(54) Title: PRODUCTION OF POLYALKANOATE

(57) Abstract

Genes encoding polyhydroxyalkanoate synthase, β-ketothiolase and acetoacetyl CoA reductase are isolated from the publicly available bacterium \textit{Chromatium vinosum}. Recombinant genomes of plants or other species of bacteria which contain these genes are capable of producing polyalkanoate polymers. The nucleotide sequences of the said three genes have been determined.
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PRODUCTION OF POLYALKANOATE

This invention relates to the production of polyhydroxyalkanoate by the culture of microorganisms which produce same.

Poly-3-hydroxybutyrate is a linear polyester of D(-)-3-hydroxybutyrate. It was first discovered in Bacillus megaterium in 1925. Polyhydroxybutyrate accumulates in intracellular granules of a wide variety of bacteria. The granules appear to be membrane bound and can be stained with Sudan Black dye. The polymer is produced under conditions of nutrient limitation and acts as a reserve of carbon and energy. The molecular weight of the polyhydroxybutyrate varies from around 50,000 to greater than 1,000,000, depending on the microorganisms involved, the conditions of growth, and the method employed for extraction of the polyhydroxybutyrate. Polyhydroxybutyrate is an ideal carbon reserve as it exists in the cell in a highly reduced state, (it is virtually insoluble), and exerts negligible osmotic pressure.

Polyhydroxybutyrate and related poly-hydroxyalkanoates, such as poly-3-hydroxyvalerate and poly-3-hydroxyoctanoate, are biodegradable thermoplasics of considerable commercial importance.

The term "polyhydroxyalkanoate" as used hereinafter includes copolymers of polyhydroxybutyrate with other polyhydroxyalkanoates such as poly-3-hydroxyvalerate.
Polyhydroxyalkanoate is biodegradable and is broken down rapidly by soil microorganisms. It is thermoplastic (it melts at 180°C) and can readily be moulded into diverse forms using technology well-established for the other thermoplastics materials such as high-density polyethylene which melts at around the same temperature (190°C). The material is ideal for the production of biodegradable packaging which will degrade in landfill sites and sewage farms. The polymer is biocompatible, as well as biodegradable, and is well tolerated by the mammalian, including human, body, its degradation product, 3-hydroxybutyrate, is a normal mammalian metabolite. However, polyhydroxyalkanoate degrades only slowly in the body and its medical uses are limited to those applications where long term degradation is required.

Polyhydroxyalkanoate, produced by the microorganism Alcaligenes eutrophus, is manufactured, as a copolymer with of polyhydroxybutyrate and polylactide by Imperial Chemical Industries PLC and sold under the Trade Mark BIOPOL. It is normally supplied in the form of pellets for thermoprocessing. However, polyhydroxyalkanoate is more expensive to manufacture by existing methods than, say, polyethylene. It is, therefore, desirable that new, more economic production of polyhydroxyalkanoate be provided.

An object of the present invention is to provide materials and a method for the efficient production of polyhydroxyalkanoate.
According to the present invention there is provided gene fragments isolated from the bacterium *Chromatium vinosum* and encoding PHA polymerase, acetoacetyl CoA reductase and \( \beta \)-ketothiolase.

Preferably the *C. vinosum* is of the strain designated D, available to the public from the Deutsche Sammlung für Mikroorganismen under the Accession Number 180.

The invention also provides a 16.5kb EcoR1 fragment of *C. vinosum* DNA, designated PP10, hybridizable to a 5.2kb SmaI/EcoR1 fragment, designated SE52 isolated from *Alcaligenes eutrophus* and known to contain all three of said genes responsible for expression of PHAs.

The invention further provides a fragment of the said PP10 fragment, designated SE45, encoding the PHA-synthase and \( \beta \)-ketothiolase genes and a region, designated SB24, encoding the acetoacetyl CoA reductase gene.

The invention also provides a recombinant genome of a microorganism, preferably a bacterium or a plant, which contains one or more of said fragments designated PP10, SE45 and region SB24.

Finally, the invention provides a method for the manufacture of PHAs, comprising culturing the microorganism *Chromatium vinosum*, or a bacterium of a different species having stably incorporated within its genome by transformation one or more PHA synthesising genes from *Chromatium vinosum*.

The biosynthesis of polyhydroxyalkanoate from the substrate, acetyl-CoA involves three enzyme-catalyse steps.

The three enzymes involved are \( \beta \)-ketothiolase, acetoactyl-CoA-reductase and polyhydroxy-
butyrate-synthase, the genes for which have been cloned from *Chromatium vinosum*. The three genes are known to facilitate production of polyhydroxyalkanoates, the reactions involved being represented as follows:

\[
\begin{align*}
\ce{O &+ \beta-ketothiolase} \\
\ce{C & C & C & C} \\
\ce{H3C & SCoA & H3C & SCoA} & \rightarrow & \ce{H3C & CH2 & SCoA} \\
\ce{NADPH} & \text{NADP-linked} \\
\ce{Acetoacetyl-CoA Reductase} \\
\ce{NADP} \\
\ce{CH3 & O} & \text{Polyhydroxybutyrate-} \\
\ce{\text{|}} & \text{synthase} \\
\ce{CH & C} & \text{[CH3 O CH3 O} \\
\ce{\text{|}} & \text{CH C CH C} \\
\ce{\text{|}} & \text{CH2 O CH2 O} \\
\text{\text{n}} & \text{.}
\end{align*}
\]
The invention will now be described with reference to the accompanying drawings, of which;

Figure 1 is the physical map of the 16.5 kb EcoR1 fragment of *Chromatium vinosum* DNA, designated PP10. The positions of the restriction sites and positions and names of the sub-fragments are shown. PHA-synthase and β-ketothiolase genes are located in fragment SE45 and acetoacetyl CoA reductase in region SB24;

Figure 2 is the map of PP10 showing the positions of the β-ketothiolase and acetoacetyl CoA reductase genes and of the PHA-synthase gene open reading frames ORF2 and ORF3.

