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Regulated Expression of the *Alcaligenes eutrophus pha* Biosynthesis Genes in *Escherichia coli*

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A novel poly-β-hydroxybutyrate (PHB) production system in which the expression and gene dosage of the *Alcaligenes eutrophus pha* biosynthetic operon were effectively regulated by cultivation temperature was constructed in *Escherichia coli*. The *pha* operon was fused to the negatively regulated *tac* promoter and cloned into a vector in which the copy number is temperature dependent. A two-phase process was employed to produce PHB during fed-batch growth. In the growth phase, the culture was maintained at a low temperature. Under this condition, the plasmid copy number was depressed and the number of LacI proteins was sufficient to repress *tac*::*pha* transcription. The production phase was initiated by temperature upshift. At the elevated temperature, the number of plasmids surpassed the number of LacI repressors, which resulted in rapid induction of *tac*::*pha* transcription, synthesis of poly-β-hydroxyalkanoate-specific proteins, and polymer synthesis. During the production phase, the PHB production rate was 1.07 g of PHB liter^{-1} h^{-1} under optimized conditions. This rate is comparable to that of bacteria which naturally produce this polymer.

Poly-β-hydroxyalkanoates (PHAs) are carbon storage polymers that are accumulated by many bacteria in response to nutrient limitation (1, 41). Much research has been focused on the production of PHA in bacteria because of its potential use as a biodegradable thermoplastic (13, 17, 30). Poly-β-hydroxybutyrate (PHB), the first known candidate of this class of biomaterials (19), is synthesized by the action of three enzymes, β-ketothiolase, acetoacetyl-coenzyme A (CoA) reductase, and PHA synthase (26, 38). β-Ketothiolase condenses two acetyl-CoA molecules to acetoacetyl-CoA, which is reduced by acetoacetyl-CoA reductase to D-(−)-β-hydroxybutyryl-CoA. Subsequent polymerization to PHB is catalyzed by PHA synthase.

The PHB biosynthetic genes of *Alcaligenes eutrophus* H16 have previously been cloned into *Escherichia coli* (29, 37, 40), and the DNA sequence of the operon has been determined (16, 28, 29). It consists of three genes, *phaC*, *phaA*, and *phaB*, which encode PHA synthase, β-ketothiolase, and acetoacetyl-CoA reductase, respectively. The transcriptional start site was determined by S1 nuclease mapping (36). The promoter is similar to the *E. coli* promoter recognized by σ^{70} (34). Studies in this laboratory have shown that *pha* genes are well expressed in *E. coli* (40) and that PHB accumulates to levels as high as 95% of cellular dry weight in some *E. coli* strains (16).

Because of this high level of expression, *E. coli* may be useful for commercial production of PHB. PHB-positive *E. coli* strains have been shown to accumulate large amounts of PHB when grown on whey (16) and to do so in a manner that is independent of nutrient limitation for induction of polymer formation. In addition, the polymer is easily purified from the cell since it can be released by osmotic lysis (20). Finally, recombinant DNA techniques are well developed in *E. coli*, allowing easy genetic modification and alternate expression strategies. However, constitutive *pha* expression places a tremendous metabolic burden on the cell, retards cell growth, and often results in rapid selection of mutations that lead to loss of the PHB-producing phenotype (44). To some extent, this genetic instability can be controlled by using antibiotics in the culture to select for plasmid-bearing cells, but even this is not totally effective and its use results in a significant cost increase for the polymer. Therefore, in order for recombinant strains to be employed in commercial applications, methodologies that reduce the metabolic stress on the cell, resulting in a stable PHB-producing phenotype, must be devised.

Many strategies have been designed to reduce the problem of metabolic burden in recombinant *E. coli* (3). Of these, the most frequently used methods are to regulate the expression of genes via a heterologous promoter or, alternatively, to regulate expression by controlling gene dosage. To control heterologous gene expression, several strong, well-regulated promoters are commonly used (2, 10, 12, 14). Among these, the *tac* promoter (7, 35) has proven to be particularly useful. Binding of the *lacI* gene product to the operator site represses transcription from the promoter; in the presence of the chemical inducer isopropyl-β-D-thiogalactopyranoside (IPTG), the repressor is converted to an inactive form and transcription is initiated (15). To control gene dosage, vectors in which the copy number is temperature dependent are used. At noninducing temperatures, the copy number of the plasmid is between 1 and 10, but at inducing temperatures, the copy number of the plasmid may increase to as high as 1,000 (25).

In this paper, we describe the construction of a temperature-regulated runaway replication vector that contains a *tac*::*pha* operon fusion to tightly control expression of the *pha* biosynthetic genes. At noninducing temperatures, the number of LacI proteins tightly represses the transcription of *pha* genes. At inducing temperatures, the copy number of *tac*::*pha* plasmids surpasses the number of repressor molecules, initiating rapid PHB production. The combination of tight control under noninducing conditions and strong expression under inducing conditions facilitates very high levels of PHB production in a system that does not need to be stabilized by an antibiotic. In addition, this expression system differs from most previously described *tac*-regulated expression systems in that the addition of a chemical inducer is not necessary.

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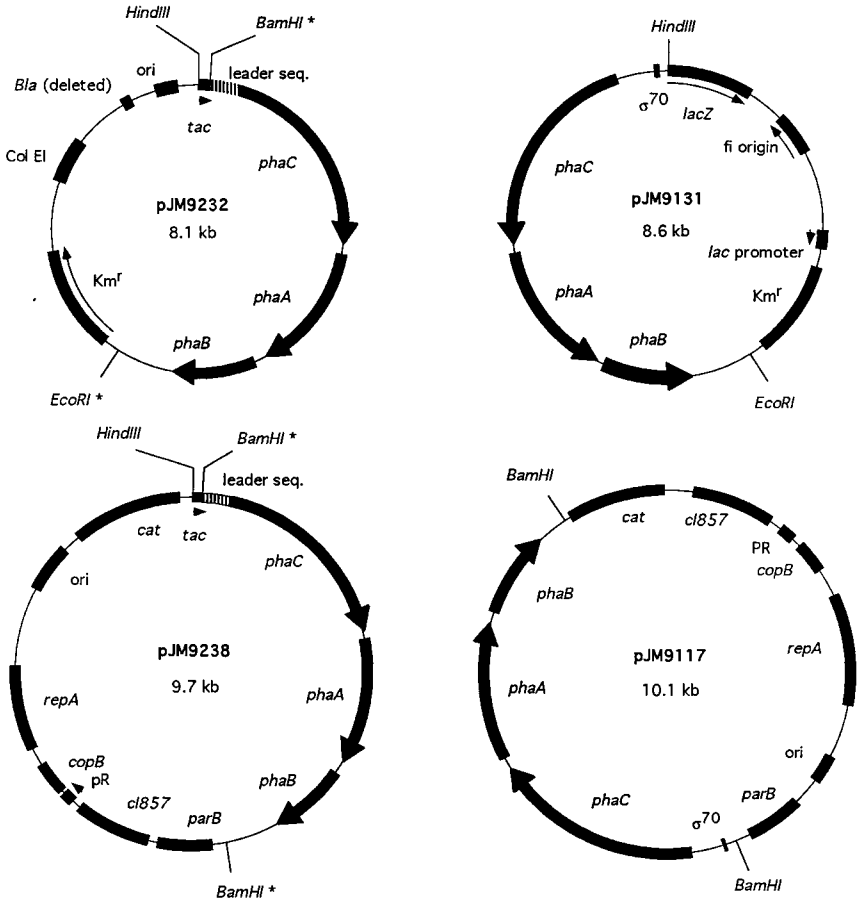

