

Kinetic Studies and Biochemical Pathway Analysis of Anaerobic Poly-(R)-3-Hydroxybutyric Acid Synthesis in *Escherichia coli*

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Poly-(R)-3-hydroxybutyric acid (PHB) was synthesized anaerobically in recombinant *Escherichia coli*. The host anaerobically accumulated PHB to more than 50% of its cell dry weight during cultivation in either growth or nongrowth medium. The maximum specific PHB production rate during growth-associated synthesis was approximately 2.3 ± 0.2 mmol of PHB/g of residual cell dry weight/h. The by-product secretion profiles differed significantly between the PHB-synthesizing strain and the control strain. PHB production decreased acetate accumulation for both growth and nongrowth-associated PHB synthesis. For instance under nongrowth cultivation, the PHB-synthesizing culture produced approximately 66% less acetate on a glucose yield basis as compared to a control culture. A theoretical biochemical network model was used to provide a rational basis to interpret the experimental results like the fermentation product secretion profiles and to study *E. coli* network capabilities under anaerobic conditions. For example, the maximum theoretical carbon yield for anaerobic PHB synthesis in *E. coli* is 0.8. The presented study is expected to be generally useful for analyzing, interpreting, and engineering cellular metabolisms.

Anaerobic cultivation is often used for producing commercial biochemicals like ethanol, lactate, succinate, and 1,3 propanediol (10, 14, 22). Anaerobic conditions also play critical roles in several environmental and ecological processes (46, 50). From a metabolic modeling perspective, anaerobic culturing conditions present an interesting system for studying cellular strategies for maintaining a redox balance. The absence of an external electron acceptor requires the transfer of reducing equivalents to metabolic intermediates which are usually easily quantifiable and provide a means of probing the intracellular workings of a microbe.

Poly-(R)-3-hydroxybutyric acid (PHB) belongs to a family of naturally occurring, biodegradable polyesters, known as polyhydroxyalkanoates (PHA) (for a recent review see reference 51). These materials act as reserve compounds for carbon, energy, and reducing equivalents and are of interest because their material properties make them a potential alternative to some petroleum-based thermoplastics. Recombinant *Escherichia coli* systems have been used extensively to study PHA production (for instance, see references 16, 24, 32, 33, 39, and 47). The effect of oxygen stress on recombinant *E. coli* cultures has been examined (52, 54); however, these studies all fed various amounts of oxygen and were not strictly anaerobic. PHB production in the absence of oxygen represents an interesting strategy for large-scale biopolymer production because anaerobic culturing typically permits simpler reactor design, control strategies, and operating conditions. For instance, since the low aqueous solubility of oxygen is not an issue, the

culture is not subject to oxygen mass transfer limitations or steep oxygen gradients, which can cause experimental variation when there is nonideal reactor mixing. Anaerobic systems also have the added benefit of potentially coproducing other valuable by-products like ethanol, lactate, succinate, or hydrogen.

PHA production under conditions of oxygen stress and in the absence of oxygen has been reported for organisms that natively accumulate PHA (3, 4, 45). Anaerobic PHA production has been studied in undefined bacterial consortia found in a wastewater treatment process known as enhanced biological phosphorous removal (for recent reviews, see references 31 and 46). These studies examined a still unclear relationship between a PHA-accumulating bacterial consortium and the removal of phosphorous compounds from wastewater streams (37).

Elementary mode analysis is used here to study theoretical aspects of anaerobic PHA production. This method uses a field of mathematics known as convex analysis to identify all possible, unique, nondivisible pathways for a network (40–43). These pathways represent the simplest, steady-state flux patterns available to a system. Under steady-state conditions, the metabolic fluxes of an organism can be expressed as a non-negative linear combination of elementary modes (12, 13). Elementary mode analysis has been used to study *E. coli* biochemical network properties (29, 41, 43, 49); however, few studies have examined the operation of foreign pathways engineered into a recombinant host (11). The network modeling approach provided a structured basis to interpret experimental results and defined explicitly the theoretical capabilities of the anaerobic *E. coli* metabolism. The presented study should therefore be generally useful for analyzing, interpreting, and engineering different aspects of cellular metabolisms.

MATERIALS AND METHODS

Experimental system. *E. coli* DH5 α (Invitrogen, Carlsbad, Calif.) was used in all experiments. Two different strains were created by transforming DH5 α with

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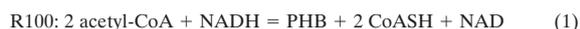
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different plasmids. Plasmid pPT500 contains the native, three-gene *Ralstonia eutropha* PHB operon from pAeT41 (32) ligated into the pCR Blunt vector (Invitrogen). The operon is efficiently expressed in *E. coli* by the native *R. eutropha* operon promoter, which resembles the *E. coli* σ^{70} system (48). This *E. coli* strain is referred to as PHB(+). The other plasmid, pCR-KT, was constructed by ligating the *R. eutropha* β -ketothiolase gene, without a promoter, into the pCR Blunt vector. The control strain harboring this plasmid does not express any PHB genes and is referred to as PHB(-).