Figure 3 is the complete nucleotide sequence of fragment SE45. The transcriptional start sites and terminators for the β-ketothiolase gene and for ORF3 and ORF3 are shown. The positions of the "-10" and "-35" sequences are also shown, as are the positions of the putative ribosome binding sites ("s/d"). Translational start and stop (*) codon are also marked and the amino acid sequences of the β-ketothiolase, ORF2 and ORF3 are given.

Figure 4 shows the alignment of the amino acid sequences of *Chromatium vinosum* ORF3 with PHA polymerase of *Alcaligenes eutrophus* and PHA polymerases 1 and 2 of *Pseudomonas oleovorans*.

Figure 5 shows the complete nucleotide sequence of the DNA encoding PHA synthesis genes from *Chromatium vinosum*. The positions of PHA polymerase (phbC), acetoacetyl CoA reductase (phbB) and ketothiolase (phbA) genes are shown, and also the positions of ORF2, ORF4, ORF5 and ORF7.

Figure 6 shows the alignment of the amino acid sequences of ketothiolases encoded by *C.vinosum*. 
(C.v.), *A. eutrophus* (A.e.), *Zoogloea ramigera* (Z.r.), *Escherichia coli* (E.c.), *Saccharomyces uvarum* (S.u.) and *Rattus norvegicus* (R.n.).

Figure 7 shows the alignment of the amino acid sequences of acetoacetyl CoA reductases encoded by *C.vinosum* (C.v.), *A.eutrophus* (A.e.) and *Z.ramigera* (Z.r.).

Figure 8 is a Table (Table 1) showing the heterologous expression in *Escherichia coli* of DNA fragments from *C.vinosum*. Activities of PHA biosynthetic enzymes expressed by the different fragments are shown. The levels of PHA accumulated in *E.coli* transformed with the fragments are also given.

**EXAMPLE**

The organism *C.vinosum* was a gift from Dr J. Imhoff of the University of Bonn, Germany.

1. Isolation of DNA fragments from *C.vinosum* encoding PHA synthesis genes

A 5.2 kb *SmaI/EcoRI* fragment (SE52), which codes for all three PHA biosynthetic genes has previously been isolated from *Alcaligenes eutrophus* [Schubert et al., *J. Bacteriol.* 170 (1988)]. This fragment was used to detect PHA biosynthetic genes of *C.vinosum*. *EcoRI* restricted genomic DNA of *C.vinosum* was blotted on to a nylon membrane and hybridized with biotinylated SE52 DNA. One signal appeared, representing a DNA fragment of 16.5 kb.

A λL47 gene bank from *C.vinosum* genomic DNA was prepared and plates with approximately 800 plaques were blotted on to nylon membranes and hybridized with biotinylated SE52 DNA. One positive recombinant phage was isolated, which harboured a 16.5 kb *EcoRI* fragment, which was
designated PP10 (Figure 1). With PP10 and a 9.4 kb EcoRI/PstI subfragment (EP94) of PP10, the phenotype of the wild type could be restored in PHA-negative mutants of A. eutrophus.

Expression studies in E. coli (see below) showed that a 4.5 kb SmaI/EcoRI (SE45) subfragment of EP94 encodes for PHA synthase and β-ketothiolase. The nucleotide sequence of this fragment was determined by the dideoxy-chain termination method of Sanger et al. with alkaline denatured double stranded plasmid DNA. The T7-polymerase sequencing kit of Pharmacia, Uppsala, Sweden, was used with 7-deazaguanosine-5'-tri-phosphate instead of dGTP. Most of the sequence was determined with a set of unidirectional overlapping deletion clones generated by exonuclease III digestion. For sequencing regions which were not covered by the deletion plasmids synthetic oligonucleotides were used.

It was not possible to clone the 4.9 kb SmaI/PstI fragment PS49 in a multi-copy vector. Therefore, fragment EP94 (Figure 1) was treated with Exonuclease Bal31, ligated to Bluescript SK and transferred to E. coli X1-1 Blue. A clone was isolated which harboured Bluescript SK with a 5.5 kb fragment (B55) and which expressed β-ketothiolase and NADH-dependent reductase activity. 3146 base pairs of B55 were part of the SE45 fragment. The other part (approximately 2350 base pairs, SB24) has been sequenced applying the primer hopping strategy. The sequence and the position of the reductase gene on SB24 are known. The results of these studies, including the organisation of the PHA biosynthetic genes in
C. vinosum and the sites of the ketothiolase, reductase and PHA synthase genes are shown in Figure 2. The determination of the full sequence of SB24 is in progress.

