Systems Biological Approach for the Production of Various Polyhydroxyalkanoates by Metabolically Engineered *Escherichia coli*

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Summary: Metabolic engineering strategies have been developed based on ever increasing molecular and genetic information of polyhydroxyalkanoate (PHA) biosynthesis in natural PHA producing bacteria, and used for developing recombinant *Escherichia coli* strains with enhanced PHA biosynthesis activity. Recently, systems level metabolic engineering approaches based on genomics, proteomics and fluxomics have been taken in designing an optimal bioprocess for the production of various PHAs in recombinant *E. coli*. For examples, *in silico* metabolic flux analysis (MFA) and proteome analysis revealed that several central metabolic enzymes including Eda, Fba and TpiA were amplified during poly(3-hydroxybutyrate)[P(3HB)] biosynthesis to support more acetyl-CoA and NADPH. Also, a genome informatics approach was successfully taken to identify various FadB homologous enzymes including YfcX, YdbU, PaaF and PaaG, and a new enoyl-CoA hydratase MaoC, which are involved in PHA biosynthesis from fatty acid in *fadB* mutant *E. coli* strains. These new systems level findings were employed to design metabolically engineered *E. coli* strains for the enhanced production of PHAs and production of novel PHAs. Therefore, systems biological approach is a robust way to improve the metabolic activities of recombinant *E. coli* locally as well as globally for the enhanced production of PHAs.

Keywords: metabolic engineering; polyhydroxyalkanoates; recombinant *Escherichia coli*; systems biology

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters, which are intracellularly accumulated as carbon and energy reserve material in numerous microorganisms when they meet unfavorable growth conditions in the presence of excess carbon sources.[1-3] Because PHAs are biodegradable and biocompatable polyesters, the material properties of

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which can be modulated by varying their monomer units, they have attracted much commercial interest as substitutes for chemically synthesized polymers. Also, recently, a new application of PHAs for the production of enantiomerically pure (*R*)-form hydroxycarboxylic acids has been developed by chemical or biological hydrolysis of PHAs.[4] (*R*)-form hydroxycarboxylic acids can be used as precursors for the manufacture of antibiotics, vitamins, perfumes and pheromones. To date, more than 150 different kinds of (R)-hydroxycarboxylic acids have been identified to be monomer units of PHAs.[5]

Along with natural PHA producing bacteria such as *Ralstonia eutropha*, *Alcaligenes latus*, *Pseudomonas aeruginosa* and *P. putida*, *Escherichia coli* has been extensively engineered to produce PHAs.[1-3] For the production of PHAs in *E. coli*, various metabolic engineering strategies have been developed so that metabolic pathways are modified to enhance metabolic capacities of *E. coli* to make them optimized for the biosynthesis of PHAs.[1-3]

Traditional metabolic engineering studies have been carried out by labor-intensive "trial-and-error" procedure, in which one should repeat experiments of constructing recombinant strains followed by examining their activities for target bioproducts until a satisfactory strain is developed. However, this strategy has often failed due to the complex and robust metabolic network of microorganism resistant to genetic and environmental perturbations. The advent of systems biology, which aims to understand the whole metabolic network of organisms at a systems level, is changing the way metabolic engineering is executed. Recent advances in high throughput experimental technologies are enabling genome, transcriptome, proteome and metabolome data to be generated at an unprecedentedly high rate. Integration of all the information and data generated through these 'omics' at the systems level is changing the strategy of biotechnology development. In this paper, even though this area is only at its beginning, we review the systems biological approaches taken for the enhanced production of PHAs in metabolically engineered *E. coli*.

