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 H₂-oxidizing bacterium Alcaligenes hydrogenophilus. H₂-oxidizing bacteria produce poly(3hydroxybutyric acid) (PHB) from CO₂, 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 CO₂ 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 CO₂ utilizers/fixers/consumers. A cluster of genes from the H₂oxidizing 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 CO₂ by H₂-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 μ g/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 MgCI₂ and MgSO₄ 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 $[\alpha^{-32}P]dCTP$ 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 *Sall* fragments (those of 0.8 and 1.1-kb) and two *Bam*HI-*Sall* fragments derived from the 1.8-kb *Sall* 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

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Α	
	phaC →
1	ATATCTAGATAGTCCAGTCGAGGTACGTATAATCCGACGCTCAACCCAGAC <u>GAGA</u> TCTCGTCAATGACGCGCGCGCGCGAGCACTCTCGCGCTAATCGGA S/D M T R A R B H S R A N R T
101	CCCTGAAGTCGATCATCGAGACCCAGGGCGAGACTGCGGCGAGGGATGATGAACCTGCTGGCGACCTGCAGCGCGGCGAGATTTCGCAAACCGACGAATC 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	GCAGTTCGTGGTCGGCAGGAATCGCGCTGCACCGAGGGGGGGG
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	GCGGCCACCAGGTGTTCCTCGTGTCGTGGCGCACAGCGGACGCATCGGTCGCCCCACAAGACCTGGGACGACTACGATGAACGAAGGCATCGTGGCACGCG 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	AGTCGATGCTGTGCAGCAGGTCAGCGGCTCCGACGAGATCAACACCGTCGGCGTCTGCGTCGGCGGCACGATGCTGCGACCGCTCTCGGTGCTTGCGGCG 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	CGCGGCGAGCATCCGGCGCTCGATGACGCTCCTCCGGCTGCTCGACGTCGTCGACGTATTTGTGGACGAGGAACGAGAACGTG R G E E 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	TTGGCGAACTCGACGCGCGCATCAGCCCGGGGCGGCGGCGGCGTACCGTTCTTGCCGCCGATCGTCGTCGTCGACCATCTGCACCATCGGCCCCGCCGCCGACAAATACGT 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	GGACAACTACCTGAAGGGCAGCACCGGCGGCGGCGCGGTTGAACCGGTGTACTGGAACAGGAACCTGACGAAGGAACCTGGCCGGATGTAAGGCGTGGTACCTG 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 H Y A W Y L
901	CGCAATACCTATCTCGAGAACCGGCTGGCGGAGGCGGGCG
1001	ACGCTCCCCCGCGAGGATCACATCGTGCCGTGGCAGACGGCCTACGCATCGACGTCGATCCTGACGGCCCCGCTGAAGTTCGTGCTGGGCGCGCGGCGGCCA 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 P V L G A S G H
1101	CATCGCCGGCGTGGTCAATCCGCCGCGAAACAGAACGGCGCCGTACTGGCCGTCAACGACGGCCGACGACGACGGCCCGACGACGACGGCCCGACGA
1201	CCGAGCAGCCGCAGCTGATGCGCTGATGCTGATGCGGCCGAGTCTGCGACGGCATCGTCGGACAGTCGTGTGGAGGGGGGCGCTACGTCACGATGAC
1301	$\label{eq:phat} phat \rightarrow \\ GCAGGATCTGCATTTACAGCGGACGATCCTTCAGCCGATCCGACGACGACGAACGA$
1401	ACCTQCGAAGATCGCGGCGCCGGAGCTGGGCCCGATGGTGATCCCGCGCGCG
1501	GCGGCCGGCCAGAACCCGGCGCCACAGTCGCCGAGTCGCCGAGCGCGGGGCGCGGGATGACGATCAACAAGTGTGCGGGTCGGG G R L G Q N P A P Q B L I K A G L P S A V P G M T I N K V C G S S G
1601	CCTGAAGGCGGTGATGCTGGCGCGAACGGCGATCATTGCCGGCGGGGGGGG
1701	GCCGGGTCGCGCAACGGGTTCCGGATGGCGACTCGAAGCTGGTCGACACGATGATCGACGGGCTGTGGGACGTGTACAACCAGTACCACATGGGAA 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	TCACGGCGGAGAACGTCGCGAAGGAATACGGGATCACGCGGAGGAGCAGGACGCATTTGCGCGCTGTCGCAGAACAAGGCGGAAGGGCGCAGAAGGCG T A B N V A K E Y G I T R B E Q D A F A R C R R T R R K G A E G G
1901	GCGCTTTAACGACGAGATCGTTCCGGTTGGCGATCCCGCAGAAGAAGGGCGAGCCGCTGCGGTTCGCGACCGAC
2001	CGGGCTGGCGGGCTGAAGCCGGCGTACGGCGAAGGGCGAACGAGGGGCTCAACGACGGACTCAACACGTCGGAGGACGACGACGACGACGACGACGACGACGACGACGA

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.