Culturing conditions. For growth studies, the strains were cultured in 2 \times YT medium: 16-g/liter tryptone (Difco, Detroit, Mich.), 10-g/liter Bacto yeast extract (Difco), and 5-g/liter NaCl (Sigma, St. Louis, Mo.) supplemented with 4% (wt/vol) glucose (Sigma) and 100- μ g/ml kanamycin (Sigma). DH5 α does not grow on minimal media due to chromosomal mutations. For nongrowth studies, the cells were harvested from the growth reactor during the mid-growth phase, washed twice with ice-cold phosphate buffer (0.2-g/liter KCl, 5-g/liter NaCl, 2.72-g/liter Na₂HPO₄·7H₂O, 0.24-g/liter KH₂PO₄ [Sigma]) and resuspended in phosphate buffer with 2% (wt/vol) glucose as the substrate and 100- μ g/ml kanamycin. All cultures were maintained at 30°C, with the pH controlled at 7.0 with 4 M NaOH and with an agitation rate of 300 rpm. Before inoculation, the reactors were sparged with nitrogen gas until the dissolved oxygen concentration reached zero. During cultivation, the reactors were sparged with 0.5 liter of nitrogen gas per min to maintain positive reactor pressure and to maintain anaerobic conditions.

Analytical techniques. PHB content was determined by the method described in reference 38. Glucose concentrations were determined with the Sigma Diagnostics D-glucose kit (Sigma). Ethanol concentrations were determined by adding 0.5 ml of culture supernatant to a gas chromatography vial along with 0.1 ml of internal standard solution (3-g/liter 1-propanol [Sigma]). Samples were run on a Hewlett-Packard (Palo Alto, Calif.) 5890A gas chromatograph with a DB-WAX 30-W capillary column and were analyzed with a flame ionization detector. Acetate levels were analyzed with the R-BIOPHARM (Darmstadt, Germany) acetic acid kit. The by-products lactate, succinate, pyruvate, and 3-hydroxybutyrate were analyzed by the procedure from reference 53 and with a Hewlett-Packard 5890A gas chromatograph and a DB-WAX 30W capillary column with a flame ionization detector. CO₂ measurements were obtained for the nongrowth cultures with a ThermoOnix Prima δ B mass spectrometer (Houston, Tex.). CO₂ production was expressed as the accumulated mass of CO₂ produced per liter of culture. This was done to facilitate comparison with other products.

Metabolic network analysis. The theoretical capabilities of the *E. coli* biochemical network were analyzed by the elementary mode model described previously (12). The reader is referred to this previous work for a detailed description of the metabolic model and for the designation of the individual reactions. Here, we describe only the modifications made to the model to realize the aim of this work. The analysis considered acetate, ethanol, lactate, succinate, and glucose as potential substrates. The designation of the appropriate transport reactions (R90, R91, R94, and R95) was changed from irreversible to reversible. In addition, the designation of reaction R55, which accounts for the interconversion of acetate and acetyl coenzyme A (CoA), was changed to reversible. The additional substrates were included because under anaerobic conditions significant amounts of these compounds are often secreted (1, 6, 15) and would be potentially available for PHB synthesis. To account for PHB synthesis, the following reaction was added to the model:



This single reaction accounts for the three-gene *R. eutropha* PHB pathway comprising the enzymes β -thiolase, reductase, and synthase. A transhydrogenase activity was assumed to be present. All simulations utilized a macromolecular biomass composition consistent with a 200-min doubling time.

The elementary mode analysis was run with the publicly available program METATOOL version 352_double (34, 42).

The Euclidean distances (44) calculated below only considered native enzymes. Network reactions that account for diffusion-controlled events like the transport of ethanol across the cell membrane were not considered. The Euclidean distance contributions for reversible reactions utilized the absolute value of the flux.

RESULTS

Kinetic studies of PHB production. A series of batch bioreactor experiments were designed to test the feasibility of growth and nongrowth-associated anaerobic PHB synthesis in

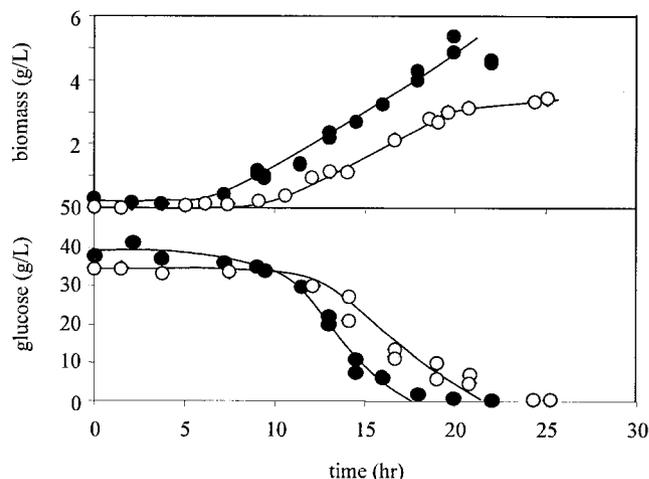


FIG. 1. Residual biomass and glucose time profiles for anaerobic *E. coli* cultures in growth media. The residual biomass is the total biomass minus the mass of PHB. Open circles, strain PHB(+); filled circles, strain PHB(-).

a recombinant *E. coli* strain. Reactor time profiles for anaerobic growth conditions are shown in Fig. 1 and 2. The control strain PHB(-) also contains a plasmid but is unable to form PHB because it does not express any of the PHB pathway enzymes. The biomass data presented for strain PHB(+) is the residual biomass. Residual biomass is defined as the total dry cell mass minus the mass of PHB.

