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Analysis of polyhydroxybutyrate flux limitations by systematic genetic and metabolic perturbations

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ABSTRACT

Poly-3-hydroxybutyrate (PHB) titers in *Escherichia coli* have benefited from 10+ years of metabolic engineering. In the majority of studies, PHB content, expressed as percent PHB (dry cell weight), is increased, although this increase can be explained by decreases in growth rate or increases in PHB flux. In this study, growth rate and PHB flux were quantified directly in response to systematic manipulation of (1) gene expression in the product-forming pathway and (2) growth rates in a nitrogen-limited chemostat. Gene expression manipulation revealed acetoacetyl-CoA reductase (*phaB*) limits flux to PHB, although overexpression of the entire pathway pushed the flux even higher. These increases in PHB flux are accompanied by decreases in growth rate, which can be explained by carbon diversion, rather than toxic effects of the PHB pathway. In chemostats, PHB flux was insensitive to growth rate. These results imply that PHB flux is primarily controlled by the expression levels of the product forming pathway and not by the availability of precursors. These results confirm prior *in vitro* measurements and metabolic models and show expression level is a major affecter of PHB flux.

1. Introduction

Growth-associated biochemical production requires a sensitive balance between product formation (specific productivity) and biomass formation (growth rate). Given that specific substrate uptake rates are finite, resources diverted to biomass and product cannot exceed a defined rate, forcing a tradeoff between the two. While product formation is the objective of a biochemical process, increasing the flux to product will eventually inhibit growth by starving the cell for resources necessary for growth, thus reducing the rate of biocatalyst production. In addition, toxicity of accumulated products or intermediates that are a result of increased diversion of resources to the product forming pathway may also hinder growth beyond what is acceptable. From an overall productivity, a precisely defined product flux is essential to maximize yield and productivity.

A carefully defined product flux is especially true in the case of growth during poly-3-hydroxybutyrate (PHB) synthesis, where the product is expected to use a large portion of glucose uptake and may be toxic due to the large intracellular PHB granules (Anderson and Dawes, 1990), thereby potentially hindering growth rate significantly. Polyhydroxyalkanoates (PHAs), the family of polyesters that include PHB, are a possible replacement for many petrochemical-based polymers that are used today. Because PHAs use renewable resources as feedstock and are biodegradable, certain co-polymer PHAs could offer 'green' replacements for commonly used polyolefins (Anderson and Dawes, 1990). PHB production in recombinant *Escherichia coli* is attractive for a number of reasons: titers as high as 75% are possible (Wang and Lee, 1997), purification of the polymer from *E. coli* is easy, and there are no known PHB depolymerases in *E. coli* (Madison and Huisman, 1999). A continuous process may be attractive for PHB production in *E. coli* because chemostats enjoy greater utilization of installed capital, lower equipment sizes, and increased volumetric productivities (when calculated including down-times inherent in batch processes), which may be helpful for this low value product. However, for continuous production simultaneous generation of both biomass and PHB is required because only a limited amount of PHB can be stored intracellularly. To date little work has explored continuous production for PHB. Previous studies have shown that PHB accumulation primarily occurs in stationary phase and very little accumulation happens in log phase growth (Wang and Lee, 1997). These characteristics would not be amenable for growth phase accumulation, and thus new engineering strategies need to be employed to improve growth phase accumulation.

PHB specific productivity (unless otherwise specified, productivity will refer to specific productivity), or flux, can be limited by available precursors to the product-forming pathway or activity of the enzymes in the pathway. The committed PHB pathway consists of three steps that convert two acetyl-CoA moieties and

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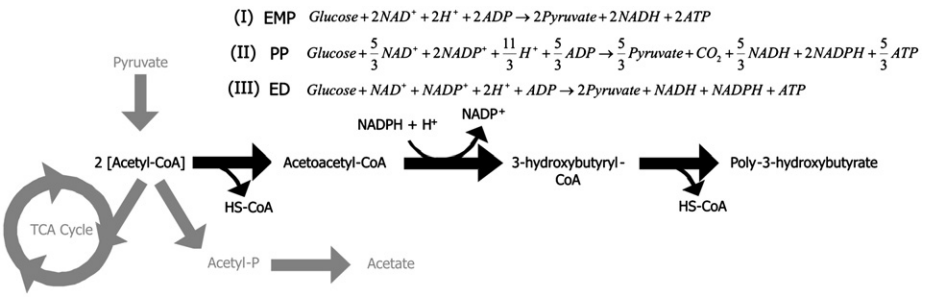

Fig. 1. Glucose metabolism to poly-3-hydroxybutyrate. Conversion of glucose to pyruvate can occur by three pathways that have different pyruvate yields and produce different reducing equivalents: (I) Embden–Meyerhof–Parnas (EMP) pathway (glycolysis), (II) pentose phosphate (PP) pathway, and (III) Entner–Doudoroff (ED) pathway. The ED pathway does not provide as much ATP, but provides the exact amount of NADPH required for PHB synthesis. [Metabolic map] The recombinant PHB pathway (black) competes for acetyl-CoA, a central node for many different biosynthesis routes, with native pathways of TCA cycle, and acetate synthesis (grey), among others. Oxidation state of the NADPH/$NADP^+$ pool and levels of acetyl phosphate may regulate the entry of acetyl-CoA to various pathways.

