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A Novel Genetically Engineered Pathway for Synthesis of Poly(Hydroxyalkanoic Acids) in *Escherichia coli*

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A new pathway to synthesize poly(hydroxyalkanoic acids) (PHA) was constructed by simultaneously expressing butyrate kinase (Buk) and phosphotransbutyrylase (Ptb) genes of *Clostridium acetobutylicum* and the two PHA synthase genes (*pha*E and *pha*C) of *Thiocapsa pfennigii* in *Escherichia coli*. The four genes were cloned into the *Bam*HI and *Eco*RI sites of pBR322, and the resulting hybrid plasmid, pBPP1, conferred activities of all three enzymes to *E. coli* JM109. Cells of this recombinant strain accumulated PHAs when hydroxyfatty acids were provided as carbon sources. Homopolyesters of 3-hydroxybutyrate (3HB), 4-hydroxybutyrate (4HB), or 4-hydroxyvalerate (4HV) were obtained from each of the corresponding hydroxyfatty acids. Various copolyesters of those hydroxyfatty acids were also obtained when two of these hydroxyfatty acids were fed at equal amounts: cells fed with 3HB and 4HB accumulated a copolyester consisting of 88 mol% 3HB and 12 mol% 4HB and contributing to 68.7% of the cell dry weight. Cells fed with 3HB and 4HV accumulated a copolyester consisting of 94 mol% 3HB and 6 mol% 4HV and contributing to 64.0% of the cell dry weight. Cells fed with 3HB, 4HB, and 4HV accumulated a terpolyester consisting of 85 mol% 3HB, 13 mol% 4HB, and 2 mol% 4HV and contributing to 68.4% of the cell dry weight.

Poly(hydroxyalkanoic acids) (PHAs) are synthesized in bacteria via different pathways. In *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) (40) and most other poly(3-hydroxybutyric acid) [poly(3HB)]-accumulating bacteria, poly(3HB) is synthesized from acetyl coenzyme A (acetyl-CoA) through three enzymes: β-ketothiolase condenses two molecules of acetyl-CoA to β-acetoacetyl-CoA, an NADPH-dependent acetoacetyl-CoA reductase catalyzes the formation of D-(−)-3-hydroxybutyryl-CoA (3HB-CoA), and PHA synthase finally polymerizes 3HB-CoA to poly(3HB) (27, 29). A modified synthesis pathway for poly(3HB) was found in *Rhodospirillum rubrum*. Instead of D-(−)-3HB-CoA, L-(+)-3HB-CoA is formed from β-acetoacetyl-CoA by an NADH-dependent reductase. L-(+)-3HB-CoA is then converted to its D-(−) isomer with enoyl-CoA hydratases (6, 18). In pseudomonads, synthesis of PHA consisting of medium-chain-length 3-hydroxyalkanoic acids occurs either through fatty acid *de novo* synthesis, which is linked to PHA synthesis by an acyl-CoA transferase (20), or through coupling to the β-oxidation pathway (10, 31). Detailed reviews on these and other PHA biosynthesis pathways are available in the literature (31–33).

Although low-molecular-weight poly(3HB) was detected in association with other molecules in *Escherichia coli* (21–23), this species does not synthesize high-molecular-weight poly(3HB) or other PHAs, nor does it accumulate such polyesters as carbon storage compounds, even if a foreign PHA synthase gene is expressed in the cells. However, by expressing the entire PHA operon from, e.g., *R. eutropha*, cells of *E. coli* synthesized and accumulated poly(3HB) (27, 29). Since the first successful cloning of the PHA operon of *R. eutropha*, many recombinant strains of *E. coli* that accumulated various other PHAs have been obtained. Besides a copolyester of 3HB and 3-hydroxyvaleric acid (3HV) [poly(3HB-*co*-3HV)] (28), recombinant strains of *E. coli* were obtained which produced polyesters containing 4-hydroxybutyric acid (4HB) such as poly(4HB) or poly(3HB-*co*-4HB) (9, 30) or polyesters containing medium-chain-length 3-hydroxyalkanoic acids such as 3-hydroxydecanoic acid (13, 19).

In this study, we present a newly designed pathway for PHA synthesis from hydroxyfatty acids by employing the genes for butyrate kinase (Buk) and phosphotransbutyrylase (Ptb) from *Clostridium acetobutylicum* and the genes for PHA synthase from *Thiocapsa pfennigii*.