Both recombinant *E. coli* strains grew anaerobically on the rich medium. Strain PHB(-) had a maximum specific growth rate of approximately $0.39 \pm 0.03 \text{ h}^{-1}$, while strain PHB(+) expressing the recombinant pathway had a slightly slower maximum specific growth rate of about $0.32 \pm 0.04 \text{ h}^{-1}$. The cultures grew exponentially for approximately the first 10 h. However, after this initial culturing period, the biomass only increased at a linear rate. The shift from exponential growth to linear growth is likely due to the accumulation of acetate in the medium or the depletion of a component in the complex medium. The cessation of biomass production coincides closely with the exhaustion of glucose. Strain PHB(-) made approximately 5 g of biomass per liter, while strain PHB(+) made approximately 3 g of residual biomass per liter before the glucose was exhausted.

The recombinant *E. coli* strain PHB(+) was capable of anaerobically synthesizing PHB. The strain accumulated PHB up to approximately 50% of its cell dry weight (cdw). The specific rate of approximately $2.3 \pm 0.2 \text{ mmol of PHB/g of residual cdw/h}$ is comparable to previously reported rates in recombinant *E. coli* strains growing on rich medium in the presence of oxygen (27, 28). The volumetric PHB production rate and PHB titer are comparable to the majority of the strains tested with batch growth in reference 28; however, improvements are likely possible by screening and selecting an *E. coli* strain best suited for the culturing conditions. High-cell-density strategies, like fed-batch cultivation, would likely further improve the volumetric production rate (27).

Cell growth is not required for anaerobic PHB synthesis. PHB was synthesized anaerobically under nongrowth conditions (see Fig. 3 and 4). The specific PHB content of the

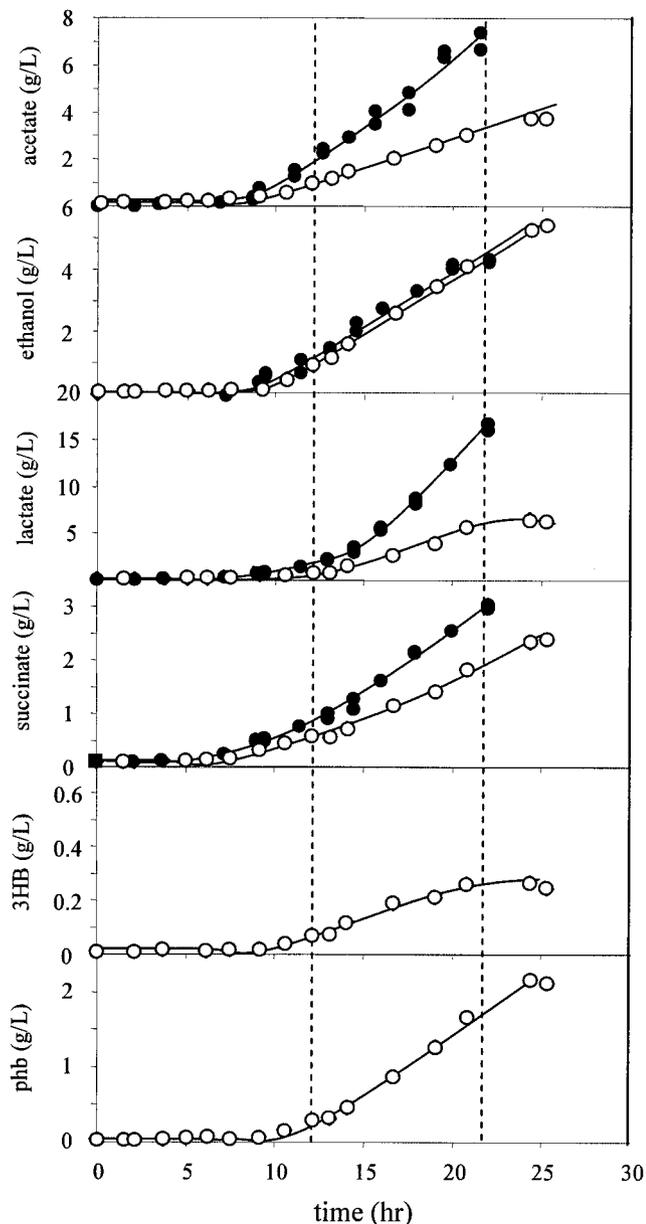


FIG. 2. Product time profiles for anaerobic *E. coli* cultures in growth medium. Open circles, strain PHB(+); filled circles, strain PHB(-). Dotted lines highlight the region used for rate and yield calculations. See text for more detail.