Metabolic Engineering for the Production of Poly(3-hydroxybutyrate)

Poly(3-hydroxybutyrate) [P(3HB)], the most ubiquitous member of the PHAs, is synthesized from acetyl-CoA by sequential reactions catalyzed by three enzymes: β-ketothiolase (PhaA) condensates two acetyl-CoA molecules to acetoacetyl-CoA, which is reduced to 3-hydroxybutylyl-CoA by acetoacetyl-CoA reductase (PhaB) using NADPH as a cofactor, and finally 3-hydroxybutylyl-CoA is incorporated into the growing chain of P(3HB).[1-3] Because the P(3HB) biosynthesis pathway inevitably competes with inherent

metabolic pathways of *E. coli* for acetyl-CoA, it is most important to make more acetyl-CoA available for P(3HB) biosynthesis without too much disruption of the metabolic activities of the host strain. Consideration of the fate of acetyl-CoA in metabolic pathways has led us to find strategies for making more acetyl-CoA available for P(3HB) biosynthesis, which consequently resulted in the increased P(3HB) yield on the carbon source and higher P(3HB) productivity. These strategies include addition of small amounts of amino acids, oleic acid and complex nitrogen sources in defined media.[6, 7] More intensive metabolic engineering strategies have been developed to achieve enhanced P(3HB) biosynthesis in two ways. In the first approach, various strategies including host strain selection, use of plasmids of different copy numbers, filamentation suppression, use of different PHA biosynthesis genes, and plasmid stabilization have been employed. Using these strategies, efficient production of P(3HB) has been demonstrated in flask, fed-batch, and pilot-scale fermentations.[1, 2, 7] Detailed results are discussed in several recent review articles.[1, 2, 7] In the second approach, the metabolic engineering strategy was more focused on modifying the inherent metabolic pathways of *E. coli* in order to increase the availability of acetyl-CoA and reducing power for the production of P(3HB). This is described below in more detail.

As mentioned earlier, the most important factor which should be considered in designing metabolic engineering strategies for the production of P(3HB) from sugars is how to make more acetyl-CoA and NADPH available for the P(3HB) biosynthesis pathway. When glucose is used as a carbon source, acetyl-CoA and NADPH can be efficiently provided from the glycolysis and pentose phosphate (PP) pathways. Lim et al.[8] employed *zwf* and *gnd* genes, which encode glucose-6-phosphate dehydrogenase and 6-posphogluconate dehydrogenase, respectively, to generate enough NADPH, which is required for reductase, for P(3HB) biosynthesis. When these enzymes were amplified, the NADPH/$NADP^+$ ratio increased by six times, and consequently the P(3HB) content increased from 23 % to 41%. However, a closer evaluation suggested that the increase of P(3HB) content was not due to enhanced P(3HB) biosynthesis but rather due to decreased cell concentration. Therefore, it is important to make a cellular status suitable for P(3HB) biosynthesis without reduction of cell growth (metabolic activity) to achieve a high concentration of P(3HB).

To elucidate the whole metabolic status of *E. coli* during P(3HB) biosynthesis, proteome analysis using 2-dimensional gel electrophoresis (2DE) and metabolic flux analysis (MFA) based on an *in silico E. coli* metabolic network model were carried out. Proteome analyses

of recombinant *E. coli* during P(3HB) biosynthesis were carried out by two different groups[9, 10] and found similar proteome patterns such as high level expression of general heat shock proteins including GroES, GroEL, DnaK and IbpAB. Besides these general aspects, proteome analysis of *E. coli* producing P(3HB) also suggested the importance of acetyl-CoA and NADPH availability for P(3HB) production. Han et al.[9] reported that the spot intensities of fructose-bisphosphate aldolase (FbaA) and triosephosphate isomerase (TpiA) in a glycolytic pathway were increased during P(3HB) accumulation by recombinant *E. coli* XL1-Blue harboring plasmid pJC4 containing the *A. latus* PHA biosynthesis genes. The reason for the increased expression of FbaA and TpiA might be due to the fact that *E. coli* modified its metabolic fluxes to increase the glyceraldehyde-3-phosphate pool, which is eventually used for P(3HB) synthesis. Also, 2-keto-3-deoxy-6-phosphogluconate aldolase (Eda), which catalyzes the final step of the Entner-Doudoroff (ED) pathway, was found to be highly up-regulated in *E. coli* XL1-Blue producing P(3HB). Even though the ED pathway has generally been considered to be inactive under normal growth conditions when glucose is used as a carbon source, it seems to become active in XL1-Blue under P(3HB) accumulating conditions. The increased demand for NADPH and acetyl-CoA during P(3HB) production seems to have made *E. coli* operate the ED pathway to modify the metabolic status to be more suitable for P(3HB) biosynthesis.