FIG. 1. Vectors used for expression of the PHA synthesis genes of *A. eutrophus* in *E. coli* HMS174. Vectors pJM9131 and pJM9232 are multicopy vectors, whereas pJM9117 and pJM9238 are runaway replication vectors. The expression of PHA synthesis genes in pJM9117 and pJM9131 is controlled by the native σ^{70} promoter of *A. eutrophus*, whereas the PHA operons in pJM9232 and pJM9238 are controlled by the *tac* promoter. Restriction sites which were removed during cloning are labeled by asterisks.

MATERIALS AND METHODS

Bacterial strains. For routine cloning, plasmids were introduced into *E. coli* DH5α [*endA1 hsdR17 supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* (ϕ80*dlacd*(*lacZ*)*M15*)] (Gibco BRL, Rockville, Md.) or *E. coli* XL1-Blue [F′::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*/*recA1 endA gyrA96* (Nal^r) *thi hsdR17 supE44 relA1 lac*] (Stratagene, La Jolla, Calif.). For subsequent experiments, plasmids were introduced into *E. coli* HMS174 (*recA1 hsdR* Rif^r) (39, 42).

Media. For batch culture studies, bacteria were grown in Luria-Bertani (LB) medium (21) that contained the appropriate antibiotics (Sigma, St. Louis, Mo.) at the following concentrations: kanamycin, 50 μg/ml; streptomycin, 10 μg/ml; chloramphenicol, 25 μg/ml. When specified, IPTG (Sigma) was added to a final concentration of 10 mM. Unless otherwise stated, all cultures were incubated at 37°C and 225 rpm in an orbital shaker and their growth was monitored by measuring optical density at 600 nm (OD_{600}).

DNA manipulations. Ligations were performed with T4 DNA ligase (Gibco BRL) as described by the manufacturer. Restriction digestions and other molecular biology techniques were performed as previously described (21). Restriction endonucleases were obtained from Gibco BRL or New England Biolabs (Boston, Mass.). Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim (Indianapolis, Ind.). T4 DNA polymerase was obtained from Gibco BRL. Linkers were obtained from New England Biolabs. DNA fragments were purified from agarose gels by using the GeneClean kit (BIO 101, La Jolla, Calif.). Plasmid DNA was purified by the alkaline lysis method (21). Plasmids were introduced into strains by electroporation as previously described (22) with the Gene Pulser (Bio-Rad, Richmond, Calif.).

Recombinant plasmids. Relevant characteristics of the *pha* expression vectors used in this study are shown in Fig. 1. Plasmid pJM9131 is a multicopy plasmid that contains the *A. eutrophus pha* operon and has been previously described (44).

A PHB$^+$ runaway replicon plasmid was constructed by cloning a 4.8-kb *Bam*HI fragment that contained the *phaC* operon and the natural promoter (from approximately base number 400 of the published sequence [16] to approximately 400 bp downstream of *phaB* [40]) into the *Bam*HI site of the runaway replication vector pRA89 (Nycomed Pharma, Copenhagen, Denmark). The resulting plasmid was designated pJM9117 (Fig. 1).

To construct a runaway replicon plasmid that contained the *tac* promoter fused to the *pha* operon, the runaway replication vector pRA90 (Nycomed Pharma) was digested with *Bam*HI and the 5′ recessed ends were filled in with T4 DNA polymerase. A 4.6-kb *Bam*HI-*Eco*RI DNA fragment that contained the *pha* operon minus its native promoter (from approximately base number 550 of the published sequence [16] to approximately 400 bp downstream of *phaB* [40]) was ligated to the *Bam*HI-*Hin*dIII-cut *tac* promoter cartridge (Pharmacia, Piscataway, N.J.) so that the *tac* promoter was in the same orientation as the native *pha* promoter that it had replaced (*Bam*HI end ligated to *Bam*HI end). The resulting *Hin*dIII-*Eco*RI 4.7-kb fragment was blunt ended with T4 polymerase and ligated into the filled-in *Bam*HI site of plasmid pRA90. The resulting plasmid contained the *tac*::*pha* operon fusion, with the *tac* promoter proximal to the *c*I857 gene on pRA90, and was designated pJM9238 (Fig. 1).

A multicopy plasmid that contained the *tac* promoter fused to the *pha* operon was constructed by cloning the 4.7-kb *tac*::*pha* fusion fragment described above (blunt ending of *Hin*dIII-*Eco*RI ends) into the *Bam*HI site (filled in with T4 polymerase) of pBluescript-SK$^+$ (Stratagene). To increase plasmid stability, kanamycin resistance GenBlock (*Eco*RI) (Pharmacia) was ligated into the filled-in *Spe*I site downstream of the *pha* operon. Then this plasmid was digested with *Dra*I to remove a 0.71-kb fragment from the *bla* structural gene and religated. The resulting plasmid was designated pJM9232 (Fig. 1).

