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Thioesterase II of *Escherichia coli* Plays an Important Role in 3-Hydroxydecanoic Acid Production

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3-Hydroxydecanoic acid (3HD) was produced in *Escherichia coli* by mobilizing (*R*)-3-hydroxydecanoyl–acyl carrier protein–coenzyme A transacylase (PhaG, encoded by the *phaG* gene). By employing an isogenic *tesB* (encoding thioesterase II)-negative knockout *E. coli* strain, CH01, it was found that the expressions of *tesB* and *phaG* can up-regulate each other. In addition, 3HD was synthesized from glucose or fructose by recombinant *E. coli* harboring *phaG* and *tesB*. This study supports the hypothesis that the physiological role of thioesterase II in *E. coli* is to prevent the abnormal accumulation of intracellular acyl-coenzyme A.

Bacteria are capable of the synthesis of a family of biopolyesters, named polyhydroxyalkanoates (PHAs). As biodegradable (11) and biocompatible (7, 29, 32) thermoplastic polyesters, PHAs are structurally simple macromolecules synthesized by many bacteria grown on various carbon sources and are believed to act as sinks for carbon and reducing equivalents (19). Over 150 different PHAs have been identified (28). All of the monomer units of PHAs are enantiomerically pure, and each was in *R* configuration (17). 3-Hydroxyalkanoic acids (3HA), as PHA monomers, may serve as intermediates for the synthesis of many valuable chemicals, such as antibiotics, vitamins, aromatics, pheromones, and (*S*)-β-amino acids (17).

3HA monomers can be prepared either by chemical synthesis or by PHA degradation. Recently, 3HA monomers, including 3-hydroxybutyric acid (8) and 3-hydroxydecanoic acid (3HD) (31), were reported to be produced by recombinant *E. coli* harboring either *phbA*, encoding β-ketothiolase, and *phbB*, encoding acetoacetyl-coenzyme A (CoA) reductase, or *phaG*, encoding (*R*)-3-hydroxydecanoyl-acyl carrier protein (3HD-ACP)–CoA transacylase (PhaG), respectively. PhaG was found to link fatty acid de novo biosynthesis to PHA production by converting 3HD-ACP to (*R*)-3-hydroxydecanoyl-CoA (3HD-CoA) (24). However, how 3HD-CoA is converted to its corresponding free-acid derivative is unclear.

Fatty acyl thioesterase activity in *E. coli* extracts was first noted by Kass et al. (13). In subsequent experiments, two separable thioesterases were confirmed (1, 2). Thioesterase I, encoded by the *tesA* gene, was found to be specific for C_{12} to C_{18} acyl-CoA esters, but it was inactive for C_6 to C_{10} acyl-CoA esters or 3-hydroxyacyl–CoA esters (20). Klinke et al. reported that cytosolic thioesterase I mediated acyl-ACP intermediates from the fatty acid de novo biosynthesis pathway to fatty acid β-oxidation in *E. coli* (14), although native thioesterase I was mainly observed as a periplasmic enzyme (6). On the other hand, thioesterase II, an enzyme encoded by the *tesB* gene that is composed of four identical subunits (3), has broader substrate specificity. Thioesterase II was reported to cleave C_6 to C_{18} acyl-CoA esters, as well as 3-hydroxyacyl–CoA esters (3, 27), while it did not function as a chain-terminating enzyme in fatty acid synthesis (21). Thus, the exact physiological function of thioesterase II in vivo is not known.

In this study, thioesterase II was coexpressed with PhaG to clarify the physiological role of thioesterase II.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and genetic techniques. All bacterial strains and plasmids used in this study are described in Table 1 and Fig. 1, while the primers used (synthesized by BioAsia Co., Shanghai, China) are listed in Table 2. Conjugation was as described by Simon et al. (26), and *Eshcrichia coli* S17-1 was employed as a donor strain. All other genetic techniques were performed following either the manufacturers' instructions or standard procedures (25).

Culture media. Mineral salts medium (23) containing 0.5 g of $(NH_4)_2SO_4$/liter and Luria-Bertani medium (25) were used in this study. All chemicals were analytical grade.

Flask fermentation conditions. Recombinant *E. coli* strains were incubated at 37°C and 200 rpm for 48 h on a rotary shaker (series 25 D; NBS, New Brunswick, N.J.) in 100 ml of Luria-Bertani medium containing 100 mg of ampicillin/liter and 50 mg of kanamycin/liter. IPTG (isopropyl-β-D-thiogalactopyranoside;1 mmol/liter), fructose (20 g/liter), and triclosan (0.1 mg/liter) were added to the culture after 9, 12, and 24 h, respectively. Just before the addition of fructose, 10 ml of broth from each culture was taken and examined for thioesterase II activity and transcriptional intensity. The cellular dry weight (CDW) and extracellular 3HD were analyzed as described below.

PHA synthase (encoded by *phaC*)-negative mutant *P. putida* GPp104 strains were inoculated in mineral salts medium containing 1 mmol of IPTG/liter and 20 g of glucose/liter at 30°C and 200 rpm for 48 h. If necessary, 50 mg of kanamycin/liter was added to the broth at the beginning of inoculation. At 12 h, 10 ml of broth from each culture was sampled for transcriptional assay of *phaG*.

