Towards Systems Metabolic Engineering of PHA Producers

Yu Kyung Jung, Sang Yup Lee, and Tran Thanh Tam

Contents

1 Introduction 64
2 Traditional Metabolic Engineering of PHA Producers 65
  2.1 Natural PHA Producers and Metabolic Engineering 65
  2.2 Engineering of Non-PHA Producers 67
3 Systems-Biological Approach for PHA Production 71
  3.1 Systems Metabolic Engineering for Strain Improvement 72
  3.2 Metabolic Engineering Based on Omics Studies 73
  3.3 Future of Systems Metabolic Engineering for PHA Production 77
4 Concluding Remarks and Future Perspectives 77
References 79

Abstract Polyhydroxyalkanoates (PHAs) are natural polyesters that accumulate in numerous microorganisms as a carbon- and energy-storage material under the nutrient-limiting condition in the presence of an excess carbon source. PHAs are considered to be one of the potential alternatives to petrochemically derived plastics owing to their versatile material properties. Over the past few decades, extensive detailed biochemical, molecular-biological, and metabolic studies related to PHA

Y.K. Jung, S.Y. Lee (✉) and T.T. Tam
Department of Chemical and Biomolecular Engineering (BK21 Program), Metabolic and Biomolecular Engineering National Research Laboratory, KAIST, 335 Gwahangno, Daejeon, 305-701, Republic of Korea
e-mail: leesy@kaist.ac.kr

Y.K. Jung, S.Y. Lee and T.T. Tam
Center for Systems and Synthetic Biotechnology, Institute for the BioCentury, KAIST, 335 Gwahangno, Daejeon, 305-701, Republic of Korea

S.Y. Lee
Department of Bio and Brain Engineering, BioProcess Engineering Research Center and Bioinformatics Research Center, KAIST, 335 Gwahangno, Daejeon, 305-701, Republic of Korea

S.Y. Lee
Department of Biological Sciences, KAIST, 335 Gwahangno, Daejeon, 305-701, Republic of Korea

G.-Q. Chen (ed.), *Plastics from Bacteria: Natural Functions and Applications*, Microbiology Monographs, Vol. 14, DOI 10.1007/978-3-642-03287_5_4,
© Springer-Verlag Berlin Heidelberg 2010

biosynthesis have been carried out. Advances in our knowledge of PHA biosynthesis led to the development of engineered strains and fermentation processes for the production of PHAs with high efficiency. Even though the traditional metabolic engineering based on our rational thinking and trial-and-error type approaches allowed development of improved strains, further enhancement in the performance is expected through systems metabolic engineering, which is metabolic engineering integrated with systems-biological approaches. In this chapter, the strategies taken for the metabolic engineering of PHA producers are briefly reviewed. Then, genomic and proteomic studies performed to understand the PHA biosynthesis in the context of whole cell metabolism as well as to develop further engineered strains are reviewed. Finally, the strategies for the systems metabolic engineering of PHA producers are suggested; these will make it possible to produce PHAs with higher efficiencies and to develop tailor-made PHAs by systems-level optimization of the metabolic network and establishment of novel pathways.

1 Introduction

As our concerns regarding the environmental problems represented by global warming are increasing, there has recently been much interest in developing bio-based processes for the production of chemicals, fuels, and materials from renewable resources. Polymers which we use everyday are no exception. Among many types of biopolymers, polyhydroxyalkanoates (PHAs) have been considered to be good alternatives to petrochemically derived polymers. PHAs are natural polyesters that accumulate in numerous microorganisms as a carbon- and energy-storage material under the nutrient-limiting condition in the presence of an excess carbon source (Anderson and Dawes 1990; Lee 1996). Since poly[(*R*)-3-hydroxybutyrate], P(3HB), homopolymer was discovered in *Bacillus megaterium* by Maurice Lemoigne in 1926, more than 150 kinds of (*R*)-hydroxyalkanoic acid monomers have been found to be incorporated into bacterial PHAs (Steinbüchel and Valentin 1995). This variety of monomers allows PHAs to have diverse material properties and broadly classifies PHAs into one of three types: thermoplastics, which are short-chain-length (SCL) PHAs consisting of C_3–C_5 monomers; elastomers, which are medium-chain-length (MCL) PHAs consisting of C_6–C_{16} monomers; and their copolymer (SCL–MCL) PHAs, which show properties of both of them and notably low density polyethylene like properties (Fig. 1). The monomer composition of PHAs depends highly on the metabolic capability of host microorganisms and on the substrate specificity of PHA synthase, and subsequently determines the physicochemical properties of PHAs (Lee 1996; Rehm 2003). This implies that tailor-made PHA production using microorganisms can be optimized by metabolic engineering and key enzyme engineering, and can be finally maximized by optimized fermentation. Recently, the genome sequences of the most common PHA producers, *Cupriavidus necator* H16 (formerly, *Ralstonia eutropha* H16) and *Pseudomonas putida* KT2440, were reported by Pohlmann et al. (2006) and Nelson et al. (2002), respectively.

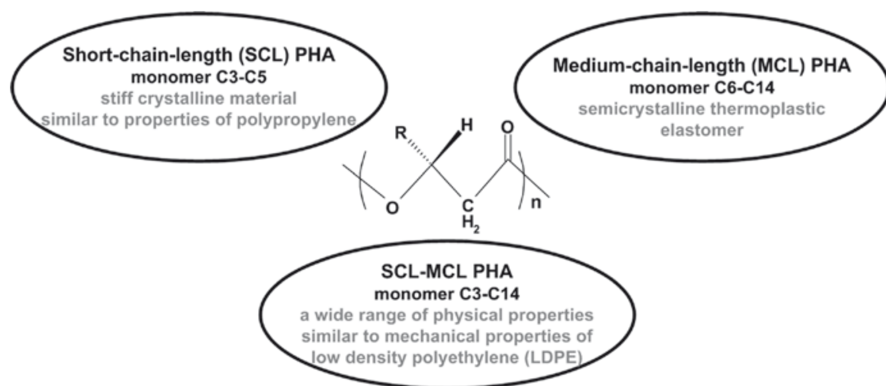

Fig. 1 General structure and three types of polyhydroxyalkanoates (PHAs), including short-chain-length (SCL) PHAs, medium-chain-length (MCL) PHAs, and SCL–MCL PHAs. Their characteristics are also summarized

Thus, PHA biosynthesis has also entered the genomics era, and strain development for novel PHA production and enhanced PHA production can be performed through systems metabolic engineering.

2 Traditional Metabolic Engineering of PHA Producers

Numerous bacteria accumulate PHAs under unfavorable growth conditions, such as the limitation of nitrogen, phosphorous, magnesium, or oxygen, in the presence of an excess carbon source. They share a common metabolism to generate core intermediates for PHA monomers from a carbon substrate, but the different characteristics of the preferred metabolism led to the generation of different monomers for PHAs (Anderson and Dawes 1990; Lee 1996). There have also been extensive studies on metabolic engineering of non-PHA-producing bacteria to make them efficiently produce PHAs to high levels (Fidler and Dennis 1992; Lee and Chang 1995; Lee et al. 1999).