2. Sequence analysis of the C. vinosum PHB

Synthetic Genes

The nucleotide sequence of SE45 is shown in Figure 3. The fragment size of SE45 is 4506 bp.

2.1 PHB synthase

The fragment sequence corresponding to the PHB synthase gene is designated as ORF3. The determination of synthase activity of deleted plasmids containing SE45 (see below) gave evidence that expression of ORF2 is also required for expression of synthase activity.

ORF2 and ORF3 are transcribed as an operon. The determination of the transcription start site of ORF2 was conducted by S1 nuclease mapping. This site occurs at bp 3059 from the 3' end of SE45. A putative "-10" site, given as 5'–ACAGAT–3' occurs at bp 3073–3078, and a "-35" site occurs at bp 3092–3099. A putative ribosome binding site occurs at bp 3040–3045. The translation start codon commences at bp 3030. The translation stop codon occurs at bp 1958.

The putative ribosome binding site of ORF3 occurs at bp 1907–1911. The translation start ATG for ORF3 occurs at bp 1899, and the translation stop codon at bp 833. Putative transcriptional terminator sites occur at hairpin structures at bp 773–786 and 796–823.

ORF2 encodes a polypeptide of 358 amino acids with a MW of 40525 da. ORF3 encodes a polypeptide of 356 amino acids with a MW of 39739 da. The gene
size of ORF3 is approximately 30% smaller as compared with the PHA polymerase genes of
A. eutrophus and P. oleovorans. The alignments of the primary structures of C. vinosum PHA polymerase,
A. eutrophus PHA polymerase and P. oleovorans PHA polymerases 1 and 2 are shown in Figure 4. Thus
the ORF3 C. vinosum polymerase is shorter than the other polymerase enzymes, lacking the first 172
amino acids from the NH₂ terminus of the
A. eutrophus PHA polymerase, and the first 148 amino
acids of the Pseudomonas polymerases. The amino
acid sequence of ORF3 exhibited an overall homology
of 25% to the polymerase of A. eutrophus, with
certain discrete regions of conserved sequence.
The amino acid sequence of ORF2 showed no
significant homology to other enzymes in the NBRF
gene bank.

2.2 β-ketothiolase

The β-ketothiolase and acetoacetyl CoA
reductase genes are transcribed in opposite
direction to ORF2 and ORF3 (Figure 2). A "-10"
site in the identified ketothiolase promoter occurs
at bp 3105-3111, and a "-35" site at bp 3082-3086.
A putative ribosome binding site occurs at bp
3167-3171. The translation start signal occurs at
bp 3181. The translation stop codon occurs at bp
4361.

The alignments of the primary structures of β
eketothiolases from Chromatium vinosum and other
sources are shown in Figure 5. Considerable
homology is apparent between the amino acid
sequences of ketothiolases from C. vinosum and other
bacterial and non-bacterial sources.
2.3 **Acetoacetyl CoA reductase**

The alignments of the primary structures of acetoacetyl CoA reductases from *C.vinosum*, *A.eutrophus* and *Z.ramigera* are shown in Figure 6. All three reductases are of similar chain length, while considerable homology is apparent between the sequences of reductases from these bacteria.

The *Chromatium vinosum* PHA synthetic genes therefore differ from the PHA synthetic genes of *A.eutrophus* and *P.oleovorans* in the following respects:

i) Whereas *A.eutrophus* PHB polymerase, acetoacetyl CoA reductase and β ketothiolase genes are all transcribed as an operon, in *C.vinosum* the ketothiolase and reductase genes are transcribed separately from the polymerase, and are transcribed in the opposite direction to the polymerase ORF3 and ORF2 genes.

ii) In contrast to *A.eutrophus*, where one gene product is required for polymerase activity, in *C.vinosum* two gene products, represented by ORF2 and ORF3 are required for expression of polymerase activity.

iii) The *C.vinosum* ORF3 polymerase is 172 amino acids shorter, at the amino terminus, than the *A.eutrophus* polymerase, and 148 amino acids shorter than the *P.oleovorans* polymerases 1 and 2. The *C.vinosum* ORF3 shows only 25% homology with the primary sequence of the *A.eutrophus* polymerase.

iv) The *A.eutrophus* acetoacetyl CoA reductase enzyme involved in PHB synthesis is NADPH specific, while the *C.vinosum* enzyme exhibits a marked preference for NADH.

Between the structural genes for ketothiolase
and acetoacetyl CoA reductase of *Chromatium vinosum*, two open reading frames (ORF4 and ORF5) appear, and downstream from the reductase gene an ORF7 has been identified (Figure 5). No additional ORFs were identified in the PHA coding region of *A.eutrophus*.

3. Expression of *C.vinosum* PHB synthetic genes in other bacteria.

With fragments PP10 and EP94 the ability to synthesise PHB could be restored to PHB negative mutants of *A.eutrophus*. Recombinant strains of the PHB negative mutant *A.eutrophus* PHB-4, transformed with these fragments, were able to synthesise polymers containing 3-hydroxybutyrate and 3-hydroxyisovalerate at significant proportions, when supplied with appropriate substrates.

Studies on expression of *C.vinosum* DNA fragments in *E.coli* are presented in Table 1. Thus *E.coli* transformed with plasmids containing fragments PP10 and EP94 expressed PHB polymerase, acetoacetyl CoA reductase and β ketothiolase activities. They also synthesised PHB up to between 10 and 12% dry weight. *E.coli* transformed with plasmids containing fragment SE45 expressed PHB polymerase and β ketothiolase, but not acetoacetyl CoA reductase, and were unable to synthesise PHB.