В	
2101	CATATIOGOCHGAAGGEAGACTGATACTICACACTGCOGGATCAAGCTACGEACACTGGACGGAGGGGATGGGGGCCOGJTGCCAGGAAGTCG Y S P E A T T D T H T A G S S Y R T L D P S V M G M G P V P A S R
2201	CANDEFICETOGRAPHACCOCCUCACACCOCCUCACCACCACCACCACCACCACCAACCA
2301	CACAAGCAGATGGGCTGGGAAGTGGAAGTGAACGTGAACGTGGAGGATGGCCATGGGCACGGGATGGGGCTGGGGGGGG
2401	COCHECTOCHOCHORMESTICAMODOCHOCHAODOCOCHICOCHECHICODOCCOCHOCHOCHOCHOCHOCHOCHOCHOCHOCHOCHOCHO
2501	СТРАНОССТРОСТОТОСООРГСКОЗОСССКОСКАЛОСССКОСТСКОЗОСССССТООРАНАССКАЛАВАНИКАЛОССКОСССТООРАНАССКАЛАВАНИКАЛОССКОССКОС * 5/D
2601	phaB→ ATGICTCAGGAATTGCTTAGTTAGGGAGGGCATGGGGCAGGACTGGCAGGGCTGTGCAAGGATGGCTTTGGTGTGGGGGGGG
2701	ACCICATERICCOCCARCTCOCCECCEGARATCCTCCARCEACEACEACEACEACEACEACEACEACEACEACEACEAC
2801	CANCERNEETICENCENDERCENDERCOCONSTICENCENCENCENCENCENCENCENCENCENCENCENCENC
2901	COCCRACERCOCCCCCCCCCAACCTAACCTCAACCTCATCCAACCAAACAAC
3001	ATCAACATCTOSTOGJIGAACGGOCAGAGGOCASTTOSGOCAGAOCAACTACTOGACGGOGGAAGGOCGGCATTCACGGCTTCACGATGTOCGTOGGGCA Q H L V G E R P E G Q F G Q T N Y S T A K A G I H G F T M S V A Q
3101	GGAASICGCGACGAAGGGCGACGGCGGACAGGCGACGGCACGGC
3201	Grosocaocaitocogitocofictococcicaocaactacticofictaticofictaticaticosciencescoccicocactice V A T I P V R R L G A P E E I G S I V A C S S N D S A S D G A D F S
3301	озстераоззозостисалитозостирасовоозалазвоезовоезоватозвортталараззосса са со стоориститозовое L N G G L H M G *
3401	
3401	S/D M T T T K K T A E R L I K K Y P N R R L
3501	TOTROGREGARICANGCANTACINGCTINACCHOTICANACANCINGTICTANACANGUGARITICANGUGARICANGUGANATUGAGUGA Y D T E T S T Y I T L T D V K Q L V L E Q E D F K V V D A K S S E
3601	NGACTGACCOSCAGACATOCTGCAGATCATOCTGGANGAGAGAGGGGGGGGGGGGGATGTTCCGTGGAGAGACGATGACGAGATCATOGCTTC D L T R S I L L Q I I L E E E S G G V P M F S S S M L S Q I I R F
3701	TROSSICHTROSATIGTIGSGRITHTIGGGCACTIGGCTIGGAGAAGAACATIGTAGGCGITICATIGGATATICTAGAACAAAGTIGGGAGAAGTAGAAGAAC Y G E A M L G M M G T Y L E K N I Q A F I D I Q N K L A D Q S K N L
3801	TSTWIG4GAACANTSCEATGAACCCCEAAGATCTG9ICCAG9ACATCCAG9GCCCCAGAGCGAATGAGCGACGCGAGCGAGCGAGCGAGCG
3901	ANGAACATGTTCGTICCHGTTGCHGGAACAAATGCHGAATCHGGCGAAGTCCHTGTTCHJTTCGJTTCAAGCCGOCTGGCTCGGHGCCGGHGAAGA
4001	AFTAAGGGENTATCOGFTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
4101	TGTOJIOGGACTCATGJICOGCTGOGATGJIGGOGCOGATGGATACOCAGGACOGJICOJITOGCATOG

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 *phaC* 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.