PHB(+) culture increased from approximately 45 to 60% of the cell dry weight. The specific PHB synthesis rate under nongrowth conditions was approximately 0.91 ± 0.2 mmol of PHB/g of residual biomass/h. The ability to produce PHB under nongrowth conditions opens the possibility of novel feeding strategies for producing PHA copolymers and for controlling PHA granule microstructure in *E. coli* (23).

Strain PHB(+) secreted significant amounts of the PHB precursor 3-hydroxybutyric acid during both growth and nongrowth conditions. During the interval between h 12 and 22 for the growth culture, the specific rate of 3-hydroxybutyryl-CoA production is approximately one-seventh the rate of PHB pro-

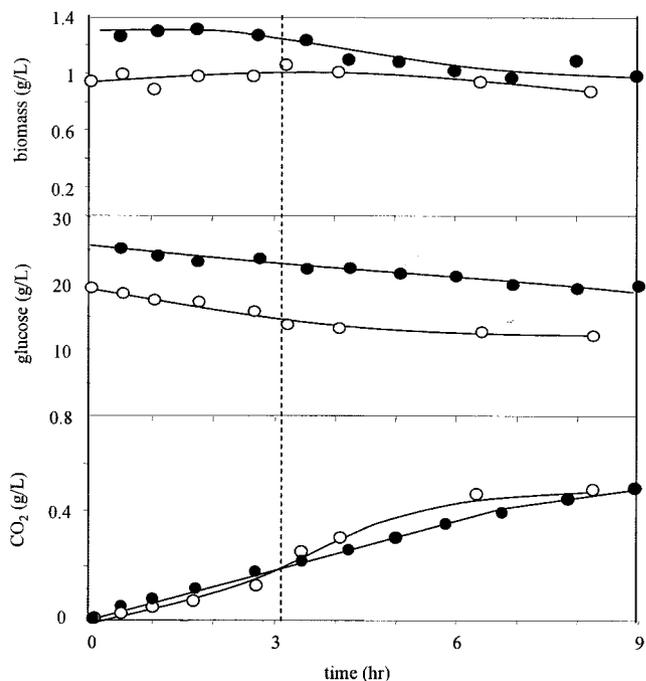


FIG. 3. Residual biomass, glucose, and CO_2 time profiles for anaerobic *E. coli* cultures in nongrowth medium. Open circles, strain PHB(+); filled circles, strain PHB(-).

duction. 3-Hydroxybutyric acid was secreted under nongrowth conditions at a rate of about 1.1 ± 0.2 mmol/g of residual biomass/h. The PHB monomer, 3-hydroxybutyryl-CoA, was likely cleaved into 3-hydroxybutyric acid and free CoA by a native *E. coli* thioesterase.

PHB synthesis had a significant effect on the production of by-products for both growth and nongrowth conditions. Strain PHB(-) accumulated significantly more acetate than strain PHB(+), while strain PHB(+) accumulated more ethanol than strain PHB(-). Under growth conditions, strain PHB(-) made significantly more lactate and succinate than strain PHB(+). These trends are likely due to the high levels of accumulated acetate.

By-product yields. The metabolic differences between the two strains were quantified by examining the by-product glucose yields. The product yield, Y , based on glucose is defined as

$$Y_{\text{glucose}}^{\text{product}} = \frac{r_{\text{product}}}{-r_{\text{glucose}}} = \frac{[\text{product produced}]}{[\text{glucose consumed}]} \quad (2)$$

where r is the rate of either by-product secretion or glucose consumption. Since the volumetric rates $\left(\frac{d[\text{product}]}{dt}\right)$ for the products were nearly linear between h 12 and 22 for the growth cultures and between h 0.5 and 3 for the nongrowth conditions, the culture yields were nearly constant during these intervals. More than 67% of all PHB synthesis occurred during these intervals. The following analysis is limited to these culturing phases.

The reactor yields for both growth and nongrowth conditions are plotted as the ratio of specific product secretion rates to the specific glucose uptake rate in Fig. 5. The rates calcu-