3HD analysis. Liquid cultures were centrifuged at 10,000 × *g* for 15 min. The cells were washed twice and dried at 80°C for 8 h to determine the CDW. An aliquot of the supernatant (5 ml) was lyophilized for 48 h. Lyophilized materials were subjected to methanolysis and assayed with a gas chromatograph (18). This analysis was performed by injecting 1 μl of sample into a Hewlett-Packard model 6890 (Plus) series gas chromatograph system equipped with a 0.25-μm-diameter HP-INNOWax capillary column 30 m in length. The standard was a commercial product (H-3648; Sigma). The gas chromatography-mass spectroscopy (GC-MS) (AntoSystem XL GC-TurboMass; Perkin-Elmer, Norwalk, Conn.) analysis was performed using the sample for GC analysis described above. 3HD production ability was presented as the ratio of 3HD to CDW.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
JM105	*supE endA sbcB15 hsdR4 rpsL thi* Δ(*lac-proAB*)	25
CH01	Thioesterase II-negative mutant of *E. coli* JM105; *tesB*::*Cm*(*r*)	This study
S17-1	*recA*; harbors the *tra* genes of plasmid RP4 in the chromosome; *proA thi-1*	26
P. putida GPp104	PHA synthase-negative mutant of *P. putida* KT2442	10
Plasmids		
pBBR1MCS	*Cm*(*r*); broad host range; *lacPOZ′*	16
pBBR1MCS-2	*Kn*(*r*); broad host range; *lacPOZ′*	15
pBluescript SK(−)	*Ap*(*r*); *lacPOZ′*	25
pGEM-T	*Ap*(*r*);T-vector	Promega Co.
pTH19ksl	*Kn*(*r*); *agp5 lacZ′ repA*$_{ts1}$	9
pLZZGPp	pBluescript SK(−) derivative, containing *phaG* gene of *P. putida* under the control of the *lac* promoter	31
pLZZH01	pGEM-T derivative containing *tesB* gene of *E. coli* JM105	This study
pLZZH08	Deletion of the BamHI-BstBI fragment of plasmid pLZZH01	This study
pLZZH09	pBBR1MCS-2 derivative, containing *tesB* gene of *E. coli* JM105 under the control of the *lac* promoter	This study
pLZZH10	Deletion of the PmlI—SnaBI fragment of plasmid pLZZGPp	This study
pLZZH11	Insertion of the 1.7-kb BamHI-BstBI fragment of pBBR1MCS into plasmid pLZZH01	This study
pLZZH12	Insertion of the SacII (filled in)-SacI fragment of pLZZH11 into plasmid pTH19ksl digested with HincII and SacI	This study

Thioesterase assay. Cells sampled as described above were washed twice with 0.1 mol of Tris-hydrochloride buffer (pH 8.0)/liter and suspended in the same buffer, followed by homogenization on ice. Crude extracts were centrifuged at 1,000 × *g* and 4°C for 5 min. The resulting supernatants were monitored for thioesterase activity. The total-protein concentration was determined by the method of Bradford (5) using Coomassie Plus Protein Assay Reagent (Pierce) with a UV–visible-light spectrophotometer (Ultraspec 3300; Biochrom) and adjusted to 10 μg/ml. The assay for thioesterase II contained (per milliliter) 0.1 mmol Tris-hydrochloride buffer (pH 8.0), 100 nmol 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma), 20 nmol decanoyl-CoA (Sigma), and 100 μl of crude cell extract. Reduction of DTNB by CoA liberated in the thioesterase reaction was measured at 412 nm (22). The initial rates were measured using a recording spectrophotometer (Specord 200; Analytik Jena AG, Jena, Germany) (22). A unit of enzyme activity was defined as the amount of the enzyme catalyzing cleavage of 1 μmol of decanoyl-CoA per min under the above-mentioned conditions. The molar extinction coefficient of reduced DTNB was taken as 13,600 (22).

RNA analysis. Total RNA was isolated and purified using commercial kits (Shenergy Biocolor Biological Science and Technology Co., Shanghai, China). Purified total RNA was quantified using an Ultraspec 3300 UV–visible-light spectrophotometer. Reverse transcriptase (RT) PCR was performed with a cDNA Cycle kit (Invitrogen). The transcriptional analysis procedure was as described by Zhang and Cronan (30) employing *Ex Taq* DNA polymerase (TakaRa; Dalian, China). Briefly, 2 μg of isolated total RNA was reverse transcribed into cDNA in a 10-μl reaction mixture. Plasmid pLZZH08 or pLZZH10 was used as the competitive DNA and was carefully diluted in a series of concentrations that were determined using an Ultraspec 3300 UV–visible-light spectrophotometer. The reverse-transcriptase reaction product (1 μl) and different concentrations of competitive DNA (as specified in the figure legends [see Fig. 5 and 6]) were added to a 20-μl PCR mixture. The products were separated on a 2.5% agarose gel and stained with ethidium bromide, and the amounts of the products were estimated by video densitometry analysis using LabWorks Analysis software (UVP). The ratios of the fluorescence intensities of the PCR products of the competitive DNA to those of the RT-PCR products were plotted as a function of the concentration of the competitive DNA (30).

Statistical analysis. The data were presented as means ± standard errors of the mean (SEM). Statistical comparisons were performed using the Student *t* test with SPSS software. *P* values of <0.05 were considered statistically significant.