λ		
	1	
		WARDARDA TO TAKE A CARDEN AND A
	-	MGTERTEPARPOPETI.REABOL.EVFROBARABLEPFE.AGOGAL.PGABLOGASEIDEMTRTLTRVAETWLEDPE.ALQ.TRLG.SFAALWA.T.TR
R.r.	1	P-IWGP.VTSVA.RAVR0POAVTA.TAEYAGRLAKI~PA.ATR
P. o. 1	1	
P.0.2	1	E.GRDLIBTLREVEROBLR-H.**.*HTAHHLLALGGQ
P. a. 1	1	I.GKDLLTBARNVL.QA.R-QHSAREVAHFSLE
P.a.2	1	
C w.	1	
т	1	
P.a.	1	
A.e.	84	-QAHAEGKAEATGPLHDRRFAGDAWRTELPY-RFAAAFYL-LEARALTELADAVEADAKTRORIRFAISOWVDANSPAHFLATNPEAORLLIESGGESLR
H.e.	101	N-OG. VTEPVVOP. PT. K RAD. SA. PV-FDLIKOS I. GRW. FEMVET. EGT EG. HEAR VI.P. LIG V. G. WH
P. 7	57	VENEROD DEDUND ADA OF DI POTT OF 188 VIENTARIA BUODEL OF MUSEUM TO THE OF A DE SUS A DE SUS AND
A		TARBET FATVER BEL. QS. FA. I BLL-QS ATR. IVEELTEAGEG PLQDERA. QFABLAFLA. SLW GVLTRAF. TAL
P.O.1	61	-KEVLL.SELAPEEDEDPSE.PLRYLQTAWRKE.QDWIGEEDLEPQDIE.GQ.V.HLMTEMAPTETLSA.VKRFFETKL
P.o.2	61	-GRVIL, DTPLOPHPR, P., SDPT, SO, PT, RGLOA., -AWOKOTRLWIEFSHL, DDD, A.AH, LTHILH, -LAPSHELL, T. VEF. FH. O. V.
P. s. 1	61	-KEVIL, OS. LEPGOD. SDP 50 PL - VPVMOT - AWPER REWISESDISDODIS CO U HITTE MEDERALE & UNDER B
		AUTT DET RANDO & ODER CONTACT ANTAL ANTAL BEN LOUD LE GULD CONTACT ANTAL, THE FROM
P	01	-GRADE.DITEGRAPQ.AQDPBL.PFRILQAAWQRQ.LAWI.KEBL.CDD.A.ALVALESVAPSWELIL.LKE.FWTIL
C.V.	1	
Ĩ.v.	1	MBBTDTB B
D .	1	
	-	TR.REBSRAWRTLEBITQT~A
A.e.	181	AGVENINEDT
	100	B WENTO BACKOT BURNER AND
a	733	
R.I.	154	K.A. YAAH%ILBRG%-LPLK-V. BDJ. T F. L.A. P. N% R. DLIBNI
P.o.1	157	D.LS.LAKE. VARG MP. VEND
P. 0. 2	157	D AUTIO BUNG TOD V D G V & A D VY WY WAR A WY C THE ALL ALL ALL ALL ALL ALL ALL ALL ALL AL
	13/	A
8.8.1	157	D. LGHLARS, VHHGSNP V. HD K. L. T
P.s.2	157	# HLL VERGMP VEKT I L. T.O R VLEXT GEROY. K
C. v.		DELTOR DYSERIO GREELIN FATORS SPECE YE D. FIN YE DE FORDEROUP TIMATE PROMET TO A TRACTICE OF Y
		BELLE AND ALL AND A
2		DELAQE. ATTERLE QGREELING DAIDIG.SPR.A-VIS.D-KLV.YK.DT.AGV-TPORTPLIVYALV.RP.MT.I.IDR TIKGLLAT.QD.Y.
P.a.	25	···NN·LLG②·Q·③····
A. B.	215	VSWREPDASMAGSTWDDYIEHAAIRAIEVARDIBGODKIEVLGFINNIGTIVSTAL-AVLAARGEEPAASVILLTTLLDFADTGILDVFVDEGEVQLRE
N.e.	297	I WVEPDERHRDK. FESYMREGIST DNIGVAT. STOVAAA. YX. 200 . LLAVT. A-YO T. BRRIK. ANF OV THA. D. K AN OTKAT.
R.r.	250	I
D - 1	28.2	T HER OLD THE WORLD' A STATUT A STATUT AND
P.0.1	233	1BFTR. ORBGLETTID.LKE. VDAVLA.T. SKDL.M. A SSALTCTALVGEYA. LGEBE-VBALVBV TTHDBQVALTL.
P.o.2	253	#PDDRHREGLSSYVQ.LEE.LEAC.SSRDP.LN.A%A&%LTNAALQGHLQ.KHQLRRVR.A%Y.VBSKFESPASL.A%TI.
P.a.1	253	
	25.3	T TERLOTET GEGEVER THE SECOND STRAND AND STRAND
		1 WEDAGARA - CONTINE. D.C B.E. T. BRBY. LA. ANALSELT . ARL. CHLOVE. OLERAVB
C.V.	105	IDGYPDQGDRALTL.DYIEGYIDRC-VDYL.EAH.VV.L.,IXQXX-AF.LMYB,LHPDKVRHLVTN.XPVDFKTPDHLLSAWVQH.XIDLAVD
T.V.	104	IDGYPDQADSALTL.DYINGYIDSC-VDYLCETHEV.OV.I10000-AF.LMYASLEPDKVKELVTM.0PVDFKTPGKLLSAWVOH.0-TDLAVD
P	120	TA V HK D PP PCT UDAVOOV S P WV WITE D S AN S WITE DAVIS
		-
λ.е.	372	ATLGGGAGAPCALLRGLELAST SFLRPHDLVWHYVVDJYLKGWTPYPFDLLWHHGÄATHLPGPWYCWYLRHTYLOËELKVPGXLTVCGVDVDÄABTDVD
х.е. М.е.	372	ATLGGGAGAPCALLRGLELABTFFLRPHDLVWWYVVDFYLKGHTPVPFDLLFHBGÄATHLPDPWYCWYLRHTYLQHELKVPGKLTVCGVPVDÄASIDVP
λ.e. M.e.	372 394	ATLGGGAGAPCALLRGIELASTSFLRPHDLVWHVVVDBYLKGSFP9PFDLLFMSGMATHL GPWYCWYLRHYLQHELKVPGKLTVCGVPVMABIDVP ERM.EHGY.E.ARM
А.е. М.е. R.r.	372 394 347	ATLGGGAGAPCALLRGLELAST SFLRPHOLVWWYVVDWYLKGHT9VPFDLLTHBGGATHL OPWYCWYLRHTYLQHELKVPGKLTVCGVPVDASIDVP RRM. EHGY. E.ARM. M. MI.SHVR.KA.AAY. ARM AAHHSFHC. B.T.AKGQHV-LGH.RL KKVK FRMRQQGF.S.K.M.GG.DHI.AKF.W.SRM.EK.AA.I.A
А.е. М.е. R.r. P.o.1	372 394 347 346	ATLGGGAGAPCALLRGLELABITSFLRPHDLVWHYVVDBYLKGHTPVPFDLLFWRGMATHL GPWYCWYLRHTYLQHFLKVPGKLTVCGVPVM ASIDVP RRM.ERGY.E.ARMA.WI.SW.VR.KA.AAT.A.RM AAHBSRC.E.S.AKGGMV-LGE.RL KKVK. FRMRQGF.S.K.M.GS.DMI.AKF.W.SRWM.KK.AAT.A.E.S.MAKHBSL.SL.GR.AKGGYV.LD.G.L.BOKCO AAKRBYGAGV.S.S.N.VG.DMI.AKF.W.S.L.K.FV.I BIS.A.AHG-D.IENFSS.FR.D.A.
λ.e. M.e. R.r. P.o.1 P.o.2	372 394 347 346 347	ATLGGGAGAPCALLRGIELASTSFLEPHOLVWHVVVDBYLKGHTPVFPDLLFHGGAATHL FRM.FHGY.K.ARM.J.HM
A.e. M.e. R.r. P.o.1 P.o.2 P.a.3	372 394 347 346 347 346	ATLGGGAGAPCALLRGLELASITSFLRPHDLVWHYVVDHYLKGHTPVPPLLHWHGGATHL OPWYCWYLRHTYLQHELKVPGKLTVCGVPVD ABIDVP RRM.ERGY.E.ARMA.MMI.SE.VR.KA.AAY.A.RMAAHSSHC.E.A.AGAW-LGB.RL KKVK. FRMRQGF.S.K.M.GS.DMI.AKF.W.SRWM.EK.AAY.A. IS.SH ANHSESL.GRI.AEGLYV-LD.G.LW HD.ACD AAKRESYCRGV.D.A.V.RT.ANMI.W.B.L.K.FV.I.B.HT.R.AAFHG-D.LDFFKL.F.R.A.BT.I. GOVCO AAKRESYCRGV.D.A.V.RT.ANMI.W.B.L.K.FA.I.Y.A.B.R.ALHG-D.LDFFKL.F.R.A.GT.I.GOVCD AAKRESYCRGV.D.A.V.RT.ANMI.W.B.L.K.FA.I.Y.A.B.R.ALHG-D.LDFFKL.F.R.AG.L.T.I.GOVCD