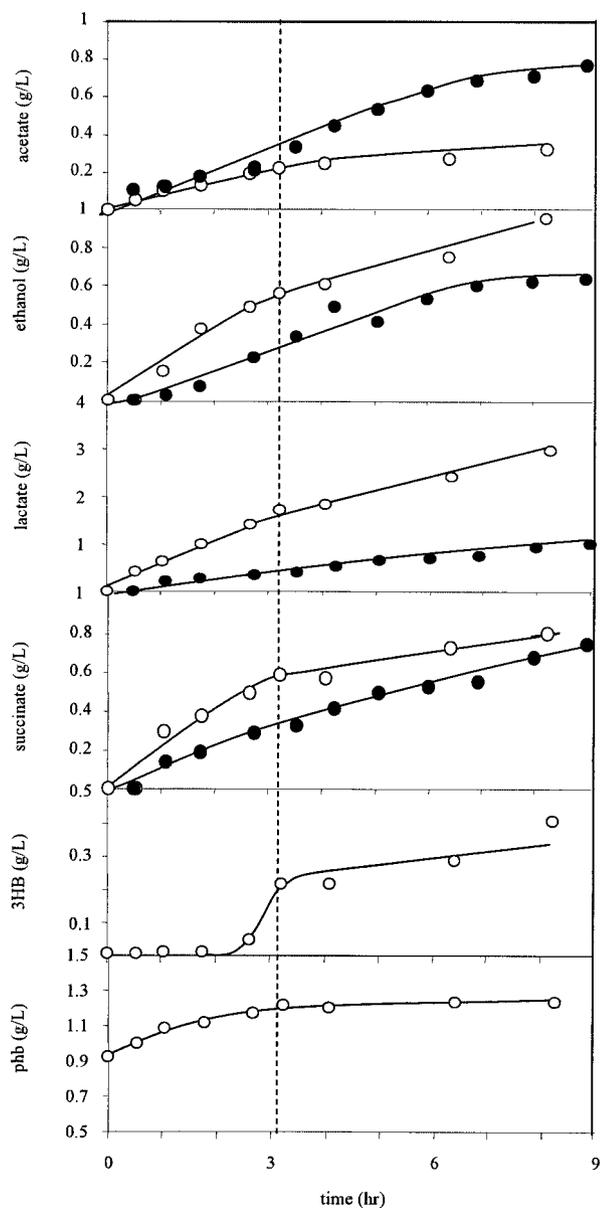


FIG. 4. Product time profiles for anaerobic *E. coli* cultures in nongrowth medium. Open circles, strain PHB(+); filled circles, strain PHB(-). Dotted lines highlight the region used for rate and yield calculations. See text for more details.

lated from the experimental cultures are shown as data points, while the slopes of the lines are equivalent to the product yield based on equation 2. The numerical value for each yield, in terms of moles of carbon (C moles) of product per C mole of glucose, is given in each plot.

The two strains utilized different ratios of metabolic intermediates as reducing equivalent sinks. Strain PHB(-) had a larger acetate, lactate, and succinate yield than PHB(+) under growth conditions, while strain PHB(+) had a higher ethanol yield under growth conditions. The growth-associated PHB yield was approximately equal to the nongrowth-associated PHB yield. The 3-hydroxybutyric acid yield was significantly higher under nongrowth conditions. This is likely due to the

high intracellular PHB content, which may have limited additional polymerization of 3-hydroxybutyryl-CoA and favored the secretion of the free acid.

Interestingly, Fig. 5 shows that even with a higher extracellular concentration of potentially toxic acetate, strain PHB(-) during growth and nongrowth conditions had a higher acetate yield on glucose.

Only trace amounts of pyruvate were detected. H₂ and formate concentrations were not determined. CO₂ concentrations were not measured for the growth conditions.

Based on the carbon yields in Fig. 5 and the CO₂ data, approximately 68 and 87% of the utilized carbon during nongrowth conditions was recovered for the PHB(-) and PHB(+) cultures, respectively. If it is assumed that one formate molecule is formed via pyruvate formate-lyase during the production of each acetate and each ethanol molecule and that each 3-hydroxybutyrate and each PHB molecule results in the production of two formate molecules, the carbon recoveries based on Fig. 5 improve to approximately 80 and 104% for the PHB(-) and PHB(+) cultures, respectively. These formate-corrected yields were used to determine a redox balance, which is expressed here as the ratio of an electron balance on the oxidized glucose to the produced fermentation products. The PHB(-) and PHB(+) cultures had ratios of 1.28 and 0.97, respectively. The carbon and redox shortcomings in the PHB(-) culture are believed to be the result of analytical error. A carbon and redox balance was not attempted for the growth conditions since the complex medium contained approximately 26 g of undefined components per liter (see Materials and Methods). Therefore, a considerable amount of the carbon is unaccounted for in the recovered by-products. However, even without more detailed data, it is apparent that anaerobic production of PHB is possible and that it has a significant effect on cellular metabolism.

DISCUSSION

The cellular redox state during anaerobic cultivation likely favors PHB production. A high NADPH/NADP ratio has been shown to stimulate PHB production (17). This is probably due to the regulation pattern of the β -ketothiolase enzyme and an increased flux through the reductase-catalyzed step in the PHB pathway which consumes reduction equivalents. This finding highlights the role of PHB not only as a carbon reserve compound but also as a redox sink. de Graf et al. (18) and Alexeeva et al. (2) studied the redox (NADH/NAD) ratios in *E. coli* under different levels of oxygen limitation, including anaerobic conditions. Under anaerobic growth conditions, the ratio of NADH to NAD is estimated to be more than 10 times higher than that under fully aerobic growth conditions. *E. coli* possesses at least two transhydrogenase systems (7, 8), so a high NADH/NAD ratio would likely favor a high NADPH/NADP ratio, which could stimulate PHB production.