RESULTS

Construction and characterization of an isogenic *tesB* knockout mutant of *E. coli*. The coding region of *tesB* (including the native ribosome binding site) from *E. coli* JM105 was amplified by PCR employing primers P1 and P3 (Table 2). The ClaI and HincII restriction sites were introduced into the PCR product. The 929-bp product was inserted into the commercial vector pGEM-T (Promega), resulting in plasmid pLZZH01 (Fig. 1a). The DNA sequence of *tesB* obtained from the inserted DNA fragment (sequenced by BioAsia Co.) exactly matched that found in the GenBank database (accession number AE000151). The isogenic *tesB* knockout mutant *E. coli* CH01 was obtained by replacing a central 200-bp region of *tesB* with a chloramphenicol resistance cassette from pBBR1MCS and employing a temperature-sensitive vector, pTH19ks1 (Fig. 1a and 2). The isogenic mutant was verified by PCR using primers P1 and P3 and primers P2 and P3, respectively (Fig. 2).

3HD is synthesized by various recombinant *E. coli* strains. In order to evaluate the functionality of thioesterase II in *E. coli*, we produced various *E. coli* recombinants and assessed the 3HA production in these cells. Extracellular 3HD was confirmed by GC-MS (Fig. 3). Plasmid pLZZH01 was digested with ClaI and HincII. The 918-bp *tesB* gene fragment was inserted into the ClaI and SmaI restriction sites in vector pBBR1MCS-2, leading to plasmid pLZZH09, which contained the *tesB* gene under the control of the *lac* promoter (Fig. 1a). Overexpression of *tesB* in recombinant *E. coli* JM105 did not result in 3HD production, and *tesB*-containing knockout mutants grew normally (Table 3). However, compared with *E. coli* JM105 harboring only *phaG*, the yield of extracellular 3HD produced by the same strain harboring both *tesB* and *phaG* was

TABLE 2. Primers used in this study[a]

No.	Sequence
P1	5′-TCC ATC GAT GCG GCA GCT TTG TTA CT-3′
P2	5′-GGA GCT AAG GAA GCT AAA ATG GAG AA-3′
P3	5′-TTA GTC GAC GGT TTT CAC CTC CGG CT-3′
P4	5′-TTG TAT GCT GCA AAA GAG ACC GT-3′
P5	5′-TAC AGC AGC CAT TCA TTC AAA TT-3′
P6	5′-ACG GCC TCG TTC GCC CAG A-3′
P7	5′-GTC CAG GAA GTG GCC CGC A-3′

[a] All primers were synthesized by BioAsia Co. (Shanghai, China).