4. Polymer Biochemistry

The specific optical rotations of methyl 3-hydroxybutyric acid liberated by methanolysis of PHB from *C.vinosum* (accumulated from acetate), from *A.eutrophus* PHB-4 pH1014::PP10 (accumulated from fructose) and *E.coli* S17-1 pSUP202::PP10 (accumulated from glucose) were all negative. The
determined values of the specific optical rotation were similar to those for PHB isolated from *A. eutrophus* (accumulated from fructose).
CLAIMS

1. Gene fragments isolated from the bacterium *Chromatium vinosum* and encoding polyhydroxy-alkanoate (PHA) synthase, acetoacetyl CoA reductase and β-ketothiolase.

2. Gene fragments as claimed in claim 1 in which the *Chromatium vinosum* is of the strain designated D, available to the public from the Deutsche Sammlung für Mikroorganismen under the Accession Number 180.

3. A 16.5kb EcoRI fragment of *Chromatium vinosum* DNA, designated PP10, hybridizable to a 5.2kb SmaI/EcoRI fragment, designated SE52 isolated from *Alcaligenes eutrophus* and known to contain the genes encoding PHA-synthase acetoacetyl CoA reductase and β-ketothiolase.

4. A fragment of the PP10 fragment claimed in claim 3, designated SE45, encoding the PHA-synthase and β-ketothiolase genes.

5. A fragment of the PP10 fragment claimed in claim 3, designated SB24, encoding the acetoacetyl CoA reductase gene.
6. A recombinant genome which contains one or more of the fragments designated PP10, SE45 and region SB24 claimed in claims 3, 4 and 5 respectively.

7. A bacterium having incorporated in its genome one or more of the fragments designated PP10, SE45 and region SB24 claimed in claims 3, 4 and 5 respectively.

8. A plant having stably incorporated in its genome by transformation one or more of the fragments designated PP10, SE45 and region SB24 claimed in claims 3, 4 and 5 respectively.

9. A method for the manufacture of poly-hydroxyalkanoates, comprising culturing the microorganism *Chromatium vinosum*, or a bacterium of a different species having stably incorporated within its genome by transformation one or more PHA synthesising genes from *Chromatium vinosum*.

10. A gene, encoding 6-ketothiolase, having the nucleotide sequence shown in Figure 3.

11. A gene encoding polyhydroxyalkanoate synthase (phbC), having the nucleotide sequence shown in Figure 5.

12. A gene encoding acetoacetyl CoA reductase (phbB) having the nucleotide sequence shown in Figure 5.
FIG. 1

Physical map of the 16.5-kb EcoRI fragment PP10 and location of B55
Region of β-ketothiolase and reductase locus and relevant open reading frames of PHB-synthase
**FIG. 3 (1/9)**