Stability of PHB phenotype and drug resistance. Strains were streaked from frozen stocks onto Luria agar plates that contained the appropriate antibiotics. Single colonies were picked, screened for the PHB-producing phenotype, and then grown to saturation in 3 ml of LB medium that contained the appropriate antibiotics. A 12.5-μl aliquot of each saturated culture was used to inoculate 50 ml of LB medium that contained 2% glucose, which was incubated in an orbital shaker at 35°C and 175 rpm for 24 h. We estimate that cultures had undergone

12 doublings upon reaching saturation. At that time, a 12.5-μl aliquot of each culture was used to inoculate 50 ml of LB medium that contained 2% glucose. This cycle was repeated until the original culture had undergone approximately 50 doublings. Each saturated culture was diluted, spread onto LB plates, and incubated at 37°C for 18 to 20 h. Colonies were picked and transferred to LB plates that contained the appropriate antibiotics to screen for the presence of the plasmid and to LB plates that contained 2% glucose to screen for the PHB-producing phenotype. Plates were incubated at 37°C for 30 h. The plates supplemented with glucose were stained for 15 min with Sudan Black B (Sigma) (9) to recognize *pha*-negative secondary mutants.

Comparison of PHB production in native and *tac* promoter *pha* clones. To more effectively control expression of the *tac* promoter in this and other experiments, the multicopy plasmid pJM9232, which contained a *tac*::*pha* fusion, was transferred to *E. coli* HMS174 which harbored plasmid pMS421 (obtained from G. Weinstock). The latter plasmid is a low-copy-number vector that confers streptomycin resistance and contains the *lacI*^{q} gene, which overproduces the Lac repressor protein (23). HMS174(pJM9131) and HMS174(pJM9232, pMS421) were each inoculated into 50 ml of LB medium that contained the appropriate antibiotics in a 250-ml Erlenmeyer flask and incubated at 37°C. HMS174 (pJM9117) and HMS174(pJM9238) were grown in the same manner except that the incubation temperature was 30°C. After 15 h, cultures were diluted to a final optical density of 0.1 in 250 ml of the same medium in a 1-liter baffled Erlenmeyer flask, glucose was added to a final concentration of 2%, and cultures were incubated as before. The *pha* operon was induced in HMS174(pJM9232, pMS421) at an optical density of 2.75 by the addition of IPTG. The *pha* operon was induced in HMS174(pJM9117) and HMS174(pJM9238) at an optical density of 0.7 by transferring cultures to a 41°C water bath for 30 min. Following induction, cultures were incubated at 37°C. Aliquots were withdrawn for dry weight determinations and quantitation of PHB production as previously described (39). Glucose concentrations were quantitated by using the Sigma diagnostic glucose assay kit according to the manufacturer's procedure (no. 635). The plasmid copy number was determined for each culture as previously described (8, 31).

Quantitation of *pha* operon gene products by SDS-PAGE. HMS174, HMS174(pJM9131), and HMS174(pJM9232, pMS421) were each inoculated into 3 ml of LB medium that contained the appropriate antibiotic(s), if necessary, and cultures were incubated at 37°C for approximately 15 h. HMS174(pJM9117, pMS421) and HMS174(pJM9238) were grown in a similar manner except that the incubation temperature was 30°C. Cultures were diluted 1:100 in 50 ml of the same medium in a 250-ml baffled Erlenmeyer flask, glucose was added to a final concentration of 1% (wt/vol), and cultures were incubated as before. The *pha* operons in HMS174(pJM9232, pMS421), HMS174(pJM9117), and HMS174 (pJM9238) were induced as described above. Following induction, cultures were incubated at 37°C and shaken at 200 rpm. Samples of 1 ml were withdrawn at regular time intervals, and cells were harvested by centrifugation, resuspended in 200 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (10% [vol/vol] glycerol, 2% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol, 0.025% [wt/vol] bromphenol blue in 62.5 mM Tris-HCl [pH 6.8]), and boiled for 5 min. Proteins were separated by SDS-PAGE on precast Mini-PROTEAN II Ready gels (Bio-Rad) (12% or 12 to 20% gradient) in a Mini-PROTEAN II electrophoresis cell (Bio-Rad) and BRL model 200 power supply. Prestained SDS-PAGE standards (catalog no. 161-0305 [Bio-Rad]) were used as molecular weight markers. Proteins were visualized by silver staining with the Bio-Rad Silver Stain Plus kit according to the manufacturer's protocol. Gels were dried between cellophane sheets (Integrated Separation Systems, Natick, Mass.), and the *pha* gene products, which were tentatively assigned by molecular mass (40.5 kDa, β-ketothiolase [16]; 26.3 kDa, acetoacetyl-CoA reductase [16]), were quantitated as a relative percentage of total protein by densitometry with an Ultrascan XL enhanced laser densitometer (Pharmacia LKB, Uppsala, Sweden).

Enzymatic analyses. For enzymatic analyses, precultures were grown for 12 h at 30°C. These precultures were used to inoculate 250-ml LB cultures that contained the appropriate antibiotics in 1-liter baffled Erlenmeyer flasks at a final OD_{600} of 0.1. Cells that contained runaway replication systems were induced at an OD_{600} of 0.7. Cells that harbored multicopy vectors were induced at an OD_{600} of approximately 2.7. A 50-ml sample was taken at the time of induction, as well as at 1, 2, 3, and 4 h after induction.

Cells were harvested by centrifugation, washed once with 0.1 M Tris-HCl (pH 7.5), resuspended in 10% volume of the same buffer, and stored at −70°C. To analyze enzyme activity, samples were thawed and cells were disrupted in 5-ml aliquots by sonication with a Fisher 300 sonicator (tip diameter, 19 mm) at 90% of the maximum setting. Sonication consisted of four cycles of a 15-s burst followed by a 15-s break. During this procedure, samples were cooled on ice. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories).

Enzyme activities were monitored at 25°C in a Shimadzu UV160U spectrophotometer (Columbia, Md.). The activity of β-ketothiolase was monitored by recording the decrease in OD_{303} (24). Briefly, 10 μl of crude extract was added to a solution of 10 μl of 7 mM acetoacetyl-CoA, 100 μl of 0.4 M $MgCl_2$, and 750 μl of 0.1 M Tris-HCl (pH 8.1). The reaction was started by adding 30 μl of 3.4 M CoA.

Acetoacetyl-CoA reductase was monitored at 340 nm by the oxidation of NADPH during reduction of acetoacetyl-CoA (38). Twenty-five microliters of crude extract and 25 μl of 10 mM NADPH were mixed with 500 μl of 120 mM potassium phosphate buffer (pH 5.5) that contained 24 mM $MgCl_2$, 1 mM dithiothreitol, and 455 μl of H_2O. The reaction was started by adding 5 μl of 7 mM acetoacetyl-CoA.