The presented work is an experimental study of anaerobic PHB production in *E. coli*. In addition, we have attempted to interpret the observed differences in by-product yields in terms of network fluxes. A biochemical pathway analysis technique known as elementary mode analysis (40-43) was utilized to provide a rational basis for interpreting the results from a systems biology basis. The analysis method was used to inves-

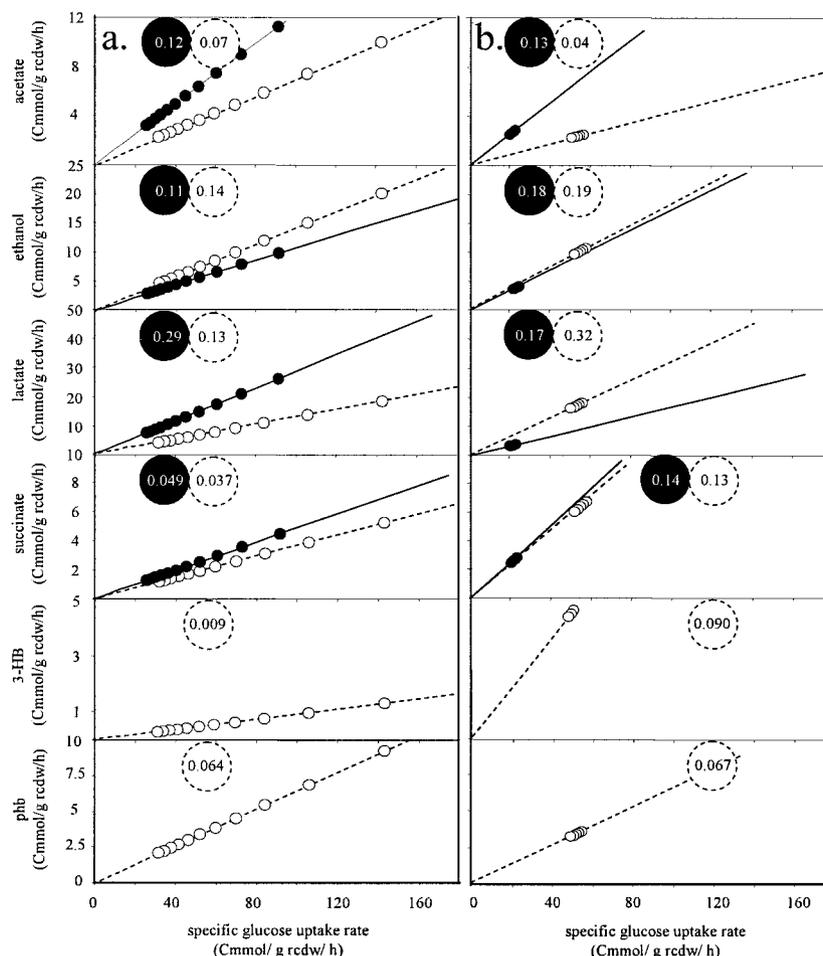


FIG. 5. Bioreactor yields for cultures under (a) growth conditions and (b) nongrowth conditions expressed as the ratio of the specific product secretion rate to the specific glucose uptake rate. All rates are expressed as C millimoles per gram of residual cell dry weight (rcdw) per hour. Filled circles and solid lines represent data from strain PHB(-), while open circles and dashed lines represent data from strain PHB(+). Numerical values for the yields are shown in the upper left of each panel. The filled circle is the product yield in terms of C millimoles per C millimole of glucose for strain PHB(-), while the open, dashed circle is the carbon yield for strain PHB(+).

tigate three questions related to the experimental results and two theoretical questions related to network properties as follows.

(i) **Anaerobic PHB production.** Elementary mode analysis was first used to determine whether the network model could predict the feasibility of anaerobic PHB production. The analysis identified a total of 202 anaerobic PHB-producing pathways. Ninety-eight modes made PHB without coproducing biomass. These results are summarized in Table 1. Product carbon

yield is used as a measure of pathway efficiency and is defined here as the ratio of the C moles in the product to the number of C moles in the substrate(s). The most efficient anaerobic PHB synthesis mode has a carbon yield of 0.8 (Fig. 6). This mode represents the upper limit of network performance. However, the mode does not account for critical processes like maintenance energy generation, which would lower the observed product yield.

(ii) **PHB synthesis lowers acetate yields and increases ethanol yields.** To explain these observations in terms of network fluxes, a method capable of estimating the minimum network perturbation was applied to the possible anaerobic, nongrowth PHB pathways. This method, known as minimization of metabolic adjustment, predicts the behavior of a perturbed system by assuming a network will minimize the readjustment of native, optimal fluxes (44). The theory was developed on an *E. coli* knockout mutant and is examined here on a recombinant host expressing a foreign pathway.