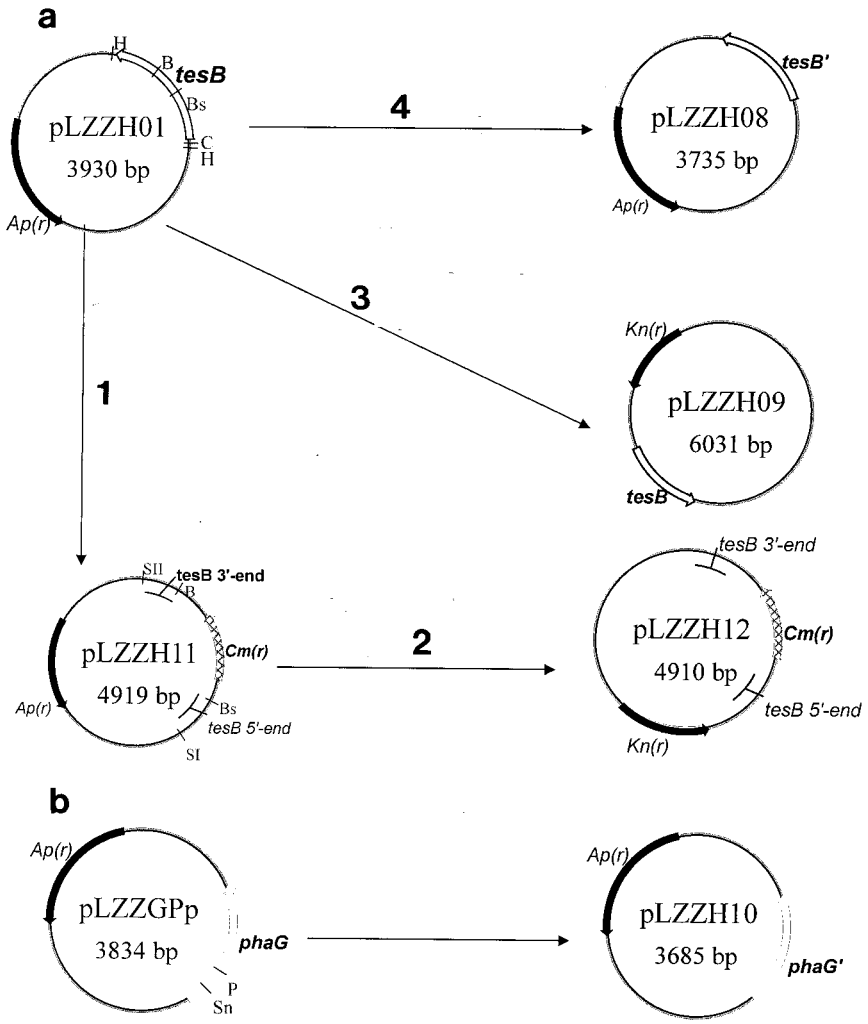

FIG. 1. Scheme of plasmids used in this study. (a) The *tesB* gene from the *E. coli* JM105 genome was amplified by PCR with primers P1 and P3 and inserted into the pGEM-T vector to yield the starting plasmid, pLZZH01. The chloramphenicol resistance cassette from pBBR1MCS replaced the BstBI/BamHI fragment of *tesB* in pLZZH01, leading to the plasmid pLZZH11 (step 1). The SacII (filled in)-SacI fragment from pLZZH11 was inserted into the HincII/SacI sites of the temperature-sensitive vector pTH19ks1, resulting in pLZZH12 (step 2), which was used to construct the *tesB* knockout mutant *E. coli* strain. The HincII/ClaI fragment of pLZZH01, containing the *tesB* gene, was introduced into the corresponding sites of the vector pBBR1MCS-2 to yield pLZZH09 (step 3). BstBI/BamHI double-digested pLZZH01 was sequentially blunt ended by T4 polymerase and ligated to obtain the competitive plasmid pLZZH08 for the *tesB* transcriptional assay (step 4). (b) pLZZGPp was digested with PmlI and SnaBI, followed by ligation, resulting in pLZZH10, used for the transcriptional assay for *phaG*. *tesB* encodes thioesterase II; *tesB′*, the BstBI/BamHI fragment of *tesB*, was deleted; *phaG* encodes 3HD-ACP–CoA transacylase; *phaG′*, the SnaBI/PmlI fragment of *phaG*, was deleted. *Ap(r)*, *Kn(r)*, and *Cm(r)*, ampicillin, kanamycin, and chloramphenicol resistance genes, respectively. B, BamHI; Bs, BstBI; C, ClaI; H, HincII; P, PmlI; SI, SacI; SII, SacII; Sn, SnaBI.

significantly increased (Table 3). Extracellular 3HD production by the *tesB*-negative recombinant *E. coli* CH01 (pLZZGPp, pBBR1MCS-2) was ~3% of the CDW, significantly lower than that of the *tesB*-positive strain *E. coli* JM105(pLZZGPp, pBBR1MCS-2), which was ~30% of the CDW. Introduction of the *tesB* gene in *E. coli* CH01 [as *E. coli* CH01(pLZZGPp, pLZZH09)] returned 3HD production to the same level as that of the *tesB*-positive control strain [as *E. coli* JM105(pLZZGPp, pLZZH09)] (Table 3). The results revealed that thioesterase II plays an important role in converting 3HD-CoA to free 3HD in *E. coli*.

Thioesterase II assay of recombinant *E. coli* strains. Sole heterogenous expression of *phaG* in *E. coli* JM105 was accompanied by a 1.5-fold increase in thioesterase II activity compared with the control strain, *E. coli* JM105(pBluescript SK(−), pBBR1MSC-2), which was 15.8 mU/mg of total protein and was defined as 100% (Fig. 4). No effect was observed when the host was a *tesB*-negative strain, *E. coli* CH01.

Transcriptional assay of *tesB*. Semiquantitative RT-PCR was employed to study the level of *tesB* transcription in *phaG*-containing *E. coli* JM105(pLZZGPp, pBBR1MCS-2) sampled from the flask cultures with *E. coli* JM105(pBluescript SK(−), pBBR1MCS-2) as a control. The principle of this method is the application of a known concentration of plasmid pLZZH08 to compete with the RT-PCR product. To obtain pLZZH08, pLZZH01 was digested with BamHI and BstBI, followed by blunt-ending and ligation processes (Fig. 1a). RT-PCR was carried out by adding known concentrations of competitive

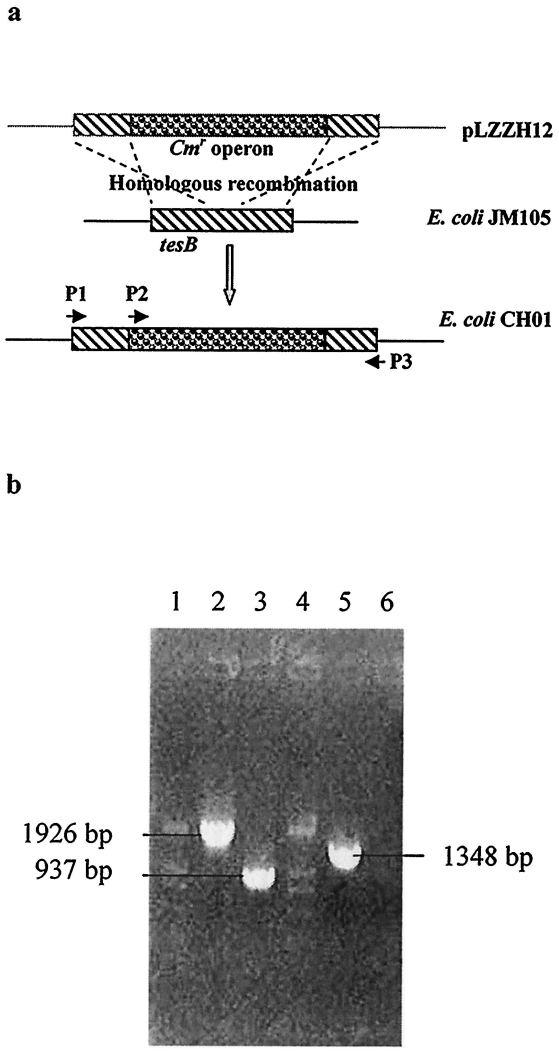

FIG. 2. Scheme of construction (a) and identification (b) of *E. coli* CH01. (a) The derivative of the temperature-sensitive vector pTh19ks1, pLZZH12, harboring a DNA fragment in which the chloramphenicol resistance cassette from pBBR1MCS replaced the BstBI/BamHI fragment of *tesB*, was introduced into *E. coli* JM105. Due to homologous recombination, a *tesB* knockout mutant strain, *E. coli* CH01, was obtained. P1 anneals with the 5′ end of *tesB*, P2 anneals with the chloramphenicol resistance gene (*Cm^r*), and P3 anneals with the 3′ end of *tesB*. (b) Lanes 1 and 4, DL2000 DNA marker (TaKaRa); lanes 2 and 3, PCR analysis of *E. coli* CH01 and *E. coli* JM105 with primers P1 and P3 (the expected product sizes determined from sequence data were 1,926 and 937 bp, respectively); lanes 5 and 6, PCR analysis of *E. coli* CH01 with primers P2 and P3 (lane5; the expected product size was 1,348 bp) and of the control strain, *E. coli* JM105, with the same primers (lane 6; no product was expected).

