig. 4. Agroase gel electrophoresis of reamplified full length (PCR) products of phb synthase gene in the presence of FLP1 and FLM1 primers.
Lane 1: (PCR) products of full lenth phbCAh
Lane M: lambda DNA digested with Hind III marke

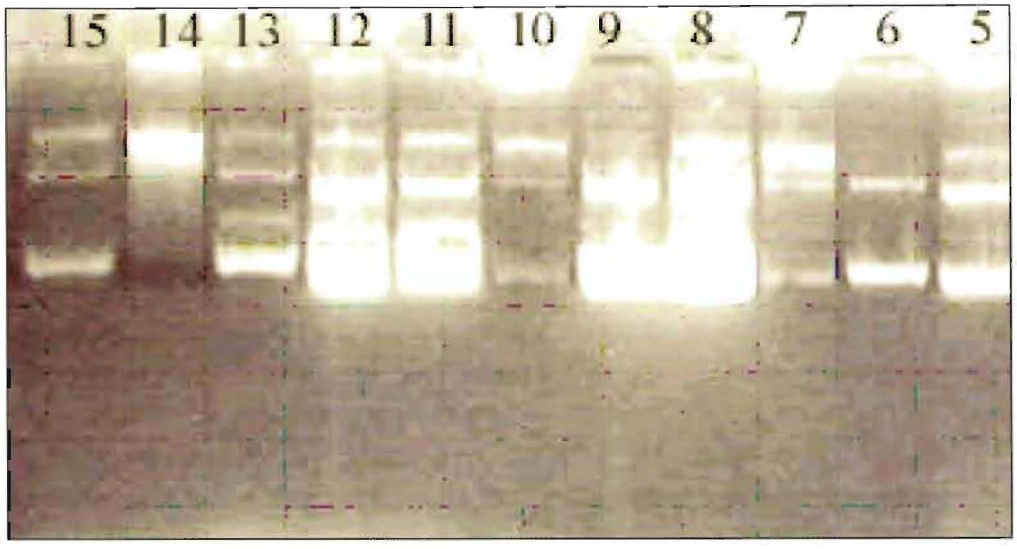

Fig. 5. Screening of putative recombinant plasmids digested with restriction enzyme, EcoR1 and separated on 1.0 % agarose gel. Lanes from 5 to 15 and lanes 1, 2, and 3 : represent non recombinant plasmids. Lane 4: represents phbCAh clone; Lane M. 1kb DNA ladder.

AGGCCATGCAGTCGCCCATGCCTCAGGCCGCGGCTGGCTCGGCTCAGACG
GTTCAGTTCGATACCCCCAAACTGCACGGGTTGCAGCAGGAATATCTGCA
GTCCGTGCAGTCGCTCCCGGATGCCAAGCAGGTTCAGGCTCTGCTGGCC
AAGGACAATCGCTTCGCCAAGCCCGAATGGAGCTCCAATCCTGTTGCGGC
GATGGCCGGAGCCAACTATCTGCTCGGCAGCCGCATGCTGACCGGCATGG
CCGAGGCCGTGCAGGGTGATGAGAAAACGCGCAACCGCGTGCGCTTTGCG
GTCGAGCAATGGGTGGCCCTCATGGCGCCCAGCATTTTACTTGCGCTGAA
TGCCGATGCGCTCAACAAGGTCGTGGAAACCAAGGGCGAGAGCCTGGCCC
ACGGCATTGCCAATCTGCTGGCCGACATGCGCCAAGGTCATGTCTCCATG
ACCGACGAATGCCTGTTCACCGTGGGACAGAACGTGGCGACCACCGAAGG
CGCGGTGGTTTACGAGAATGAGCTGTTCCAGCTCATCGAGTACAAGCCGC
TGACAGCCAAGCTCTTCGAGAAGCCTTTACTGATGGTGCCGCCGTGCATCT

Figure 6 . Partial nucleotide sequence of phbCAh of *A. hydrophila*

Conclusion

The isolation of Polyhydroxybutyrate synthase gene is a first step toward cloning of full length gene, and isolation of other two genes (acetoacetyl-coA reductase and 3-ketothiolase) responsible for biosynthesis of polyhydroxybutrate polymer that used instead of non-degradable polymer in the synthesis of eco-friendly thermoplastic, this work was conducted for complete molecular charterization of these genes.

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