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Effects of recombinant modulation of the *phb*CAB operon copy number on PHB synthesis rates in *Ralstonia eutropha*

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Abstract

We have increased the gene dosage of the poly(3-hydroxybutyrate) biosynthesis operon in *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) to test whether PHB synthesis rates may be increased by recombinant methods. The native *R. eutropha phb*CAB operon was inserted into the broad-host-range vector pKT230. This PHB operon-containing plasmid, and a control plasmid containing the identical broad-host-range replicon but not the PHB genes, were transferred to *R. eutropha* H16. Analysis of whole-cell lysates indicated that the strain harboring the operon-containing plasmid possessed β-ketothiolase and acetoacetyl-CoA reductase specific activities that were 6.0 and 6.2 times elevated, respectively, as compared to the control strain with a single operon. After growth on fructose, PHB synthesis rates were sharply dependent on the type of carbon source offered during the PHB accumulation phase under nitrogen limitation. In the case of the strain harboring the control plasmid, and in comparison to fructose as carbon source, PHB accumulation was 2.15, 2.83, and 2.60 times faster when resuspended in nitrogen-free medium with lactate, acetate, or 3-hydroxybutyrate, respectively. The strain harboring the PHB operon-containing plasmid synthesized PHB at a lower specific rate in each case. During exponential growth on fructose, the strain harboring the control plasmid was again more efficient at forming PHB. These results suggest that increasing the intracellular concentration of PHB precursors may be a superior alternative to raising the levels of PHB enzymes for enhancing PHB productivity in *R. eutropha*.

Keywords: PHB synthesis rates; Poly(3-hydroxybutyrate); *Ralstonia eutropha*

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1. Introduction

Petroleum-based plastics are ubiquitous and vital materials in modern society, but they are nondegradable in the environment, and they are manufactured from nonrenewable resources. As a result, a substantial market for biodegradable substitutes exists. Poly(3-hydroxybutyrate) (PHB) is a biopolymer formed as granular inclusion bodies in numerous species of bacteria where it is used for storing carbon, energy and reducing equivalents (Steinbüchel, 1991). PHB has attracted considerable interest as a natural, biodegradable, and biocompatible plastic with the potential to be produced economically by microbial cultivation or in other biological systems. *Ralstonia eutropha* is an industrially-utilized microorganism that is able to produce PHB homopolymer at levels up to 80% of cellular dry weight. The more versatile poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer is also produced when propionic acid is included in the feed (Byrom, 1992). *R. eutropha* accumulates PHB most efficiently under nutrient limitation, such as nitrogen or phosphorous, in the presence of excess carbon source (Oeding and Schlegel, 1973).

PHB is synthesized from acetyl-CoA in *R. eutropha* by means of three enzymatic reactions catalyzed by a β-ketothiolase (EC 1.1.1.36), an NADPH-dependent acetoacetyl-CoA reductase (EC 2.3.1.9) and PHB synthase (no EC number assigned). In this reaction series, one molecule of NADPH is oxidized and must be re-reduced via cell metabolism. The genes encoding these enzymes (*phb*A, *phb*B, and *phb*C, respectively) have been isolated from *R. eutropha* and are found to be clustered in a single *phb*CAB operon (Schubert et al., 1988; Slater et al., 1988; Peoples and Sinskey, 1989).

Polymer accumulation rates in *R. eutropha* could conceivably be increased by introducing a plasmid carrying an extra copy of one or more of the PHB genes, thus raising the levels of enzymes necessary for catalyzing the final reactions leading to PHB formation. If it is not possible to increase PHB synthesis rates by overexpressing all three enzymes, the results suggest that some strategy besides manipulating the PHB genes may be preferable for raising PHB productivity in *R. eutropha*.

2. Materials and methods

2.1. Organism and growth media

The strain used in this study was *R. eutropha* H16 (ATCC 17699), which will be referred to as wild-type in this communication. 2 × YT medium was composed of 16 g tryptone, 10 g yeast extract, and 5 g NaCl l^{-1} (Sambrook et al., 1989). Dilution buffer consisted of 0.85% (w/v) NaCl and 0.01% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20). Phosphate buffer contained 6.7 g $Na_2HPO_4 \cdot 7H_2O$ and 1.5 g KH_2PO_4 l^{-1}. Each liter of mineral salts medium, which was based on that described by Ramsay et al. (1990), contained 6.7 g $Na_2HPO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 6 mg ferrous ammonium citrate, 7.5 mg $CaCl_2$, and 1 ml of trace element solution. Trace element solution consisted of 0.3 g H_3BO_3, 0.2 g $CoCl_2 \cdot 6H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$, 30 mg $MnCl_2 \cdot 4H_2O$, 20 mg $NiCl_2 \cdot 6H_2O$, 30 mg $NaMo_4 \cdot 2H_2O$, and 0.1 g $ZnSO_4 \cdot 7H_2O$ l^{-1}. The carbon source for mineral salts medium was 2% (w/v) fructose, 1% (w/v) DL-lactic acid (sodium salt), 0.05% (w/v) sodium acetate, or 0.05% (w/v) DL-3-hydroxybutyric acid (sodium salt). All media ingredients, and all other chemicals except where noted, were purchased from Sigma (St. Louis, MO).