MFA is a useful tool to evaluate cellular metabolic conditions and to suggest strategies for increasing the product yield under a given condition. *E. coli* producing P(3HB) has been analyzed by MFA,[11, 12] which also suggested the importance of acetyl-CoA and NADPH availability for P(3HB) production. Furthermore, the MFA of *E. coli* producing P(3HB) during the fed-batch culture explained the reasons why oxygen limitation had a positive effect on P(3HB) production.[11] It was found that under the oxygen limiting condition, additional carbon flux through pyruvate formate lyase increased without any change of the pyruvate dehydrogenase flux, which is used under normal growth conditions, resulting in the accumulation of twice as much acetyl-CoA, which was subsequently used for P(3HB) biosynthesis.[11] Since P(3HB) biosynthesis is affected by many central metabolic pathway fluxes, it will be important to optimize the flux distributions in such ways that the flux towards P(3HB) is maximized while the fluxes towards byproducts such as acetic, lactic, and formic acids are minimized. Also, cultivation strategies should be carefully optimized because a too-early accumulation of P(3HB) results in low final cell concentration (due to

the growth inhibition) while a too-late accumulation of P(3HB) results in low P(3HB) content, both of which result in low P(3HB) productivity.

Metabolic flux distributions in wild-type *E. coli* and recombinant *E. coli* producing P(3HB) were compared using the *in silico E. coli* metabolic network constructed with 154 reversible and 156 irreversible reactions and 295 metabolites. The results suggested that the ED pathway flux increased significantly under a P(3HB) accumulating condition,[12] which agreed well with the results obtained with proteome analysis.[9] These MFA results were confirmed by comparing the abilities of P(3HB) synthesis in *E. coli* KS272 and its *eda* mutant. The P(3HB) concentration and the P(3HB) content were lower in the *eda* mutant *E. coli* strain than those obtained in a non-mutant strain.[12] Therefore, at least in the *E. coli* KS272 strain, Eda, the ED pathway plays an important role in P(3HB) biosynthesis. Metabolic pathways engineered in *E. coli* for the production of PHAs are summarized in Figure 1.