PHA synthase assay was done by a modified method according to Valentin and Steinbüchel (43). Twenty-five microliters of crude extract was mixed with 965 μl of 25 mM Tris-HCl (pH 7.5) that contained 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid). The reaction was started by adding 10 μl of 10 mM DL-3-hydroxybutyric acid (Sigma). The increase was monitored at OD_{412}.

Optimization of PHB production in HMS174(pJM9238) batch culture. Two parameters were optimized to maximize PHB production in HMS174(pJM9238): incubation temperature and cell density at induction. In the first set of optimization experiments, cultures were grown at different temperatures under steady-state conditions. This strain was inoculated into 50 ml of LB medium that contained chloramphenicol, and the culture was incubated in an orbital shaker at 30°C and 200 rpm for approximately 15 h. This culture was used to inoculate six 50-ml LB cultures, each to an initial optical density of 0.10. Each of these cultures was incubated at a different temperature (30, 32, 34, 36, 38, or 40°C) at a speed setting of 175 rpm. Aliquots were withdrawn from each culture during mid-log phase to determine PHB yield and dry weight.

In the second set of optimization experiments, PHB synthesis was thermally induced in HMS174(pJM9238) at various cell densities. An overnight culture of this strain was used to inoculate 250 ml of LB medium that contained 2% (wt/vol) glucose and chloramphenicol in a 1-liter baffled Erlenmeyer flask (prewarmed to 36°C) to an initial optical density of 0.1. This culture was incubated at 36°C and 175 rpm. At regular time intervals during growth, the optical density was measured and 20-ml aliquots of culture were withdrawn and added to sterile 250-ml baffled Erlenmeyer flasks that had been prewarmed to 38°C. These cultures were incubated at 175 rpm for 24 h and harvested. PHB yield and dry weight were determined for each culture.

PHB production during fed-batch growth of HMS174(pJM9238). HMS174 (pJM9238) was used for PHB production during fed-batch growth on an ES10 Biostat E 10-liter fermentor (B. Braun Biotech, Allentown, Pa.). Parameters were controlled with the Micro-MFCS computer control system (B. Braun Biotech). HMS174(pJM9238) was inoculated into 50 ml of LB medium that contained chloramphenicol and was grown at 30°C for 8 h. Then 25 ml of this culture was inoculated into 250 ml of LB medium in a 1-liter baffled Erlenmeyer flask, and this culture was incubated for 10 h at 30°C. This culture was inoculated into a fermentor vessel that contained 5 liters of medium containing the following components: anhydrous Na_2HPO_4, 6 g/liter; anhydrous KH_2PO_4, 6 g/liter; $(NH_4)_2SO_4$, 5 g/liter; $MgSO_4 \cdot 7H_2O$, 0.35 g/liter; trace elements (33), 5 ml/liter; yeast extract, 5 g/liter; and chloramphenicol, 25 μg/ml. The feed medium was composed of the following components and amounts: $(NH_4)_2SO_4$, 33 g/liter; glucose, 400 g/liter; $MgSO_4 \cdot 7H_2O$, 7 g/liter; trace elements, 5 ml/liter; and yeast extract, 5 g/liter. Culture was incubated at 30°C to an optical density of about 20, at which time the temperature was shifted to 38°C. Aliquots were harvested at approximately 2-h intervals for determinations of dry weight, PHB content, and glucose concentration. Ammonia concentrations were determined with a model 95-12 ammonia electrode (Orion Research Inc.) and a Fisher Scientific Accumet pH meter 915 according to the manufacturer's protocol.

PHB molecular weight determination. The molecular weight of the PHB polymer was determined by intrinsic viscometry as previously described (4), with an Ostwald-Fenske size 50 viscometer tube (Kimble).

RESULTS

Stability of PHB phenotype and drug resistance. The stability of the PHB phenotype and drug resistance was monitored for approximately 50 generations during growth in batch culture. In multicopy clones, both kanamycin resistance and the PHB phenotype were more stable in HMS174(pJM9232, pMS421) than in HMS174(pJM9131) (Fig. 2A and B). In HMS174(pJM9131) culture, only 42% of cells were kanamycin resistant and 16% retained the PHB phenotype after 12 generations (Fig. 2B), while in HMS174(pJM9232, pMS421) culture, 98% of cells were kanamycin resistant and 72% retained the PHB phenotype after a similar cultivation period (Fig. 2A). After 35 generations, less than 10% of the cells retained kanamycin resistance and produced PHB.

In runaway replication clones, chloramphenicol resistance was more stable in HMS174(pJM9117) and the PHB phenotype was more stable in HMS174(pJM9238) (Fig. 2C and D). For HMS174(pJM9117) culture, all of the colonies screened were chloramphenicol resistant, but only 2% produced PHB after 36 generations (Fig. 2D); in HMS174(pJM9238) culture, 90% were chloramphenicol resistant and retained the PHB

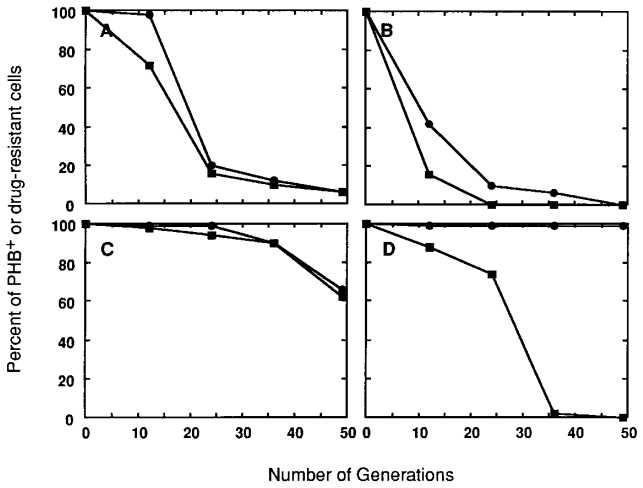

FIG. 2. Stability of PHB production and antibiotic resistance. Antibiotic resistance (●) and stability of the PHB^+ phenotype (■) of native *pha* operon and *tac*::*pha* fusion clones were monitored in LB liquid medium that contained 2% (wt/vol) glucose. (A) *E. coli* HMS174(pJM9232, pMS421); (B) HMS174 (pJM9131); (C) HMS174(pJM9238); (D) HMS174(pJM9117).