2.1 Natural PHA Producers and Metabolic Engineering

Generally, many naturally PHA producing bacteria, including *C. necator* (Peoples and Sinskey 1989; Schubert et al. 1988; Slater et al. 1988; Doi et al. 1992), *Alcaligenes latus* (Braunegg and Bogensberger 1985; Hangii 1990; Hrabak 1992; Yamane et al 1996), *Pseudomonads* (Gross et al. 1989; Fritzsche et al. 1990; Huisman et al. 1991; Timm and Steinbüchel 1992; Timm et al. 1994), *Aeromonas* (Doi et al. 1995; Lee et al. 2000), *Rhodococcus ruber* (Pieper and Steinbüchel 1992), and *Syntrophomonas*

wolfei (McInerney et al. 1992), can synthesize a wide range of PHAs depending on the carbon source and the cultivation condition employed. Among them, *C. necator* and *P. putida* have been most widely used for PHA production.

C. necator has been studied extensively because of its great ability to accumulate P(3HB), up to 80–90 wt%, from simple carbon sources (Repaske and Mayer 1976; Heinzle and Lafferty 1980; Kim et al. 1994a). By applying nitrogen or phosphorous limitation, *C. necator* can accumulate a large amount of P(3HB) from a simple carbon source such as fructose or glucose. When both glucose and propionate are added during the polymer accumulation phase, poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate], P(3HB-*co*-3HV), can be produced in *C. necator*. Depending on the ratio of glucose to propionate fed into the medium, the (*R*)-3-hydroxyvalerate (3HV) fraction of this copolymer can be varied from 0 to 30 mol%, and a polymer content of about 75 wt% is obtained (Byrom 1992; Kim et al. 1994b). When 22 $g\,l^{-1}$ propionate was used as the sole carbon source during the polymer synthesis phase, the 3HV fraction reached up to 43 mol%, whereas the polymer content was only 35 wt% in *C. necator* (Doi et al. 1986). *C. necator* possesses class I PHA synthase, which preferentially utilizes and incorporates SCL (*R*)-3-hydroxyalkanoates into PHAs. However, it has been proved that *C. necator* has the capability to accumulate MCL PHAs consisting of (*R*)-3-hydroxyhexanoate (3HHx) and (*R*)-3-hydroxyoctanoate (3HO) as well as SCL PHAs when a β-oxidation inhibitor, sodium acrylate, is added and sodium octanoate is used as a carbon source (Green et al. 2002).

Although *C. necator* belongs to the naturally PHA producing bacteria, a number of engineered *C. necator* strains have been developed to improve PHA productivity as well as to control monomer compositions of PHAs. By introducing the genes encoding crotonyl-coenzyme A (CoA) reductase from *Streptomyces cinnamonensis*, and PHA synthase and (*R*)-specific enoyl-CoA hydratase from *Aeromonas caviae* into a *C. necator* PHA-negative mutant strain, the engineered strain was able to produce poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate], P(3HB-*co*-3HHx), having 1.5 mol% 3HHx monomer up to 48 wt% from fructose (Fukui et al. 2002). Production of up to 87 wt% P(3HB-*co*-3HHx) having a constant level of 5 mol% 3HHx was achieved by cultivating a *C. necator* PHA-negative mutant strain harboring the *A. caviae* PHA synthase gene on palm kernel oil (Loo et al. 2005). Moreover, the 3HHx fraction could be altered in the range 0–5.1 mol% when several *A. caviae phaC* ($phaC_{Ac}$) mutant genes were introduced into the *C. necator* PHA-negative mutant strain, which resulted in the production of various P(3HB-*co*-3HHx) having different thermal properties of interest (Tsuge et al. 2004).

In pseudomonads belonging to the ribosomal RNA homology group I, the de novo fatty acid biosynthesis and degradation pathways generate intermediates including enoyl-CoA, 3-ketoacyl-CoA, (*S*)-3-hydroxyacyl-CoA, 3-ketoacyl-CoA, and 3-hydroxyacyl-acyl carrier protein (ACP), which can eventually serve as the precursors of MCL PHAs (Lee 1996; Madison and Huisman 1999; Witholt and Kessler 1999; Park and Lee 2003). Various enzymes, including enoyl-CoA hydratase (Fiedler et al. 2002; Fukui and Doi 1998), 3-ketoacyl-ACP reductase (Park et al. 2002; Ren et al. 2000; Taguchi et al. 1999), epimerase (Madison and Huisman 1999), and 3-hydroxyacyl-ACP:CoA transacylase (Rehm et al. 1998;

Hoffmann et al. 2000), play important roles in connecting the fatty acid metabolism and PHA biosynthesis. Since a novel metabolic link between fatty acid biosynthesis and PHA biosynthesis by the 3-hydroxydecanoyl-ACP:CoA transacylase (PhaG) was identified in *P. putida* (Rehm et al. 1998), there have been several reports showing that most pseudomonads use PhaG to accumulate PHAs containing MCL units from unrelated carbon sources such as sugar, except for *P. oleovorans* and *P. fragi* (Hoffmann et al. 2000; Fiedler et al. 2000). It was found that the expression system of the *P. oleovorans phaG* gene ($phaG_{Po}$) did not function properly in *P. oleovorans*. Reverse transcriptase polymerase chain reaction (PCR) of $phaG_{Po}$ obviously showed that $phaG_{Po}$ was not transcribed even when gluconate was used as a carbon source. However, the *P. oleovorans phaG* gene was expressed well using the *lac* promoter, which enabled *P. oleovorans* to accumulate MCL PHA up to 74 wt% (Hoffmann et al. 2000). Another non-PHA-producing *Pseudomonas* strain, *P. fragi*, equipped with the *phaC1* gene from *P. aeruginosa* and the *phaG* gene from *P. putida*, was able to accumulate 14 wt% MCL PHA composed mainly of about 60 mol% (*R*)-3-hydroxydecanoate, and additional constituents of 2 mol% 3HHx, 21 mol% 3HO, 11 mol% (*R*)-3-hydroxydodecanoate (3HDD), 4 mol% (*R*)-3-hydroxydodecenoate, and 1 mol% (*R*)-3-hydroxytetradecanoate (3HTD), when cultivated on gluconate as the sole carbon source (Fiedler et al. 2000).

It was reported that *P. putida* CA-3 was able to accumulate MCL PHA from the aromatic hydrocarbon styrene as the sole carbon source under nitrogen limitation through styrene degradation, de novo fatty acid biosynthesis, and β-oxidation pathways (O'Leary et al. 2001, 2005; Ward and O'Connor 2005). Recently, the *fadBA* mutant strain of *P. putida* KT2442 led to the enhanced production of MCL PHA having a dominant 3HDD or 3HTD fraction (up to 50 mol%) with higher crystallinity and tensile strength than typical MCL PHA, showing improved properties for various applications (Ouyang et al. 2007; Liu and Chen 2007).

2.2 Engineering of Non-PHA Producers

Although natural PHA producers have the ability to accumulate PHAs during their life cycle, they often have relatively low growth rate and low optimal growth temperature, and also possess PHA degradation pathways, which are disadvantageous to PHA production. On the other hand, *Escherichia coli*, a non-PHA-accumulating bacterium, is capable of neither PHA synthesis nor its degradation. *E. coli* grows fast at a relatively high temperature, and is easy to lyse, which allows a shorter cycle time for PHA production and cost savings associated with the cooling of the fermentor and the PHA purification process (Madison and Huisman 1999). Furthermore, *E. coli* has been intensively studied for PHA production because it was able to be metabolically engineered to synthesize PHAs thanks to versatile tools for genetic engineering (Fig. 2).