2.2. Evaluation of antibiotic effects on plasmid retention and growth rates

For the experiments used to determine an appropriate antibiotic concentration, mineral salts medium containing 2% (w/v) fructose was utilized, containing varying levels of kanamycin. Cultures were grown at 30°C with shaking at 250 rpm, as described in the legend for Table 1.

2.3. Plasmid construction

Fig. 1 summarizes the construction of the plasmids used in this study. All restriction enzymes

Table 1
Data from experiments used to determine an appropriate antibiotic concentration for growth of plasmid-bearing strains[a]

Initial kanamycin (μg ml^{-1})	Plasmid-containing cell fraction (%)	μ (h^{-1})
0	7	0.292 ± 0.009
50	9	
100	14	0.214 ± 0.004
150	18	
200	25	0.155 ± 0.008
250	31	

[a] In the first experiment, a set of six culture tubes containing 0–250 μg ml^{-1} kanamycin were inoculated with *R. eutropha*/pKA40, shaken for 36 h, and the plasmid-containing cell fractions were determined. A second experiment was performed to determine specific growth rates for the same strain by monitoring growth as A_{436} in 250 ml baffled shake flasks containing 0, 100, or 200 μg ml^{-1} kanamycin. The two experiments were independent, although the identical strain and medium were utilized. Errors are standard deviation about the line of a plot of $\ln(A_{436})$ vs time.

were purchased from Life Technologies™ (Gaithersburg, MD). The entire PHB biosynthesis operon has been cloned into an *E. coli* vector as a *Bam*HI/*Eco*RI insert (Peoples and Sinskey, 1989) and was donated to this laboratory as pAeT41 by A. Sinskey. pKT230 (ATCC 37294), which is based on the IncQ/P4 group pRSF1010 (GenBank™ Acc. M28829), was chosen as a broad-host-range vector for use here since it was reported to replicate stably in *R. eutropha* (Park et al., 1995a). pKA40 was created by digesting pAeT41 and pKT230 with *Bam*HI/*Eco*RI and subsequent ligation of the unmodified *phb*CAB operon (including promoter) into the broad-host-range vector. pKA40 is similar to the vector pHB523 which was created by Park et al. (1995b). The vectors differ in that they removed 1958 bp from the pRSF1010 portion of pKT230, whereas we removed only 104 bp, and the operon was also inserted in the opposite orientation. pZR10 was constructed from pCR®-Blunt (Invitrogen, Carlsbad, CA) and pKT230 with a *Pst*I/*Eco*RI digest of both plasmids and subsequent ligation. pZR10 contains the high copy number ColE1 replicon, two useful cloning regions, and encodes resistance to three antibiotics.

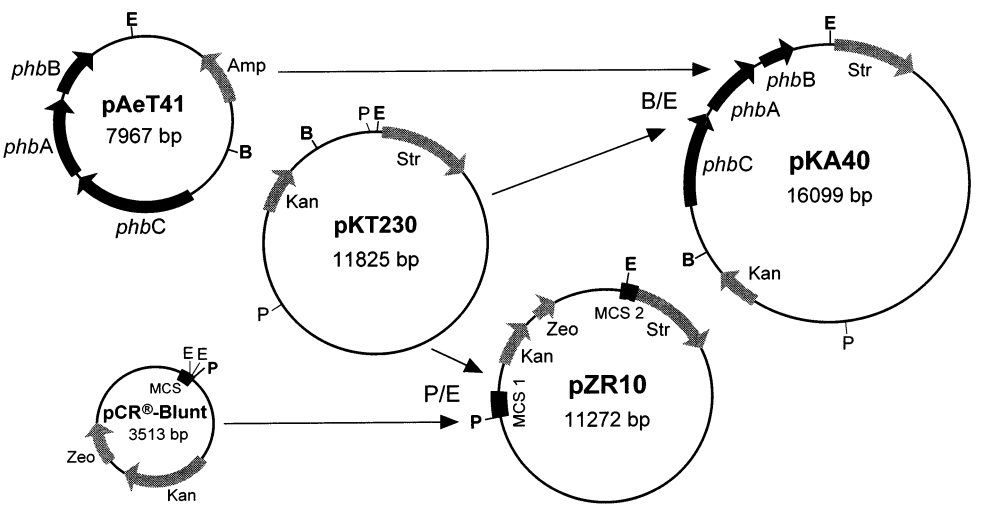

Fig. 1. Plasmid constructs used in this study. Amp = ampicillin, Kan = kanamycin, Str = streptomycin, Zeo = zeocin. B = *Bam*HI, E = *Eco*RI, P = *Pst*I. Unique restriction sites are shown in bold. Between the *Eco*RI and *Pst*I sites shown in pKA40 and pZR10 in this diagram, the two vectors are identical and both contain almost the entire broad-host-range portion of pKT230. In pZR10, multiple cloning site (MCS) 1 contains the unique sites *Apa*I, *Bsa*I, *Bsr*GI, *Pst*I, *Pvu*I, *Sna*BI, *Xba*I, and *Xho*I. MCS 2 contains the unique sites *Bam*HI, *Eco*RI, *Hin*dIII, *Hpa*I, *Kpn*I, *Mlu*I, *Sac*II, and *Spe*I.

