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Cloning and Sequence Analysis of the Poly(3-Hydroxyalkanoic Acid)-Synthesis Genes of *Pseudomonas acidophila*

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ABSTRACT

Pseudomonas acidophila can grow with CO_2 as a sole carbon source by the possession of a recombinant plasmid that clones genes that confer chemolithoautotrophic growth ability derived from the H_2-oxidizing bacterium *Alcaligenes hydrogenophilus*. H_2-oxidizing bacteria produce poly(3-hydroxybutyric acid) (PHB) from CO_2, but recombinant *P. acidophila* can produce the more useful biopolymer poly(3-hydroxyalkanoic acid) (PHA). In this study, the *pha* genes of *P. acidophila* were cloned and a sequence analysis was carried out. A gene library was constructed using the cosmid vector pVK102. A recombinant cosmid carrying the *pha* genes was selected by the complementation of a PHB-negative mutant of *Alcaligenes eutrophus* H16. The resulting recombinant cosmid pIK7 contained a 14.8-kb DNA insert. Subcloning was done, and the recombinant plasmid pEH74 was selected by hybridization with the *A. eutrophus* H16 *pha* genes. *Escherichia coli* possessing pEH74 produced PHB, indicating that pEH74 contained the *pha* genes of *P. acidophila*. The nucleotide sequences of the PHA-synthesis genes *phaA* (β-ketothiolase), *phaB* (acetoacetyl-CoA reductase), and *phaC* (PHA synthase) in pEH74 were determined. The homologies of *phaA*, *phaB*, and *phaC* between *P. acidophila* and *A. eutrophus* H16 were 64.7, 76.1, and 56.6%, respectively.

Index Entries: polyhydroxyalkanoate; polyhydroxybutyrate; nucleotide sequence; *Pseudomonas acidophila*.

INTRODUCTION

A large variety of bacteria accumulate polyhydroxyalkanoates (PHAs) in their cells under nutrient-limited conditions. PHAs are synthe-

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sized as a carbon and energy storage compound or as a sink for reducing equivalents *(1,2)*. Since these polyesters are thermoplastic and biodegradable in natural environments, they are of interest to the chemical industry for the biotechnological production of PHAs for various applications *(3)*. Today, PHA production from renewable feedstock is becoming prevalent. In particular, CO_2, which is increasing in the atmosphere and causing the greenhouse effect, is seen as a promising carbon source for PHA production. It will be very useful if a usable product that harmonizes with the environment can be produced from an environment-damaging material.

H_2-oxidizing bacteria, which are autotrophs, grow with CO_2 as a sole carbon source using H_2-oxidizing energy. H_2-oxidizing bacteria are rapid growers and reach a high cell concentration under chemolithoautotrophic conditions *(4)*, characteristics that make these bacteria excellent candidates for use as CO_2 utilizers/fixers/consumers. A cluster of genes from the H_2-oxidizing bacterium *Alcaligenes hydrogenophilus* encoding its chemolithoautotrophic growth ability was cloned in vivo using a transferable R-plasmid, R68.45, as a cloning vector *(5)*. The ability to grow chemolithoautotrophically was transferred to a Gram-negative bacterium, *Pseudomonas acidophila*, using the recombinant plasmid pFUS *(6)*. *P. acidophila*, which accumulates PHA copolymers from low-carbon-number organic compounds such as formate and acetate, could grow under chemolithoautotrophic conditions as a consequence of the possession of pFUS, and synthesized PHA copolymers from CO_2 *(7)*. This result is considered very significant because PHA production from CO_2 by H_2-oxidizing bacteria had previously been restricted to the homopolymer poly(3-hydroxybutyric acid) (PHB), which is one of the PHAs *(8)*. PHA copolymers are worth producing because they can confer distinct properties on polyesters *(9)*.

In our previous study, a gene library of *P. acidophila* genomic DNA was constructed and the *pha* genes were cloned to obtain more information on PHA production from CO_2 *(7)*. In the present work, the nucleotide sequences of the *pha* genes were determined and the three structural genes of the PHA synthetic pathway *(phaA, phaB,* and *phaC)* were analyzed.

MATERIALS AND METHODS

Bacterial Strain and PHA Accumulation Conditions

PHA synthesis was carried out by a two-step cultivation of recombinant *Escherichia coli* JM109 carrying the *pha* genes from *P. acidophila* IFO13774. Recombinant cells were first grown in Luria-Bertani (LB) broth under air at 37°C overnight. The cells were then harvested and washed twice with sterilized water. To promote PHA synthesis, cells were inoculated into 300 mL of a nitrogen-free mineral salts medium *(10)* supplemented with a carbon source at 1% (w/v), at an initial cell concentration of 1 optical density at 660 nm. Cultivation was carried out aerobically at

37°C for 48 h. Ampicillin was added to the medium at a final concentration of 100 μg/mL for the maintenance of the plasmid.

Analysis of PHA

The polymer was isolated from lyophilized cells, and the composition of bacterial PHA was determined by NMR analysis as described previously *(7)*.

Transformation

For transformation, *E. coli* was cultivated in LB broth containing 20 mM each $MgCl_2$ and $MgSO_4$ at 37°C. Competent cells were prepared and transformed by the calcium chloride procedure *(11)*.