: Nucleotide sequence of fragment SE45

```
EcoRI
1   GCTTAAGTAGGTCCCGGTGTAGAGGGTACACGGCCTAACCTTCACTGGCTTTCTAAGACTTGG
101  CCGCGAAGTGGGCTGCCCTTGCGGCTAGGAAGGGTCGCTAGGCTGG AGAG
201  TCACGAGGTAACTAGGACGCCTCCTTAGGCTGCGGGCCCGGGGCTTGCTGTC
301  GCCAGGCCTAAGCGCTACTGCAGTCTCGCCCCGCAAGAGGCCTATAGGC
401  GGCTGGGGCTACACGCAGCCACAGCGCGCCGGGACCAGCGCGACCGCGTGC
501  ACGGCCTCTCGATCGAGGGGACCAGCAGCTACGGGCACGCTAGGC
601  ACGGCTCGCTCTGAGGCTGAGGCTACGGGCTACGGGCAGCTACGGGGCC
701  AATTGCACCTTCGGGTCCAGGCTTACGAGGCAGCTACTCGTGCTGCGGGCC
801  GCGGGCTTAACGGGCGGCGCGGCGCTAATTGCAGGCTAGCAGCACTCGGT
     * R E N L W
901  CGGTCACTGGCCGCCCATGCGCCCATCCGAGGCCACATCGGCGCGACCGAC
     G H I G G F P F A L E T Y D P S S
1001 TCGCGTCTCTACACTCGTGCGGCGGTCCTACAGGAAGCTCAGGCTTGA
     L A F I N L V P C T I D K L D V
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FIG. 3 (2/9)

AAGCTAAGGTCTGGGACAGTGACTTTGAGGTCTACTCACAGACGAGTACTCATG
CGCGCCCTACAGCTAAACAAACTAGCCTCAGTTCTCCTGACTCGGCGGCCAGCTAAAGGAC
GGAAACGCTGACCAGCGGCGAAAGGACTCAGATGACTGGAAACGGCGACGC
GTAGGGGGGTAGTGCTAGTGCTAGGTTCCAACTACCAACCCGACATTTGTCGGC
GACCACCTTGATGACCGGTGCAGCCGCTAGCGGTACGGCGACGCTACGGCCCG
CTAAGGCTGCAGGGAACGACGCTAGGTTCTCTCCAGAAGGCCCACCTCCGTC
GACCGCTGTAAGATGTAGGTTCTAAGTTCTAGGTTCCGACCATATCCCCGCCG
ACTCAGGGGTCGCAGTAGACCTACGCGAAGGCTGCTGCTGCTGCTGCTG
GAATGCGCTACCAGCGGCACCTGAAGAAAGACGCAGCGGGAACCGCGACTGTATCTA
KGIAPTVEKQAKGVSYI

CAGTCAGGGAACCTCGGGGTTCTCGGTAGGGCGGCCTGTTCCACTAGGACC
TLGKLARSDAPPVVLHDQ
GGACAGGCGGTGTTGGTCCGCGGGCTAAGCTCCTCGGCAACAAAGACCATCT
EQGGLVVGGGNLFGNNQY
FIG. 3 (4/9)

GCTTCTAGGTTAAAGAGGTACGCGTCTTCAAGAACTGGAACAGGCCAGCAG
   PFIFWKEMRLFNKVKDPDD

GAACGTGCTGTCTTCCAGGTCAAGTCTCGAGTGCCCCTACAAACGGTAC
   KLSTFTWNLLEGPINGM

TCCAACAGGGCCCAGAACTCTCGTGGCGGCACTGGTACCAGTGCTCCAACAG
   LNDPTKFDVPVTVMTVLN

GAACGTGCTATGGCTGCTTAACGTGAACAGCTGCGCCACCCGAAGCGCGTC
   GQCIgLNNVKDVGHAE vitality

CTCGCGCGCGACGGGACTAGGCTATCGGGTCAGCTAGTCCATCTGCAGG
   LARDAPDYPYGWDILYVD

AGCCAGTACATCCCCGCAACTGGTGCCCATGTAGTCTCCGTGCGCTGCC
   DTMYPRNVLAYVILLPV

CGAACAGGAGCGAGCATCTGCGGGACGAACCCGACTGCGCCACAGCTACC
   LKDSEYVAPSKSVGTIDA

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GAACGTGCGAGTGCGGCTAACCAGAAACCACCCACCGCGCGACGCCCTC
   *DAPNTKTTTRRRAP

GGCCGAGGAGTGCGCGTCCGCCGACGCTGCGCTGCAAGGTCTAAGCGCGTCGG
   APEEGALAVRRELDDRLS
FIG. 3 (5/9)

2101 CACTGCGTCCCGGAAACAAGAGCAGCTGCCTGCAGAGGACCTCGGCGCAGGACC
HRLAKNERRTEQRLRDQ

2201 AGCTGGTCTTAGCTGTCGGAGGAACCTCGAGGTAGACTCGCAACTGGT
DVLSMRKQLAMQANV

2301 CGGAAGGCTGTCGCTCCGCTGGGTCAACACAGCATCTCAGCGCGCGCGCTGAG
AGECCAUVNDYLRASD

2401 CTGGCGTAAATCGGCTCGAACACATCTGCAACACCACATAGGACCTCG
VSKMGLKTYVNYTEQL

2501 AGGAGCGCCACATCGGCTTGGCCGGCGGCTCTCCGAGCTAGTCTGG
EERTYGGLGPASLARDL

2601 CGCTGTAAGCTCCTGAGTCGAGCTCGAAGCTCAGCTCGCGCTCAGGTT
PSSSSMMRRQWNDLPLEW

2701 CTTCGGCGAAGACGTACGAGCTCGAAGCTACGCTGAGGTTAGGC
FRKQMDELTKTWWLELG

2801 TTCTTTAAAGAAGCGGAGGTAGTAGGAAAGGTACTCCTCGCAGT
FFNKGQEMMKEMFSRSS

2901 GGGTCCCGCAACCCGGCTTGACCTCAGCGACACGGTCTGTTACCCGAA
EWPTTASSASDLGMAK

3001 TAGCAGTAACTCTTTATATCAAACAGAGTTACCTCGATGGGAGGAGGCACC
DDNFFNTNSM

ORF2 S/D

SUBSTITUTE SHEET
FIG. 3 (7/9)

3101 TACGCATCTTAGGTTGTCGGCGCTGGGTATAGCCCGCTGCAGCAGAGGTA

VDAGRSAIGTFGGGSLS

3201 GCAGCTGGCGGCGGGTCACGGGTAAGCTTGGGAAGGCGGCGGTCAGACGC

RTGLAPEQIDEVILGQV

3301 GCATGGCCTGACGGCCGCTTTGTCTAGCTGCTCCACTAAAGAGCGCGGTCC

GLPHSVPAMTIINKVCG

3401 GCCCGGATGGCGTAAGCCACAGCAGGCGGTACTGTTAGTTGGTTCCAGACGCC

ADIVIAGGQESMQSS

3501 ACGGCTGTAGCATAGCGCCGGCCAGTCCTCTCGTACGTCAGGAGC

KDTMIVDGGLWDADFNNYH

3601 TTCTCTGTCTACGCTGGAGACACCATACGGAAGTTGGTTGATAG

QQDFAAASQKTEAAR

3701 TCCTCGTCTCCTGCCGAAGCGGCGGAGGCTCGTCCTCTGCTCCCCGC

RKGDPKVFDADEPFRH

3801 CGCGTTCGCCGGCTAGGCTTCCACAAACTACGGCTGCTCAAGGGCGCAGTG

GSVTAGNASGINDGAAM

3901 CCGTGCAGTGCCCGCCCATGCGGAGGCGGTATGTTTGCTGCCGCGGCGGT