The plasmid-based expression of many genes for PHA synthesis in *E. coli* resulted in the production of various types of PHAs. The recombinant *E. coli* har-

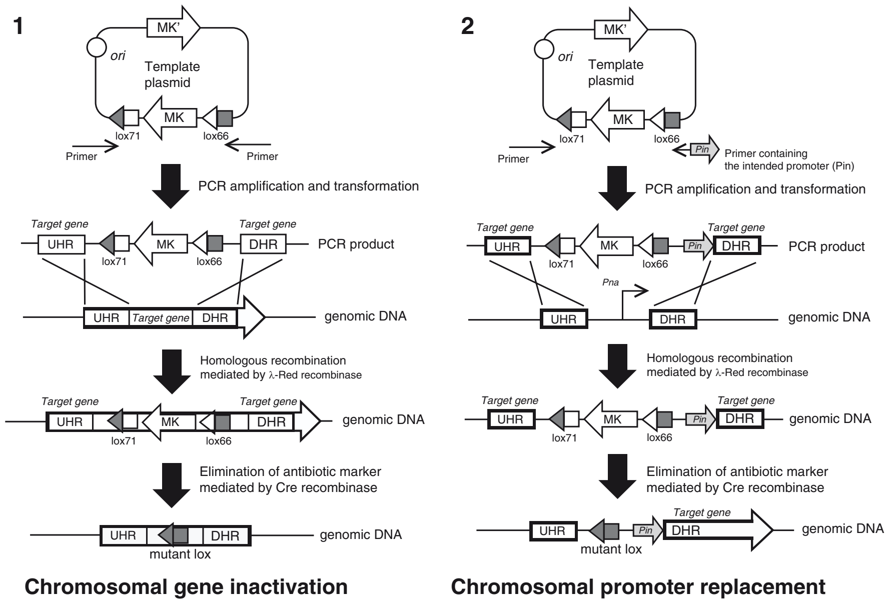

Fig. 2 PCR-mediated homologous recombination using the λ-Red recombinase as a typical *Escherichia coli* chromosomal manipulation method (Datsenko and Wanner 2000; Palmeros et al. 2000; Yuan et al. 2006). **1** Chromosomal gene inactivation, **2** chromosomal promoter replacement. *ori* replication origin, *MK* antibiotic resistance marker, *UHR* upper homologous region, *DHR* down homologous region, *Pna* native promoter

boring the *A. latus* PHA biosynthesis genes (*phaCAB*$_{Al}$) was able to produce the highest amount of P(3HB) compared with *C. necator* H16 wild-type strain and recombinant *E. coli* harboring *C. necator* H16 PHA biosynthesis genes (*phaCAB*$_{Cn}$) (Genser et al. 1998; Choi et al. 1998). The P(3HB) concentration and productivity obtained from the recombinant *E. coli* harboring the *phaCAB*$_{Al}$ genes were as high as 141.6 g l^{-1} and 4.63 g l^{-1} h^{-1}, respectively (Choi et al. 1998). More interestingly, a filamentation-suppressed recombinant *E. coli* harboring the *phaCAB*$_{Al}$ genes and the *E. coli ftsZ* gene encoding the earliest acting cell division protein, FtsZ, accumulated P(3HB) up to 82.4 wt%, which was higher than that obtained with the recombinant *E. coli* harboring only the *phaCAB*$_{Al}$ genes (Choi and Lee 1999a).

In addition to P(3HB) homopolymer, P(3HB-*co*-3HV) copolymer could be produced in recombinant *E. coli* (Slater et al. 1992; Yim et al. 1996; Choi and Lee 1999b; Wong et al. 2007). When the PHA biosynthesis genes from *C. necator* (*phaCAB*$_{Cn}$) were introduced into *E. coli* LS5218 (*fadR atoC*(Con)), which can constitutively express the enzymes involved in the utilization of short-chain (C_4–C_6) fatty acids, P(3HB-*co*-3HV) could be produced from glucose and propionate as carbon sources. The P(3HB-*co*-3HV) content and the 3HV monomer fraction could be varied depending on the concentrations of glucose and propionate in the medium (Slater et al. 1992). Also, the recombinant *E. coli* XL1-Blue strain equipped with the *phaCAB*$_{Cn}$

genes was able to produce P(3HB-*co*-3HV) from glucose and propionate. Moreover, it was indicated that induction with acetate and/or oleate as well as propionate for the activation of propionate metabolism increased the PHA concentration and the 3HV fraction (Yim et al. 1996). P(3HB-*co*-3HV) could also be produced in recombinant *E. coli* harboring the *Salmonella enterica prpE* gene encoding propionyl-CoA synthetase and the $phaCAB_{Cn}$ genes. The 3HV monomer fraction in the copolymer varied from 5 to 18 mol%, depending on the expression level of PrpE, which was under the control of the isopropyl β-D-1-thiogalactopyranoside inducible *tac* promoter (Wong et al. 2007).

Production of MCL PHAs was first investigated in recombinant *E. coli* by Langenbach et al (1997). A number of strategies have been developed to improve MCL PHA productivity by providing PHA precursors from the fatty acid β-oxidation pathway (Fig. 3; Park et al. 2004). The β-oxidation pathway has been engineered by the overexpression of enoyl-CoA hydratase (Fiedler et al. 2002; Fukui and Doi 1998) or 3-ketoacyl-ACP reductase (Park et al. 2002; Ren et al. 2000;

