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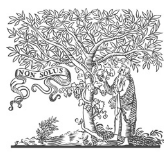

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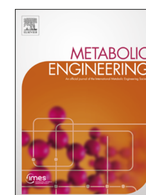

Regulatory effects on central carbon metabolism from poly-3-hydroxybutryate synthesis

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ABSTRACT

Poly-3-hydroxybutyrate (PHB) synthesis in *Escherichia coli* elicits regulatory responses that affect product yield and productivity. We used controlled, steady-state cultures (chemostats) of a genetically stable strain to determine growth-independent metabolic flux regulation. We measured flux and steady-state intracellular metabolite concentrations across different dilution rates (0.05, 0.15, 0.3 h^{-1}), limitations (glucose, gluconate and nitrogen), and operon copy counts of the PHB pathway (0, 6, 17, and 29). As PHB flux increases, specific substrate consumption and lactate secretion increase while formate and acetate secretion decreases in N-limited, glucose-fed conditions.

To understand the regulatory mechanisms that resulted in these macroscopic changes, we used a flux balance analysis model to analyze intracellular redox conditions. Our model shows that under N-limited conditions, synthesis of PHB creates excess reducing equivalents. Cells, under these conditions, secrete more reduced metabolites in order to recycle reducing equivalents. By switching to a more oxidized substrate (gluconate) that decreased excess reducing equivalents, PHB flux yield increased 1.6 fold compared to glucose-fed fermentations. High flux of PHB ($\sim$1.2 mmol/g DCW h) was maintained under these steady-state, oxidized conditions. These results imply redox imbalance is a driving force in industrial production of PHB, and substrates that are more oxidized than glucose can increase productivity.

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1. Introduction

Optimizing yields and productivity requires product flux to be maximized while byproducts and biomass are minimized. Recent literature highlights the two-way coupling between growth rate and transcriptomic, proteomic, and metabolomic regulation (Klumpp et al., 2009; Matsumoto et al., 2013; You et al., 2013). This suggests that any engineering strategy must consider its effects on growth rate (biomass flux) and growth's effect on product synthesis. In the case of poly-3-hydroxybutryate (PHB) synthesis, an increase in percent PHB of cellular dry weight (CDW) could be due to an increased flux toward product or to a reduction in growth. Both would show an increase in percent PHB in CDW. Previous measurements in growth-controlled chemostats suggest many PHB engineering strategies increase PHB content by reducing growth (Tyo et al., 2010). PHB flux may affect growth through several mechanisms. At a minimum, carbon that is converted to PHB cannot be used to make cellular components. Beyond this, regulatory effects are likely important. Decoupling growth rate from PHB production will be essential to understanding the regulatory effects. We used steady-state cultures (via chemostat cultivation) with precise control over heterologous pathway expression (via the Chemically Induced Chromosomal Evolution, CIChE, method described below).

The PHB pathway in *Escherichia coli* has three enzymes, β-ketothiolase, acetoacetyl-CoA reductase (AAR), and PHB synthase, and are encoded by the genes *phaA*, *phaB*, and *phaC* or *phaEC* respectively (Anderson and Dawes, 1990; Madison and Huisman, 1999). Two equivalents of acetyl-CoA are converted to acetoacetyl-CoA by β-ketothiolase. Using a reducing equivalent (NADPH), acetoacetyl-CoA is converted to 3-hydroxybutyryl-CoA by AAR and then elongated to poly-3-hydrobutyrate by PHB synthase. In total, the PHB pathway converts two acetyl-CoA molecules and one NADPH to one unit of PHB (Peoples and Sinskey, 1989; Schubert et al., 1988).

To systematically measure the metabolic response to PHB flux, we must control pathway expression level with stability over many generations. Plasmids, the typical vehicle for introducing heterologous pathways to organisms, do not have the genetic stability required for continuous cultures (Keasling, 1999). We turned to genomic integration of the PHB operon. To precisely vary activity of the PHB pathway, the copy number of the operon on the genome can be varied using the CIChE method. Particular copy numbers are

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made as tandem repeats on the genome and are not subject to copy number variation (as extrachromosomal plasmids are) that is present both as (a) a distribution of copy numbers in a population and (b) the average copy number varying with growth phase, environment, and a host of other factors. Because the PHB pathway exists as repeats on the genome, where the genome copy number is strictly controlled, we ensure that the expression is relatively constant across the different chemostat conditions. Specific PHB productivity was shown to remain through 80–100 generations and thus suitable for chemostats (Tyo et al., 2009).

To better understand how PHB pathway expression perturbs central carbon metabolism, we measured fluxes under a variety of growth rates and nutrient conditions. Different growth rate have different intracellular conditions, such as different ribosome concentrations and overall metabolic productivity to meet growth demands (Maaløe, 1979). Likewise, the C/N ratio affects a number of metabolic processes. Therefore, we cultivated *E. coli* at steady state with varying dilution rates (0.05 h^{-1}, 0.15 h^{-1}, and 0.3 h^{-1}), PHB pathway operon copies (0, 6, 17, and 29 copies), and nutrient conditions (carbon and nitrogen limitation). We measured metabolite uptake/secretion and used Flux Balance Analysis (FBA) to calculate redox synthesis/consumption rates. Combined with intracellular metabolite measurements, we could observe substantial metabolic regulation across conditions due to PHB synthesis. Our study suggests more oxidizing conditions (in particular more oxidized substrates) will increase PHB yield in *E. coli* bioprocessing because less carbon is secreted to maintain redox balance. We demonstrate such using N-limited chemostats with gluconate (a more oxidized substrate compared to glucose) where PHB flux increased 1.6 fold.