DNA to the reaction mixtures as described in Materials and Methods. Total-RNA preparations from *E. coli* JM105(pBluescript SK(−), pBBR1MCS-2) and *E. coli* JM105(pLZZGPp, pBBR1MCS-2) were used as RT reaction templates, and the concentration of cDNA formed was taken to be proportional to the mRNA concentration. P4 and P5 were annealed to central sequences of the *tesB* gene (Fig. 5a); thus, the products of *tesB* transcripts were 611 and 415 bp for pLZZH08 (Fig. 5b). The ratios of the fluorescence intensities of the PCR products

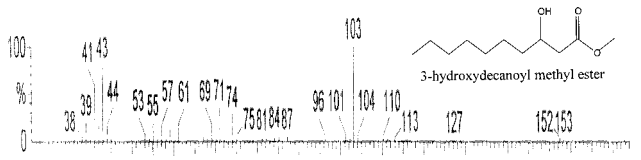

FIG. 3. Identification of extracellular 3-hydroxydecanoic acid. Supernantant (5 ml) was lyophilized for 48 h. The lyophilized material was subjected to methanolysis, and a GC-MS assay was performed (AntoSystem XL GC-TurboMass).

to those of the RT-PCR products were plotted as a function of the concentration of the competitive DNA (Fig. 5c). When the ratio was 1, the molar concentration of the competitive DNA added to the reaction mixture was identical to the molar concentration of the *tesB* cDNA, which was proportional to the *tesB* mRNA concentration (30). This result revealed a remarkable increase in transcription of *tesB* when *phaG* was expressed in *E. coli* JM105.

Heterologous expression of *tesB* in *Pseudomonas putida* GPp104. Heterologous expression of *tesB* in *P. putida* GPp104 led to the extracellular accumulation of 3HD, while no 3HD was detected in the growth media inoculated with *P. putida* GPp104 and recombinant *P. putida* GPp104 harboring only the vector pBBR1MCS-2 (Table 4). On the other hand, pLZZH10, the derivative plasmid of pLZZGPp (Fig. 1b), was used as the competitive template for semiquantitative RT-PCR assay of *phaG* with the primer pair P6 and P7 (Fig. 6a). The PCR product of pLZZH10 was 496 bp, while the RT-PCR product was 645 bp (Fig. 6b). Interestingly, heterologous expression of *tesB* in *P. putida* GPp104 led to a significant increase in *phaG* transcription (Fig. 6c).

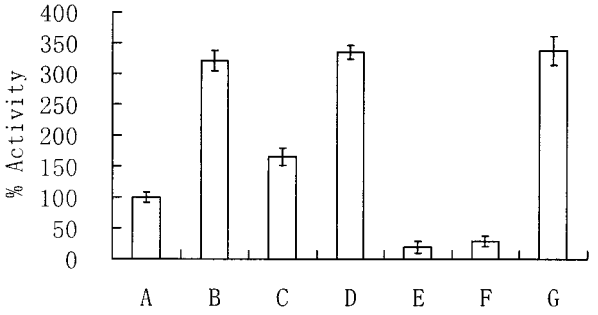

FIG. 4. Enzyme activity assay of thioesterase II enzymes from recombinant *E. coli* strains. The mean of three independent samples of *E. coli* JM105(pBluescript SK(−), pBBR1MCS-2) was 15.8 mU/mg of protein, which was defined as 100%. Bars: A, *E. coli* JM105(pBluescript SK(−), pBBR1MCS-2); B, *E. coli* JM105(pBluescript SK(−), pLZZH09); C, *E. coli* JM105(pLZZGPp, pBBR1MCS-2); D, *E. coli* JM105(pLZZGPp, pLZZH09); E, *E. coli* CH01(pBluescript SK(−), pBBR1MCS-2); F, *E. coli* CH01(pLZZGPp, pBBR1MCS-2); G, *E. coli* CH01(pLZZGPp, pLZZH09). *E. coli* CH01 is the isogenic *tesB*-negative mutant of *E. coli* JM105. Plasmid pLZZGPp is a derivative of the vector pBluescript SK(−) harboring *phaG* under the control of the *lac* promoter, while pLZZH09 is a derivative of vector pBBR1MCS-2 harboring *tesB* under the control of the *lac* promoter. The data are shown as means ± SEM ($n = 3$).