one reducing equivalent (NADPH) to one unit of PHB (Fig. 1). The three enzymes in the PHB pathway are β-ketothiolase, acetoacetyl-CoA reductase (AAR), and PHB synthase, encoded by the genes *phaA*, *phaB*, and *phaEC* (or *phaC*), respectively. PHB metabolized from glucose has an excess of reducing equivalents (shown as H_2) as shown in Eq. (1). The chemical reactions in Fig. 1(I–III) show that *E. coli* can metabolize glucose in a variety of ways that provide different forms of reducing cofactors and energy as byproducts while providing acetyl-CoA for PHB production.

$$n C_6H_{12}O_6 \rightarrow (C_4H_6O_2)_n + 2nCO_2 + 3nH_2 \quad (1)$$

PHB content, the most commonly reported and industrially relevant parameter, can be misleading when trying to interpret biological mechanisms. PHB content can be increased by either increasing PHB flux or retarding growth rate. Metabolic engineering schemes often have significant effects on growth rate, independent of the PHB pathway. It is possible for flux to PHB to be unchanged, while a decreased growth rate will give higher PHB accumulation. Increased PHB content is always good; however, overall productivity is best improved by perturbations that increase PHB flux directly. From an industrial standpoint, this would reduce batch times or allow for continuous operation.

The literature offers conflicting viewpoints as to what limits PHB flux. Some have implicated NADPH as a limiting reagent under growth conditions. These studies have focused on altering the redox balance in the cell through (a) phosphoglucose isomerase (*pgi*) deletion, (b) overexpression of pentose phosphate (PP) pathway enzymes (*zwf*, *gnd*, *talA*, and *tktA*), (c) gluconate feeding, or (d) transhydrogenase overexpression (Jung et al., 2004; Lim et al., 2002; Sanchez et al., 2006; Shi et al., 1999; Song et al., 2006). These act through (a) preventing glucose from being metabolized through the Embden–Meyerhof–Parnas (EMP) pathway, and instead metabolized through the PP pathway or the Entner–Doudoroff (ED) pathway, making NADPH instead of NADH, (b) increasing activity of PP pathway causing the same redirection as in (a), (c) feeding substrates naturally metabolized by the PP or ED pathways, and (d) catalyzing electron exchange from NADH to NADP+.

While the above studies did increase NADPH supply, it is unclear whether the observed increases in flux to PHB were a result of overcoming a NADPH shortage to the PHB pathway, making more acetyl-CoA available for PHB biosynthesis by downregulating other acetyl-CoA consuming reactions, or general growth retardation, which would allow a higher PHB cell content. For PHB production on glucose, measurements of NADPH/NADP+ ratio was 3 and NADPH concentration was 225 μM (van Wegen et al., 2001). This is higher than the NADPH K_m of AAR, 19 μM (Steinbuchel and Schlegel, 1991), which should be sufficient to supply NADPH. Hong et al. (2003) have shown that carbon is directed down the ED pathway to provide more NADPH for PHB production (unlike the EMP pathway, which produces NADH), implying NADPH may not be limiting. On the other hand, several pathways that consume acetyl-CoA (Fig. 1, acetyl-CoA node) are regulated in a NADPH-dependent manner. TCA cycle consumption of acetyl-CoA is linked to redox in the cell through product inhibition by NADPH of isocitrate dehydrogenase (Dean and Koshland, 1993), and allosteric inhibition by high NADPH/NADP+ ratios that have been associated with lowered citrate synthase activity for growth on glucose (Wang and Lee, 1997; Lim et al., 2002). The acetate pathway is regulated, in part, by the need to regenerate NAD^+ (Wolfe, 2005). If NADH levels are lowered by an increased diversion of reducing equivalents to NADPH, the flux to acetate may be downregulated. In aggregate, NADPH-directed perturbations may also increase availability of acetyl-CoA for the PHB pathway by NADPH-induced downregulation of competing pathways.

Attempts to directly improve the supply of acetyl-CoA may also have unexpected effects that further confuse experimental observations. Acetate secretion is observed in PHB producing *E. coli* under aerobic conditions (Sanchez et al., 2006), implying an excess of acetyl-CoA is available. Phosphate acetyltransferase (*pta*) and acetate kinase (*ack*) deletions, which should decrease acetate production, have also decreased PHB accumulation, implying that enzyme competition for the acetyl-CoA pool may not be the determining factor (Shi et al., 1999). Instead, the presence of acetyl phosphate may be required in order to activate PHB synthase (Miyake et al., 2000) and without acetyl phosphate the PHB pathway may thus be downregulated. Shi et al. (1999) also showed that addition of α-methyl-glucoside, a glucose analog that retards glucose uptake through non-toxic competitive inhibition, decreases acid secretion, but does not change PHB accumulation. PHB flux appears insensitive to glucose uptake, implying the pathway activity may exert metabolic control over the flux from acetyl-CoA to PHB.

The previous work has resulted in impressive PHB titers in late stationary phase (see the review by Madison and Huisman (1999) for many examples). It may be possible to shorten the batch time necessary to achieve these high titers by analyzing the rates of production, i.e. PHB flux. Pathway enzyme activities have been implicated as a possible flux limitation. *In vitro* measurements of PHB pathway enzyme activities and kinetic models have predicted that PHB flux is limited by enzyme activity, not precursor supply (Sim et al., 1997; van Wegen et al., 2001), but to date, no systematic, *in vivo* study has been undertaken to this end. In this work, we examined the effects of stepwise overexpression of the PHB pathway genes on pathway flux. Separately, the growth rate was varied through dilution rate in a nitrogen-limited chemostat