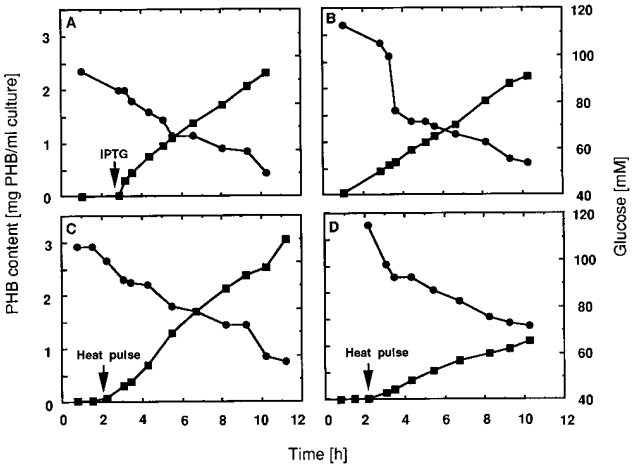

FIG. 3. PHB accumulation and glucose utilization. Native *pha* operon and *tac*::*pha* fusion clones were analyzed for PHB accumulation and utilization of the carbon source during growth in batch culture. Samples were withdrawn at regular time intervals to determine PHB yields per milliliter of culture (■) and glucose concentrations (●). (A) HMS174(pJM9232, pMS421); (B) HMS174(pJM9131); (C) HMS174(pJM9238); (D) HMS174(pJM9117).

phenotype after the same amount of growth (Fig. 2C). PHB-producing HMS174(pJM9117) colonies grown on LB plates that contained 2% (wt/vol) glucose took up far less lipophilic strain than did PHB-producing HMS174(pJM9238) colonies grown on the same medium. Examination of these cells indicated that HMS174(pJM9117) cells contained significantly less PHB than did HMS174(pJM9238) cells.

PHB production of native *pha* operon and *tac*::*pha* fusion clones in batch culture. To analyze the influence of the *lac* promoter and the runaway replication system on PHB accumulation, *E. coli* HMS174(pJM9232, pMS421), HMS174 (pJM9131), HMS174(pJM9238), and HMS174(pJM9117) were grown in batch cultures on LB medium that contained 2% (wt/vol) glucose and the appropriate antibiotics. The PHB content and the concentration of the carbon source were analyzed prior to and after induction. The PHB accumulation rates obtained were comparable for all clones except *E. coli* HMS174(pJM9117), which accumulated PHB at a rate of 0.12 g of PHB $liter^{-1}$ h^{-1} (Fig. 3). The accumulation rates were 0.24, 0.27, and 0.33 g $liter^{-1}$ h^{-1} for HMS174(pJM9131), HMS174(pJM9232, pMS421), and HMS174(pJM9238), respectively. No significant PHB accumulation was seen prior to induction in clones that harbored the temperature-controlled runaway replication vectors (pJM9117 and pJM9238) or the *tac*-controlled multicopy vector (pJM9232). However, the multicopy vector that contained the native constitutive promoter (pJM9131) exhibited PHB accumulation virtually from the time of inoculation. The rates of glucose utilization were similar in all cultures, except for HMS174(pJM9117), in which it was lower, which corresponds to the low rate of PHB accumulation in this strain (Fig. 3). The low rate of PHB accumulation in HMS174(pJM9117) did not correlate with plasmid copy number, since its copy number was comparable to the copy numbers of multicopy vectors (pJM9232 and pJM9131) 4 h after induction and went even higher later in the growth phase (Fig. 4).

Induction of *pha* operon gene products. Enzymatic data indicated significant increases in activity for all three enzymes after induction of *tac* promoter-controlled vectors (pJM9232 and pJM9238) and weak induction in the runaway replication vector (pJM9117) (Fig. 5). In general, the activity profiles could be divided into two similar groups. With the exception of PHA synthase activity, the activities of HMS174(pJM9131) and HMS174(pJM9117) were similar (Fig. 5B and D). This is expected since they both express enzymes from the native promoter. On the other hand, HMS174(pJM9131) manifested substantially higher PHA synthase levels than did HMS174 (pJM9117), a fact that is difficult to explain. However, these data are supported by PHB accumulation data, for HMS174 (pJM9117) accumulated much less PHB than did HMS174 (pJM9131) (Fig. 3B and D). The only other significant differ-

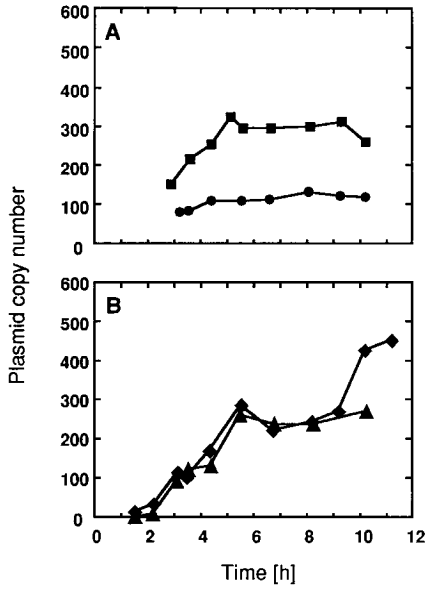

FIG. 4. Plasmid copy number. The copy numbers of multicopy plasmids pJM9131 (■) and pJM9232 (●) (A) as well as those of runaway replication plasmids pJM9117 (▲) and pJM9238 (◆) (B) were monitored during growth of batch cultures. Cells were grown in shake flasks, and the *pha* operon was induced as described in Materials and Methods.