The set of all 98 possible anaerobic PHB pathways was analyzed for their Euclidean distance from the most efficient,

TABLE 1. Summary of elementary mode analysis of a recombinant *E. coli* biochemical network

Mode	No. of modes
Total aerobic and anaerobic.....	10,269
Total anaerobic.....	836
Total biomass (anaerobic).....	461
Biomass (anaerobic, no PHB).....	357
Total PHB (anaerobic).....	202
PHB (anaerobic, no biomass).....	98

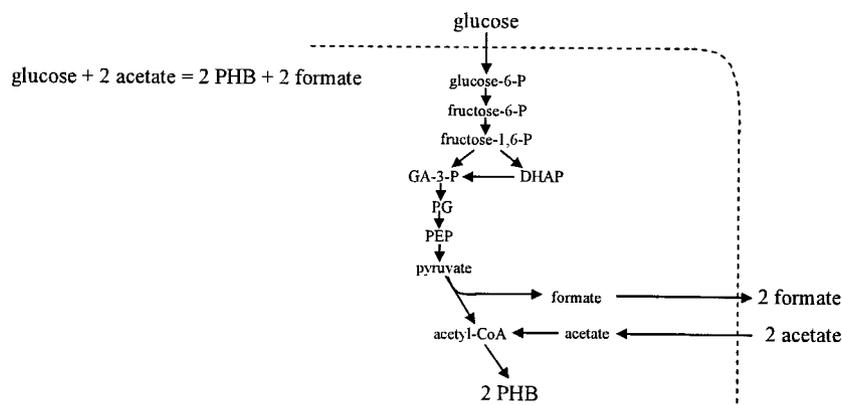


FIG. 6. Most efficient anaerobic PHB-synthesizing pathway for the recombinant *E. coli* biochemical network. PHB carbon yield is 0.8.

native ATP-producing pathway, which was identified as M_4^{ATP} in reference 12. This pathway was examined because under non-growth conditions it is heavily utilized to satisfy maintenance energy requirements. The Euclidean distance, D_j , between a given anaerobic PHB pathway, M_j^{PHB} , and the optimal anaerobic energy pathway, M_4^{ATP} , is defined as follows

$$D_j(M_j^{PHB}, M_4^{ATP}) = \sqrt{\sum_{i=1}^R (m_{i,j}^{PHB} - m_{i,4}^{ATP})^2} \quad j = 1, 98 \quad (3)$$

where R is the number of reactions in the pathway model and m is the flux through reaction i of elementary mode j . All pathways were normalized to reflect a flux of 1 glucose mol prior to comparison. Smaller Euclidean distances represent a smaller perturbation of native fluxes. Smaller Euclidean distances could therefore be a characteristic of a recombinant pathway which is likely to be utilized. The calculated distances varied between 1.11 and 30.25 mol/mol of glucose.

The overall stoichiometries of the six pathways with the shortest Euclidean distances to the energy pathway M_4^{ATP} are shown in Table 2. These pathways show two trends consistent with experimental results. Four of the pathways (modes 2 to 5) consume acetate along with glucose to make PHB. These pathways suggest that PHB production would lower the observed acetate yield. Four of the pathways (modes 1, 3, 4, and 6) coproduce ethanol along with PHB. These pathways suggest that PHB production would increase the observed ethanol production. During both growth and nongrowth experiments,

strain PHB(+) accumulated less acetate and accumulated more ethanol than strain PHB(-) (Fig. 2 and 4). Thus, the concept of the smallest Euclidean distance appears to be consistent with the observed behavior.

As discussed in reference 13, maintenance energy pathways are also active during biomass production. It is therefore reasonable to assume that the results of this analysis apply as well to growth conditions.

(iii) Production of lactate and succinate. The most efficient pathway for anaerobic growth and energy production in terms of glucose carbon yield (0.5 ATP per C mol of glucose) produces only acetate, ethanol, and formate as by-products (pathway M_4^{ATP} ; see Table 2) (12). However, there is often substantial accumulation of lactate and succinate during anaerobic culturing (Fig. 2 and 4). The next most efficient pathways have an ATP/C mol of glucose yield of 0.33 and produce either lactate or a combination of succinate, acetate, and formate (pathways not shown). Lactate production in *E. coli* has been linked to cultivation at acidic pHs because, as compared to acetate, lactate is less toxic (19, 25). Lactate is also actively transported out of the cell, which may permit faster NAD^+ regeneration fluxes than diffusion of acetate and ethanol through the cell boundaries (5, 30). The production of succinate, which is also actively transported out of the cell, likely represents a strategy similar to the lactate-producing mode.

(iv) Optimal pathways for coproducing PHB and another valuable or less toxic compound like ethanol, lactate, succinate, or H_2 . From a practical standpoint, it could be advanta-

TABLE 2. Anaerobic PHB-producing pathways that represent the shortest Euclidean distance from the optimal anaerobic energy-producing pathway^a

Pathway	Euclidean distance (mol/mol of glucose)
M_4^{ATP} glucose = 3 ATP + acetate + ethanol + formate.....	0
1. 3 glucose = 6 ATP + 2 ethanol + 6 formate + 2 PHB.....	1.11
2. glucose + 2 acetate = 2 formate + 2 PHB.....	2.0
3. 3 glucose + 5 acetate = 4 ethanol + 5 formate + 3 CO ₂ + 3 PHB.....	4.22
4. 3 glucose + 3 acetate = ethanol + 6 formate + 4 PHB.....	4.44
5. 3 glucose + 3 acetate + CO ₂ = succinate + 5 formate + 4 PHB.....	5.0
6. 3 glucose = 3 ATP + 2 ethanol + 6 formate + 2 PHB.....	5.11

^a The modes are listed in ascending order based on the Euclidean distance. The most efficient anaerobic energy-generating mode is listed at the top for comparison. See text for more details.