Nucleotide Sequence Analysis

DNA sequencing was performed by the dideoxy-chain-termination method of Sanger et al. *(12)* with alkaline-denatured double-stranded plasmid DNA *(13)* and with [α-^{32}P]dCTP using a Δ Tth polymerase DNA sequencing PRO kit (Toyobo, Japan) according to the manufacturer's protocol. Subclonings were performed by standard procedures *(11)*. Deletion mutants were prepared using a kilosequence deletion kit (Takara Shuzo, Japan).

Analysis of Nucleotide and Amino Acid Sequences

Nucleic acid sequence data and deduced amino acid sequences were analyzed with the Genetyx-Mac program (Software Development, Japan). Homology searches were performed using the Genbank (release 3/96) database.

RESULTS

Subcloning of *pha* Genes

In our previous study, a gene library of *P. acidophila* IFO-13774 genomic DNA was constructed using the cosmid vector pVK102. A recombinant cosmid, pIK7, containing a 14.8-kb *Hind*III fragment, was selected by heterologous complementation of a PHB-negative mutant, *A. eutrophus* PHB-4, which lacked active PHB synthase *(7)*. The 14.8-kb *Hind*III fragment was hybridized with a probe containing the *phbA*, *phbB*, and *phbC* genes from *A. eutrophus* H16. The 14.8-kb *Hind*III fragment was digested with *Eco*RI, and a 7.4-kb *Eco*R1-*Hind*III fragment that was hybridized with the probe was subcloned using plasmid pUC19 as a vector. The resulting recombinant plasmid pEH74 contained a sequence of three *Sal*I fragments, of 0.8,

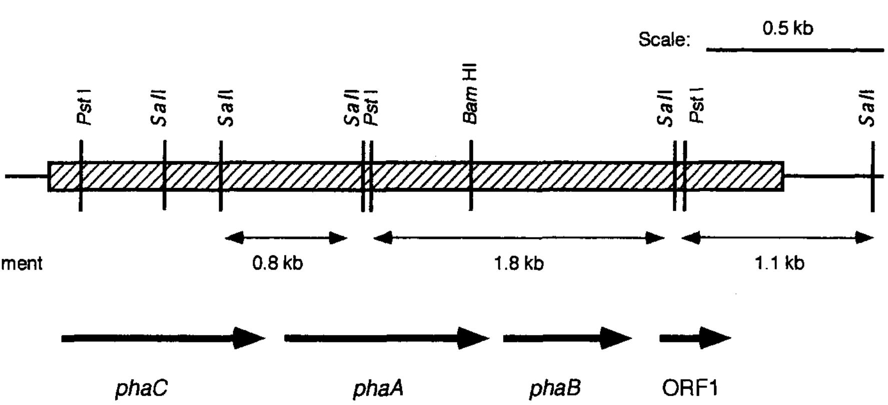

Fig. 1. Physical map of the *P. acidophila pha* gene locus and adjacent region in pEH74. The nucleotide sequence of the shaded region is given in Fig. 2. The locations and directions of the β-ketothiolase *(phaA)*, acetoacetyl-coenzymeA reductase *(phaB)*, and PHA polymerase genes *(phaC)* are indicated by arrows.

1.8, and 1.1-kb, which were hybridized with the probe (Fig. 1). For the sequencing of this region, subclonings of two of the *Sal*I fragments (those of 0.8 and 1.1-kb) and two *Bam*HI-*Sal*I fragments derived from the 1.8-kb *Sal*I fragment digested with *Bam*HI were performed using pUC19 as a vector. If necessary, deletion mutants were constructed.

Nucleotide Sequence Analysis of *pha* Genes and Their Flanking Regions

A 4169-bp region of the locus of the *P. acidophila pha* genes was sequenced using the subfragments mentioned above (Fig. 2). Four open reading frames (ORFs) were found and were identified by homology searching. First ORF: the 1152-bp structural gene of *P. acidophila* PHA synthase *(phaC)* mapped from position 64 to 1216. It encoded for a protein of 384 amino acids, and the M_r of the putative translational product was 42,779. Second ORF: the 1152-bp structural gene of *P. acidophila* β-ketothiolase *(phaA)* running from position 1350 to 2502. It encoded for a protein of 384 amino acids, and the M_r of the putative translational product was 40,200. Third ORF: the 726-bp structural gene of *P. acidophila* acetoacetyl-CoA reductase *(phaB)* starting at position 2601 and ending at 3327. It encoded for a protein of 242 amino acids, and the M_r of the putative translational product was 25,860. Fourth ORF (ORF1): this began at position 3443 and ended at 3825. It encoded for a protein of 144 amino acids, and the M_r of the putative translational product was 16,634. ORF1 identified downstream of *phaB* was compared with known DNA sequences to establish its function. Significant DNA sequence homology to known DNA sequences was not found. The overall GC content of the 4169-bp region was 63.1% (mol/mol). The four

A

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                                                                       phaC →
    1   ATATCTAGATAGTCCAGTCGAGGTACGTATAATCCGACGCTCAACCCAGACGAGATCTCGTCAATGACGCGCGCGCGCGAGCACTCTCGCGCTAATCGGA
                                                           S/D         M  T  R  A  R  E  H  S  R  A  N  R  T