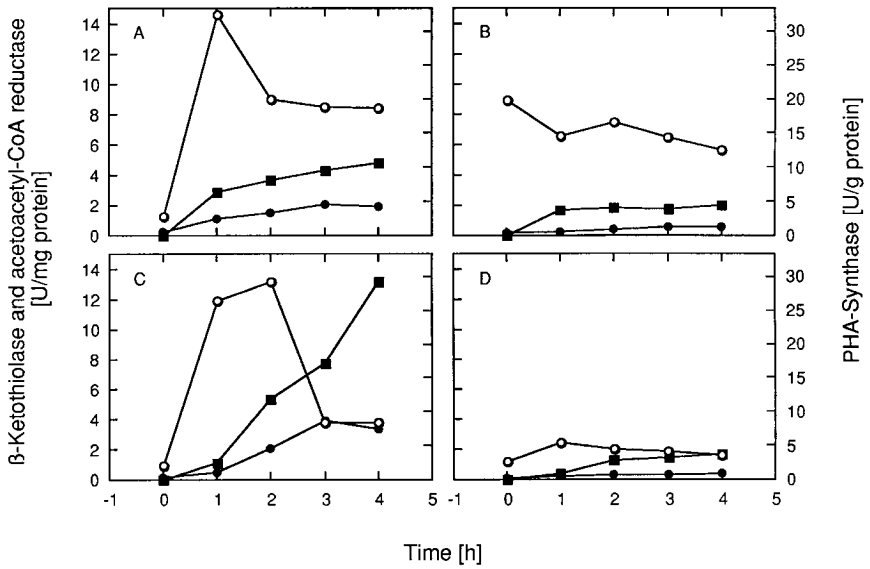

FIG. 5. Induction of enzyme activities in native *pha* operon and *tac*::*pha* fusion clones during growth in batch cultures. Samples were withdrawn from cultures at regular time intervals and analyzed as indicated in the text. β-Ketothiolase (■) and acetoacetyl-CoA reductase (●) were quantitated in units per milligram of protein, whereas PHA synthase (○) was quantitated in units per gram of protein. (A) HMS174(pJM9232, pMS421); (B) HMS174(pJM9131); (C) HMS174(pJM9238); (D) HMS174(pJM9117).

ence between these two vectors was the timing of the appearance of β-ketothiolase and acetoacetyl-CoA reductase; the enzyme levels of the runaway replication vector (pJM9117) took slightly longer to reach their maxima (Fig. 5D).

The enzyme activity profiles for cells that contained *tac* promoter-controlled vectors were also similar. PHA synthase activity attained its maximum very quickly and then decreased, in contrast to the profile of HMS174(pJM9131), which had its maximum at the first time point and decreased thereafter. The final β-ketothiolase and acetoacetyl-CoA reductase levels in cells that harbored the runaway replication *tac*-controlled vector (pJM9238) were at least twice as high as those in cells that carried the multicopy *tac*-controlled vector (pJM9232), even though there was a lag phase in induction of the enzyme (Fig. 5C).

SDS-PAGE gels were run with samples obtained from each time point, and a representative gel for time point no. 4 is displayed in Fig. 6). From this gel, only β-ketothiolase was putatively identified on the basis of its known molecular mass (40.5 kDa [16]) and overexpression. Densitometry was conducted, and the relative amounts of β-ketothiolase were determined (Fig. 7). The densitometry data are in agreement with the enzymatic data for each strain (Fig. 5).

Additional bands that might be PHB-specific proteins were seen on these gels. These bands included a putative PHA synthase at a relative molecular mass of 65.4 kDa and a putative acetoacetyl-CoA reductase at 26.6 kDa. In addition, there were strongly expressed proteins at 23.5 and 57.2 kDa in HMS174(pJM9238) and at 26.6 kDa in HMS174(pJM9117) (Fig. 6).

Optimal conditions for PHB production in HMS174 (pJM9238). Because HMS174(pJM9238) was clearly superior in its PHB production capabilities, protein expression, and stability, we conducted further experiments under optimized conditions. Previous experiments indicated that thermal induction at 34°C and an optical density of 0.7 was optimal for PHB production in HMS174(pJM9117) (11a). *E. coli* HMS174 (pJM9238) was grown at different temperatures under steady-state conditions. During late logarithmic phase, the percent PHB per dry cell weight was 10.6% at 36°C, 43.1% at 38°C, and 19.1% at 40°C (at temperatures below 36°C, PHB was below 10% of dry cell weight). These results show that the optimal condition for incubating culture during the preinduction growth phase is at or below 36°C, and the optimal temperature for inducing PHB production is 38°C.

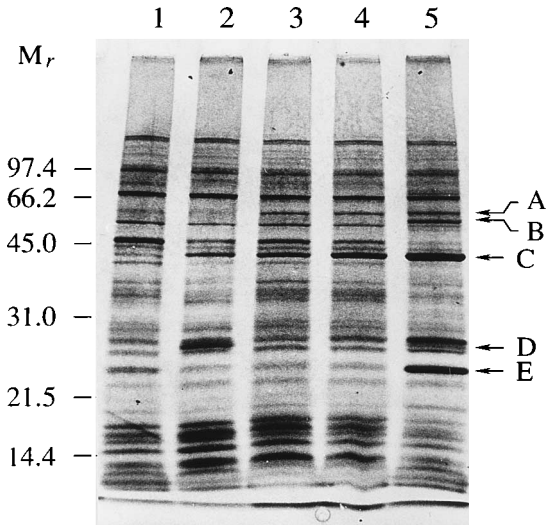

FIG. 6. Separation of total cellular proteins by SDS-PAGE. Samples were withdrawn 4 h after induction and separated by SDS–12% PAGE. Protein bands indicated by C (40.3 kDa) and D (26.5 kDa) represent putative β-ketothiolase (40.5 kDa [16]) and possibly acetoacetyl-CoA reductase (26.3 kDa [16]), respectively. The bands indicated by A (65.4 kDa) and B (57.2 kDa) may represent PHA synthase (63.9 kDa [29]) and a putative degradation product. The band indicated by E (23.5 kDa) occurred in all strains but was much stronger in crude extracts of *E. coli* HMS174(pJM9238). The gel was loaded with crude extracts of *E. coli* HMS174 (lane 1), HMS174(pJM9117) (lane 2), HMS174(pJM9131) (lane 3), HMS174(pJM9232, pMS421) (lane 4), and HMS174(pJM9238) (lane 5).