A.e. M.e. R.r. P.o.1 P.o.2 P.a.1	372 394 347 346 347 346	ATLGGGAGAPCALLRGIELASTSFLRBHOLVWHVVUDFYLKGFFPFPLFDLFHGGAATEL GPWYCWYLRHTYLGHELKVPGKLTVCGVPUB ABIDVP REM EHGY.E.ARM. J WM. I S. W.V.KA.AA
A.e. M.e. R.r. P.o.1 P.o.2 P.a.1 P.a.2	372 394 347 346 347 346 347	ATLGGGAGAPCALLRGIELASTSFLRPHOLVWHYVVDBYLKGHTPYFPLLTHGGAATHL GDWYCWYLRHTYLGHFLKVDGKLTVCGVPVD ASIDVF FRM EHGY E ARM. A. MM
A.e. M.e. P.o.1 P.o.2 P.a.1 P.a.2 C.v.	372 394 347 346 347 346 347 200	ATLGGGAGAPCALLRGLELABT SFLRPHOLVWHYVVD BYLKGHTVPFDLLFMBGBATHL GPWYCWYLRHTYLQHELKVPGKLTVCGVPVD ABIDVP ERM EHOY E ARM. A. M
A.e. M.e. P.o.1 P.o.2 P.a.1 P.a.2 C.v. T.v.	372 394 347 346 347 346 347 200 199	ATLGGGAGAPCALLRGIELASTSFLPHOLVWHVVDDFVLKGHTPVFPDLLWHGGATTL THE RGGAGAPCALLRGIELASTSFLPHOLVWHVVDDFVLKGHTPVFPDLLWHGGATTL THE RGVUS R.ARH.J.HWI.S
A.e. M.e. P.o.1 P.o.2 P.a.1 P.a.2 C.v. T.v.	372 394 347 346 347 346 347 200 199 216	ATLGGGAGAPCALLRGIELABITSFLRPHOLUWWYVVDBYLKGHTPVPFDLLFWRGBATHL GDWYCWYLRHTYLQHELKVPGKLTVCGVPVBASIDV RAM.ERGY.E.ARMA.WMI.SW.Y.RAAAT.A. RM AAHHSIHC.E.T.AKQGMY-LOB.RL KKVK FRMRQGF 5.K.M.GG.DHI.AKF.W.SRWM.KK.AAAT.A.E.S.WA ANHHSISL.GRI.AEGLY-LO.G.LW HD.ACD AAKRBSYCAGV.S.A.K.G.NKV.AWMI.W.B.L.K.FV.I.B.T.R.AAHHG-LUFFKS.P.R.A.F.S.T.I.C.KVKID AAKRBSYCGGV.J.A.V.RI.AMMI.W.B.L.K.FAA.IY.A.S.R.AAHHG-LUFFKS.P.R.A.F.S.T.I.C.KVKID AAKRBSYCGGV.J.A.V.RI.AMMI.W.B.L.K.FAA.IY.A.S.R.AAHHG-LUFFKS.P.R.A.F.S.T.I.C.KVKID AAKRBSYCGGV.J.A.V.RI.AMMI.W.S.L.K.FAA.IY.A.S.R.AAHHG-LUFFKS.P.R.A.F.S.T.I.KVKID AAKRBSYCGGV.J.A.V.RI.AMMI.W.S.L.K.FAA.IY.B.R.AAHHG-LUFFKS.P.R.A.F.S.T.I.KVKID AAKRBSYCGGV.J.R.MV.AWMI.W.S.L.K.FAA.IY.B.R.R.AAHHG-LUFFKS.P.R.A.F.S.T.A. KVAID AAKRBSYCGGV.J.R.M.KV.AWMI.W.S.L.K.PAA.IY.B.R.R.AFHG-LUDFFKS.P.R.A.F.S.T.A.S.KVAID SAKRBSYCGV.J.R.M.KV.AWM.J.L.W.B.J.C.G.PAA.IY.B.F.R.AAFHG-LUDFFKS.P.R.A.F.S.T.G.KVAID M.FIP.JLLWFTLGLKFFLTGCKYVM.VD-HL-DDPDKVKBFLNHKFKI.F.BPDQ.STRCFTKDF.CO.G.GF-LW.G.VLG.G.KD.TC. M.HIP.JLLWFTLGLKFFLTGCKYVM.VD-HL-DDPDKVKBFLNHKK.FI SPDQ.STRCFTKDF.CO.G.GF-LW.G.KLG.GE.KD.KS.S.K.KSK.G.G.G.F.M.G.K.G.K.S.KSK.S.G.G.F.J.K.KSKWID DARGBYWANDA ABUT ABUT ABUT ABUT ABUT ABUT ABUT ABU
A.e. M.e. P.o. 1 P.o. 2 P.a. 1 P.a. 2 C.v. T.v. F.a.	372 394 347 346 347 346 347 200 199 216	ATLGGGAGAPCALLRGIELASTSFLRBHOLVWHVVDDFVLKGFYPFPFDLLWHGGATHL GPWYCWYLRHTYLGHELKVPGKLTVCGVPVD ABIDVP FRM EHGY. E.ARM. J WK. I S. U.Y.KA.AAY A. RM AARBS. SL.GR.AGUIY-LGH.RL KKVK. FRM EHGY.S.AN
A.e. M.e. R.r. P.o.1 P.o.2 P.a.1 P.a.2 C.v. T.v. F.a.	372 394 347 346 347 346 347 200 199 216	ATLGGGAGAPCALLRGIELASTSFLRPHOLVWHVVVDBYLKGHTPFFDLLFHGGÄATHL GPWYCWYLRHTYLGHELKVDGKLTVCGVPVD ASIDVF FRM.EHGY.K.ARM.A.MM
A.e. M.e. R.r. P.o. 1 P.a. 2 P.a. 1 P.a. 2 C.v. T.v. F.a.	372 394 347 346 347 346 347 200 199 216	ATLGGGAGAPCALLRGIELASTSFLRPHOLVWHVVUDBYLKGSFVPPPPDLLFMBGBATHL GPWYCWYLRHYLQHELKVPGKLTVCGVPVD ABIDVP ERM EHGY E. ARMA. MM
A.e. M.e. R.r. P.o.2 P.a.1 P.a.2 C.v. T.v. F.a. A.e.	372 394 347 346 347 346 347 200 199 216 472	ATLGGGAGAPCALLRGIELASTSFLRPHOLVWHVVDDFVLKGFT9FFDLJTHGGATTL_GPHVCWYLRHTVLGHELKVPGKLTVCGVPVDASIDV FRM.EHGY.E.ARM.J.M.W
A.e. M.e. P.o.1 P.o.2 P.a.1 P.a.2 C.v. T.v. F.a. A.e. M.e.	372 394 347 346 347 346 347 200 199 216 472 488	ATLGGGAGAPCALLRGIELASTSFLRPHOLVWEVVVDFVLKGHTPFPFDLLTHSGMATHL GPWYCWYLRHTYLOHFLKVDGKLTVCGVPVD ASIDVF FRM.EHGY.E.ARM.A.MM
A.e. M.e. P.o.1 P.o.2 P.a.1 P.a.2 C.v. T.v. F.a. A.e. M.e.	372 394 347 346 347 200 199 216 472 488	ATLGGGAGAPCALLRGIELABTSFLRBHOLVWHVVUDFYLKHFT9FPDLLFWHGEMITEL GPWYCWYLRHTYLGWELKVPGKLTVCGVPVE ABIDV FRM EHGY. E. ARM. J WK. I S. U.Y.KA.AM. Y A. AM ANHEST. HC. H.A.KOQNV-LHERKLTVCGVPVE ABIDV FRM EHGY. E. ARM. J WK. I S. U.Y.KA.AM. Y A. AM ANHEST. HC. H.A.KOQNV-LHERKLTVCGVPVE ABIDV FRM EHGY. E. AN GG DWI AK. F. W. SAWM. FK.AM. I A I S. WM ANHEST. HC. H. A.KOQNV-LHERKLTVCGVPVE ABIDV AKRHSTQAGV. S.K. GG DWI AK. F. W. SAWM. FK.AM. I A I S. WM ANHEST. HC. H. ANHEST. HC. H. ANGANY-LHERKLTVCGVPVE ABIDV AKRHSTQAGV. S.K. GG DWI AK. F. W. SAWM. FK.AM. I A I S. WM ANHEST. HC. H. ANHEST. HC. H. ANGANY-LHE. K.YVK. AKRHSTQAGV. D.A.K. RIAMM. I W. B. L.K. FW I K. AANHEG-LUFFKS J.H. AG. E. T. I QVXED
A.e. M.e. R.r. P.o.1 P.a.1 P.a.1 P.a.2 C.v. T.v. F.a. A.e. M.e. R.r.	372 394 347 346 347 200 199 216 472 488 441	ATLGGGAGAPCALLRGIELASTSFLEPHOLVWHVVUDBYLKGHTPVFPDLLFHGGATHL GPWYCWYLRHTYLGHELKVDGKLTVCGUPVDABSIDVF FRM.FHGY.K.ARM.J.M.M