ARLVAFASAGVDPAIM

4001 ACCGCGACGACACCAGGAAGCGGTGCGGCCACAGCTAGGGCGCTAGTA
F1G. 3

Ketothiolase

S/D

M S E N I V I

AAGGTAGTCTTAGGTCTCCTGTAGGTGCTTACTCGCTCTTTGTATGAGAT

SL S A T E I G T A V L K G L L A
AGTGAAGAGCGTGGTGCTCTAGGGCGGGACGAGTTCCTCCGGACGCCGC

L T A G V G Q N P A R Q T T L H A
ACGACTGGGCGGCGACCCCGTGCTTGGGGGGCCGGCATCTGTTGTCCACCGTGC

S G L K A V H L A M Q A I A C G D
GTGCCAGACTTCGCGGCCACGTAGACCCTACGTCCGGTACCGGAGACGCCCT

H V L P R S R D G Q R M G D W S M
GTCAGGACGGCGCAAGCGGCGCTTGCCAGTCCGGTACCCCGTGCACAGCTAC

M G T T A E N I A Q K Y G F T R E
TGTCCTCGGCTCTTGTATAGCGGGCTTCTTCATGCCGAAATGCGCGC

Q K A G R F Q D E I I P I E I P Q
CGTCTTCGACCGGCGAAGGTCTCGTCTCTGTAAGGCTAGCTCTAGGCGT

G T T A E S L G K L R P A F S K D
CCGTTGGCCCGCTCTCAGACCAGGCTTCGGACGAGGCGGGAAGAGCTCTCTG

V V V M K E S K A K E L G L K P M
ACCAGCACCACACTACTTCTCAAGGTCCGCTCTTGACACAGACTTCCTCGGCT

G T G P I P A S T K C L E K A G W
CCCCTGCCCGGGCTAGGGCCGCAGTGGTTCAACGGACCTCTTCCGGCCGAC

SUBSTITUTE SHEET
FIG. 4 (1/3)

PHB polymerase 173 E S G G E S L R A G V R N M E D L T R - - G K I S O T D E S A F E V -
PHB polymerase 2 173 E S G G E S L R A G V R N M E D L T R - - G K I S O T D E S A F E V -
PHA polymerase 1 149 E T G G K S L L D G L S N L A K D L V N N G G M P S Q V N M D A F E V -
PHA polymerase 2 149 E T G G K S L L D G L S N L A K D L V N N G G M P S Q V N M D A F E V -
PHA polymerase 3 1 1 M F P I D I R P D K L T Q E M L O Y S R - - - K L G Q G M E N L L N A E
PHA polymerase 1 184 G K N L G T S E G A V V Y R N D V L E L I Q Y K P I T E - - Q V H A R P
PHA polymerase 2 184 G K N L G T S E G A V V Y R N D V L E L I Q Y K P I T E - - Q V H A R P
PHA polymerase 3 34 A I D T G S V S P K Q A V V S E D K L V L Y D R P E G A P E A Q P V V
PHA polymerase 1 184 G K N L G T S E G A V V Y R N D V L E L I Q Y K P I T E - - Q V H A R P
PHA polymerase 2 184 G K N L G T S E G A V V Y R N D V L E L I Q Y K P I T E - - Q V H A R P
PHA polymerase 3 70 A I D T G S V S P K Q A V V S E D K L V L Y D R P E G A P E A Q P V V
PHA polymerase 1 218 G K N L G T S E G A V V Y R N D V L E L I Q Y K P I T E - - Q V H A R P
PHA polymerase 2 218 G K N L G T S E G A V V Y R N D V L E L I Q Y K P I T E - - Q V H A R P
PHA polymerase 3 240 G K N L G T S E G A V V Y R N D V L E L I Q Y K P I T E - - Q V H A R P
PHA polymerase 1 254 S W R N P D A S M A G S T W D D Y E H A A I R A I E V A R D I S G Q D
PHA polymerase 1 254 S W R N P D A S M A G S T W D D Y E H A A I R A I E V A R D I S G Q D
FIG. 5 (2/20)

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CGGCCTACAGCTAAACAACTAGCTTCTGACTCGGCCGGACCTAAGGAC
AAACGCTGACCAGGCGCGAAAGGACTCATAGTGACTGGAAGACGGCCGACGC
AGGCCGGTCACTCACTGTAGTTCAACTACAAACCGGCATTGTGCACGC
CCACCTTGTGACCCCGGTGCTAGGCGTACGGCGCACGTAAAGGCGCCG
AAGCCTGCGCGGAAGCAGCTAGCTCTCCAGAAGGCCCACCTCCTGC
CCGCTGTAAGATGTAGTGCTCTAAGTTATTCGGAACCATATCCCACGG

TCAGGGGCTCGCAGTAGCAGCCTAGGCAAGCTGCGCGACTGCTCTCGCTCG

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FIG. 5 (5 / 20)

1601 ACCGGCACCCGTCGTCAGGGAACTACCACTACCTGCCCAGAAGGACCTA
QGTLALKITSRDEREQI

1701 CGACCCGAAGCCCCGCGCGGGAGCCCAAGCGTGATCGTATCTCCTGG
PQAEPAGERPDRYRYLYV

1801 GAGCCGCAACTCGTCTAAAGGGAAGGTACGGACTGGGTCGAACGCGAC
EAANLNLNEMGQGLKRS

1901 TAGCGACAGCAGACTAAGACAGACCACTCGGACGACAAGCTAGTCGG
S/D

2001 GGAACCGCAGCCCGCCGCCCCGCTGACTCGTCACCGTCGGCAACAC
AKAAPAPSRLLATAPKT

2101 CACTCGTCGCCGGAAACAGAGCGCTGCCAGGACTCTCGGCCAGGA
HLAKNERRETEQLRD

2201 AGCTGGTCTAGCTGTCGCGGAAGAAGCTCAGGGTAGACTCGCAACTG
DVLSMRKKLMQANV
| 2301 | GCGGAAGCGTGGTGGGTCGAACAGCATCTCGGACGCGCGGGCCTC AGGCCAVWNDELARAS |
| 2401 | CTGGCTGAAGTACGGCTCAGCACCACATCTGCAACCCACATAAGGACCT VSKMGLKTYVNTYEQ |