2.4. Plasmid isolation

Plasmids were routinely amplified in, and isolated from *E. coli* strain DH5α (Life Technologies™, Gaithersburg, MD) using a rapid alkaline lysis miniprep technique (Xiang et al., 1994). For isolation of pRSF1010-based plasmids, a boiling miniprep was used (Engenbrecht et al., 1995) since it resulted in plasmid DNA that could be cut completely in restriction enzyme digests. For plasmid isolation from *R. eutropha*, it was necessary to modify the alkaline lysis procedure cited above: cell pellets from at least 10 ml of culture volume were kept on ice throughout the procedure, all reagent volumes were doubled, and extraction with an equal volume of phenol:chloroform (Amresco, Solon, OH) was performed just before the DNA precipitation step. The plasmid DNA resulting from this procedure was used to transform competent DH5α, and the plasmid's identity then confirmed by restriction digests.

2.5. Introduction of plasmids into R. eutropha

Electroporation (Park et al., 1995a) was performed to introduce pZR10 into *R. eutropha*, and triparental mating (Leong et al., 1982) was used for pKA40. Triparental mating was carried out as follows, utilizing the helper plasmid pRK2073 (ATCC 37339). DH5α/pKA40, DH5α/pRK2073, and wild-type *R. eutropha* were each streaked across the entire surface of a 2 × YT plate containing 100 μg ml^{-1} kanamycin, 100 μg ml^{-1} streptomycin, and no antibiotic, respectively. These plates were then grown overnight to confluence. Several loopfuls of all three cultures were mixed together in a 3 cm diameter circle on a 2 × YT nonselective plate, and incubated 48 h at 30°C. This mixture was then scraped off the plate, and serially diluted using ice cold dilution buffer. Several dilutions were spread onto chemolithoautotrophic plates made from mineral salts medium containing no carbon source, 0.5 g l^{-1} Na_2CO_3, and 100 μg ml^{-1} kanamycin. These plates were incubated 72 h at 30°C in a hydrogen incubator, which held 80% (v/v) H_2, 10% (v/v) CO_2, and 10% (v/v) O_2. Since *E. coli* could grow on *R. eutropha* exudate on these plates, single colonies from the initial attempt were streaked out and incubated again on identical plates. This was sufficient to isolate *R. eutropha* harboring the plasmid of interest.

2.6. Optical density and cellular dry weight

Optical density was measured at 436 nm using a Hewlett Packard (Palo Alto, CA) 8452A Diode Array Spectrophotometer and 1 cm Acryl-Cuvettes (No. 67.740, Sarstedt, Newton, NC). Samples were diluted so that the spectrophotometer reading was always less than 1.0. Cellular dry weight was determined by vacuum-filtering a known volume of culture medium through a preweighed Supor-200® filter disc (Gelman Sciences, Ann Arbor, MI), followed by a 5 ml water rinse. These filter discs were then dried at 80°C for 48 h at atmospheric pressure, and weighed to determine the cellular dry weight.

2.7. Plasmid stability

Plasmid stability was assessed in triplicate by serially diluting cell culture using ice cold dilution buffer, and spreading 50 μl of several dilutions on parallel 2 × YT plates containing 100 μg ml^{-1} kanamycin or no kanamycin. The number of colony forming units (CFUs) on both types of plates were determined after 48 h incubation at 30°C. The plasmid-containing cell fraction was then calculated as the ratio of the viable counts. This procedure was performed during early stationary phase for the bioreactor experiments that used only fructose as carbon source, and immediately after re-suspension in nitrogen-free medium for the fed-batch experiments.

2.8. Enzyme assays

Cell culture samples of 15 ml volume were used for enzyme assays. Whole-cell lysates were prepared by French press, and the insoluble fraction was removed by centrifugation at 15 000 × *g* for 1 h at 4°C in a centrifuge (model J2-21, Beckman, Fullerton, CA). The cuvette holder was maintained at 30°C, buffers were kept at room temperature, and other reaction ingredients were kept on

ice. In each case, the reaction mixture was first allowed to equilibrate in the cuvette holder for 2 min, and the reaction was then initiated by addition of enzyme. One unit (U) of enzyme is the amount required to catalyze the utilization of 1 μmol of substrate per minute. The thiolysis of acetoacetyl-CoA was used to assay β-ketothiolase activity (Nishimura et al., 1978), with a millimolar extinction coefficient of 12.1 (Haywood et al., 1988a). The reduction of acetoacetyl-CoA was used to assay acetoacetyl-CoA reductase activity (Haywood et al., 1988b), with a millimolar extinction coefficient of 6.2 (Kuchel and Ralston, 1988). A colorimetric method (Park et al., 1995b) was used to assay PHB synthase activity.