A.e. M.e. R.r. P.o.2 P.a.1 P.a.2 C.v. T.v. F.a. A.e. M.e. R.r. F.o.1	372 394 346 347 346 347 200 199 216 472 488 441 443	ATLGGGAGAPCALLRGIELASTSFLRPHOLVWHVVUDBYLKGSTPUPPDLLFWBGBATHL GPWYCWYLRHTYLQHELKVPGKLTVCGVPVD ABIDV ERM EHGY E. ARM
A.e. M.e. R.r. P.o.1 P.a.1 P.a.2 C.v. T.v. F.a. A.e. M.e. R.r. P.o.1 P.o.1	372 394 346 346 347 200 199 216 472 488 441 443	ATLGGGAQAPCALLRGIELASTSFLRPHOLVWHVVDDFVLKGHFPVFPDLLWHGGATEL GPWYCWYLSHTYLGHELKVPGKLTVCGVPVDASIDV FRH.EHGY.E.ARH.J.HK
A.e. M.e. R.r. P.o.1 P.a.1 P.a.1 P.a.2 C.v. T.v. F.a. A.e. M.e. R.r. R.r. P.o.2	372 394 346 346 347 200 199 216 472 488 441 443 444	ATLGGGAGAPCALLRGIELASTSFLEPHOLVWHVVUDBYLKGHTPVFPDLLFHGGÄTTEL GPWYCWYLRHTYLGHELKVDGKLTVCGVPVDASIDVF FRM.EHGY.K.ARM.J.M.M
A.e. R.r. P.o.1 P.a.1 P.a.1 P.a.2 C.v. T.v. P.a. A.e. R.r. P.o.1 P.o.2 P.a.1	372 394 346 346 347 200 199 216 472 488 441 443 444 443	ATLGGGAGAPCALLRGIELABTSFLRPHOLUWHVUUDFYLKGHFUPFDLLFHBGBATHL GDWYCWYLRHTYLGHELKUPGKLTUCGUPUBABIDU FRH EHGY, E.ARM. J WK. I.S. U.Y.KA.AAY A. RH AAHBS. SC. B.Y.KAQAV-LGH RL.KXVK. FRH EHGY, E.ARM. J WK. I.S. U.Y.KA.AAY A. RH AAHBS. SC. B.Y.KAQAV-LGH RL.KXVK.
A.e. R.r. P.o. 2 P.a. 1 P.a. 2 C.v. T.v. P.a. A.e. M.e. R.r. P.o. 2 P.a. 2 P.a. 2 P.a. 2 P.a. 2 P.a. 1 P.o. 2 P.a.	372 394 347 346 347 200 199 216 472 488 441 443 444 443	ATLGGGAGAPCALLRGIELASTSFLEPHOLVWHVVUDBYLKGHTPVFPDLLWHGGATHLOPHVCWYLRHTYLGHELKVDGKLTVCGVPVDABIDVF FRM.EHGY.E.ARM.J.M.MI.S., N.W.KA.AAY.J. M.M.AMHESTBC.S.AKGONV-LGERLJ.KKVK. FRM.EHGY.E.A.M.G.DKI.AKF.W.SHM.EKA.A.I.A.E.S.M.AMHESTBC.S.AKGONV-LGERLJ.KKVK. FRM.EGOFS.S.K.M.G.DKI.AKF.W.SHM.EKA.A.I.A.E.S.M.AMHESTBC.S.G.R.A.AGGIV-LGERLJ.KKVK.
A.e. R.r. P.o. 2 P.a. 1 P.a. 2 C.v. T.v. F.a. A.e. M.e. R.r. P.o. 1 P.a. 1 P.a. 2 C.v.	372 394 346 346 347 200 199 216 472 488 441 443 444 443 444 443	ATLGGGAGAPCALLRGIELASTSFLRPHOLVWHVVVDFVLKGHTPFPDLITHGGMATHL GPWYCWYLRHTYLONFLKVDGKLTVCGVPVD ASIDVF FRM.EHGY.E.ARM.A.MM
A.e. R.r. P.o. 2 P.a. 1 P.a. 2 C.v. T.v. P.a. R.c. R.c. P.o. 2 C.v. P.a. 2 C.v. P.a. 2 C.v. P.a. 1 P.a. 2 C.v. R.v. P.a. 1 P.a. 2 C.v. R.v. P.a. 1 P.a. 2 C.v. R.v. P.a. 2 C.v. R.v. 1 P.a. 2 C.v. R.v. 1 P.a. 2 C.v. R.v. 1 P.a. 2 C.v. 2 C.v. 2 C.v. 2 R.v. 1 P.a. 2 C.v. 2 C.v. 2 R.v. 1 P.a. 2 C.v. 2 C.v. 2 R.v. 1 P.a. 2 C.v.	372 394 347 346 347 200 199 216 472 488 441 443 444 443 444 293	ATLGGGAGAPCALLRGIELABTSFLEPHOLVWHVVDDFVLKGFT9FFDLJWHGGATEL GPWYCWYLRHTYLGHELKVPGKLTVCGVPVDABIDV FRH EHGY E.ARH. J. W I.S. H. VR.KA.AAY.A. RH ANHES. EC. H.T.AKGQNV-LGH.RL.SYCGVPVD.ABIDVE FRH EHGY E.ARH. J. W W. SNWH. K.AA.I.Y.A. SH ANHES. EC. H.T.AKGQNV-LGH.RL.SYCGVPVD.ABID.CC
A.e. R.r. 1 P.o. 2 P.a. 2 C.v. P.a. A.e. M.e. R.r. 1 P.o. 2 P.a. 2 C.v. P.a. 2 C.v. P.o. 1 P.o. 2 P.a. 2 C.v. P.a. 2 C.v. P.a. 2 C.v. P.a. 2 P.a. 2 C.v.	372 394 347 346 346 346 346 347 200 216 472 488 441 443 444 443 444 294 293	ATLGGGAGAPCALLRGIELASTSFLEPHOLVWHVVUDBYLKGHTPFFPDLUTHGGÄNTEL GPWYCWYLRHTYLGHELKVDGKLTVCGUPVDGABIDVF FRM. FHGY. R.ARM. A. MM
A.e. R.r. P.o. 2 P.a. 1 P.a. 2 C.v. T.v. A.e. R.r. P.o. 2 P.a. 2 C.v. T.v. P.a. 2 C.v. T.v. P.a. 1 P.a. 1 P.c. 1 P.c. 1 P.a. 1 P.a. 1 P.a. 1 P.a. 1 P.a. 1 P.a. 1 P.a. 2 C.v. T.v. 1 P.a. 1 P.a. 2 C.v.	372 394 347 346 347 346 347 200 199 216 472 488 441 443 444 444 294 293 310	ATLGGGAGAPCALLEGIELASTSFLEPHOLVWEVVVDFVLKGHTPFFDLIFHGGÄTEL GPWYCWYLRHTYLONELKVDGKLTVCGVPVD ASIDVF FRM.EHGY.E.ARM.A.MM
A.e. R.r. P.o.2 P.a.2 C.v. P.a. A.e. A.e. R.r. P.o.2 P.a.2 C.v. P.a.2	372 394 347 346 346 346 346 347 200 216 472 488 441 443 444 443 444 294 293 310	ATLGGGAGAPCALLRGIELASTSFLEPHOLVWHVVDDFVLKGHT9VFDLLWHGGATEL GPWYCWYLRHTYLGHELKVPGKLTVCGVPVDASIDV FRM.EHGY.E.ARM.J.M.W
A.e. R.r. P.o.2 P.a.2 C.v. F.a. A.e. M.e. P.o.1 P.a.2 P.a.2 C.v. F.a. P.o.2 P.a.2 C.v. F.a. P.o.2 P.a.2 C.v. F.a. P.o.2 P.a.2 C.v. F.a. P.o.2 P.a.2 C.v. F.a. A.e. M.e. P.o.2 P.a.2 C.v. F.a. A.e. M.e. P.o.2 P.a.2 C.v. F.a. A.e. M.e. P.o.2 P.a.2 C.v. F.a. A.e. P.o.2 P.a.2 C.v. F.a. A.e. P.o.2 P.a.2 C.v. F.a. A.e. P.o.2 P.a.2 C.v. F.a. A.e. P.o.2 P.a.2 C.v. F.a. A.e. P.o.2 P.a.2 C.v. F.a. A.e. P.o.2 P.a.2 C.v. F.a. A.e. P.o.2 P.a.2 P.a.2 P.a.2 P.a.2 P.o.2 P.a.2	372 394 347 346 347 346 347 200 199 216 472 488 441 443 444 444 294 310	ATLGGGAGAPCALLRGIELASTSFLEPHOLVWHVVUDBYLKGHTPFFDLIFHGGÄTTL GPWYCWYLRHTYLGHELKVDGKLTVCGUPVDÄASIDVF FRM.FHGY.K.ARM.J.M.M