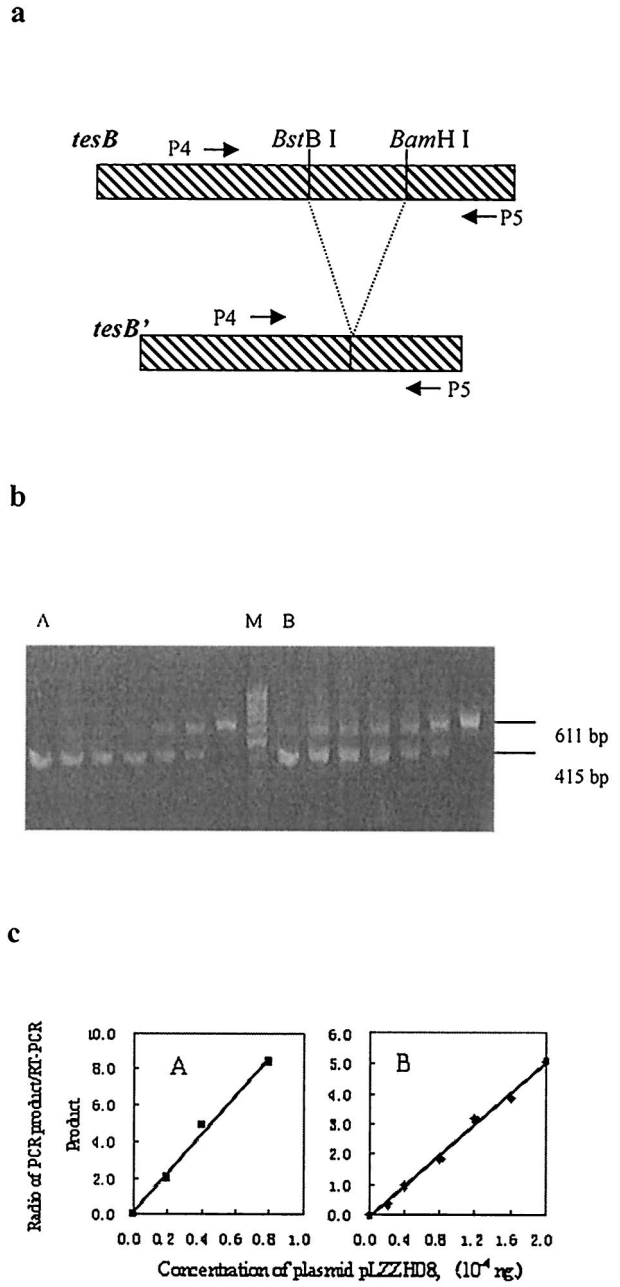

FIG. 5. Semiquantitative RT-PCR assay of *tesB* transcription in both the *phaG*-negative strain *E. coli*(pBluescript SK(−), pBBR1MCS-2) (A) and the *phaG*-containing strain *E. coli* JM105(pLZZGPp, pBBR1MCS-2) (B). The strains were sampled from the flask cultures (see the text for details). (a) RT-PCR and PCR were performed by employing primers P4 and P5. Plasmid pLZZH08, harboring a DNA fragment, *tesB'* (*tesB* without a central BstBI/BamHI fragment), was used as competitive DNA. (b and c) Competitive DNA (2.0×10^{-4}, 1.6×10^{-4}, 1.2×10^{-4}, 0.8×10^{-4}, 0.4×10^{-4}, 0.2×10^{-4}, and 0 ng) was added to lanes from left to right, respectively, in gels A and B. Lane M is a GeneRuler 50-bp DNA ladder (MBI). The fluorescence intensities of the bands on the agarose gel in each lane were quantified by densitometry following ethidium bromide staining. The ratio of the intensity of the PCR product (415 bp) of competitive DNA to that of the RT-PCR product (611 bp) was calculated for each reaction, and these ratios were plotted as a function of the competitive DNA concentration (c). The data are shown as means of six experiments. The error bars for SEM are not visible because they are smaller than the symbol size.