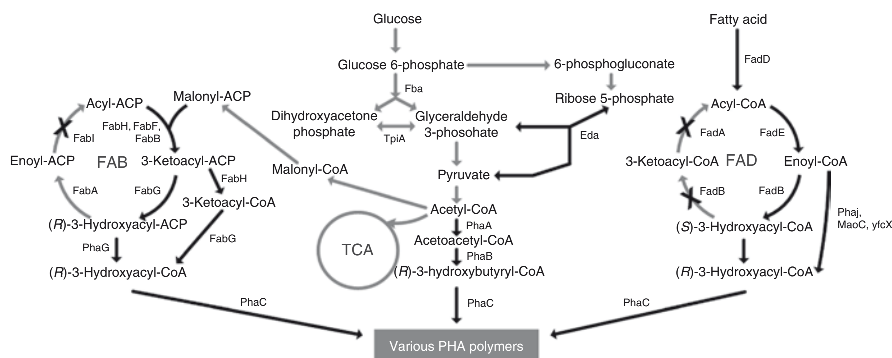

Fig. 3 Rational metabolic engineering for PHA production in *E. coli*. *Black arrows* and *crosses* represent genetic manipulations of overexpression and inhibition, respectively, for the enhancement of targeted monomers. The enzymes related to PHA production are shown. The enzymes are listed below along with the representative sources of the foreign enzymes: FabA, β-hydroxyl- acyl carrier protein (ACP) dehydrase; FabB, β-ketoacyl-ACP synthase/malonyl-ACP decarboxylase; FabF, β-ketoacyl-ACP synthase; FabG, β-ketoacyl-ACP reductase (Park et al. 2002); FabH, β-ketoacyl-ACP synthase/acetyl coenzyme A (CoA):ACP transacylase (Taguchi et al. 1999; Nomura et al. 2004); FabI, enoyl-ACP reductase (Rehm et al. 2001); FadA, β-ketoacyl-CoA thiolase (Park et al. 2003); FadB, multifunctional enzyme encoding hydratase and dehydrogenase activities (Park et al. 2003); FadD, fatty acyl-CoA synthetase (Park et al. 2003); FadE, acyl-CoA dehydrogenase (Park et al. 2003); MaoC, enoyl-CoA hydratase (Park and Lee 2003); YfcX, enoyl-CoA hydratase (Snell et al. 2002); Fba, fructose bisphosphate aldolase (Han et al. 2001); TpiA, triosephosphate isomerase (Han et al. 2001); Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase (Hong et al. 2003; Han et al. 2001); PhaA, β-keththiolase from *Cupriavidus necator* (Lee 1996); PhaB, acetoacetyl-CoA reductase from *C. necator* (Lee 1996); PhaG, (*R*)-3-hydroxydecanoyl-CoA:ACP transacylase from *Pseudomonas putida* (Rehm et al. 1998); PhaJ, enoyl-CoA hydratase from *Aeromonads* (Fukui and Doi 1998); PhaC, PHA synthase from types I, II, III, and IV (Rehm 2003). *TCA* tricarboxylic acid cycle, *FAB* de novo fatty acid biosynthesis pathway, *FAD* fatty acid β-oxidation pathway

Taguchi et al. 1999), and/or by the deletion of FadB or FadA (Langenbach et al. 1997; Snell et al. 2002).

It has been reported that *E. coli* possesses enzymes belonging to the crotonase superfamily, including YfcX, PaaF, PaaG, and YgfG, all of which are highly homologous to FadB. A number of recent studies reported increasing the enoyl-CoA hydratase activity by the overexpression of one of these enzymes in *E. coli* (Haller et al. 2000; Snell et al. 2002; Fiedler et al. 2002; Park and Lee 2003, 2004). The *E. coli fadB yfcX* mutant strain harboring only the *P. oleovorans phaC1* gene ($phaC1_{Po}$) was not able to produce MCL PHA when grown in the presence of fatty acids. However, introduction of the *yfcX* gene along with the $phaC1_{Po}$ gene into an *E. coli fadB yfcX* mutant strain restored the capability of producing MCL PHA in this strain. Moreover, the PHA content increased significantly up to 27.7 wt%, which is 3 times more than that obtained with the *E. coli fadB* mutant strain (Snell et al. 2002). Furthermore, *E. coli* FadB homologous enzymes, such as PaaG, PaaF, BhbD, SceH, and YdbU, identified by a protein database search, were recently taken into consideration in an *E. coli* W3110 *fadB* mutant, WB101 strain (Park and Lee 2004). It is noticeable that no PHA accumulation was observed when each of these genes was coexpressed with the *Pseudomonas* sp. 61-3 *phaC2* gene ($phaC2_{Ps61\text{-}3}$) in *E. coli* W3110. The probable reason is that *E. coli* W3110 wild-type strain possesses a completely functional fatty acid β-oxidation pathway and does not use these enzymes to produce PHAs. On the other hand, coexpression of these genes along with the $phaC2_{Ps61\text{-}3}$ gene in *E. coli* WB101 led to MCL PHA accumulation. When the *paaG, paaF*, and *ydbU* genes were individually coexpressed with the $phaC2_{Ps61\text{-}3}$ gene in *E. coli* WB101, the PHA concentrations obtained were 0.37, 0.25, and 0.33 $g\,l^{-1}$, respectively, from 2 $g\,l^{-1}$ sodium decanoate, which was higher than that obtained (0.16 $g\,l^{-1}$) with *E. coli* WB101 expressing only the $phaC2_{Ps61\text{-}3}$ (Park and Lee 2004).

E. coli MaoC, which is homologous to *P. aeruginosa* (*R*)-specific enoyl-CoA hydratase (PhaJ1), was identified and found to be important for PHA synthesis in *E. coli* WB101. When the $phaC2_{Ps61\text{-}3}$ gene was introduced, *E. coli fadB maoC* mutant produced 43% less MCL PHA from decanoate, compared with *E. coli fadB* mutant WB101. The plasmid-based expression of the *maoC* gene was able to restore the PHA biosynthetic capability. Also, *E. coli* W3110 wild-type strain harboring the *maoC* gene and the $phaC2_{Ps61\text{-}3}$ gene was able to produce MCL PHA from decanoate. This study suggested that MaoC is an enoyl-CoA hydratase supplying (*R*)-3-hydroxyacyl-CoA, (*R*)-3HA-CoA, from the fatty acid β-oxidation pathway to the PHA synthesis pathway in *E. coli* (Park and Lee 2003).

The *P. aeruginosa phaJ1* and *phaJ2* genes ($phaJ1_{Pa}$ and $phaJ2_{Pa}$) have also been studied in *E. coli* for PHA production. Six recombinant *E. coli* LS5218 strains equipped with a PHA synthase gene ($phaC_{Ac}$ or $phaC1_{Ps61\text{-}3}$) alone or its combination with a hydratase gene ($phaJ1_{Pa}$ or $phaJ2_{Pa}$) were compared with one another for PHA production. Among them, four strains harboring both PHA synthase and hydratase genes accumulated much more PHA (up to 29 wt%) than the two strains harboring the PHA synthase gene alone. This result proved that both the $phaJ1_{Pa}$ gene and the $phaJ2_{Pa}$ gene play an important role in supplying the monomer (*R*)-3HA-CoAs for PHA synthesis in *E. coli* LS5218 (Tsuge et al. 2000).

The de novo fatty acid biosynthesis pathway has also attracted a great deal of attention as a means to direct PHA precursors in a desired way. The 3-hydroxydecanoyl-ACP:CoA transferase encoded by the *phaG* gene plays a role in bridging the fatty acid metabolism and PHA biosynthesis. Unlike the *Pseudomonas* fatty acid biosynthetic pathway, the *E. coli* fatty acid biosynthetic pathway does not efficiently provide MCL PHA precursors from unrelated carbon sources, such as glucose and gluconate, resulting in poor MCL PHA production in *E. coli.* Expression of the *phaG* gene from several *Pseudomonas* strains alone was not able to support (*R*)-3HA-CoAs in recombinant *E. coli* from unrelated carbon sources (Rehm et al. 1998). The strategy of chemical inhibition of fatty acid biosynthesis, which works well in some microorganisms, did not allow more efficient MCL PHA production in recombinant *E. coli.* When cerulenin, which specifically inhibits FabB (β-ketoacyl-ACP synthase I) and FabF (β-ketoacyl-ACP synthase II), was added to the culture of recombinant *E. coli* harboring the *P. putida phaG* gene and the *P. aeruginosa phaC1* gene, MCL PHA was not synthesized from gluconate. However, when FabI (enoyl-ACP reductase) was inhibited by a specific inhibitor, triclosan, a small amount (2–3 wt%) of MCL PHA could be synthesized in the same recombinant *E. coli* from gluconate (Rehm et al. 2001). Instead of PhaG in *Pseudomonas*, *E. coli* has thioesterase I (TesA) as a major link between the fatty acid metabolism and PHA biosynthesis. By expression of the *P. oleovorans phaC2* gene and the *E. coli tesA* gene in *E. coli*, MCL PHA could be synthesized with a content of 2.3 wt% from gluconate (Klinke et al. 1999).