MATERIALS AND METHODS

Strains, plasmids, media, and cultivation. All strains and plasmids used in this study are listed in Table 1. Strains were cultivated at 37°C and were maintained in Luria-Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) or M9 mineral salts medium (1) according to Sambrook et al. (25); ampicillin (100 mg/liter) was added when needed. Additional carbon sources were applied from filter-sterilized stock solutions as indicated in the text.

Genetic manipulation. Routine isolation of plasmid was done by the alkaline lysis method (25); plasmid restriction enzymatic digestion, agarose gel electrophoresis, and DNA ligation were performed by following procedures in a labo-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli		
JM109	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*(lac-proAB)*	Stratagene
K2006	F⁻ *his*⁻ *fadR16 fadA30 atoC49 atoA28;* source of pJC7	11
DH1	F⁻ *endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA96 relA1* λ⁻*;* source of pBR322	7
Plasmids		
pBR322	Ap^r	New England Biolabs
pJC7	Ap^r *buk*$^+$*ptb*$^+$	4
pBPP1	Ap^r *buk*$^+$ *ptb*$^+$ *phaEC*$^+$	This study
pBPP2	Ap^r *buk*$^+$ *ptb*$^+$ *phaEC*$^-$	This study
pBPha	Ap^r *phaEC*$^+$	This study

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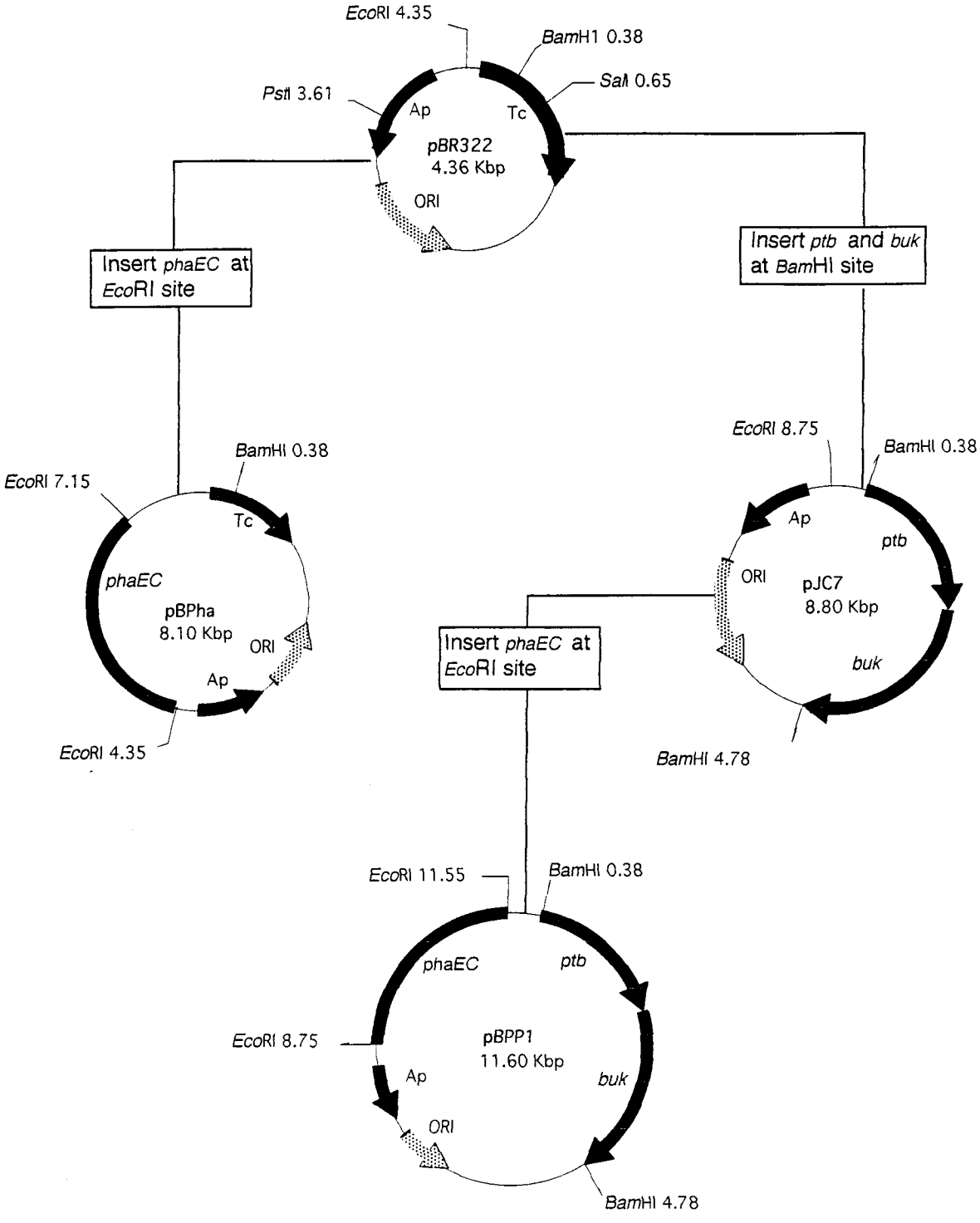