A.e. Rr. 1 P.o. 2 P.o. 2 P.a. 2 C.v. T.v. P.a. A.e. Rr. 1 P.o. 2 P.a. 2 C.v. T.v. P.a. 2 C.v. A.e. Rr. 1 P.o. 2 P.o. 2 P.a. 2 C.v. A.e. A.e. P.o. 2 P.a. 2 C.v. A.e. A.e. P.o. 2 P.a. 2 C.v. A.e. A.e. P.o. 2 P.a. 2 C.v. A.e. P.a. 2 C.v. A.e. P.o. 2 P.a. 2 C.v. A.e. A.e. P.o. 2 P.a. 2 C.v. A.e. P.o. 2 P.a. 2 C.v. A.e. P.o. 2 P.o. 2 P.a. 2 C.v. A.e. P.o. 2 P.o. 2 P.a. 2 C.v. P.a. 3 A.e. A.e	372 394 347 346 347 346 200 199 216 472 488 443 444 443 444 293 310	ATLGGGAQAPCALLRGIELABTSFLEPHOLVWHVVDDFLKGHFYDFFDLJWHGGATEL GPWYCWYLBHTYLQHELKVPGKLTVCGVPVDABIDV FRH EHGY E.ARH. J. W
A.e. M.e. Rro.1 Pro.2 Pro.2 Pro.2 T.v. R.c. R.c. R.c. R.c. P.o.2 P.o. A.e. R.c. P.o.2 P.o. A.e. R.c. P.o. A.e.	372 394 347 346 347 200 199 216 472 488 441 443 444 443 444 443 294 293 310 565	ATLGGGAGAPCALLRGIELASTSFLEPHOLVWHVVUDBYLKGHTPVFPDLLWHGGATEL OPWYCWYLRHTYLGHELKVOGKLTVCGVPVDABIDV FRM.FHGY.E.ARM.J.M.MI.S., N.K.A.A.AY. A. IM ARMESTE.S. AKGONV-LGE.RL.XKYK. FRM.FHGY.E.A.M.G.DKI.A.F.W.SHWM.FK.A.A.I.A.F.S.M.ARMESTBC.S.A.G.R.A.AGGNV-LGE.RL.XKYK.
A.e. Rr. 1 P.o. 2 P.o. 2 P.a. 2 C.v. R.c. P.a. 2 C.v. P.a. 2 C.v. P.a. 2 C.v. A.e. M.e. P.o. 1 P.a. 1 P.a. 1 C.v. A.e. A.e. P.o. 1 P.o. 2 P.o.	372 394 347 346 347 209 216 472 488 441 443 444 444 294 310 565 581	ATLGGGAGAPCALLRGIELASTSFLRPHOLVWHVVUDBYLKGHTPFFDLITHGGMATHL GDWYCWYLRHTYLOWELKVDGKLTVCGVPVDSASIDVF FRM.EHGY.E.ARM.A.MM
A.e. R.r. P.o. 1 P.o. 2 P.o. 1 P.o. 2 P.o. 1 P.o. 2 P.o. 2 P.	372 394 347 346 347 346 200 199 216 472 484 443 444 443 444 443 444 443 310 565 581 540	ATLGGGAQAPCALLRGIELASTSFLEPHOLVWHVVDDFVLKGHT9VFDLLWHGGATEL GPWYCWYLBHTYLGBELKVPGKLTVCGVPVDAASIDV ERM.EBGY.E.ARM.J.M.W
A.e. Rro.1 Pro.2 Pro.2 Pro.2 Pro.2 Rro.2 Rro.2 Rro.2 Rro.2 Rro.2 Rro.2 Pro.2 Rro	372 394 347 346 347 200 199 472 488 441 443 444 444 444 444 294 310 565 581 546	ATLGGGAGAPCALLRGIELASTSFLEPHOLVWHVVUDBYLKGHTPFFDLITHGGÄNTEL OPWYCWYLRHTYLGHELKVDGKLTVCGVPVDBABIDVF FRM.EHGY.E.ARM.J.M.M
A.e. R.c. 12 P.c. 21 P.c. 2	372 394 347 346 347 2009 216 488 441 444 444 444 293 310 565 5810 536 537	ATLGGGAGAPCALLRGIELABTSFLRPHOLVWHVVVDFVLKGHTPFFDLITHGGÄTTL GPWYCWYLRHTYLGWELKVDGKLTVCGVPVBASIDVF FRM.EHGY.E.ARMA.MM
A. e. M. e. R. r. 12 P. 0.12 P. 0.12 C. v. P. 0.12 C. v. P. 0.12 C. v. P. 0.12 C. v. A. e. M. e. P. 0.12 P. 0.12 C. v. P. 0.12 P. 0.12 C. v. P. 0.12 P. 0.12 P. 0.12 C. v. P. 0.12 P. 0.12 P. 0.12 C. v. P. 0.12 P. 0	372 394 347 346 347 2009 216 472 4881 443 444 443 444 443 444 293 5651 5816 5837	ATLGGGAGAPCALLRGIELASTSFLPHOLVWHVVUDFVLKGHT9FPJFDLIFHGGATEL OPWYCWYLRHTYLGHELKVDGKLTVCGUPVDASIDV FRM.FRGVF.A.R.M.J.M.MI.S., N.W.KA.AAY.X.M.MAHABFRC.S.T.AGGNV-LGERLSINKTK FRM.FRGVGFS.K.M.G.DKI.K.F.W.SHMM.FK.A.A.I.A.E.S.M.ARHBFRC.S.T.AGGNV-LGERLSINKTK
A.e. Rro.12 Pr.4.2 Tr.4. A.e. Rro.2 Tr.4. A.e. Rro.2 A.e. Rro.2 A.e. Rro.2 A.e. Rro.2 A.e. Rro.2 A.e. Rro.2 A.e. Rro.2 A.e. Pr.4.2 C.T. A.e. Rro.2 A.e. Pr.4.2 C.T. A.e. Rro.2 A.e. Pr.4.2 C.T. A.e. Pr.4.2 C.T. A.e. Pr.4.2 C.T. A.e. Pr.4.2 C.T. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. A.e. P.C. A.e. P.C. A.e. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. P.C. A.e. A.e. A.e. P.C. A.e. A.	3794 39476 3447 34467 34467 2009 212 728 4443 4443 4443 4443 4443 55810 55810 55810 558367 55367 53361	ATLGGGAGAPCALLRGIELASTSFLEPHOLVWHVVUDBYLKGHTPFFDLITHGGÄNTEL GPWYCWYLRHTYLGWELKVDGKLTVCGVPVDÄASIDVF FRM.EHGY.E.ARM.J.M.M
A. e. M. e. R P. O. 1 P. P. a. 1 P. C. 1 P. C. 1 P. C. 1 P. C. 1 P. C. 2 T. P. C. 1 P. C. 1	372 394 347 346 347 209 216 472 4881 443 4443 4443 4443 4443 55310 53810 5387 5337	ATLGGGAGAPCALLRGIELABTSFLRPHOLVWHVVVDFVLKGHTPVFPDLIFWGGATEL GDWYCWYLRHTYLOWELKVPGKLTVCGVPVDASIDV FRM EHGY E.ARM. A.MM
A. e. M. e. P. O. 1 P. A. 1 P. A. 1 C. V. P. A. C. V. P. A. A. e. M. e. P. C. 1 P. A. 1 P. 2 C. V. P. A. 1 P. A. 2 C. V. P. A. 1 P. A. 2 C. V. P. A. 2 C. V. P. A. 2 C. V. 2 P. A. 2 C. V. 1 P. A. 2 C. V. 1	3724 3947 3447 3447 3447 3447 3447 2199 216 472 4881 4443 4443 4443 42943 5651 55376 53367 53376	ATLGGGAGAPCALLRGIELASTSFLPHOLVWHVVUDBYLKGHTPFFDLITHGGÄTTL OPPVCWYLRHTYLGHELKVDGKLTVCGVPVDBABIDVF FRM.FRGV.F.A.RMA.MM
A. e. M. e. P. 0. 12 P. 0. 12 P. 0. 12 P. 0. 12 C. v. P. 0. 12 P. 0. 12 C. v. A. H. R. C. P. 0. 21 P. 0.	372 346 3476 35810 5387 3556 3556	ATLGGGAGAPCALLRGIELASTSFLRPHOLVWHVVUDFVLKGHTPFFDLITWSGMATEL GDWVCWYLRHTVLOWELKVDGKLTVCGVPVDSASIDVF FRM.EHGY.E.ARMA.MM