  101   CCCTGAAGTCGATCATCGAGACCCAGGGCGAGACTGCGGCAGGGATGATGAACCTGCTCGGCGACCTGCAGCGCGGCAAGATTTCGCAAACCGACGAATC
          L  K  S  I  I  E  T  Q  G  E  T  A  A  G  M  M  N  L  L  G  D  L  Q  R  G  K  I  S  Q  T  D  E  S

  201   GCAGTTCGTGGTCGGCAGGAATCGCGCTGCACCGAGGGGAGCGGTCGTCTACGAGAACGACCTGATCCAGCTGATCCAGTACAAGCCGACGAAGGAGAAG
         Q  F  V  V  G  R  N  R  A  A  P  R  G  A  V  V  Y  E  N  D  L  I  Q  L  I  Q  Y  K  P  T  K  E  K

  301   GGAGACGTGTCGAAGCATCTCATCGTGCCGCCTGCGATCAACAAGTTCTACATCCTCGATCTGCACGCGAAAATTCCGCGGTCCGAACGACGCTGTCGTC
        G  D  V  S  K  H  L  I  V  P  P  A  I  N  K  F  Y  I  L  D  L  H  A  K  I  P  R  S  E  R  R  C  R  R

  401   GCGGCCACCAGGTGTTCCTCGTGTCGTGGCGCACAGCGGACGCATCGGTCGCCCACAAGACCTGGGACGACTACGATGAACGAAGGCATCGTGGCACGCG
          G  H  Q  V  F  L  V  S  W  R  T  A  D  A  S  V  A  H  K  T  W  D  D  Y  D  E  R  R  H  R  G  T  R

  501   AGTCGATGCTGTGCAGCAGGTCAGCGGCTCCGACGAGATCAACACCGTCGGCTTCTGCGTCGGCGGCACGATGCTGCGACCGCTCTCGGTGCTTGCGGCG
         V  D  A  V  Q  Q  V  S  G  S  D  E  I  N  T  V  G  F  C  V  G  G  T  M  L  R  P  L  S  V  L  A  A

  601   CGCGGCGAGCATCCGGCGTCGATGACGCTGCTCACGGCGATGCTCGACTTCTCCGATACGGGCGTCGTCGACGTATTTGTGGACGAGGAACGAGAACGTG
        R  G  E  H  P  A  S  M  T  L  L  T  A  M  L  D  F  S  D  T  G  V  V  D  V  F  V  D  E  E  R  E  R  V

  701   TTCGCGAACTCGACGCCGCGCATCAGCCCGGGCGGCGTACCGTTCTTGCCGCCGATCGTCTGTTCGCGCATCTGCACGTGCGCCTCGTCGACAAATACGT
          R  E  L  D  A  A  H  Q  P  G  R  R  T  V  L  A  A  D  R  L  F  A  H  L  H  V  R  L  V  D  K  Y  V

  801   GGACAACTACCTGAAGGGCAGCACGCCGGCGCCGTTCGACCTGCTGTACTGGAACAGCGACTCGACGAACCTGCCTGGCCCGATGTACGCGTGGTACCTG
         D  N  Y  L  K  G  S  T  P  A  P  F  D  L  L  Y  W  N  S  D  S  T  N  L  P  G  P  M  Y  A  W  Y  L

  901   CGCAATACCTATCTCGAGAACCGGCTGCGTGAGCCGGGCGGGCTGACCGTGTGCGGCGAAGCGGTCGACCTGTCGCTGATCGACTTGCCGACGTTCATCT
        R  N  T  Y  L  E  N  R  L  R  E  P  G  G  L  T  V  C  G  E  A  V  D  L  S  L  I  D  L  P  T  F  I  Y

 1001   ACGGCTCGCGCGAGGATCACATCGTGCCGTGGCAGACGGCCTACGCATCGACGTCGATCCTGACGGGCCCGCTGAAGTTCGTGCTGGGCGCGTCGGGCCA
          G  S  R  E  D  H  I  V  P  W  Q  T  A  Y  A  S  T  S  I  L  T  G  P  L  K  F  V  L  G  A  S  G  H

 1101   CATCGCCGGCGTGGTCAATCCGCCGCGAAACAGAAGCGCACGTACTGGCGTCAACGACGACAGCCTGCCCGATCGGCCGACGACTGGCCCGCCGGCCCGA
         I  A  G  V  V  N  P  P  R  N  R  S  A  R  T  G  V  N  D  D  S  L  P  D  R  P  T  T  G  P  P  A  R

 1201   CCGAGCAGCCGCAGCTGATGCGCTGATCGATGCTGATCAGCGGCCGAGTCTGCGACGGCATCGTCGGACAGTCGTGTCGAGGGCGCTACGTCACGATGAC

                                                         phaA →
 1301   GCAGGATCTGCATTTACAGCGGACGATCCTTCAGCCGATCCGAGAAGCAATGACGGACGTAGTGATCGTATCGGCCGGACCGGTCGGTAAATTCGGCGGC
                                                 S/D     M  T  D  V  V  I  V  S  A  G  P  V  G  K  F  G  G