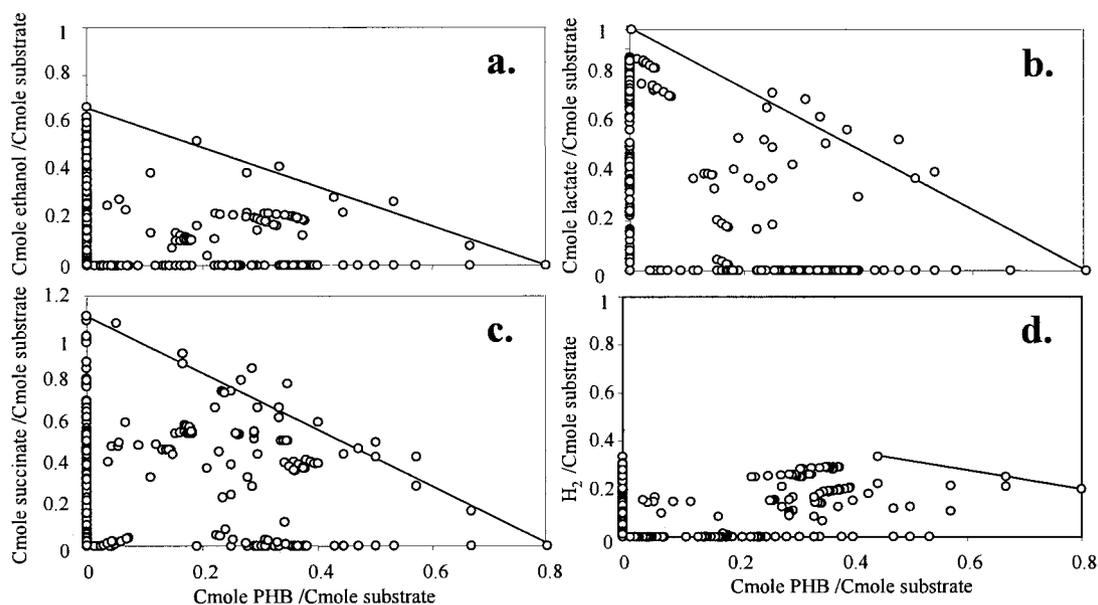


FIG. 7. Relationship between product carbon yields for multiple products. Carbon yield was defined as the ratio of C moles in product to the number of C moles in the substrate(s). The H_2 yield represents the moles of H_2 produced per C mole of substrate(s). a, Ethanol and PHB; b, lactate and PHB; c, succinate and PHB; and d, H_2 and PHB. The line represents the yield performance of linear combinations of the two highest-yielding single-product pathways. Modes above this line represent a higher conversion of substrate carbon into coproducts.

geous to direct fluxes away from toxic compounds like acetate toward either less toxic or more valuable by-products. Figure 7 examines the theoretical capabilities of the *E. coli* biochemical network to anaerobically coproduce ethanol (Fig. 7a), succinate (Fig. 7b), lactate (Fig. 7c), and H_2 (Fig. 7d) along with PHB. Some of the carbon yields for succinate are greater than 1 due to the assimilation of CO_2 via PEP carboxylase. H_2 production is assumed to occur from formate via formate hydrogen-lyase.

A straight line is used to connect the highest-yielding anaerobic PHB mode with the highest-yielding mode for the other considered by-products. This line represents the relationship between the yields of the two products during coproduction if only linear combinations of the two end point modes are considered. When coproduction of two products is desired, the plot reveals that linear combinations of the two highest-yielding, single-product modes is not always the most efficient use of the metabolic network. Modes that lie above the line represent strategies that are more efficient in terms of total substrate carbon recovery than those using linear combinations of the most efficient modes for each single product. While Fig. 7 represents the substrate carbon yield, other considerations like the commercial value of each product can also be explored.

(v) **Comparison of PHB production in *E. coli* and *Saccharomyces cerevisiae*.** Numerous studies have examined PHA expression in different recombinant systems with the intention of taking advantage of each unique biochemical network (for example, see references 20, 26, 35, 36, 47). A comparison of the most efficient anaerobic PHB-producing modes in *E. coli* and *S. cerevisiae* reveals very different theoretical network capabilities. The most efficient *E. coli* pathway has a theoretical PHB carbon yield of 0.8 (Fig. 6), while the most efficient *S. cerevisiae* pathway has a theoretical PHB carbon yield of only 0.24 (11).

The topological features of both biochemical networks are similar, but the absence of two key enzymatic activities in *S. cerevisiae* lowers its PHB carbon yield. Unlike *E. coli*, *S. cerevisiae* does not possess a transhydrogenase system (9, 21) nor does it possess a pyruvate formate-lyase activity. The lack of these enzymatic activities lowers the anaerobic PHB yield.

The anaerobic synthesis of PHB was demonstrated in a recombinant *E. coli* strain under both growth and nongrowth conditions. The recombinant pathway had a significant effect on by-product secretion patterns. The system represents a potentially interesting strategy for PHA synthesis and provides insight into the *E. coli* central metabolism.

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