| 2501 | AGGAGCGCCACATCGGCTCTGGCCCGGCTCTCCCGAGCTAGTTC EERTYGGLGAPSLARDL |
| 2601 | CGCTGTAGCTCCTGTAGTACGGCAGCTCAACAGCTCGCCGTCGAGG PSSSSMMRMQWNDLPLE |
| 2701 | CTTCGCGAAGACGTACAGGAAGCTCGCAGAACCAGGTCTCGAGGTTCG FRKQMDLTEKTKTWLEL |
2801 TTCTTCAGAAGACGGACGGCAGGATGAGATAGAAGGATCTTGCTCGCAGCTG
       F F N K G Q E M M K E M F S R S

2901 GGGTCCCGCACAACCGGGCTTGACCTCCCGCGACAGGTCCTGGGTACCCGGA
       E W P T T A S S S A S D L G M A K

ATCGTCATTGAAAGAAATGATGGTCATGGACGTACCCTCCTCTGTTG
3001 TAGCAGTAACTTCTTTTATCAAACGAGTACCTGCTATGGGAGGACGACCC
       D D N F F N T N S M S/D

ORF2

"-10"

mRNA

ATCGGTAGAATCCACAGGGCCGCGACCCCATATCGGGGACGCTCGTCCAT
3101 TACGCTTCTTAGGTGTCCGCGCCTGGGTATAGCCCTTCGAGAGGTA

VDAGRSAIGTFGGGSLS
3201 CGTCGACGCCCGCGCGCAGTGGGCTCCGACAGCCTCGGCAGTGCAGT

R T G L A P E Q I D E V I L G Q V
3301 CGTACCGGACTCGCGCGCGGAAACAGATCGAGGGAAGGTGATTTCGCGCGAGG

GLPHSVPAMTINKVCG
3401 CGGGGCTACCCGCTCCTGGTGCGCCGGCATGACCATCAAAAAGGTCTCGCGG
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<td>DSDITRSILLQIMLEEE ATGACAGGCGATATCACCGGTTCCATCCGTCCAGATCATGCTGGAGGAGGAG</td>
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<td>IRFYGGTTLQGFARLEL CCGCTTCTACGGCGACCCCTTCAGGGCACCTGCGCCGCTATGGAATCTT</td>
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FIG. 5

4801  SLDLFAKQQQEVTKA  
      CACTCGACCTGTTCGCCAAGCAGCAACAGGAAAGTGACCAAGGCACTC

4901  IWADLQDELMRAAGFP  
      CTGGGCTGATCTCCAGGACGAACTCATGCGCGCGGCTGGCTTTCCGG

5001 “-10” (?)  
      GCCGTCGGTCACAGCTTTATTGTGCAATGCAACATTGTGCACCTGCA

5101  EWTNKSVERTSFGE  
      ACGAGTGGACCAACAAAGAGCGTCGAGCGCATGACCAGCTTCGTTGAG

5201  LYMDSMRLMKLATES  
      CCTGTACATGGATCACAGCATGCCTGATGAAGCTGGCCACCCGAT

5301  SERVMAESKATMQFFG  
      AGCGGCCGTCATGCGGCAGAGCAAGGCGACCATGCAAGTCTTTCCGG

5401  EDLRKSVAV  
      GCGAAGATCTGCGCAAGGCGTCGGCCGTCTAAAGAGCCGCAGCCTCTG
FIG. 5

L T D N P F G T V T R L T Q K N V E
ACCGACAATCCCTTCCGGGACGGTGACACGGCTGACCTCAAGAAGAACGTGAGAT

V A P R K K E *
TCGCGCCGCCGCAAGAAAAAAAAGATAATGGAGATTTGGCGAAAATTTCGCACTGACG

ORF5
S/D
CAAACCTTACGAGATGATCATGAACACCCACCACCGACAGCCCTCAAGAAGACGTCA

M N T D S L K T V N
LNVRLFEKLAARQMDAVN
CTGAACGTGCCTCGTTCGAGAGCTGGCGCCGCGCTCAGATGGACGCCCGCGTAA

KGYNDLFLKKGQVDATKEL
CCAAGGGTTACAATGACCTCTTCAAGGGTCAGGTCGAGCGCCACCAAGGAACTG

DARDEYRWWWFEKSLNDVS
CGATGCCGCCGACGAATACCCCGGCTGTGGTTGAGAAGAGAGCCTGAAACGCACGTCA

GGCCATCGCGATCCAGGGATGGCATCGCCATTGTCATGCTTCCGGATCGGCCG
FIG. 5 (20/20)

---

R M A Q P N E I A A A I A F L A G D
TCGCA T GGC T CAG C T A AT GAG AT C G C G C C G C A T C G C T T T T C T G C C G G C G A

L F M H
CTGTTCATG C AT T GAT TTAGATCATAC ACC G G C G C A A AT A CAAA A CACT G A A AT G

ATGAGACGT TTACAGCCGC GCACGC G C G GTTTTTTTTTGTGAGAATCGAAT

R Y C D D V L D A A R F A D Y A P N
CGCTACTG CGATGACGTGCTCGACGC GC GC CGCTCGC C GAC TAT GC GC CGAA T

V T A S A A L I E A A A I A E H A D A
GGTGACGC CGC AGCG C CGCG TTGTGATCGAGG GC GC GATCGCGGA GC ACG CGACG

L I G I K G Q R A R T L L S A G V S
CCTGATCG CCAT CAA G GGC AGCG C GCA AAGG ACATT GCT CAC GC CGGG GTG TAG

N N A T L G R R L D F I D M E P T A
AA CA ATG C C A C A CT CG CTG C C G C G GCT CGATT T T CAT CG A C AT G G A ACC GA CC G C A

C V L H G A C L A
CCTGCCCG C C T T CAC G GAC ATG T C T C GC AT C
**FIG. 6**

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<th>C.v. 346</th>
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### Homology to β-ketothiolase of *C. vinsonum*:

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<tr>
<td>A.e. 381</td>
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<tr>
<td>Z.r. 380</td>
<td>43.0%</td>
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<tr>
<td>E.c. 375</td>
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<tr>
<td>S.u. 387</td>
<td>42.6%</td>