 1401   ACCTGCGAAGATCGCGGCGCCGGAGCTGGGCCGATGGTGATCCGCGCGGTGCTGGAGCGCGCGGGCGTGAAGCCGGAGCAGGTGAGCGAAGTGATCCCTG
        T  C  E  D  R  G  A  G  A  G  P  M  V  I  R  A  V  L  E  R  A  G  V  K  P  E  Q  V  S  E  V  I  P  G

 1501   GCGGCCGGCTCGGCCAGAACCCGGCGCCGCAGTCGCTGATCAAGGCCGGGCTGCCGAGCGCGGTGCCGGGGATGACGATCAACAAGGTGTGCGGGTCGGG
          G  R  L  G  Q  N  P  A  P  Q  S  L  I  K  A  G  L  P  S  A  V  P  G  M  T  I  N  K  V  C  G  S  G

 1601   CCTGAAGGCGGTGATGCTGGCGCGAACGGCGATCATTGCCGGCGAGGCGGACATCGTGATTGCGGGCGGGCAGGAGAACATGAGCGCGGCGCGACGTGCT
         L  K  A  V  M  L  A  R  T  A  I  I  A  G  E  A  D  I  V  I  A  G  G  Q  E  N  M  S  A  A  R  R  A

 1701   GCCGGGTCGCGCAACGGGTTCCGGATGGGCGACTCGAAGCTGGTCGACACGATGATCGTAGACGGGCTGTGGGACGTGTACAACCAGTACCACATGGGAA
        A  G  S  R  N  G  F  R  M  G  D  S  K  L  V  D  T  M  I  V  D  G  L  W  D  V  Y  N  Q  Y  H  M  G  I

 1801   TCACGGCGGAGAACGTCGCGAAGGAATACGGGATCACGCGCGAGGAGCAGGACGCATTTGCGCGCTGTCGCAGAACAAGGCGGAAGGGCGCAGAAGGCGG
          T  A  E  N  V  A  K  E  Y  G  I  T  R  E  E  Q  D  A  F  A  R  C  R  R  T  R  R  K  G  A  E  G  G

 1901   GCGCTTTAACGACGAGATCGTTCCGGTTGGCGATCCCGCAGAAGAAGGGCGAGCCGCTGCAGTTCGCGACCGACGAGTTCGTACACGGCGTGACGGCGGA
         R  F  N  D  E  I  V  P  V  G  D  P  A  E  E  G  R  A  A  A  V  R  D  R  R  V  R  T  R  R  D  G  G

 2001   CGGGCTGGCGGGCTGAAGCCGGCGTTCGCGAAGGACGGCACGGTGACGGCGGCGAACGAGTCGGGGCTCAACGACGGACTCAACACGTCCGACGACGTAT
        R  A  G  G  L  K  P  A  F  A  K  D  G  T  V  T  A  A  N  E  S  G  L  N  D  G  L  N  T  S  D  D  V  S
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Fig. 2. Nucleotide sequence of the *P. acidophila pha* gene locus and the adjacent region. Amino acids deduced from the nucleotide sequence of the tentative genes are specified by the standard one-letter abbreviations. Putative ribosome binding sites (Shine-Dalgarno sequences, S/D) are underlined.

ORFs were preceded by tentative ribosome-binding sites upstream of the respective ATG start codons. These data show that the three enzymes of the *P. acidophila* PHA synthetic pathway are encoded by the three genes organized as *phaC-phaA-phaB*, as illustrated in Fig. 1.