2.9. PHB assay

A hydrochloric acid propanolysis method was utilized for gas chromatographic quantification of PHB (Riis and Mai, 1988), as described previously (Hahn et al., 1997).

2.10. Protein assays

The Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) was used to measure protein content in whole-cell lysates for enzyme assays. For bioreactor experiments, whole cell protein was measured by a modified technique of Lowry et al. (1951). Reagent A was made by adding 3 ml of 2% (w/v) $CuSO_4 \cdot 5H_2O$ and 3 ml of 4% (w/v) sodium potassium tartrate to 100 ml of 13% (w/v) Na_2CO_3. Reagent B was Folin and Ciocalteu's Phenol Reagent. A 1 ml culture sample was centrifuged at high speed in a microcentrifuge for 1 min, and the supernatant was discarded. The cells were then resuspended in 0.5 ml of 0.85% (w/v) NaCl, and 50 μl of 16% (w/v) NaOH was added. The microcentrifuge tube was heated for 10 min in a boiling water bath, cooled quickly, and 0.5 ml of Reagent A was introduced. This mixture was incubated 10 min at room temperature, followed by addition of 150 μl of Reagent B. A_{626} was recorded after a further 30 min incubation. Bovine serum albumin fraction V was used as a standard for both protein assays.

2.11. Bioreactor experiments

Wild-type *R. eutropha* (without plasmid) was grown in a 7 l bioreactor (model MGF 7, New Brunswick Scientific, Edison, NJ) using mineral salts medium with fructose as carbon source. *R. eutropha*/pKA40 and *R. eutropha*/pZR10 were grown in two 2 l bioreactors (model 502D, LH Fermentation, Hayward, CA), containing mineral salts medium (fructose) with the following changes: nitrogen was reduced by 50% to ensure cells would have an excess of fructose, and freshly mixed kanamycin was included at 150 μg ml^{-1}. Standard bioreactor parameters for these experiments and also for the fed-batch experiments were 700 rpm agitation, 1 vvm aeration, and 30°C. Dissolved oxygen was monitored, but not controlled. pH was monitored, and maintained at 7.0 using a base solution of 1 M NaOH, and an acid solution of 4% (v/v) phosphoric acid during growth on fructose. A different acid solution was used for pH control of the fed-batch bioreactors, which is described below.

2.12. Fed-batch bioreactor experiments

The 2 l bioreactors described above were also used for each fed-batch experiment. These experiments were performed in two stages. Cells from the first stage bioreactor, which contained mineral salts medium (fructose) and 150 μg ml^{-1} freshly mixed kanamycin, were harvested at $A_{436} = 4.5$–5.0 (i.e. mid-exponential growth). The cells were washed twice by centrifuging at $3000 \times g$ for 15 min at 4°C in a centrifuge (model J2-21, Beckman, Fullerton, CA), and then vortexing in 1 l ice cold phosphate buffer. The cells were centrifuged once more, resuspended in 80 ml of medium taken from the second stage bioreactor, and added to that bioreactor. The second stage bioreactor held mineral salts medium containing a low concentration of organic acid as described above, but containing no nitrogen or kanamycin. Acid feed solutions for the second stage bioreactors were prepared by dissolving 2 mol of the specified organic acid in 800 ml water, titrating to pH 7.0 with NaOH or HCl, adding 1 mol of H_2SO_4, and then adding water to a final volume of 1 l. Use of

such an acid feed solution resulted in slow addition of the organic acid, while avoiding toxic levels.

2.13. Sampling procedures

R. eutropha wild-type samples were withdrawn manually. For all other experiments, an automatic sampling device was used. The device comprised a peristaltic pump programmed using a personal computer interfaced with a DT2805 data acquisition board (Data Translation, Marlborough, MA) to withdraw bioreactor samples at fixed intervals. The software employed was LabTech Notebook version 9.0 (Laboratory Technologies, Wilmington, MA). Consecutive samples were deposited by a microfractionator (model FC-80K, Gilson, Middleton, WI) into 13×100 mm borosilicate glass tubes and maintained at 2°C.

2.14. Plasmid copy number estimate

For a multicopy gene with no differences in promoter efficiencies, the specific enzyme activity found in a whole-cell lysate is expected to be proportional to the average number of copies of the gene that exist in a single cell (Bailey and Ollis, 1986). For the case of any one of the three PHB genes, in *R. eutropha*/pKA40, where every cell possesses at least one copy of the PHB operon and a substantial proportion of the cells possess extra copies, the following equation should be valid:

$$m_t S_K = m_p \alpha (C + 1) + m_n \alpha \quad (1)$$

where m_t (mg) is the total mass of protein; m_p (mg) is the mass of protein from plasmid-containing cells; m_n (mg) is the mass of protein from plasmid-free cells; S_K (U mg^{-1}) is the specific enzyme activity of *R. eutropha*/pKA40; α(U mg^{-1} · cell (gene copies)$^{-1}$) is a proportionality constant; and C(gene copies (cell)$^{-1}$) is the average copy number of plasmids in plasmid-containing cells. The term $m_n\alpha$ is also implicitly multiplied by 1. Since cells of a *R. eutropha*/pZR10 culture have only one chromosomal copy of each gene, α is numerically equivalent to S_Z(U mg^{-1}), the specific enzyme activity of whole-cell lysate from *R. eutropha*/pZR10. Since $m_p + m_n = m_t$, a numerical estimate for the plasmid copy number of pKA40 in *R. eutropha* may be found by applying

$$C = \frac{1}{F}\frac{S_K - S_Z}{S_Z} \quad (2)$$

where F is the fraction of cells in a *R. eutropha*/pKA40 culture that possess the plasmid.