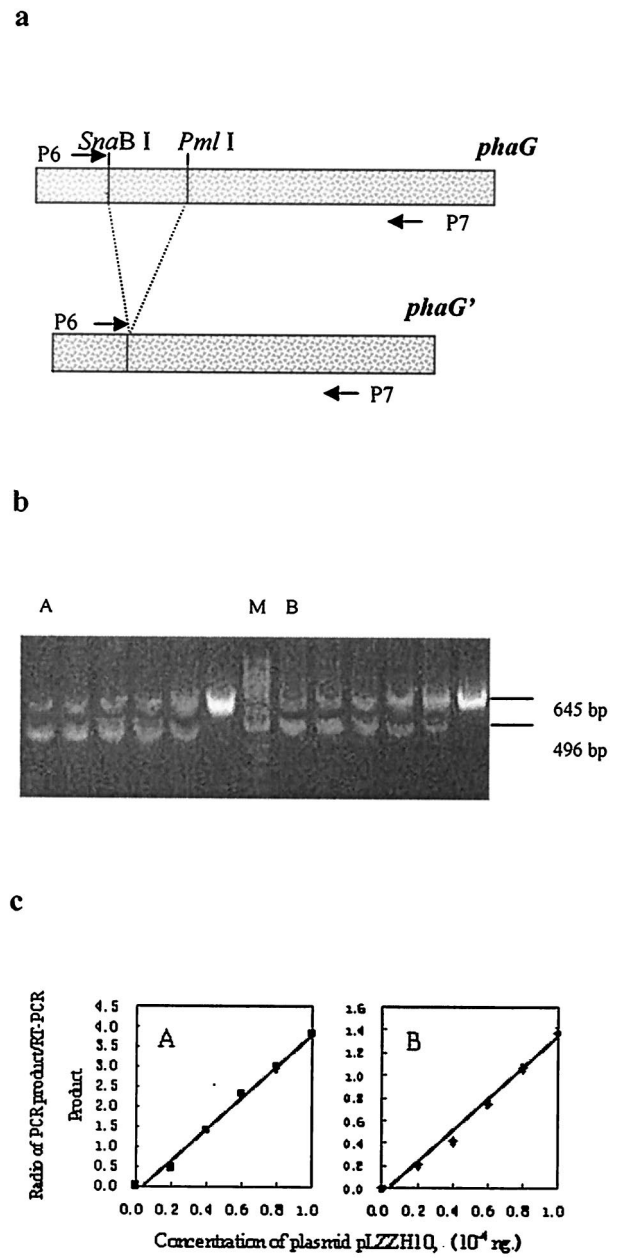

FIG. 6. Semiquantitative RT-PCR assay of *phaG* transcription in the *tesB*-negative strain *P. putida* GPp104(pBBR1MCS-2) (A) and the *tesB*-containing strain *P. putida* GPp104(pLZZH09) (B). The strains were sampled from the flask cultures (described in Materials and Methods). (a) RT-PCR and PCR were performed by employing primers P6 and P7. Plasmid pLZZH10, harboring a DNA fragment, *phaG'* (*phaG* without a central SnaBI/PmlI fragment), was used as competitive DNA. (b and c) Competitive DNA (1.0×10^{-4}, 0.8×10^{-4}, 0.6×10^{-4}, 0.4×10^{-4}, 0.2×10^{-4}, and 0 ng) was added to lanes from left to right, respectively, in gels A and B. Lane M is a GeneRuler 50-bp DNA ladder (MBI). The fluorescence intensities of the bands on the agarose gel in each lane were quantified by densitometry following ethidium bromide staining. The ratio of the intensity of the PCR product (496 bp) of competitive DNA to that of the RT-PCR product (645 bp) was calculated for each reaction, and these ratios were plotted as a function of the competitive DNA concentration (c). The data are shown as means of five experiments. The error bars for SEM are not visible because they are smaller than the symbol size.

TABLE 3. Extracellular accumulation of 3HD by various recombinant *E. coli* strains[a]

E. coli strain	Plasmid	CDW (g/liter)	3HD (mg/liter)	%3HD/CDW (wt/wt)
JM105	pBluescript SK(−), pBBR1MCS-2	1.84 ± 0.05	ND[b]	
	pBluescript SK (−), pLZZH09	1.75 ± 0.03	ND	
	pLZZGPp, pBBR1MCS-2	2.11 ± 0.04	642 ± 12	30.4 ± 0.5
	pLZZGPp, pLZZH09	2.20 ± 0.04	1,021 ± 22	45.9 ± 0.8
CH01	pBluescript SK(−), pBBR1MCS-2	1.81 ± 0.03	ND	
	pLZZGPp, pBBR1MCS-2	2.10 ± 0.05	72 ± 6	3.4 ± 0.1
	pLZZGPp, pLZZH09	2.21 ± 0.03	956 ± 18	43.3 ± 0.6

[a] Recombinant *E. coli* strains were cultivated in 500-ml conical flasks containing 100 ml of Luria-Bertain medium supplemented with 100 mg of ampicillin/liter and 50 mg of kanamycin/liter according to the resistance properties of the plasmids (Table 1 and Fig. 1). *E. coli* CH01 is the isogenic *tesB*-negative knockout mutant of *E. coli* JM105. Plasmid pLZZGPp is a derivative of the vector pBluescript SK(−) harboring *phaG* under the control of the *lac* promoter, while pLZZH09 is a derivative of the vector pBBR1MCS-2 harboring *tesB* under the control of the *lac* promoter. Cells were grown at 37°C and 200 rpm for 48 h; 1 mmol of IPTG/liter, 20 g of fructose/liter, and 0.1 mg of triclosan/liter were added to the culture after 9, 12, and 24 h, respectively. The data are shown as means ± SEM ($n = 6$).
[b] ND, not detectable.

DISCUSSION

As monomers of PHA, 3HA have great potential for many applications as intermediates for the synthesis of valuable compounds (17). The introduction of β-ketothiolase (*phbA*) and acetoacetyl-CoA reductase (*phbB*) genes from *Ralstonia eutropha* and phosphotransbutyrylase (*ptb*) and butyrate kinase (*buk*) genes from *Clostridium acetobutylicum* into *E. coli* established a pathway for extracellular 3-hydroxybutyric acid production starting from acetyl-CoA (8). Expression of PhaG could lead to extracellular accumulation of valuable medium-chain-length-3HA, such as 3HD, by recombinant *E. coli* from glucose or fructose (31). This study demonstrated that PhaG directs the fatty acid de novo biosynthesis pathway to 3HD production, as well as to PHA synthesis (24). However, medium-chain-length-3HA synthesis utilizing PhaG involves a complex process.

In this study, for the first time, *tesB* of *E. coli* was expressed in vivo together with *phaG* in order to understand how the two genes affect 3HD biosynthesis. The engineered pathway used carbon sources from glycolysis, followed by fatty acid de novo biosynthesis. Next, PhaG converted 3HD-ACP to 3HD-CoA (24). Finally, the thioester of 3HD-CoA was cleaved to yield 3HD (Fig. 7). As a cytosolic tetrameric protein (3), thioesterase II plays an important role in this pathway, as demonstrated by three facts: (i) in the presence of PhaG, overexpression of *tesB* in *E. coli* led to increased 3HD synthesis; (ii) in contrast to a *tesB*-positive strain, 3HD production was lowered significantly in a *tesB*-negative *E. coli* strain harboring *phaG*; and (iii) the reintroduction of *tesB* restored 3HD production ability (Table 3).