The *E. coli fabH* gene encoding 3-ketoacyl-ACP synthase III and the *E. coli* and *Pseudomonas* sp. 61-3 *fabD* genes encoding malonyl-CoA:ACP transacylase were found to have 3-hydroxybutyryl-ACP:CoA transferase activity (Taguchi et al. 1999). The *E. coli* FabH with a limited substrate specificity (C_2–C_4) was engineered to have a broader substrate specificity (C_4–C_{10}). As a result, recombinant *E. coli* strain harboring the mutant *fabH* gene and $phaC1_{Ps61\text{-}3}$ was able to induce the production of monomers of C_4–C_{10}, and subsequently to produce PHA copolymers containing SCL and MCL units up to 2.6 wt% from glucose as a carbon source. This study indicated that the composition of PHA copolymers could be controlled by the activity of monomer-supplying enzymes present in the fatty acid metabolism (Nomura et al. 2004).

3 Systems-Biological Approach for PHA Production

The traditional metabolic engineering strategies described so far have worked out quite nicely in enhancing PHA production or producing novel PHAs. Recent advances in systems-biological studies are providing us with the tools that can be used to further improve the performance of PHA producers by optimizing the metabolic, regulatory, and signaling networks at the systems level. Some examples of employing omics and systems-biological tools in improving the strains are described in this section. Also, general strategies of performing systems metabolic engineering are described (Lee et al. 2005; Park et al. 2008).

3.1 Systems Metabolic Engineering for Strain Improvement

Traditional strain improvement was performed by random, laborious, and time-consuming procedures to develop the mutant strain overproducing the target bio-products. However, these might cause unwanted alteration of the cellular metabolism, which makes it difficult to make further improvements when the conditions change (Lee et al. 2005). Rational metabolic engineering has been found to be a successful strategy for improving the ability of a microorganism to overproduce the desired bioproducts through modification of the cellular metabolism by using various recombinant DNA technologies and rational target identification (Lee and Papoutsakis 1999).

As the complete genome sequences of an increasing number of organisms are becoming available, postgenome research (the "omics" studies) has rapidly been advancing. Comparative analysis of genomes provides a powerful way to identify the genes which need to be introduced, deleted, and/or modified to develop the desired strain (Ohnishi et al. 2002; Rückert et al. 2003; Lee et al. 2005). In addition, *in silico* analysis including the construction of an *in silico* genome-scale metabolic model and metabolic simulation are able to predict the effects of genetic and/or environmental perturbations on cellular metabolism, which is also useful for the design of strategies for strain development (Wiechert 2002; Ishii et al. 2004; Lee et al. 2005; Park et al. 2008). Transcriptomics allows the simultaneous monitoring of relative messenger RNA expression levels in multiple samples by using high-throughput DNA microarrays. By comparing transcriptome profiles among different strains, time points, and culture conditions, one can further engineer potential target genes and regulatory circuits (Choi et al. 2003; Ohnishi et al. 2003; Tummala et al. 2003). Proteomics allows analysis of all the proteins of the cell or its parts by using two-dimensional gel electrophoresis (2DGE) or chromatography coupled with various mass spectrometry (MS) methods (Han and Lee 2006). Considering that most cellular metabolic activities are directly or indirectly regulated by proteins, proteome profiles give us valuable clues to further understand and engineer cellular metabolism (Han et al. 2001, 2003; Kabir and Shimizu 2003). Metabolomics allows quantitative analysis of cellular metabolites and metabolic intermediates by using chromatography coupled with MS or nuclear magnetic resonance. Fluxomics allows analysis of metabolic flux profiles based on flux and isotopomer balances. Comparative analysis of metabolic flux profiles under various genetic and environmental conditions allows us to understand the physiological status of the cells (Stephanopoulos 2004; Wittmann and Heinzle 2001). There have been several successful studies on strain improvement based on omics analyses (Yoon et al. 2003; Askenazi et al. 2003; Lee et al. 2003, 2005; Park et al. 2008).

Systems biotechnology makes it possible to comprehensively collect global cellular information, including omics data, and to integrate these data through metabolic, signaling, and regulatory networks, followed by the construction of computational models of the biological system (Lee et al. 2005; Park et al. 2008). This allows us to understand the cellular metabolism at a global scale and rationally engineer the strain for the enhanced production of the desired bioproducts (Fig. 4).

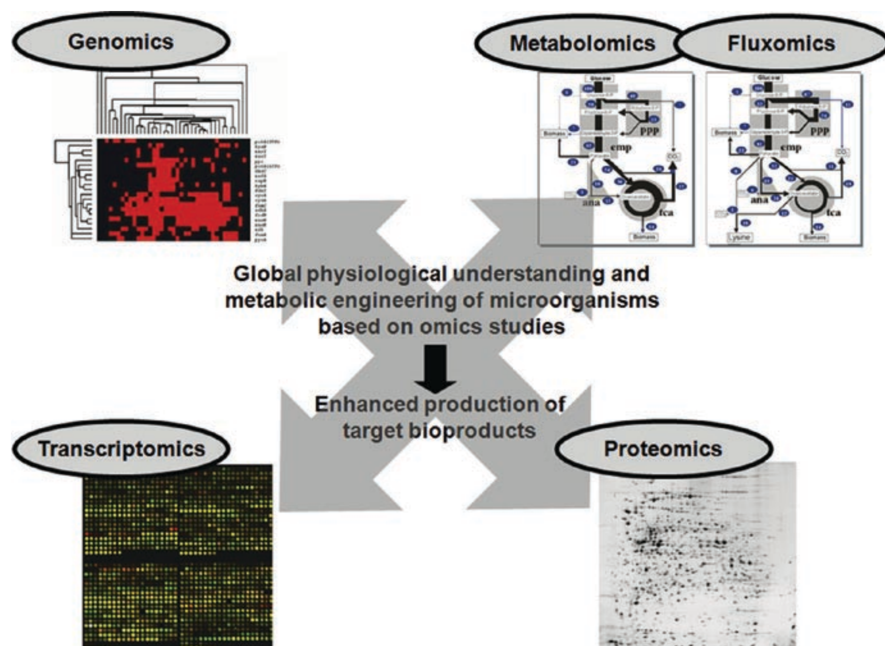

Fig. 4 Strain improvement based on the omics studies. Various omics analyses can be used to decipher the cellular physiological status in a comparative manner, which can subsequently be used to identify gene and pathway targets to be engineered

3.2 Metabolic Engineering Based on Omics Studies

Along with natural PHA producers, *E. coli*, a non-PHA-producing bacterium, has been metabolically engineered to produce PHAs. During the last few decades, various strategies, including host strain selection, change of plasmid copy number, filamentation suppression, use of PHA biosynthesis genes from different sources, and plasmid stabilization, have been employed (Park and Lee 2005). Also, the inherent metabolic pathways of *E. coli* were engineered to increase the availability of precursors for polymer synthesis.