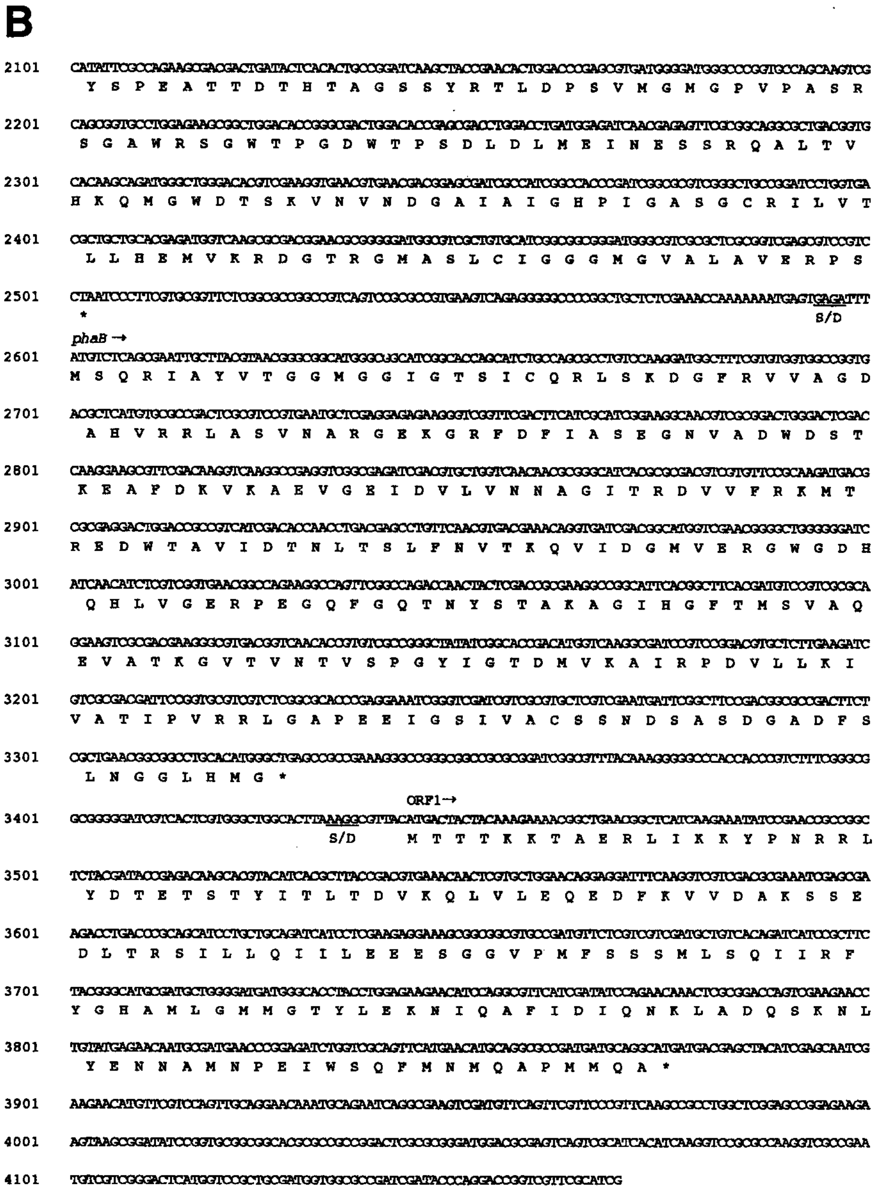

Fig. 2. *(continued)*

Comparison of PHA Polymerase, β-Ketothiolase, Acetoacetyl-CoA Reductase, and ORF1 Product

The deduced amino acid sequences of the *phaC*, *phaA*, *phaB*, and ORF1 genes from *P. acidophila* were compared with those from other microorganisms.

In an alignment of the sequences, the *pha*C product from *P. acidophila* showed 20.5 to 56.6% homology with the PHA polymerases from *A. eutro-*

phus (14), Methylobacterium extorquens (15), Rhodococcus ruber (16), Pseudomonas oleovorans (1 and 2) *(17), Pseudomonas aeruginosa* (1 and 2) *(18), Chromatium vinosum (19),* and *Thiocystis violacea (20)* (Fig. 3).

The deduced amino acid sequence of the *phaA* gene from *P. acidophila* exhibited 64.7, 47.3, and 51.9% homology with the β-ketothiolases from *A. eutrophus (21), Zoogloea ramigera (22),* and *C. vinosum (19),* respectively (Fig. 4).

The deduced amino acid sequence of the *phaB* gene from *P. acidophila* was 76.1, 46.3, and 48.8% homologous with the acetoacetyl-CoA reductases from *A. eutrophus (21), Z. ramigera (22),* and *C. vinosum (19),* respectively (Fig. 5).

The deduced amino acid sequence of ORF1 of *P. acidophila* was compared with those of ORF4 of *C. vinosum (19)* and *T. violacea (20)* (Fig. 6). The homology was 49.3 and 53.4% with the ORF4 sequences from *C. vinosum* and *T. violacea,* respectively.

Heterologous Expression of *pha* Genes in *E. coli*

E. coli JM109 was transformed with pEH74, which contained *phaA, phaB,* and *phaC* from *P. acidophila.* Polymer accumulation from various carbon sources was tested in the recombinant *E. coli* JM109 carrying pEH74. The *pha* genes from *P. acidophila* were expressed in *E. coli* and conferred on it the ability to synthesize polymer. The polymer content of the cells in *E. coli* varied between 2.9 and 62.1% of the cellular dry mass (Table 1). The polymer type produced by *E. coli* was PHB homopolymer with all carbon sources.

DISCUSSION

The recombinant cosmid pIK7 selected by the complementation experiment in our previous study was confirmed to contain the three structural genes of PHA synthesis. The deduced amino acid sequences of the *P. acidophila pha* genes were highly homologous with those from *A. eutrophus:* 56.6% for PHA polymerase, 64.7% for β-ketothiolase, and 76.1% for acetoacetyl-CoA reductase (Figs. 3–5).

It has been proposed that the mechanism for PHB polymerase involves two partial reactions: the formation of an acyl-S-enzyme intermediate as a first step followed by the transesterification of a primer acceptor *(23).* Two cysteine residues conserved in PHA polymerase appear to be important in acyl-S-enzyme intermediate formation and transesterification *(14).* In this study, *P. acidophila* PHA polymerase contained three cysteine residues, of which Cys165 and Cys297 were appropriate cysteine residues. Two highly conserved segments that seemed to be important for the polymerization reaction *(24)* and a sequence, NXXGXCXGG, which incorporates the lipase consensus sequence (lipase-box), were identified in *P. acidophila* PHA polymerase.