FIG. 1. Construction of plasmids used in this study. Plasmid pJC7 was generated by Cary et al. (4).

ratory manual (25) and instructions from the manufacturers. DNA fragments were purified by using a Qiaquick gel extraction kit (catalog no. 28704; Qiagen, Hilden, Germany).

Measurements of enzyme activity. Buk activity was assayed in the direction of butyryl phosphate formation according to Hartmanis (8) modified from Rose (24). Enzymatic analysis was done in a total volume of 0.5 ml. After 5 to 10 min, the reaction was stopped by addition of 0.5 ml of 10% (wt/vol) trichloroacetic acid, and then 2 ml of acidic $FeCl_3$ solution was added. The absorbance at 540 nm was measured, and enzyme activity was calculated on the basis of a molar extinction coefficient of 0.169 mM^{-1} cm^{-1} (4).

Ptb activity was measured in the direction of butyryl-CoA to butyryl phosphate conversion, by monitoring the reaction of CoA with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at 412 nm (39). The assay contained in a total volume of 0.5 ml 0.1 M potassium phosphate, 0.2 mM butyryl-CoA, and 1.0 mM DTNB. An extinction coefficient of 13.61 mM^{-1} cm^{-1} was used.

PHA synthase was determined spectrophotometrically by monitoring the release of CoA at 412 nm (37). The assay contained 1 mM DTNB, 20 mM $MgCl_2$, and 0.4 mM 3HB-CoA in 150 mM Tris-HCl buffer (pH 7.5).

Accumulation of PHAs. For examination of PHA accumulation, strains were cultivated at 37°C in 300-ml Erlenmeyer flasks containing 50 ml of medium and

TABLE 2. Specific activities of relevant enzymes in recombinant strains of *E. coli* JM109 strains harboring different plasmids[a]

Strain	Sp act (U/mg of protein)		
	Buk	Ptb	PHA synthase
JM109(pJC7)	18	3.0	ND[b]
JM109(pBPP1)	10	3.8	0.1
JM109(pBPP2)	15	2.5	ND
JM109(pBPha)	ND	ND	0.5

[a] Strains were cultivated in 300-ml Erlenmeyer flasks containing 50 ml of LB medium with 1 mM isopropyl-β-D-thiogalactopyranoside and 100 mg of ampicillin per liter at 37°C and at 200 rpm for 24 h.
[b] ND, not detected.

were agitated at 200 rpm. LB or M9 medium containing 1% (wt/vol) glucose was used. Various hydroxyfatty acids were added at the beginning of or during cultivation to final concentrations of 0.2 or 0.4% (wt/vol) as indicated in the text.

Isolation and analysis of polyesters. Cells from 50 ml of medium were harvested after 2 to 3 days cultivation by centrifugation (4,000 rpm for 10 min in a Minifuge RF, type no. 3360 [Heraeus Septech]) and washed three times. For quantative determination of PHAs in the cells and analysis of the constituents, 5 to 10 mg of lyophilized cells was subjected to methanolysis in the presence of 15% sulfuric acid, and the resulted hydroxyacyl methylesters were analyzed by gas chromatography as described previously (3, 35).

Determination of protein concentrations. Protein concentrations were determined according to Bradford (2).

Chemicals. D-(−)-3HB and 4HB were purchased from Sigma (Deisenhofen, Germany). 4HV was prepared by alkaline hydrolysis of 4-hydroxyvalerolactone. NaOH (10 N) was added to 4-hydroxyvalerolactone to a final volume of 50% while stirring. The pH of the suspension was adjusted between 11 and 12. The reaction proceeded under stirring on ice until the pH was stable and no phase separation occurred. After the reaction was completed, the hydrolysate was adjusted to a pH of 7.0 with 1 N HCl.