Interestingly, when *phaG* was expressed in *E. coli*, both the activity and the transcription level of thioesterase II increased (Fig. 4 and 5). It appeared that the abnormal accumulation of 3HD-CoA induced by PhaG up-regulated *tesB* gene transcription. On the other hand, when *tesB* was expressed in the *phaC*-negative strain *P. putida* GPp104, 3HD produced by the recombinant strain was detected in the culture, and the transcription level of *phaG* was remarkably increased (Table 4 and Fig. 6). These results indicate a mechanism for 3HD synthesis like that described in Fig. 7. As is known, in a *phaC*-

TABLE 4. Extracellular accumulation of 3HD by recombinant *P. putida* GPp104 strains from glucose[a]

Plasmid	CDW (g/liter)	3HD (mg/liter)
	1.13 ± 0.06	ND[b]
pBBR1MCS-2	0.77 ± 0.05	ND
pLZZH09	0.97 ± 0.03	208 ± 10

[a] Recombinant *P. putida* GPp104 strains were cultivated in 500-ml conical flasks containing 100 ml of mineral salts medium containing 1 mmol of IPTG/liter and 20 g of glucose/liter. If necessary, 50 mg of kanamycin/liter was added to the broth at the beginning of fermentation. Plasmid pLZZH09 was the derivative of the vector pBBR1MCS-2 harboring *tesB* under the control of the *lac* promoter. The cells were grown at 30°C and 200 rpm for 48 h. The data are shown as means ± SEM ($n = 5$).
[b] ND, not detectable.

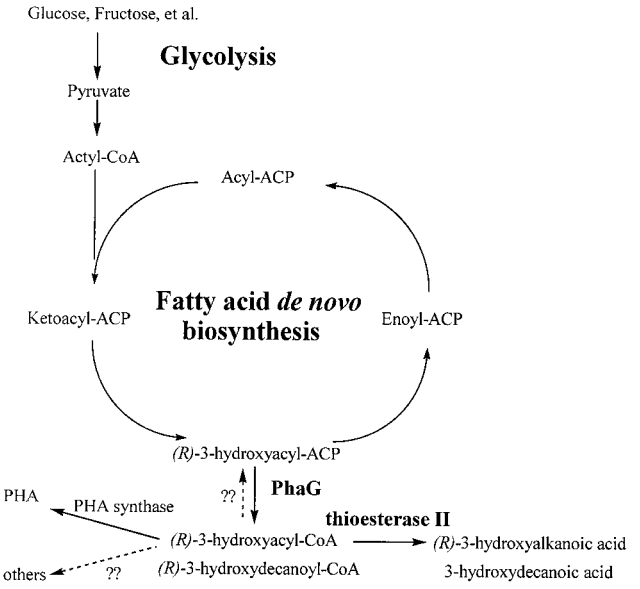

FIG. 7. Schematic illustration of biosynthesis pathway for 3HD and PHA from unrelated carbon sources, such as glucose and fructose. 3HD-ACP–CoA transacylase (PhaG) is encoded by the gene *phaG* from *P. putida*, while thioesterase II is encoded by the gene *tesB* from *E. coli*. This engineered pathway began with glycolysis, followed by fatty acid de novo biosynthesis. Under the catalysis of the transacylase, 3HD-ACP is converted to 3HD-CoA. 3HD-CoA is either cleaved by thioesterase II to yield 3HD or polymerized by PHA synthase to yield PHA. In native PHA synthase-negative *P. putida* GPp104, 3HD-CoA may be converted to other intermediates or back to 3HD-ACP, due to the absence of thioesterase II.

positive *P. putida* strain, 3HD-CoA was removed by PHA synthase, resulting in the accumulation of PHA polymers as a sink for reducing power (19). When PhaG was introduced in recombinant *E. coli*, 3HD-CoA accumulated to an abnormally high level. Due to the absence of PHA synthase in *E. coli*, thioesterase II seems to play a role in reducing the accumulation of 3HD-CoA by removing CoA ester to release its free-acid derivative (Fig. 7). In *phaC*-negative *P. putida* GPp104, there should be another mechanism to reduce CoA ester accumulation, while the heterologous expression of *tesB* led to increased transcription of *phaG* by removing the product of the transacylation reaction catalyzed by PhaG (Fig. 6 and 7). Thus, both PhaG and thioesterase II are essential for extracellular production of 3HD.

Surprisingly, two thioesterases whose activities are similar to those of *E. coli* were found in *Rhodopseudomonas sphaeroides* (4). Similar thioesterase complementation occurring in dissimilar bacteria suggests that these enzymes play important roles (22). However, the physiological function of thioesterase II was not fully understood, as no obvious physiological or biochemical defect was observed in *E. coli* with *tesB* overexpression or deletion (21, 22). However, the high specificity for acyl-CoA substrates suggests that one role of this enzyme could be to prevent the accumulation of intracellular acyl-CoA (27), although the normal cellular enzyme level is generally sufficient to preclude acyl-CoA accumulation (12). In the present study, by employing a direct 3HD biosynthesis pathway for high-level accumulation of intracellular 3HD-CoA, thioesterase II was indeed found to play a role in preventing the accumulation of intracellular acyl-CoA and thus in maintaining an appropriate acyl-CoA pool in vivo.

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