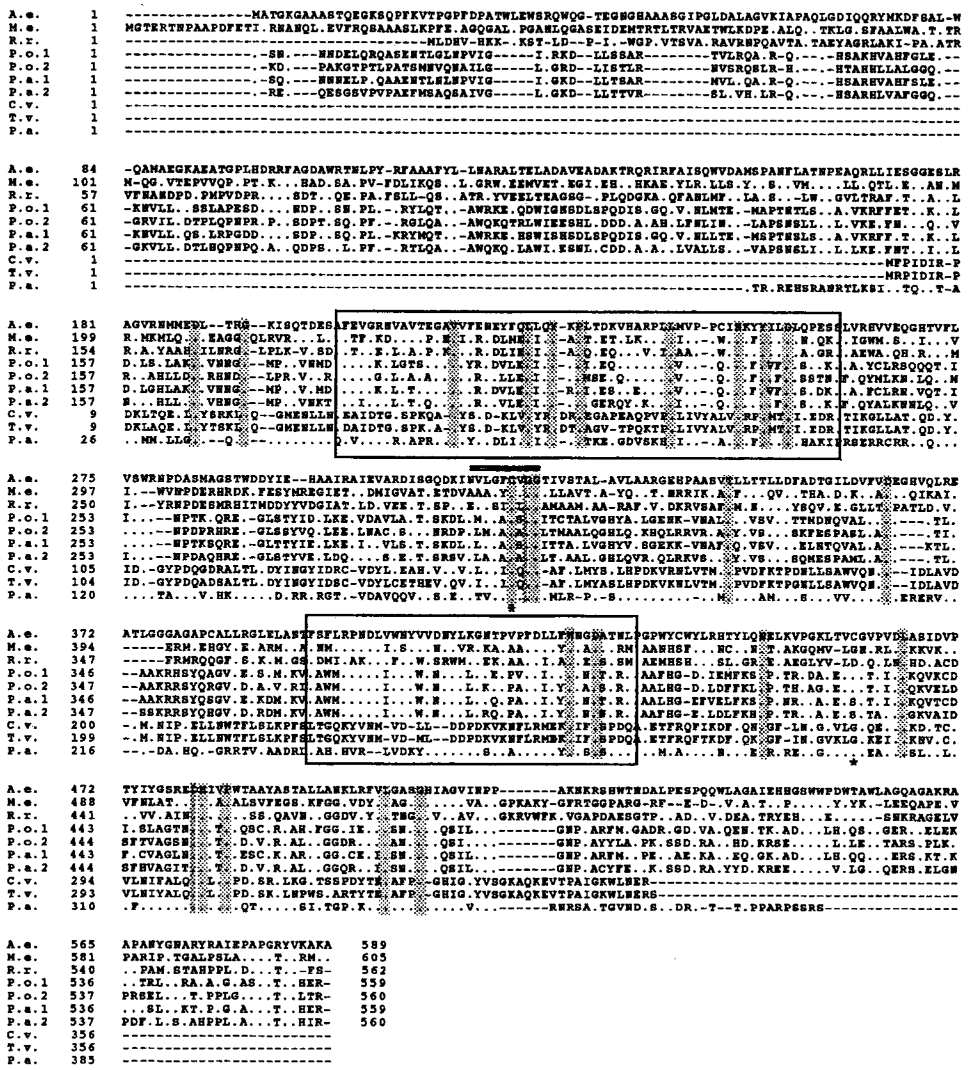

Fig. 3. Alignment of various PHA polymerases. The PHA polymerases are as follows: A. e., *A. eutrophus;* M. e., *M. extorquens;* R.r., *R. ruber;* P.o.1 and P.o.2, *P. oleovorans;* P.a.1 and P.a.2, *P. aeruginosa;* C.v., *C. vinosum;* T.v., *T. violacea;* P.a., *P. acidophila.* Dots indicate amino acids identical to the *A. eutrophus* sequence; dashes signify gaps introduced to maximize the alignment of the sequences; shading shows identical residues present in all the sequences. The cysteine residues at positions 319 and 459 in the *A. eutrophus* sequence, which have been proposed as candidates for the residues involved in the formation of an acyl-S-enzyme intermediate and in transesterification, were marked by asterisks. Lipase-box like sequences are indicated by a double line above the sequences. Highly conserved segments are boxed. Cysteine residues that have been proposed to be involved in the polymerization reaction are marked by asterisks.

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P.a.   1  MT-DVVIVSAG--PVGKFGGT-CEDRGAGAGPMVIRAVLERAGVKPEQVSEVIPGGRL----GQNPAPQSLIKAGLPSAVPGMTINKVCG
A.e.   1  ..-.......ARTA......SLAKIPAPEL.AV..K.A...............H.QV.TAGS.....R.AA......AM..A........
Z.r.   1  .STPSIVIASARTA..S.N.AFANTPAHEL.AT..S........AAGE.N...L.QV.PAGE.....R.AAM...V.QEATAWGM.QL..
C.V.   1  .SENI...D..RSAI.T...SLSSLSATEI.TA.LKGL.A.T.LA...ID...L.QV.TAGV.....R.TTLH....HS..A........