RESULTS

Construction of plasmids. Plasmid pJC7, which contained a 4.4-kbp genomic fragment encoding the Buk and Ptb genes from *C. acetobutylicum* in the *Bam*HI site of pBR322 (4), was used to ligate a 2.8-kbp *Eco*RI restriction fragment of pHP1014:B28 encoding the PHA synthase genes (*phaEC*) of *T. pfennigii* (16) into this plasmid. The resulting hybrid plasmid, pBPP1, harbored all four genes for Buk, Ptb, and PHA synthase (Fig. 1). In parallel, plasmid pBPha was constructed by inserting only the PHA synthase genes into pBR322 (Fig. 1).

Analysis of Buk, Ptb, and PHA synthase activities confirmed that all three enzymes were expressed in functionally active form in the recombinant *E. coli* JM109(pBPP1). Plasmids pJC7 and pBPha conferred only Buk and Ptb activities or PHA synthase activity, respectively, to the recombinant strains of JM109 (Table 2).

During construction of the plasmids, we also found a plasmid, referred to as pBPP2, that conferred Buk and Ptb activities but no PHA synthase activity to the cells (Table 2). Restriction analysis of the plasmid revealed that the 2.8-kbp *Eco*RI fragment harboring *phaEC* existed in this plasmid, but opposite the direction in pBPP1, indicating that the orientation of *phaEC* genes is relevant for expression of the PHA synthase.

Detection of poly(3HB) in different media. As found in our experiments, accumulation of PHAs depended on the supply of hydroxyfatty acids as carbon sources. When cultivated in LB or M9 medium with 1% glucose, *E. coli* JM109(pBPP1) accumulated only traces of poly(3HB) (Table 3). However, when 3HB was fed in addition to glucose, JM109(pBPP1) accumulated significant amounts of poly(3HB), amounting to more than 40% of the cell dry matter (Table 3) if cultivation was done in M9 medium. In LB medium supplemented with glucose and 3HB, poly(3HB) was also accumulated, but to only approximately 5% of the cell dry matter.

TABLE 3. Accumulation of poly(3HB) by recombinant strains of *E. coli* JM109 harboring different plasmids[a]

Medium	Poly(3HB) content (% of cell dry wt)		
	JM109 (pJC7)	JM109 (pBPP1)	JM109 (pBPha)
M9	0.2	0.4	0.3
LB	0.2	0.2	0.3
M9 + glucose (1%)	0.3	0.3	0.3
LB + glucose (1%)	0.3	0.3	0.3
M9 + glucose (1%) + 3HB (0.4%)	0.4	43.3	0.5
LB + glucose (1%) + 3HB (0.4%)	0.4	5.1	0.4

[a] Strains were cultivated in 300-ml Erlenmeyer flasks containing 50 ml of medium with the indicated carbon source, 1 mM isopropyl-β-D-thiogalactopyranoside and 100 mg of ampicillin per liter at 37°C and at 200 rpm for 72 h.

Accumulation of homopolyesters other than poly(3HB). *E. coli* JM109(pBPP1) was also examined for the capability to synthesize and accumulate PHAs from 4HB or 4HV (Table 4). Poly(4HB) accumulated to up to 4.8% of the cell dry matter when 0.2% 4HB was added two times at the beginning and after 24 h of cultivation to cultures in M9 medium containing 1% glucose. Under the same conditions, the strain accumulated poly(4HV) to 1.3% of the cell dry weight if 4HV instead of 4HB was provided. Obviously, poly(4HB) and poly(4HV) were not as efficiently accumulated as poly(3HB) (Tables 3 and 4).

Accumulation of copolyesters by JM109(pBPP1). Besides the ability to accumulate the homopolyesters poly(3HB), poly (4HB), and poly(4HV), the strains were also investigated for the capability to accumulate copolyesters (Table 4). When 3HB and a second hydroxyfatty acids were fed, the cells accumulated large amounts of copolyesters. Although 3HB was the predominant constituent in all experiments done, 4HB or 4HV contributed 12 or 6 mol% of the constituents if 4HB or 4HV was the second carbon source, respectively. When all three hydroxyfatty acids were fed at equal amounts, a terpolyester consisting of 85 mol% 3HB, 13 mol% 4HB, and 2 mol% 4HV was accumulated. These results indicated that the three enzymes involved in this novel PHA biosynthesis pathway preferred 3HB as substrates.