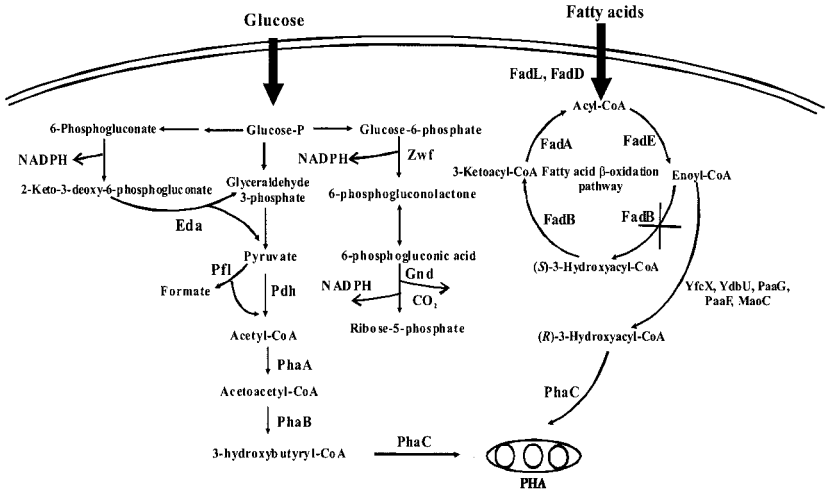

Figure 1. Metabolic pathways engineered in *Escherichia coli* for the biosynthesis of PHAs. In the case of P(3HB) biosynthesis, glycolysis, PP pathways and ED pathways are modified to make more acetyl-CoA and NADPH available for P(3HB) biosynthesis. In the case of engineering β-oxidation pathway for the production of MCL-PHAs, (*R*)-3-hydroxyacyl-CoAs are supplied by FadB homologous enzymes including YfcX, YdbU, PaaG and PaaF, and an enoyl-CoA hydratase homologous enzyme MaoC in a *fadB* mutant *E. coli* strain. Abbreviations are: Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; FadB, enoyl-CoA hydratase; FadD, acyl-CoA synthetase; FadE, acyl-CoA dehydrogenase; FadL, membrane bound fatty acid transport protein; Gnd, 6-phosphogluconate dehydrogenase; Pdh, pyruvate dehydrogenase; Pfl, pyruvate formate lyase; PhaA, β-ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC, PHA synthase; Zwf, glucose-6-phosphate dehydrogenase.

Global information obtained from 'omics' approaches can be used to investigate the whole metabolism at a systems level and to design optimal metabolic engineering strategies, which cannot be easily predicted by rational thinking. Systems biological approaches for the examination of metabolic pathway redistribution of recombinant *E. coli* producing P(3HB) by various 'omics' approaches are schematically represented in Figure 2.

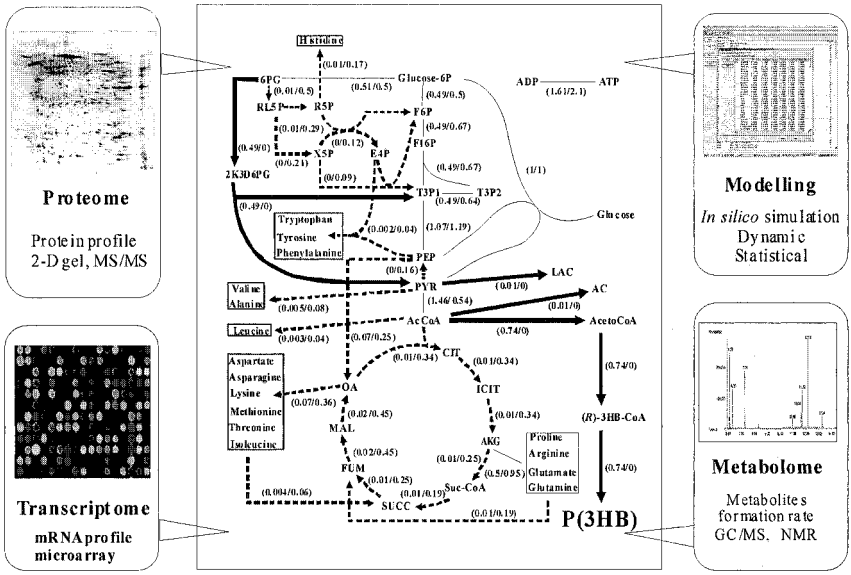

Figure 2. Pictorial representation of systems biological approaches for unraveling metabolic redistribution of recombinant *E. coli* producing PHAs. Information obtained through various 'omics' approaches including genomics, transcriptomics, proteomics, metabolomics, and fluxomics can be integrated to design optimal strain and bioprocess for the enhanced production of PHAs. The numbers in the center box are, in order, the intracellular fluxes obtained for recombinant *E. coli* producing P(3HB) having the P(3HB) content of 78% and non-P(3HB) producing *E. Coli.*[12] Bold arrows indicate the fluxes that are increased by more than 2-fold, while dashed arrows indicate the fluxes that are decreased to less than a half. The center box was redrawn from ref. 12.

Metabolic fluxes of *E. coli* during P(3HB) production resolved by MFA through the *in silico* organism model can be confirmed and reconsidered using transcriptome, proteome and metabolome data to suggest more concrete metabolic engineering strategies for the efficient production of P(3HB).