P.a.  83  SGLKAVMLARTAIIAGEADIVIAGGQENMSAARRA-AGSRNGFRMGDSKLVDTMIVDGLWDVYNQYHMGITAENVAKEYGITREEQDAFA
A.e.  90  .........AN..M..D.E..V..........PHVLP...D......A....................................A..E..
Z.r.  91  ...R..A.GMQQ.AT.D.S.IV...M.S..M.PHC-.H-LA.VK...F.MI....K...T.AFYG....T.......QWQLS.D......
C.V.  91  ......H..MQ..AC.D..........S..QSSHVLPR..D.Q....WSMK..........AF.N....T....I.QK..F...Q.....

P.a. 172  RCRRTRRKGAE-GGRFNDEIVPVGDPAEEGRAAAVR-DRRVRTRRDGGRAGGLKPAFAKDGTVTAANESGLNDGLNTSDDVSYSPEATTD
A.e. 180  VGSQNKAEA.QKA.K.DE.....LI.QRK.DPV.FKT.EF..QGATLDSMS......D.A.......A......AAAVVVM.AAKAKELG
Z.r. 179  VASQNKAEA.QKD...K.....FIVKGRK.DITV-DA.EYI.HGATLDSMAK.R...D.E.....G.A......AAAALLM.EAEASRRG
C.V. 181  AASQQKTEA.QKA...Q...I.IEI.QRK.DPKVFDA.EFP.HGTTAESL.K.R...S.D.S...G.A..I...AAMVVVMKE.KAKELG

P.a. 260  -THTAG-SSY-RT-LDPSVMGMGPVPASRSGAWRSGWTPGDWTPSDLDLMEINES-SRQALTVHKQMGWDTSKVNVNDGAIAIGHPIGAS
A.e. 270  L.PL.TIK..ANAGV..K..........K----.-ALSRAE...Q.........AFAA...A..Q............G............
Z.r. 268  IQPLGRIV.WATVGV..K...T..IPA--.--RK-ALERAG.KIG....V.A..AFAA..CA.N.DL...P.I....G............
C.V. 271  LKPM.RLVAFASAGV..AI..T..IPA--.--TK-CLEKAG...A....I.A..AFAA..MS.NQD....L......G............

P.a. 345  GCRILVTLLHEMVKRDGTRGMASLCIGGGMGVALAVERPS    384
A.e. 355  ............KR..AKK.L.................K-    393
Z.r. 353  .A...N...F..KR.GARK.L.T.........MCI.SL-    391
C.V. 356  .A.V.....Y..Q...AKK.L.T......Q........M-    394
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Fig. 4. Alignment of various β-ketothiolases. The β-ketothiolases are as follows: P.a., *P. acidophila;* A. e., *A. eutrophus,* Z. r., *Z. ramigera* C. v., *C. vinosum.* Dots indicate amino acids identical to the *P. acidophila* sequence; dashes signify gaps introduced to maximize the alignment of the sequences. The cysteine residue at position 81, which functions as the first step in the thiolase reaction, and that at position 369, which functions as the active-site base in deprotonation in the condensation reaction, are shaded.

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P.a.   1 MSQRIAYVTGGMGGIGTSICQRLSKDGFRVVAG-DAH-VRRLASVNARGEKGRFDFIASEGNVADWDSTKEAFDKVKAEVGEIDVLVNNA
A.e.   1 .T...............A.....A.........CGPNSP..EKWLEQQKAL.-................T......S....V...I...
Z.r.   1 .-S.V.L....SR...AA.SIA.KAA.YK.A.S-Y.GNDDAAKPFK-A-.T-G--IAVYKWD.SSYEACV.GIA..E.DL.P.......
C.v.   1 .-A...L....I.......T..A...CT...NCHPSEAAAAEEWKQARAAEG..IAVFTAD.SSF.DSARMVREITEQ..P..I...C.

P.a.  89 GITRDVVFRKMTREDWTAVIDTNLTSLFNVTKQVIDGMVERGWGDHQHLVGERPE-GQFGQTNYSTAKAGIHGFTMSVAQEVATKGVTVN
A.e.  90 .............A..D.....................AD....RIVNISSVNGQK.............L.....AL.............
Z.r.  84 ...K.AM.H...PDQ.N...N....G...M.HP.WS..RD.SF.RIVNISSINGQK..M..A...A....DL...KAL...G.A..I...
C.v.  90 .....KT.K..EQAH.E...NV..N.V....R..W...L...F.RIINISSVNGQR....A...A....M.....AL...G.S.......

P.a. 178 TVSPGYIGTDMVKAIRPDVL-LKIVATIPVRRLGAPEEIGSIVA-CSSNDSA-SDGADFSLNGGLHMG  242
A.e. 180 .......A........Q...-D........K...L....A..C.WL..EE.GF.T.............  246
Z.r. 174 AIC......E..R..PEK..NER.IPQ...G...E.D..AR..VFLA.DEAGFIT.STI.A...QFFV  241
C.v. 180 .I....VE.A.TL.MND..-RNS.ISG..M..MAQ.N..AAAI.FLAGDE.GYMT..NLPV....F.H  246
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Fig. 5. Alignment of various acetoacetyl-coenzymeA reductases. The acetoacetyl-coenzymeA reductases are as follows: P. a., *P. acidophila;* A. e., *A. eutrophus;* Z. r., *Z. ramigera;* C. v., *C. vinosum.* Dots indicate amino acids identical to the *P. acidophila* sequence; dashes signify gaps introduced to maximize the alignment of the sequences. The NAD(P) binding region is shaded.