There have been many successful cases of the development of metabolically engineered *E. coli* strains for the production of P(3HB), which is one of the best characterized PHAs. P(3HB) synthesis is initiated by condensation of two acetyl-CoA molecules into acetoacetyl-CoA, subsequently followed by reduction to 3-hydroxybutyryl-CoA using NADPH as a cofactor, and finally 3-hydroxybutyryl-CoA is incorporated into the growing chain of P(3HB) (Lee 1996). Because the P(3HB) synthesis pathway competes with inherent metabolic pathways needing acetyl-CoA, it is very important to increase the acetyl-CoA pool available for the P(3HB) synthesis reaction, resulting in increased P(3HB) yield and productivity.

Glucose is metabolized to generate acetyl-CoA and NADPH through the glycolytic and pentose phosphate pathways. The increase of the NADPH pool, required for the reduction reaction as mentioned earlier, was accomplished by overexpressing the *zwf* and *gnd* genes which encode glucose 6-phosphate and 6-phosphogluconate dehydrogenase, respectively (Lim et al. 2002). As a result, the NADPH to NADP+ ratio increased sixfold, and the P(3HB) content increased from 23 to 41%. But, the increase of P(3HB) content was actually due to the decreased cell concentration, which implies active cellular status is also critical to ultimately achieve a high concentration of P(3HB).

Integrated cellular responses of metabolically engineered *E. coli* to the accumulation of P(3HB) in the early stationary phase have been studied intensively by proteome analysis using 2DGE (Han et al. 2001). Out of 20 proteins showing the altered expression levels during the P(3HB) accumulation phase, 13 proteins were identified by MS. Among them, three heat shock proteins (GroEL, GroES, and DnaK) were remarkably upregulated in P(3HB)-accumulating cells, which indicated that the cells are under stress owing to the intracellular P(3HB) accumulation. Protein encoded by the *yfiD* gene, which was known to be expressed at low pH (Blankenhorn et al. 1999), was greatly induced with the accumulation of P(3HB). In the presence of glucose, EF-Tu, which is one of the most abundant cytosolic proteins and plays a key role in protein biosynthesis, decreased drastically, which retarded the protein biosynthesis process. Also, the levels of fructose bisphosphate aldolase (Fba), triosephosphate isomerase (TpiA), and 2-oxo-3-deoxy-6-phosphogluconate aldolase/2-keto-4-hydroxyglutarate aldolase (Eda) were increased in P(3HB)-accumulating cells. The increased expression of Fba and TpiA might be due to the fact that *E. coli* modified its metabolic fluxes to increase the glyceraldehyde 3-phosphate pool, which is subsequently used for P(3HB) synthesis. On the other hand, Eda catalyzes the final reaction of the Entner–Doudoroff (ED) pathway to supplement glyceraldehyde 3-phosphate and pyruvate, and finally to increase the acetyl-CoA pool. NADPH is simultaneously provided from the ED pathway. It could be concluded that cellular demand for the large amounts of acetyl-CoA and NADPH for P(3HB) biosynthesis caused the increased synthesis of two enzymes of the glycolytic pathway and one enzyme of the ED pathway (Fig. 3). In addition, the accumulation of P(3HB) in *E. coli* acted as a stress on the cells, which reduced the cellular metabolic activity and induced the expression of various protective proteins such as chaperones. On the basis of these results, a fermentation strategy should be developed in such a way that cells do not synthesize P(3HB) too early during the cultivation (Han et al. 2001).

P(3HB)-granule-associated proteome in recombinant *E. coli* harboring the *phaCAB* genes was also analyzed (Han et al. 2006). It was found that five proteins (EF-Tu, PhbA, IbpA, IbpB, and YbeD) out of seven spots identified were related to function of translation, heat-stress responses, and P(3HB) biosynthesis. Among them, IbpA and IbpB were already known to bind to the inclusion bodies of recombinant proteins in *E .coli* (Han et al. 2004). With use of immunoblotting and immunoelectron microscopy, it was found that IbpA and IbpB seem to have the function of phasins in *E. coli,* which affect the morphology of the granules and prevent other proteins

from binding to P(3HB) granules. In natural PHA producers, phasins are known as amphipathic proteins (12–28 kDa) which dominantly bind to the surface of PHA granules and form a layer on PHAs. Since there are no known phasins in *E. coli*, unlike in natural PHA producers, P(3HB) production in *E. coli* may cause more problems related to the direct exposure of hydrophobic P(3HB) granules to cytosolic proteins. In a previous study, during the P(3HB) accumulation, a heat-shock-like response was observed with increased levels of GroEL, GroES, and DnaK (Han et al. 2001). In this study, IbpA and IpbB were the major proteins on the surface of P(3HB) granules. Most of the P(3HB) granules accumulated in *E. coli* XL1-Blue harboring the *phaCAB* genes were smooth and round. Interestingly, the P(3HB) granules in *E. coli* XL1-Blue *ibpAB* mutant (XIB101) harboring *phaCAB* genes were significantly distorted and shrunken with wrinkles. The original shapes that were observed in the wild-type strain could be restored when the *ibpAB* genes were reintroduced into the XIB101 strain. Thus, IbpA and IpbB were found to be important in recombinant *E. coli* producing P(3HB) by stabilizing the interface between the hydrophobic P(3HB) granules and the hydrophilic cytoplasm. Furthermore, IbpA and IbpB act like phasins in the recombinant *E. coli*, affecting the morphology of the P(3HB) granules, and reducing the amount of cytosolic proteins bound to the P(3HB) granules (Han et al. 2006).

The *in silico* metabolic network of *E. coli* was constructed and was used to simulate the distribution of metabolic fluxes in the wild-type *E. coli* and recombinant *E. coli* producing P(3HB) (Hong et al. 2003). The acetyl-CoA flux into the tricarboxylic acid cycle, which competes with the P(3HB) biosynthesis pathway, decreased significantly during P(3HB) production. It was notable to find from *in silico* analysis that the ED pathway flux increased significantly under P(3HB)-accumulating conditions. To prove the role of the ED pathway in P(3HB) production, *E. coli eda* mutant strain (KEDA) was examined as a host strain for the production of P(3HB). The P(3HB) content obtained with *E. coli* KEDA harboring the *phaCAB* genes was lower than that obtained with its parent strain *E. coli* KS272 harboring the *phaCAB* genes. The reduced P(3HB) biosynthetic capacity of *E. coli* KEDA harboring the *phaCAB* genes could be restored by coexpression of the *E. coli eda* gene. Thus, the ED pathway was found to play an important role in P(3HB) synthesis in recombinant *E. coli* as predicted by metabolic flux analysis, which agreed well with the result of the aforementioned proteomic analysis of P(3HB)-producing *E. coli* (Fig. 3; Han et al. 2001; Hong et al. 2003).