Engineering of Fatty Acid β-oxidation Pathways for the Production of PHAs

The intermediates of fatty acid metabolism are good precursors for the medium-chain-length (MCL) PHAs consisting of monomer units having 6-12 carbon atoms. Enoyl-CoA, 3-ketoacyl-CoA, 3-hydroxyacyl-CoA, and the 3-hydroxyacyl-acyl carrier protein (ACP) are good precursors, which are easily converted by various enzymes such as enoyl-CoA hydratase[13-17] and ketoacyl-CoA reductase[18-20] to (*R*)-3-hydroxyacyl-CoA, the most favorable substrate of MCL-PHA synthase. Therefore, metabolic engineering strategies for the modification of fatty acid metabolism have been developed to efficiently produce MCL-PHAs in recombinant *E. coli*.

Since the first demonstration that a *fadB* mutant *E. coli* expressing the *Pseudomonas aeruginosa phaC1* gene could synthesize MCL-PHA from decanoic acid,[21] the *fadB* and *fadA* mutant *E. coli* strains have been used for the production of PHAs from various fatty acids.[1-3] Also, artificial links between PHA biosynthesis and functional β-oxidation pathways have been constructed by expressing the enoyl-CoA hydratase or ketoacyl-CoA reductase gene from various bacteria.[13-20]

Even though impaired *β*-oxidation pathways seem to be essential for supplying (*R*)-3-hydroxyacyl-CoAs from fatty acid, there has been no clear explanation for which enzymes are truly responsible for these conversion reactions. Recently, bioinformatics tools including the search for biological databases were employed to address this question.

Based on the amino acid sequence homology of proteins, several enzymes in *E. coli* belonging to the crotonase superfamily highly homologous to FadB were identified.[22] These enzymes were found to share the same active site amino acid sequence as that of FadB.[22] Until now, YfcX, PaaF, PaaG and YdbU have been characterized at molecular levels for their roles in PHA biosynthesis from fatty acid in the *fadB* mutant *E. coli* strains.[23-25] When one of the genes encoding these enzymes was deleted from the chromosome of the *fadB* mutant *E. coli* strain, the ability to synthesize PHAs was reduced to a different extent (unpublished results for the cases of PaaF, PaaG, and YdbU). Deletion of YfcX was found to most significantly reduce the extent of PHA production.[23-25] Alternatively, amplification of one of these enzymes in a *fadB* mutant *E. coli* strain enhanced PHA biosynthesis from fatty acids.[25] These results clearly show that bioinformatics can be very useful in elucidating important enzymes in metabolic pathways and constructing new metabolic pathways for the enhanced production of PHAs.

Besides the identification of new FadB homologous enzymes involved in PHA biosynthesis from fatty acids in *E. coli*, protein database searches also revealed that a new enoyl-CoA hydratase similar to that of natural PHA producing bacteria also exists in *E. Coli.*[23] In natural PHA producing bacteria, PHA biosynthesis from fatty acid is suggested to occur through (*R*)-specific hydration of enoyl-CoAs by (*R*)-specific enoyl-CoA hydratase (PhaJ). In *E. coli*, putative aldehyde dehydrogenase MaoC was found to be homologous to *P. aeruginosa* PhaJ1 and involved in PHA biosynthesis by hydration of enoyl-CoA into (*R*)-3-hydroxyacyl-CoA in a *fadB* mutant *E. Coli.*[23] Interestingly, intrinsic expression of the *maoC* gene from the chromosome was not enough to support PHA biosynthesis in the presence of functional FadB. This seems to be due to the fact that the activity of MaoC as an enoyl-CoA hydratase might be rather weak compared with FadB. Nevertheless, MaoC was found to play an important role in MCL-PHA biosynthesis from fatty acid in a *fadB* mutant *E. coli* strain. In Figure 1, the engineered β-oxidation pathways for the production of MCL-PHAs in recombinant *E. coli* are summarized.

Conclusion

In this paper, we briefly reviewed how systems biological approaches have been employed to improve metabolic activities of *E. coli* for the production of PHAs. Until now, enhanced production of PHAs has been addressed by the development of strains through simple genetic engineering and optimization of the fermentation process. Thanks to the availability of high throughput experimental tools and quantitative analysis technologies, it is now possible to take systems biological approaches to develop more robust metabolic engineering strategies for the enhanced production of PHAs or even tailored PHAs.

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