The catalytically essential cysteine residues discussed for the *Z. ramigera* enzyme *(19)* were conserved at Cys81 and Cys369 in *P. acidophila* β-ketothiolase. The former would participate in the formation of the acyl-enzyme intermediate as the first step in the β-ketothiolase reaction, whereas the latter would function as an active-site base in deprotonation in the condensation reaction.

The amino acid sequence TGGXXG has been found in acetoacetyl-CoA reductases, and is thought to participate in binding the ADP moiety

Table 1
Heterologous Expression of *Pha* Genes from *P. acidophila* in *E. coli*

Carbon source	Polymer weight (mg/liter)	Polymer content (% of dry wt)
Formate	76	23.6
Acetate	67	20.5
Propionate	18	5.4
Malate	18	4.9
Arabinose	46	14.2
Succinate	132	29.7
Gluconate	83	20.7
Glucose	184	41.9
Heptanoate	10	2.9
Octanoate	59	19.3
Dodecanoate	205	62.1

E. coli JM109 carrying recombinant plasmid pEH74 was used. For the polymer accumulation conditions, see MATERIALS AND METHODS.

```
P.a.   1  MTTTKKTAERLIKKYPNRRLYDTETSTYITLTDVKQLVLEQEDFKVVDAKSSEDLTRSIL
C.v.   1  .----N-S..I.............V.R....A..RD..MSGQP.R.L.SANDS.I.....
T.v.   1  .----N-SD.I.............V.R....A..RN..MDCTS.....TANES.I.....

P.a.  61  LQIILEEESGGVPMFSSSMLSQIIRFYGHAMLGMMGTYLEKNIQAFI-DIQ--NK-LADQ
C.v.  56  ...M....T..Q.L..AN..A.......GTL..TFAR...SSLDL.AKQQ.EVT.A.T.N
T.v.  56  ...M....T..E.L..A...A.......GTL...FAR...SSLDL.AKQQ.DMT.T.G.N

P.a. 117  S-KNLYENNAMNPEIWS--Q--FM---NMQ-AP-MMQA 144
C.v. 116  PFGTVTRLTQK.V...ADL.DEL.RAAGFPV..RKKKE 153
T.v. 116  PFEAMTRMTQK.V...ADM.EE------F--------- 138
```

Fig. 6. Alignment of the deduced amino acid sequences of ORF1 of *P. acidophila* and ORF4 from *C. vinosum* and *T. violacea*. Dots indicate amino acids identical to *P. acidophila*; dashes signify gaps introduced to maximize the alignment of the sequences. Highly conserved segments are boxed.

of NAD (25). This sequence was found near the N-terminus of *P. acidophila* acetoacetyl-CoA reductase.

ORF1-PHB-binding proteins are divided into 4 groups (26): PHA polymerase, intracellular PHA depolymerase, a protein called phasin that stabilizes the structure of PHA, and other proteins. The surface of the PHA granule is considered to be covered with a phospholipid, but the amount is not sufficient to cover the surface perfectly. Phasin seems to partially complement the phospholipid deficiency. The deduced amino acid sequence of ORF1 from *P. acidophila* was compared with the ORF4 sequences from *C. vinosum* and *T. violacea*, the functions of which have not been clarified. They showed high homology, and a highly conserved segment was observed in all the sequences. Further studies are necessary to determine whether these ORFs are phasin-encoding ORFs.

In *A. eutrophus*, three *pha* genes are organized in one operon (*pha*C-*phaA-phaB*) (14). The transcription start point is mapped 307-bp upstream from the translational initiation point of the *phbC* gene (27). The −35 region (TTGACA) and the −10 region (AACAAT) identified directly upstream of the transcription start site of *phbC* were identical (TTGACA) or very similar (TATAAT) to the corresponding sequences of the *E. coli* σ^{70} consensus promoter sequences (28). The order of the *pha* genes in *P. acidophila* was *phaC-phaA-phaB*, as it is in *A. eutrophus*. No promoter-like sequence was detected in the 4169-bp of the *P. acidophila pha* locus. The length of the upstream region of *phbC* analyzed was approx 50-bp. It might be necessary to conduct further sequencing to detect the promoter of the *pha* genes. In *A. eutrophus*, the expression of all three genes of the pathway (*pha*C-*phaA-phaB*) in *E. coli* results in the accumulation of significant levels of PHB in this bacterium. The expression of *phbC* alone in *E. coli* produces neither PHB nor significant levels of PHB polymerase activity (14). In this study, *E. coli* carrying pEH74 produced considerable amounts of PHB (2.6–62.1%) (Table 1). It is probable that the 7.4-kb *Eco*RI-*Hin*dIII fragment inserted in pEH74 contains the promoter that works for the effective expression of the three *pha* genes in *E. coli*.

P. acidophila has novel characteristics that enable it to produce PHA copolymers from CO_2. However, the deduced amino acid sequences of PHA synthetic enzymes were not specific to the bacterium, but similar to those reported for other PHB-producing bacteria. In heterologous expression in *E. coli* and *A. eutrophus* (7), the products were PHB homopolymer. Future studies will show the factors affecting the polymer type.

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