Natural PHA producers have advantages in that they have inherent metabolic and regulation systems for PHA synthesis, which makes them attractive as a powerful platform for PHA production. Recently, the systematic analysis of *C. necator*, one of the most widely used PHA producers, was performed. The transcriptional analysis of the *phaA, phaB, phaC, phaP, phaR, phaZ1a, phaZ1b*, and *phaZ1c* genes related to P(3HB) homeostasis in *C. necator* H16 was carried out during three-stage cultivation [cell growth, P(3HB) biosynthesis, and P(3HB) utilization stages] by employing reverse transcriptase quantitative PCR and western blotting (Lawrence et al. 2005). It was known that these genes somehow correlate with P(3HB) formation and P(3HB) utilization in cells. However, how these genes work together in harmony and how they depend on P(3HB) granule assembly and breakdown are still

poorly understood. Therefore, it is necessary to construct a detailed general model of the dynamic granule-forming regime. A model having three distinct patterns of transcription observed during the cell growth, and P(3HB) biosynthesis and utilization phases was suggested (Lawrence et al. 2005). The first one is the transcriptional pattern of a group of five genes (*phaR*, *phaA*, *phaB*, *phaC*, and *phaZ1a*) which showed the same transcriptional trend, decreasing during the period of ammonium consumption and keeping a steady transcriptional state after the absence of ammonium (Lawrence et al. 2005). The second one is the transcriptional pattern of two coupled genes, *phaP* and *phaR*. Phasins (PhaP) are commonly known as stabilizing proteins which predominantly bind to the surface of P(3HB) granules in *C. necator*. The transcript level of the *phaP* gene was observed to increase sixfold within 1 h of the shift to P(3HB) production and rapidly decrease during the utilization phase. Moreover, very little transcript of the *phaP* gene was observed in the absence of P(3HB) synthase. Hence, these observations suggested that PhaP was coupled to P(3HB) biosynthesis, as previously proposed in several studies (York et al. 2001; Potter et al. 2002). On the other hand, it has been well established that PhaR is subject to autoregulation, and negatively regulates the accumulation of PhaP, which was also shown at the transcriptional level (York et al. 2002; Potter et al. 2002; Lawrence et al. 2005). If the *phaR* gene were regulated in the same manner as the *phaP* gene, transcript levels similar to those of the *phaP* gene should be observed; however, that is not the case. In addition, the transcript levels of the *phaR* gene did not depend on the P(3HB) accumulation. Thus, it could be concluded that the *phaR* gene is autoregulated, differently from the regulation of the *phaP* gene (Lawrence et al. 2005). The transcript level of the *phaZ1b* gene was classified as the third pattern. The transcription of the *phaZ1b* gene increased sharply, more than tenfold at 1–2 h after the onset of P(3HB) production, and remained at a high level during the P(3HB) production phase. It was undetectable in the P(3HB) utilization phase. However, even in the absence of PHA synthase, the *phaZ1b* gene showed a transcript level similar to that seen during the P(3HB) production phase, indicating that it was not involved in the catabolism of P(3HB), which did not match the expectation for the expression of an intracellular depolymerase (Lawrence et al. 2005).

Proteomic analysis using 2DGE and the genome sequence of *C. necator* H16 enabled the detection and identification of proteins that were differentially expressed during the different phases of P(3HB) metabolism. Samples from three different phases, including the exponential cell growth phase, the stationary growth phase for P(3HB) biosynthesis, and the P(3HB) utilization phase, were analyzed. Among several proteins changing quantitatively during the time course of cultivation, flagellin, which is a major protein of bacterial flagella, was identified. The flagella formation in *C. necator* changed significantly depending on the life cycle, nutritional supply, and, especially, P(3HB) metabolism. Cells were strongly flagellated in the exponential cell growth phase and lost flagella during the transition to the stationary phase. Then, in the stationary phase for P(3HB) biosynthesis (an excess of the extracellular carbon source; nutrient limitation), flagellation of the cells stagnated. In the P(3HB) utilization phase (addition of a nitrogen source to the cells that

were carbon-deprived, but filled with P(3HB); carbon limitation), cells significantly increased the degradation of their flagella, or stopped flagellin synthesis while normal degradation continued. Interestingly, in contrast to the wild-type cells, the phasin (PhaP)-negative mutant cells remained flagellated under the P(3HB) utilization phase, which suggested the assumption that a linkage between the loss of phasins with P(3HB) accumulation and flagellation. Moreover, *C. necator* H16 wild-type and the phasin-negative mutants were not flagellated when cultivated in complex and nutrient-rich medium, where cells were not exposed to nutritional limitation and obviously did not require much motility (Raberg et al. 2008).

3.3 Future of Systems Metabolic Engineering for PHA Production

As already described, there have been only several reports on the systems-level analysis and systems metabolic engineering of PHA producers. It is expected that an increasing number of studies will be performed to improve the PHA production system. In general, the following objectives will be pursued: utilization of inexpensive carbon sources, high PHA concentration, high PHA productivity, high PHA yield, and production of novel PHAs and tailor-made PHAs having desired properties. Examples of systems metabolic engineering for the enhanced production of amino acids recently reported can serve as guidelines for the approaches to be taken for the strain development (Park et al. 2007; Lee et al. 2007). Not only the metabolic network but also the regulatory circuits can be optimized together to achieve the desired level of performance. Furthermore, all steps for PHA production, from the upstream process (strain development) to the midstream process (fermentation and other unit operation) to the downstream process (recovery), should be optimized. Considering these processes all together, termed “systems biotechnology” (Lee et al. 2005), one can rationally engineer the strain for the production of desired PHAs with high efficiency (Fig. 5).

4 Concluding Remarks and Future Perspectives

Over the past few decades, PHA production in various microorganisms has been considerably improved by metabolic engineering. Recently, systems-level analysis of metabolic, signaling, and regulatory networks makes it possible to comprehensively understand the global physiological processes of the cells accumulating PHAs. Through systems-biological studies including omics and computational studies, new targets and strategies for the improvement of PHA production can be developed, followed by the construction of a new metabolic system for novel PHAs with desired