3. Results

3.1. Plasmid retention

Use of an antibiotic to select for a plasmid typically reduces both the growth rate of a culture and the rate of formation of a growth-associated product. To gauge the true effect of the plasmid bearing the PHB operon (pKA40) on PHB synthesis rates in *R. eutropha*, it is important to use for comparison a control strain that harbors a vector lacking the PHB operon. This control vector should also contain the identical broad-host-range origin of replication, as well as antibiotic resistance. Differences in PHB synthesis rates that are observed should then be attributable to variations in operon gene dosage, as plasmid-associated effects are expected to be similar in both strains. Hence, the control vector pZR10, containing the identical pRSF1010-based broad-host-range replicon as pKA40, was constructed. In addition, this vector has increased utility since it possesses a high-copy number *E. coli* replicon, facilitating purification of plasmid DNA for subcloning and electroporation, and it has supplementary unique cloning sites. Although the kanamycin resistance genes in pKA40 and pZR10 are not identical, they both encode aminoglycoside 3′-phosphotransferases (Yamamoto and Yokota, 1980).

Table 1 summarizes the experiments used to determine appropriate antibiotic concentrations for the growth of plasmid-bearing strains. The data indicate that there is a clear correlation between kanamycin concentration and plasmid retention, and that increasing the concentration of kanamycin reduces growth rates. On the basis of

Table 2
Specific growth rates, plasmid-containing cell fractions, and specific enzyme activities in all bioreactor experiments utilizing plasmid-bearing strains[a]

Plasmid	Stationary phase C source	μ (h^{-1})	Plasmid-containing cell fraction	β-Ketothiolase activity (U mg^{-1})	Reductase activity (U mg^{-1})
pKA40	Fructose	0.174 ± 0.004	0.49	4.61 ± 0.58	8.51 ± 1.68
pZR10	Fructose	0.177 ± 0.005	0.45	1.05 ± 0.43	1.85 ± 0.41
pKA40	Lactate	ND	0.62 ± 0.12	9.24 ± 0.31	10.43 ± 0.67
pZR10	Lactate	0.240 ± 0.004	0.30 ± 0.07	0.83 ± 0.41	1.18 ± 0.08
pKA40	Acetate	0.205 ± 0.004	0.39 ± 0.05	5.13 ± 0.35	9.17 ± 1.26
pZR10	Acetate	0.247 ± 0.006	0.16 ± 0.06	1.04 ± 0.12	1.22 ± 0.18
pKA40	3HB	0.175 ± 0.008	0.60 ± 0.14	5.43 ± 0.19	8.97 ± 0.81
pZR10	3HB	ND	0.29 ± 0.05	1.46 ± 0.09	2.24 ± 0.30

[a] In all cases, cells were initially grown utilizing fructose as carbon source. For this table, specific growth rates during exponential phase were determined by monitoring A_{436}. For the experiments with organic acids, cells in mid-exponential phase were harvested, washed twice, and resuspended in nitrogen-free medium with a small amount of organic acid. The organic acid was then fed additionally through the pH control mechanism as described in Section 2. Enzyme activities for all experiments presented here are normalized to total protein content. Activities from the PHB operon-bearing strain would have been higher if normalized instead to the plasmid-containing cell fraction. 3HB = 3-hydroxybutyrate.

these trials, 150 μg ml^{-1} kanamycin was chosen for use in bioreactor experiments as a compromise between high growth rates and high plasmid retention.

The plasmid-containing cell fractions as measured in stationary phase for the bioreactor experiments using only fructose as carbon source, and for initial samples in the nitrogen-free fed-batch bioreactor experiments, are shown in Table 2. The average retention across all experiments was $52.2 \pm 10.4\%$ for the plasmid containing the PHB operon (pKA40), and $29.8 \pm 6.2\%$ for the control plasmid (pZR10). Although retention was not high for either plasmid, the plasmid bearing the PHB operon was apparently more stable than the operon-free control plasmid. A more stable plasmid system could perhaps be achieved through addition of a partitioning (*par*) locus, as has been done with recombinant *E. coli* harboring the PHB biosynthetic genes (Haigermoser et al., 1993; Lee et al., 1994).