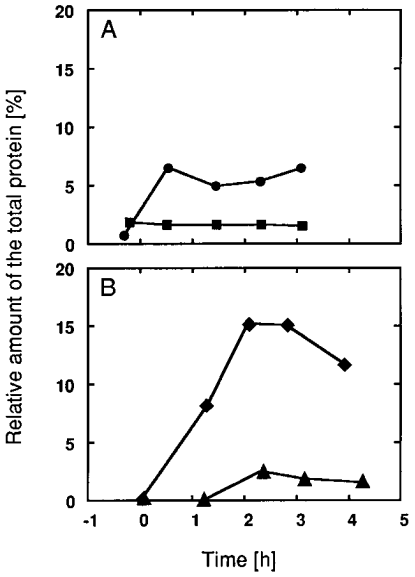

FIG. 7. Relative amounts of putative β-ketothiolase protein by SDS-PAGE after silver staining. The *pha* gene products of native *pha* operon and *tac*::*pha* fusion clones were induced during growth in batch cultures. Samples were withdrawn from cultures at regular time intervals and analyzed by SDS-PAGE. The putative β-ketothiolase was quantitated as a relative percentage of total protein by laser densitometry. (A) Clones of *E. coli* HMS174 that contained the multicopy plasmids (pJM9232, pMS421 [●] and pJM9131 [■]; (B) clones of HMS174 that harbored the runaway replication vectors (pJM9238 [◆] and pJM9117 [▲]).

The optimal cell density for inducing PHB accumulation was determined (Fig. 8). PHB production in HMS174(pJM9238) was highest when culture was induced at low cell densities during early log phase and was lowest when culture was induced at higher cell densities during mid-log phase. PHB production slightly increased when culture was induced at the highest cell densities during late log phase.

PHB production during fed-batch growth of HMS174 (pJM9238). To obtain an idea of the PHB production capabilities of HMS174(pJM9238), it was grown in fed-batch culture in a 10-liter fermentor. Culture was inoculated at an initial OD_{600} of 0.7 and allowed to grow until it entered early log phase (OD_{600} of 12.5). At this point, induction was carried out by raising the temperature to 38°C. Immediately before thermal induction, the PHB concentration was less than 1 mg/ml of culture. At the 25-h time point, 15 h after induction, the PHB concentration was approximately 16 mg/ml of culture. During the accumulation period, the PHB concentration increased at a rate of approximately 1.07 g of PHB liter^{-1} h^{-1} (Fig. 9). At that time, the ammonia concentration in culture was in the range of 2 to 4 mM and the glucose concentration was approximately 5 to 20 mM. The decrease in ammonia and glucose levels stopped abruptly after the rate of PHB production decreased at 30 h (data not shown). The average molecular weight of a sample of the PHB polymer taken after PHB production ceased was 1.15×10^6.

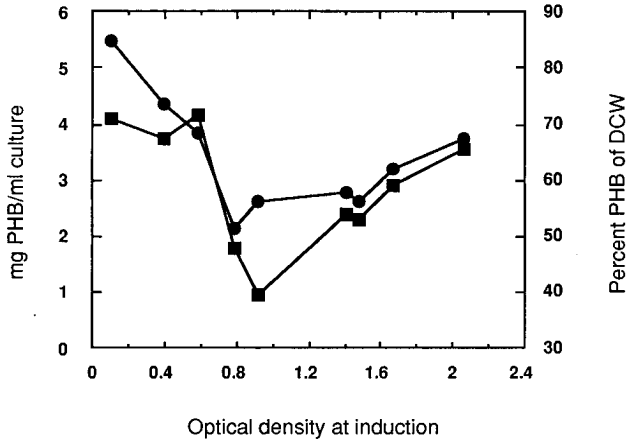

FIG. 8. Optimization of PHB production in HMS174(pJM9238). The PHB level (in milligrams per milliliter of culture) (●) and PHB yield as a percentage of dry cell weight (DCW) (■) for each culture were determined after 24 h of incubation.

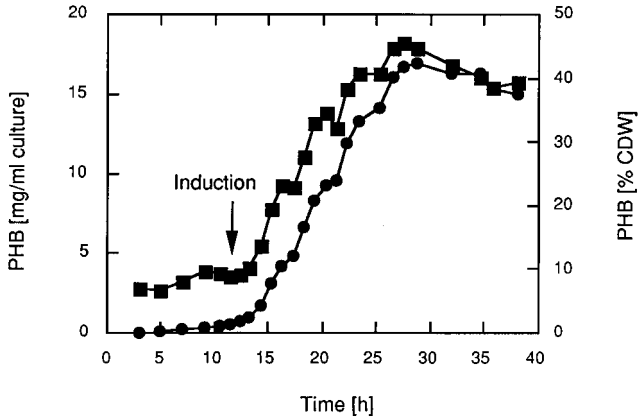

FIG. 9. PHB accumulation of HMS174(pJM9238) in fed-batch culture. PHB production was initiated by shifting the temperature to 38°C at the time indicated by the arrow. PHB production (in milligrams of product per milliliter of) culture (●) and PHB yield as a percentage of cellular dry weight (CDW) (■) were determined during growth and accumulation phases.

DISCUSSION

Efficient expression of products in recombinant bacteria relies not only on high-level expression of the genes but also on controlling expression of the genes in order to minimize the metabolic burden on the cell. To this end, we have constructed *tac* promoter transcriptional fusions to the *A. eutrophus pha* biosynthetic operon to regulate PHB production in *E. coli*. A *tac*::*pha* fusion was cloned into a high-copy-number plasmid and a plasmid in which copy number is temperature dependent.

These vectors exhibited significant differences in stability and, therefore, PHB$^+$ phenotype. One would expect any genetic strategy that limited PHB production to result in increased stability. Since stability of the PHB$^+$ phenotype can be attained by employing either the *tac* promoter or the runaway replication vector, it is not surprising that *E. coli* HMS174(pJM9238), which combined both characteristics, exhibited the highest stability. These results suggest that the combination of tight control of the *pha* operon by the *tac* promoter with low gene dosage is effective in stabilizing PHB production in recombinant *E. coli*. Similar results have been described by Borel and coworkers (6), who constructed *trc* promoter fusions to the *serU* and *supD* genes in *E. coli* and cloned the fusions onto a runaway replication vector to increase stability. These expression systems are stable at noninducing temperatures, and tRNA^{Ser} levels increase by more than 20-fold at the inducing temperature.