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Comparison of amino acid sequences of the β-ketothiolases encoded by *Chromatium vinsonum* (C.v.), *Alcaligenes eutrophus* (A.e.), *Zoogloea ramigera* (Z.r.), *Escherichia coli* (E.c.), *Saccharomyces uvarum* (S.u.) and *Rattus norvegicus* (R.n.).
Comparison of amino acid sequences of reductases encoded by C. vinonsum (C.v.), A. eutrophus (A.e.) and Z. ramigera (ZZR).

**Homology to reductase of C. vinonsum:**

| C.v. | 215 |
| 214 |
| 210 | MG |

56.4% 48.3%
# FIG. 8 (1/2)

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<th>Strain (plasmid)</th>
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<td>LB-Tc-Glu</td>
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<td><em>phbA</em>⁺, <em>phbB</em>⁺, <em>phbC</em>⁺, ORF2⁺</td>
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<td>S17-1 (pSUP202)</td>
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<td>XL1-Blue (KS⁻)</td>
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<td>LB-Ap-Glu</td>
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### FIG. 8 (2/2)

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<th>NADPH-dependent</th>
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**INTERNATIONAL SEARCH REPORT**

**I. CLASSIFICATION OF SUBJECT MATTER**

According to International Patent Classification (IPC) or to both National Classification and IPC

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<th>Int.Cl. 5</th>
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**II. FIELDS SEARCHED**

Minimum Documentation Search

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Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

**III. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<tr>
<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to Claim No.</th>
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<tr>
<td>A</td>
<td>ARCHIVES OF MICROBIOLOGY vol. 155, no. 5, 1991, pages 415 - 421, LIEBERGESSL, M., ET AL. 'Formation of poly-3 hydroxyalkanoates by phototrophic and chemolithic bacteria', see the whole document</td>
<td>1-12</td>
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<td>A</td>
<td>TRENDS IN BIOTECHNOLOGY vol. 5, no. 9, September 1987, pages 246 - 250, BYROM, D. 'Polymer synthesis by microorganisms: technology and economics', see page 248, left column; table 2</td>
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<td>A</td>
<td>WO,A,9 100 917 (MIT) 24 January 1991, see the whole document</td>
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**IV. CERTIFICATION**

Date of the Actual Completion of the International Search: 09 OCTOBER 1992

Date of Mailing of this International Search Report: 19.10.92

International Searching Authority: EUROPEAN PATENT OFFICE

Signature of Authorized Officer: Maddox A.D.
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<td>JOURNAL OF BIOLOGICAL CHEMISTRY vol. 264, no. 26, 15 September 1989, BALTIMORE, MD US pages 15293 - 15297 PEOPLES, O.P., ET AL. 'Poly-beta-hydroxybutyrate biosynthesis in Alcaligenes eutrophus H16' see figures 5,6</td>
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<td>A</td>
<td>SCIENCE vol. 245, 15 September 1989, LANCASTER, PA US pages 1187 - 1189 POOL, R., ET AL. 'In search of the plastic potato' see page 1189, column 2 - column 3</td>
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<td>J. BACTERIOLOGY vol. 170, no. 12, December 1988, pages 5837 - 5847 SCHUBERT, P., ET AL. 'Cloning of the alcaligenes eutrophus genes for the synthesis of PHB in Escherichia coli' see the whole document</td>
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