Plasmid instability was also evident from optical density measurements of cells growing exponentially on fructose in both sets of bioreactor experiments. Since growth conditions were identical and the density of inocula was always less than $A_{436} = 5.0$, the specific growth rates of both strains should have been consistent. However, as indicated in Table 2, a high degree of variability was observed. Fig. 2 illustrates that greater degrees of plasmid loss appear to be correlated

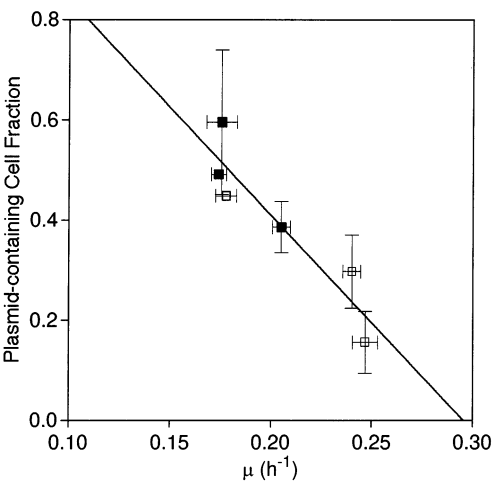

Fig. 2. Correlation between plasmid-containing cell fraction and specific growth rate μ (h^{-1}). The latter was measured as A_{436} during exponential growth on fructose in both sets of bioreactor experiments. Closed squares = *R. eutropha* harboring the plasmid containing the PHB operon (pKA40); open squares = *R. eutropha* harboring the control plasmid (pZR10). A linear curve fit is also shown. Ordinate error bars are one standard deviation ($n = 3$), and abscissa error bars are standard deviation about the line of a plot of $\ln(A_{436})$ vs time.

with higher specific growth rates in both strains. This correlation also emphasizes that under similar levels of plasmid retention the two strains have similar growth rates, substantiating the adequacy of pZR10 as a control vector.

3.2. Enzyme activity

To assess the gene dosage effect of the operon-bearing plasmid, enzyme activities were determined from whole-cell lysates. Enzyme assay results for β-ketothiolase and acetoacetyl-CoA reductase are shown in Table 2. *R. eutropha* harboring the plasmid containing the PHB operon (pKA40) exhibited much higher enzyme levels than *R. eutropha* harboring the control plasmid (pZR10). Averaging across all experiments, β-ketothiolase activity was 6.0 times higher and reductase activity 6.2 times higher in *R. eutropha*/pKA40 than in *R. eutropha*/pZR10. Even though the plasmids were not particularly stable, retention was sufficient to increase enzyme levels significantly. Using Eq. (2), which takes plasmid loss into account, the plasmid copy number of pKA40 was estimated at a value of 10.0 ± 4.6.

The PHB synthase assay did not yield reliable results, even for wild-type *R. eutropha*. A negligible level of PHB synthase in *R. eutropha* lysates is commonly observed, and is probably due to the association of the enzyme with the insoluble PHB granules which are sedimented with cell debris during lysate preparation (Haywood et al., 1989; Steinbüchel and Schlegel, 1991; Gerngross et al., 1993). Because of this difficulty, the strains used in this study were not routinely tested for the presence of PHB synthase. However, functional synthase activity associated with pKA40 was demonstrated by transforming this vector by electroporation into both *E. coli* DH5α and the *R. eutropha* PHB-4 strain, a mutant that lacks PHB synthase activity (Schlegel et al., 1970). Both strains then produced substantial quantities of PHB (data not shown), signifying that a functional, normally expressed synthase was present on the PHB operon in pKA40.

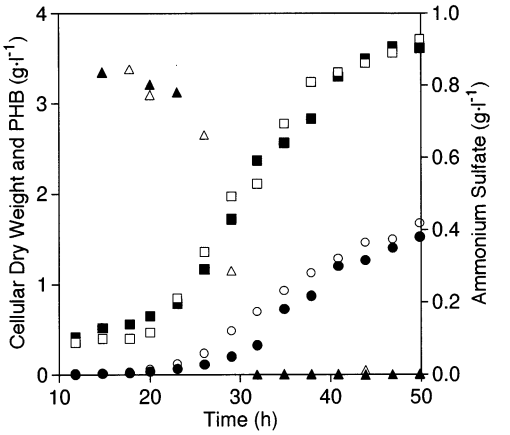

Fig. 3. Data from the plasmid-bearing strains in bioreactor experiments using only fructose as carbon source. Closed symbols are *R. eutropha* harboring the plasmid containing the PHB operon (pKA40), and open symbols are *R. eutropha* harboring the control plasmid (pZR10). Squares = cellular dry weight; circles = PHB; triangles = ammonium sulfate.

3.3. Bioreactor experiments using only fructose as carbon source

Estimates for the specific rates of residual (non-PHB) biomass formation during exponential growth phase during cultivation in bioreactors on mineral salts medium with fructose as carbon source were 0.270 ± 0.008 h^{-1} for wild-type *R. eutropha*, 0.104 ± 0.012 h^{-1} for *R. eutropha* harboring the plasmid containing the PHB operon (pKA40), and 0.143 ± 0.015 h^{-1} for *R. eutropha* harboring the control plasmid (pZR10). Fig. 3 shows data acquired for the two plasmid-bearing strains during these experiments. The plasmid-bearing strains also required over twice the time as the wild-type strain to complete the exponential growth phase.