The *tac* promoter effectively regulated PHB accumulation in the multicopy plasmid clone (pJM9232, pMS421). Before induction, the PHB concentration in the *tac*::*pha* fusion clone (pJM9232, pMS421) was 10-fold lower than that in the native *pha* operon clone (pJM9131) at similar cell densities. After induction, PHB production rates were similar. The *tac* promoter also significantly increased the PHB production rates in runaway replication clones. Before induction, the PHB concentrations in the *tac*::*pha* fusion clone (pJM9238) and the native *pha* operon clone (pJM9117) were similar. After induction, the PHB yield and rate of PHB production were two- and threefold higher, respectively, in the *tac*::*pha* fusion clone than those in the native *pha* operon clone at similar cell densities. These results are consistent with previous studies in which Bittner and Vapnek (5) reported a 40-fold increase in β-galactosidase activity upon thermal induction of a runaway replication plasmid that contained the *lacZ* gene and Arfman and coworkers (2) showed that the *Zymomonas mobilis adhA*, *adhB*, and *pdc* genes can be regulated in *E. coli* by fusing them to the *tac* promoter and reported a 66-fold increase in alcohol dehydrogenase II activity by the addition of IPTG.

Expression of β-ketothiolase synthesis is clearly regulated by the *tac* promoter. In both *tac*::*pha* fusion constructs, this protein was rapidly synthesized to high levels following induction. This is in agreement with data obtained by enzymatic analysis. Because thiolase was expressed at such high levels (as much as 13% of total cell protein), cells were examined for inclusion bodies, but none were found. However, the recognition of inclusion bodies would have been extremely difficult because of PHB granules in cells. Data obtained from the protein patterns of crude extracts indicated less influence of the *tac* promoter on expression of acetoacetyl-CoA reductase in that clearly overexpressed proteins that corresponded to the size of acetoacetyl-CoA reductase were not present. This may be due to two stem-loop structures, with one located in the *phaA* structural gene and the other in the intergenic region between *phaA* and *phaB* (16), that may reduce expression of the *phaB* gene. PHA synthase also was not discernible as a clearly overexpressed protein that corresponded to the correct molecular weight but was easily seen (on the basis of enzymatic activity) to be induced in a manner similar to that of β-ketothiolase. However, it differed from β-ketothiolase in that after it had reached a maximum level of enzyme activity, it decreased significantly, suggesting degradation of the protein.

Crude extracts of *E. coli* HMS174(pJM9238) exhibited additional protein bands with relative molecular masses of 65.4, 57.2, and 23.5 kDa. The 65.4- and 57.2-kDa proteins may be related to PHA synthase. Peoples and Sinskey have identified a protein having a molecular mass of 58 kDa as PHB synthase (29). The calculated molecular mass of *A. eutrophus* PHA synthase is 63.9 kDa (29), a figure that corresponds closely to the 65.4-kDa protein. Recently, in overexpression studies of PHB-specific proteins we found two proteins with relative molecular masses of 61 and 55 kDa (16) which may be the same proteins. Therefore, it may be that active PHA synthase is 63.9 kDa and that a degradation product of that synthase (inactive) is approximately 58 kDa. The kinetics of appearance for the 65.4-kDa protein correlated well with expression of PHA synthase activity.

The function of the 23.5-kDa protein is not known; however, it was the second most prominent protein found in crude extracts of HMS174(pJM9238). Whether overexpression of this protein correlates with PHA accumulation remains unclear and has to be analyzed in further investigations.

In previous studies with runaway replication vectors and *pha* genes under native promoter control, we found that the optimum induction point was mid-log phase. Induction at time points prior to or following this stage of growth resulted in severe reductions in PHB yield (18). This result agrees with the usage suggested by the supplier of these runaway replication vectors (Nycomed Pharma). In contrast, we have found in this study that a runaway replication vector that contains the *pha* biosynthesis operon under *tac* promoter control (pJM9238) is best induced at low cell densities. The reason for this difference is unclear. Upon initial consideration, induction at low cell densities might seem to be a problem because induction of the PHA operon results in a decrease or complete cessation of cell growth and drastic reductions in total yield. This does not seem to be the case in that final PHB production levels in induced strains [HMS174(pJM9232) and HMS174(pJM9238)] are similar to or slightly higher than that in an uninduced strain [HMS174(pJM9131)]. Perhaps, in logarithmic growth phase, the rate of cell division is sufficiently high to keep ahead of polymer production. It is clear that the point at which the *pha* genes are induced is a critical factor in optimizing PHB synthesis and needs to be empirically determined for each strain and expression system.

A fed-batch growth study of the *tac*::*pha* fusion runaway replicon strain has shown that this system has potential for use in large-scale PHB production. In this study, the rate of PHB production during fed-batch growth was 1.07 g of PHB liter^{-1} h^{-1}. The rate of PHB production in *A. eutrophus* grown under similar conditions with glucose as the carbon source was 1.0 g of PHB liter^{-1} h^{-1} (32). In *Azotobacter vinelandii* grown on molasses, the rate of PHB production was approximately 1.09 g of PHB liter^{-1} h^{-1} (27).

Our results suggest that expression of PHA genes in these strains appears to involve complex regulatory mechanisms. In previous studies, we have used the effects of high gene dosage to obtain high PHB levels (11, 39). On the basis of our current results, we suggest that high-level PHB production can be obtained through either high gene dosage, use of heterologous promoters to increase the number of transcripts from the operon, or a combination. Though these gene products are highly expressed (Fig. 6 and 7), the activity levels detected for each enzyme and PHB accumulation are not much different than those found in the native host, *A. eutrophus* (39). In fact, the PHB accumulation rates of HMS174(pJM9131), HMS174(pJM9232, pMS421), and HMS174(pJM9238) were quite similar. This may indicate that a metabolic rate-limiting step in *E. coli* does not allow an increase in PHB production, despite a large excess of PHB proteins. The most likely possibility for this rate-limiting step is in the expression of PHA synthase activity, which despite being overexpressed, is still approximately 10 times lower than that in *A. eutrophus* (43). This unexpectedly low synthase activity may be due to instability of the protein or to a lack of sufficient amounts of processing enzymes that are found in abundance in *A. eutrophus*. The enzyme activity of β-ketothiolase was also unexpectedly low. Though the protein amount was up to 15% of total cellular protein in strain HMS174(pJM9238), the β-ketothiolase activity was no higher than that reported for *A. eutrophus*. The reason for this remains unclear. However, it might be caused by incorrect protein folding, an inactivation process, or its presence in insoluble form in inclusion bodies. Finally, it may be that the metabolism of the cell simply cannot provide substrate fast enough to push PHB accumulation rates much beyond 1 to 2 g liter^{-1} h^{-1}. For a better understanding of the rate-limiting steps of PHA accumulation, researchers need to address the regulation of cellular metabolism as well as control of the *pha* operon.

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