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.

P.a. 1 MT-DVVIVSAG--PVGKFGGT-CEDRGAGAGPMVIRAVLERAGVKPEQVSEVIPGGRL----GQNPAPQSLIKAGLPSAVPGMTINKVKG λ.e. Z.r. c.v. .SENI...D..RSAI.T...SLSSLSATEI.TA.LKGL.A.T.LA...ID...L.QV.TAGV.....R.TTLE....BS..A.... P.a. 83 SGLKAVHLARTAIIAGEADIVIAGGQENMSAARRA-AGSRNGFRNGDSKLVDTNIVDGLWDVINQIHNGITAENVAKEYGITREEQDAFA P.a. 172 RCRRTRRKGAE-GGRFNDEIVPVGDPAEEGRAAAVR-DRRVRTRRDGGRAGGLKPAFAKDGTVTAANESGLNDGLNTSDDVSISPEATTD A.e. 180 VGSQNKAEA.QKA.K.DE....LI.QRK.DFV.FKT.EF..QGATLDSNS.....D.A.....A.A.VVVM.AAKAKELG 2.r. 179 VASQNKAEA.QKD...K....FIVKGRK.DITV-DA.EYI.HGATLDSMAK.R...D.E....G.A.....AAALLM.EAEASRRG 2.r. 179 C.V. 181 AASQQKTEA.QKA...Q...I.IEI.QRK.DPKVFDA.EFP.EGTTAESL.K.R...S.D.S...G.A..I...AANVVVMKE.KAKELG P.a. 260 -THTAG-SSY-RT-LDPSVMGMGPVPASRSGAWRSGWTPGDWTPSDLDLMEINES-SRQALTVHKQMGWDTSKVNVNDGAIAIGHPIGAS C.V. 271 LKPM.RLVAFASAGV..AI..T..IPA--.--TK-CLEKAG...A....I.A..AFAA..MS.NQD....L......G........ P.a. 345 GCRILVTLLHEMVKRDGTRGMASLEIGGGMGVALAVERPS 393 391 394