2. Materials and methods

2.1. Chemicals, media and other materials

All chemicals were purchased from Sigma-Aldrich, qPCR reagents from Bio-Rad, and primers from IDT unless otherwise noted.

Luria–Bertani broth was used for cell transformation. Sterile MR media was used for inoculum preparation and continuous cultures. MR media was composed of 3 g/L $(NH_4)_2HPO_4$, 22 g/L KH_2PO_4, 0.875 g/L citric acid monohydrate, 5 ml/L trace metals mix, 0.7 g/L $MgSO_4 \cdot 7H_2O$, and 10 mg/L thiamin (Wang and Lee, 1997). Trace metals solution was comprised of 5 M HCl, 10 g/L $FeSO_4 \cdot 7H_2O$, 2.2 g/L $ZnSO_4 \cdot 7H_2O$, 1 g/L $CuSO_4 \cdot 5H_2O$, 0.5 g/L $MnSO_4 \cdot 4H_2O$, 2 g/L $CaCl_2$, 0.02 g/L $Na_2B_4O_7 \cdot 10H_2O$, and 0.1 g/L $Na_2B_4O_7 \cdot 10H_2O$. 30 g/L glucose was added for N-limited conditions, and 8 g/L was used for C-limited. For the gluconate cultures, an equimolar amount of sodium gluconate was substituted for glucose.

2.2. Strain construction

Four strains were used in this study (see Table 1). All strains were constructed using the CIChE system (Tyo et al., 2009). In this system, a gene cassette containing the PHB pathway genes (*phaA*, *phaB*, and *phaEC*) and chloramphenicol resistance marker is flanked by two homologous regions recognized by *recA* recombinase. Through subculturing the strain in increasing concentrations of chloroamphenicol, we can select for higher copy numbers of the PHB gene pathway in consequent generations. At a chloroamphenicol concentration corresponding to our desired copy count, P1 phage transduction was used to knockout *recA* and to confer kanamycin resistance by transfecting a *recA: kan* allele from BW26547. With *recA* knocked out, the copy number of the PHB pathway was stabilized due to the lack of recombinase. Strain KS29, selected out of a maximum chloramphenicol concentration of 1.5 mg/mL, was used in a previous study (Tyo et al., 2010). Strain KS00 is K12 with a *recA: kan* allele. Strains KS06 and KS17 were subcultured to 0.54 mg/mL and 1.22 mg/mL chloramphenicol respectively from a one copy strain using CIChE. These strains were subsequently transfected with the *recA*: kan^R cassette as described earlier.

Table 1
E. coli strains used in this study.

Strain	PHB operon copy number	PHB production in shake flask (g/L)	Source
KS00	0.0545 ± 0.007	None detectable	Tyo et al. (2009)
KS06	6.20 ± 0.22	0.0126 ± 0.0003	This report
KS17	16.76 ± 2.70	0.399 ± 0.009	This report
KS29	28.54 ± 0.93	1.30 ± 0.03	Tyo et al. (2009)

2.3. Copy count characterization

All strain copy numbers were characterized by quantitative PCR using the Bio-Rad (Hercules, CA) CFX96 real-time PCR detection module with C1000 thermal cycler. Primers are listed in Table S1. Genomic copy number was determined by the measurement of the PHB pathway gene *phaA*, and the gene *bioA* served as a single copy reference control for the genome. Genomic DNA was purified from the strains using the Promega Wizard Genomic DNA Purification Kit. Standards were created via PCR using the genomic DNA from a strain with the PHB operon. Gene copy count was calculated as $C_{\mathrm{phaA}}/C_{\mathrm{bioA}}$.

2.4. Shake flask experiments

PHB production was determined from strains KS00, KS06, KS17, and KS29 from cells grown in 50 mL of MR media with 25 mg/L kanamycin in 250 mL shake flasks. Flasks were inoculated to an OD of 0.05 and incubated at 37 °C with 225 rpm shaking. Flasks were sampled after 72 h.

2.5. Chemostat experiments

Experiments were performed in 1 L stirred bioreactors (Sartorius Stedim Biotech, Frankfurt, Germany) with 0.5 L working volume. Temperature, pH, and dissolved oxygen (DO_2) were all measured with appropriate probes (Hamilton Company, Reno, NV, USA). Culture pH was maintained at 6.8 ± 0.05 pH with the addition of 2 N NaOH. Temperature was maintained at 37 °C using heating and cooling water jacket. Air inflow was maintained at 0.25 slpm. dO_2 control ensured that that the dO_2 content did not fall below 20% by adjusting the stir rate from 200 to 600 rpm and oxygen supplementation into the air inflow. CO_2 and O_2 offgas were recorded (BlueSens, Herten, Germany). Reactors were inoculated to an OD of 0.02 and operated in batch mode for 14 h after the fresh media flow was initiated. Cell culture effluent level were controlled by placing the outlet line at a height that corresponds to a working volume of 0.5 L. Growth rate was monitored with CO_2 offgas measurements. Steady-state was assumed to be four residence times and confirmed by steady, constant CO_2 output. Once steady-state was achieved, culture samples were collected through a sampling port. All data represents triplicate bioreactors at each condition.

2.6. Glucose, biomass, and fermentation product quantification

10 mL samples were withdrawn from the bioreactor at steady-state. Samples were centrifuged at 5000*g* for 10 min at 2 °C. Supernatant was tested for the presence of ammonia in C-limited and

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