DISCUSSION

For the first time, Buk and Ptb of *C. acetobutylicum* were coupled *in vivo* to the PHA synthase of *T. pfennigii* to establish

TABLE 4. Accumulation of PHAs by recombinant strains of *E. coli* JM109 harboring plasmid pBPP1 during cultivation on hydroxyfatty acids[a]

Carbon source(s) in addition to glucose	PHA content (% of cell dry wt)	Composition of PHA (mol%)		
		3HB	4HB	4HV
3HB	40.8	100	ND[b]	ND
4HB	4.8	ND	100	ND
4HV	1.3	ND	ND	100
3HB + 4HB	68.7	88	12	ND
3HB + 4HV	64.0	94	ND	6
3HB + 4HB + 4HV	68.4	85	13	2

[a] Cells were cultivated in 300-ml Erlenmeyer flasks containing 50 ml of M9 medium with 1% (wt/vol) glucose, 1 mM isopropyl-β-D-thiogalactopyranoside and 100 mg of ampicillin per liter at 37°C and at 200 rpm for 3 days; 0.2% (wt/vol) each fatty acid was fed after 24 and 48 h.
[b] ND, not detected.

a novel PHA biosynthesis pathway that was expressed in a functionally active form in recombinant *E. coli*. This engineered metabolic pathway allowed the synthesis and accumulation of polyesters containing not only 3HB but also 4HB and/or 4HV as constituents. Synthesis of PHAs containing these constituents depended greatly on the provision of the respective hydroxyfatty acids as carbon sources. If more than one hydroxyfatty acid was provided, copolyesters and even a terpolyester were synthesized. The lower efficiencies for the incorporation of 4HB and 4HV into PHAs reflected the substrate preference of the enzymes involved. We noticed that recombinant strains of *Pseudomonas putida* expressing PHA synthase of *T. pfennigii* accumulated PHAs containing significant portions of 3-hydroxyhexanoate or 4HV (15, 26), which suggested a rather broad substrate specificity of this PHA synthase. The lower molar ratio of 4HV of the PHAs synthesized in this study (2 to 6 mol% versus 20 mol% in previous reports) is most probably due to the low conversion of 4HV to 4HV-CoA in *E. coli*. We recently demonstrated low activities of Buk and Ptb toward 4HV and 4-hydroxyvaleryl phosphate, respectively (17). From this study and the literature, it became also evident that the PHA synthase of *T. pfennigii* is unlikely to use 4HV-CoA as a substrate.

In addition, this study clearly demonstrated that a PHA biosynthesis pathway engineered in a previous study by an *in vitro* approach (17) is functionally active *in vivo* in *E. coli*. Therefore, *in vitro* engineering of pathways may be a useful strategy to evaluate whether the establishment of a particular pathway in a bacterium by *in vivo* metabolic engineering is feasible. This is a general conclusion from this study in addition to having obtained a better poly(4HB)-producing strain.

Production of homopolyesters or copolyesters of 4HB in bacteria has attracted many researchers. Synthesis of copolyesters containing 4HB was found in *R. eutropha* (11, 12, 36, 38), *Hydrogenophaga pseudoflava* (5), in recombinant strains of *E. coli* (9), and in several other bacteria. The high amounts of the copolyester (up to 68.4% by weight) in *E. coli* JM109(pBPP1) made this strain competitive to other producers. Further studies to increase the ratio of 4HB in these copolyesters will be conducted.

PHAs have been detected in several anaerobic heterotrophic bacteria such as *Clostridium botulinum*, *Desulfovibrio sapovarans*, and *Syntrophomonas wolfei* (for a review, see reference 31). PHAs were also detected in biological samples from anaerobic environments (for a review, see reference 31). Neither molecular nor enzymatic studies on the biosynthesis of PHAs in these bacteria have been done, and it is not known whether these anaerobes use PHA synthesis pathways similar to those found in *R. eutropha* or other well-studied aerobic bacteria. This study indicates a putative alternative pathway to synthesize hydroxyacyl-CoA thioesters, which are the substrates of PHA synthase, presuming that hydroxyfatty acids are produced in anaerobic bacteria.

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