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.

P.a. A.e. Z.r. C.v.	1 1 1 1	MSQRIAYVIGGMGGIGTSICQRLSKDGFRVVAG-DAE-VRRLASVNARGEKGRPDFIASEGNVADWDSTKEAFDKVKAEVGEIDVLVNNA .TS
P.a. A.e. Z.r. C.v.	89 90 84 90	GITRDVVFRKMTREDWTAVIDTNLTSLFNVTKQVIDGHVERGWGDHQHLVGERPE-GQFGQTNSTAKAGIHGFTMSVAQEVATKGVTVN A.D
P.a. A.e. Z.r. C.v.	178 180 174 180	TVSPGYIGTDMVKAIRPDVL-LKIVATIPVRRLGAPEEIGSIVA-CSSNDSA-SDGADFSLNGGLHMG 242 AQDKLA.C.WL.EE.GF.T

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

Heterologous Expression of Pha Genes from P. aciaophila 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	

 Table 1

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

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

P.a. C.v.	1 1	MTTTKKTAERLIKKYPNRRLYDTETSTYITLTDVKQLVLEQEDFKVVDAKSSEDLTRSIL N-S.IIV.RA.RD.MSGQP.R.L.SANDS.I
T.v.	1	N-SD.ITANES.I
P.a.	61	LQIILEEESGGVPMFSSSMLSQIIRFYGHAMLGMMGTYLEKNIQAFI-DIQNK-LADQ
C.v.	56	MTQ.LANAGTLTFARSSLDL.AKQQ.EVT.A.T.N
T.v.	56	MTE.LAAGTLFARSSLDL.AKQQ.DMT.T.G.N
P.a.	117	S-KNLYENNAMNPEIWSOFMNMQ-AP-MMOA 144
C.v.	116	PFGTVTRLTOK.VADL.DEL.RAAGFPVRKKKE 153
T.v.	116	PFEAMTRMTQK.VADM.EEF 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 (phaCphaA-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 (phaCphaA-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 EcoRI-HindIII 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 CO₂. 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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