The change in the ratio of PHB to residual biomass during exponential growth in these experiments has also been calculated, and is presented in Fig. 4. This figure shows that the wild-type strain exhibited nearly balanced growth, since the ratio of PHB to residual biomass was constant over the entire exponential growth phase. However, in both plasmid-bearing strains this ratio was not constant. The amount of PHB per residual biomass increased more rapidly in the strain

harboring pZR10 than in the strain harboring pKA40. Combined with the observation that residual biomass also increased faster in *R. eutropha*/pZR10, it seems that additional copies of the PHB operon did not enhance PHB accumulation during exponential growth with fructose as carbon source.

3.4. Nitrogen-free fed-batch bioreactor experiments

A representative graph of data collected from the nitrogen-free fed-batch bioreactor experiments is shown in Fig. 5. Cellular dry weight and PHB showed linear increases that were essentially parallel, and both residual biomass and protein levels remained constant. The average initial PHB content in all these experiments was $15.0 \pm 1.5\%$, indicating that initial conditions within both strains were nearly identical when the experiments were started. The results of this series of experiments are summarized in Fig. 6. All measures for PHB used indicate that for all carbon sources except fructose (where the two strains were approximately equivalent), *R. eutropha* harboring the operon-free control plasmid (pZR10) synthesized PHB at rates higher than *R. eutropha* harboring the plasmid containing the PHB operon (pKA40). The effect was most pronounced when 3-hydroxybutyrate was used as carbon source.

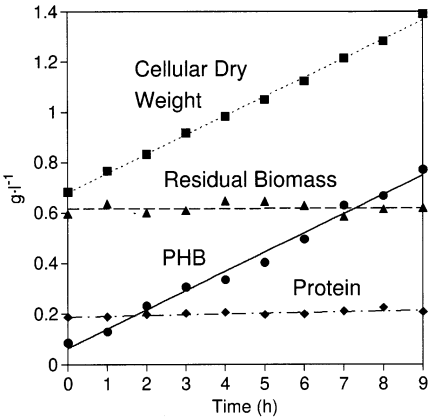

Fig. 5. Sample data and linear curve fits from *R. eutropha*/pZR10 resuspended in nitrogen-free mineral salts medium with acetate as carbon source. In this series of fed-batch experiments, an organic acid was initially supplied and later supplemented during the experiment through pH control as described in Section 2. Squares = cellular dry weight; circles = PHB; triangles = residual biomass; diamonds = protein.

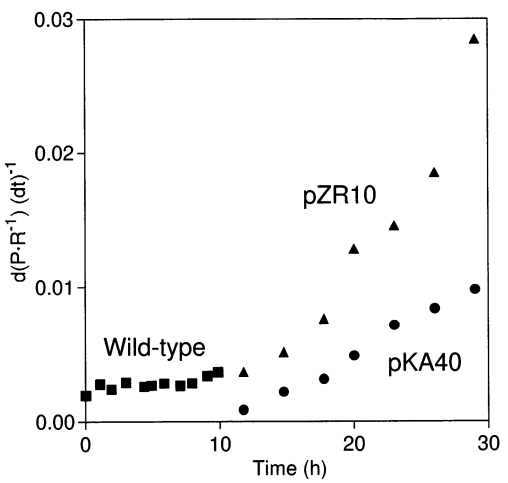

Fig. 4. Rates of change of PHB accumulation per residual biomass during exponential growth phase with fructose as carbon source, obtained from the relationship

$$\frac{d(P/R)}{dt} = \frac{1}{R}\frac{dP}{dt} - \frac{P}{R}\mu_R.$$

Estimates for $dP(dt)^{-1}$ and μ_R were obtained through exponential curve fits. PHB (P) and residual biomass (R) have been determined experimentally. Exponential growth of wild-type *R. eutropha* lasted approximately 10 h, but this period extended to approximately 30 h in *R. eutropha* strains harboring plasmids. Squares = wild-type *R. eutropha*; circles = *R. eutropha* harboring the plasmid containing the PHB operon (pKA40); triangles = *R. eutropha* harboring the control plasmid (pZR10).

The additional use of various organic acids as carbon source increased the flux through the PHB biosynthesis pathway significantly during the PHB accumulation phase when compared to experiments using only fructose as carbon source. In comparison to fructose, PHB synthesis rates were increased an average of 2.15, 2.83, and 2.60 times in *R. eutropha* harboring the control vector pZR10, when the strain was resuspended with lactate, acetate, or 3-hydroxybutyrate as carbon source, respectively. While flux increased in both plasmid-bearing strains during exposure to these different carbon sources, the strain harboring the plasmid containing the PHB operon (pKA40) was apparently at a disadvantage. In average, *R. eu-*

tropha/pZR10 had 1.15, 1.27, and 1.96 times higher PHB synthesis rates compared to *R. eutropha*/pKA40, for lactate, acetate and 3-hydroxybutyrate as carbon source, respectively.

4. Discussion

Resuspending either of the two recombinant strains in nitrogen-free medium with various organic acids as carbon source resulted in significantly increased PHB synthesis rates during stationary phase, when compared to fructose. Since the enzyme activities within either strain should be comparatively constant during exposure to the different carbon sources, one has to conclude that the concentrations of the primary substrates of the pathway, acetyl-CoA and NADPH, were increased in both strains when organic acids were used. Evidently not one of the PHB enzymes was saturated by substrate during the PHB accumulation phase with fructose, lactate, or 3-hydroxybutyrate as carbon source. The lower rate of PHB synthesis on the basis of fructose is likely attributable to the inhibition of glucose 6-phosphate dehydrogenase by high NADH levels which is thought to regulate the flux through the Entner–Doudoroff pathway (Bowein et al., 1974).

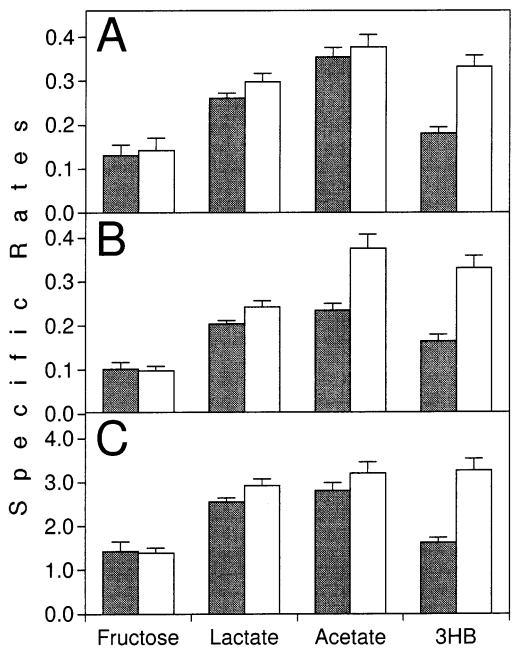

Fig. 6. Specific rates of increase in (A) cellular dry weight, (B) PHB content, and (C) optical density (A_{436}) on various carbon sources. All increases are due to accumulation of PHB, and are expressed here per unit protein present in the bioreactor. Wild-type *R. eutropha* was also evaluated on fructose, and was similar to the two plasmid-bearing strains. Gray columns = *R. eutropha* harboring the plasmid containing the PHB operon (pKA40); white columns = *R. eutropha* harboring the control plasmid (pZR10). Error bars are standard deviation about the line.

The presence of added copies of the *phb*CAB operon resulted in a decrease in PHB synthesis rates on various carbon sources, despite a significantly increased enzyme level of all pathway enzymes. According to enzyme kinetics, an increase in the concentrations of these enzymes should lead to at least a small increase in PHB synthesis rates provided that precursors and cofactors are present in the same concentrations (Leaf and Srienc, 1998). The decrease in flux to PHB that we observed probably corresponds to a reduction of the precursors available to the PHB enzymes. This in turn was likely caused by a decrease in the activity of at least one upstream enzyme, which limited PHB synthesis. The decreased rate of PHB synthesis in *R. eutropha*/pKA40, when compared to *R. eutropha*/pZR10, is then a consequence of metabolic burden, since the former strain was subjected not only to plasmid replication requirements and transcription/translation of resistance genes, but also to increased transcription/translation of the PHB operon genes.

A similar study (Park et al., 1995b,c, 1997) claims that PHB synthesis rates can be increased by raising the gene dosage of the pathway enzymes. We did not observe such behavior. Our results suggest that cell metabolism in *R. eutropha* was affected through mechanisms similar to those often observed during attempts to express heterologous proteins in other host microorganisms. In prokaryotic protein expression systems a strong, constitutive promoter is often detrimental to essential host cell functions (Glick and Pasternak, 1994). The most active expression systems, which also have strong translation

initiation domains, are lethal. Therefore, inducible promoters with little expression during exponential growth and weaker translation initiation domains are typically utilized in industry (Gold, 1990).

Additional examples exist to illustrate that production rates are not necessarily enhanced by simple pathway manipulations (Schaaff et al., 1989; Niederberger et al., 1992) It is also interesting to note that one of the PHB enzymes probably *does* catalyze a rate-limiting step when the PHB operon is expressed in *E. coli*. High plasmid copy numbers (ca. 100) are required to give PHB production levels and enzyme activities approximating those of wild-type *R. eutropha*, which has just a single chromosomal operon (Fidler and Dennis, 1992; Lee et al., 1994, 1996).

Increasing PHB synthesis rates in *R. eutropha* would be conducive to making production of this biodegradable polymer more economically attainable. The current study suggests that this may be done more readily by raising the concentrations of PHB precursors than by modulating the levels of the PHB enzymes. The observation that changing the carbon source can effectively increase PHB pathway flux suggests that the PHB enzymes as normally expressed are capable of sustaining higher flux than is usually observed during growth on fructose, and that increasing their activities may have a relatively small effect on the synthesis of PHB. When we did substantially increase the activities of the PHB enzymes, we found that there is a balance between these and critical upstream enzymes which is easily disturbed. This study also reaffirms the observation that amplifying a subset of enzymes independently from the rest of a pathway and from cell metabolism can have results that are difficult to predict.

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