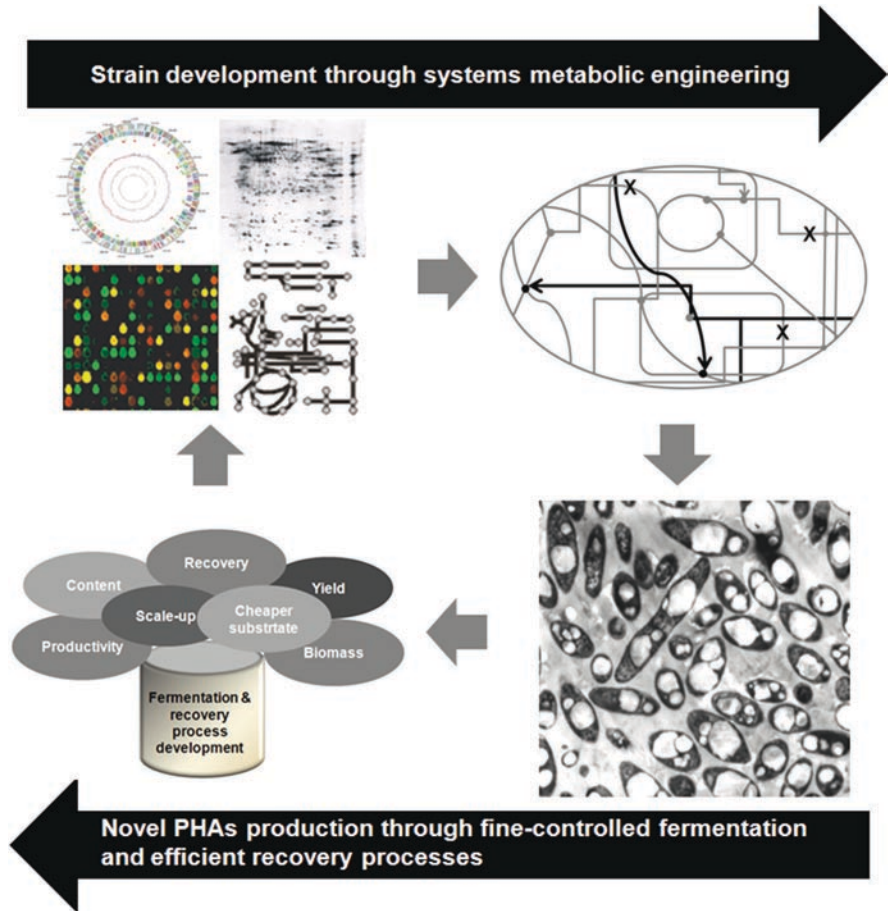

Fig. 5 Systems biotechnology for the development of an efficient PHA production system. Optimization of all steps, from the upstream process (strain development) and the midstream process (fermentation) to the downstream process (recovery), is required for the economic production of PHAs having desired characteristics

monomer compositions and molecular mass. The constructed strains should be further metabolically engineered to produce PHAs to a sufficiently high concentration with high productivity and yield from the most inexpensive carbon source through fine-controlled fermentation. Systems biotechnology is the strategy of choice for the development of a PHA production system that allows efficient and economically competitive production of polymers that can replace the petroleum-derived polymers without leaving a carbon footprint. It relies on successful systems metabolic engineering of microorganisms for the optimal performance by integrated analysis of midstream and downstream processes as well.

Acknowledgements Our work described in this paper was supported by the Korean Systems Biology Research Project (M10309020000–03B5002–00000) of the Ministry of Education, Science and Technology. Further support by the LG Chem Chair Professorship and Microsoft is appreciated.

References

Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Mol Biol Rev 54:450–472

Askenazi M, Driggers EM, Holtzman DA, Norman TC, Iverson S, Zimmer DP, Boers ME, Blomquist PR, Martinez EJ, Monreal AW, Feibelman TP, Mayorga ME, Maxon ME, Sykes K, Tobin JV, Cordero E, Salama SR, Trueheart J, Royer JC, Madden KT (2003) Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains. Nat Biotechnol 21:150–156

Blankenhorn D, Philips J, Slonczewski JL (1999) Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. J Bacteriol 181:2209–2216

Braunegg G, Bogensberger B (1985) Zur Kinetik des Wachstums und der Speicherung von Poly-D(-)-3-hydroxybuttersaure bei *Alcaligenes latus*. Acta Biotechnol 5:339–345

Byrom D (1992) Production of poly-β-hydroxybutyrate: poly-β-hydroxyvalerate copolymers. FEMS Microbiol Rev 103:247–250

Choi JI, Lee SY (1999a) Production of poly(3-hydroxybutyrate) with high P(3HB) content by recombinant *Escherichia coli* harbouring the *Alcaligenes latus* P(3HB) biosynthesis genes and the *Escherichia coli ftsZ* gene. J Microbiol Biotechnol 9:722–725

Choi JI, Lee SY (1999b) High-level production of poly(3-hydroxybutyrate-*co*-3- hydroxyvalerate) by fed-batch culture of recombinant *Escherichia coli*. Appl Environ Microbiol 65:4363–4368

Choi JI, Lee SY, Han K (1998) Cloning of the *Alcaligenes latus* polyhydroxyalkanoate biosynthesis genes and use of these genes for enhanced production of poly(3-hydroxybutyrate) in *Escherichia coli*. Appl Environ Microbiol 64:4897–4903

Choi JH, Lee SJ, Lee SJ, Lee SY (2003) Enhanced production of insulin-like growth factor I fusion protein in *Escherichia coli* by coexpression of the down-regulated genes identified by transcriptome profiling. Appl Environ Microbiol 69:4737–4742

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97:6640–6645

Doi Y, Kunioka M, Nakamura Y, Soga K (1986) Nuclear magnetic resonance studies on poly(β-hydroxybutyrate) and a copolyester of β-hydroxybutyrate and β-hydroxyvalerate isolated from *Alcaligenes eutrophus* H16. Macromolecules 19:2860–2864

Doi Y, Kawaguchi Y, Koyama N, Nakamura S, Hiramitsu M, Yoshida Y, Kimura U (1992) Synthesis and degradation of polyhydroxyalkanoates in *Alcaligenes eutrophus*. FEMS Microbiol Rev 103:103–108

Doi Y, Kitamura S, Abe H (1995) Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules 28:4822–4828

Fidler S, Dennis D (1992) Polyhydroxyalkanoate production in recombinant *Escherichia coli*. FEMS Microbiol Rev 103:231–236

Fiedler S, Steinbüchel A, Rehm BHA (2000) PhaG-mediated synthesis of poly(3-hydroxyalkanoates) consisting of medium-chain-length constituents from nonrelated carbon sources in recombinant *Pseudomonas fragi*. Appl Environ Microbiol 66:2117–2124

Fiedler S, Steinbüchel A, Rehm BHA (2002) The role of the fatty acid beta-oxidation multienzyme complex from *Pseudomonas oleovorans* in polyhydroxyalkanoate biosynthesis: molecular characterization of the *fadBA* operon from *P. oleovorans* and of the enoyl-CoA hydratase genes *phaJ* from *P. oleovorans* and *Pseudomonas putida*. Arch Microbiol 178:149–160

Fritzsche K, Lenz RW, Fuller RC (1990) Production of unsaturated polyesters by *Pseudomonas oleovorans*. Int J Biol Macromol 12:85–91

Fukui T, Doi Y (1998) Expression and characterization of (*R*)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*. J Bacteriol 180:667–673

Fukui T, Abe H, Doi Y (2002) Engineering of *Ralstonia eutropha* for production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from fructose and solid-state properties of the copolymer. Biomacromolecules 3:618–624

Genser KF, Renner G, Schwab H (1998) Molecular cloning, sequencing and expression in *Escherichia coli* of the poly(3-hydroxyalkanoate) synthesis genes from *Alcaligenes latus* DSM1124. J Biotechnol 64:123–135

Green PR, Kemper J, Lee S, Guo L, Satkowski M, Fiedler S, Steinbüchel A, Rehm BHA (2002) Formation of short chain length/medium chain length polyhydroxyalkanoate copolymers by fatty acid oxidation inhibited *Ralstonia eutropha*. Biomacromolecules 3:208–213

Gross RA, DeMello C, Lenz RW, Brandl H, Fuller RC (1989) Biosynthesis and characterization of poly(β-hydroxyalkanoates) produced by *Pseudomonas oleovorans*. Macromolecules 22:1106–1115

Haller T, Buckel T, Retey J, Gerlt JA (2000) Discovering new enzymes and metabolic pathways: conversion of succinate to propionate by *Escherichia coli*. Biochemistry 39:4622–4629

Han MJ, Lee SY (2006) The *Escherichia coli* proteome: past, present, and future prospects. Microbiol Mol Biol Rev 70:362–439

Han MJ, Yoon SS, Lee SY (2001) Proteome analysis of metabolically engineered *Escherichia coli* producing poly(3-hydroxybutyrate). J Bacteriol 183:301–308

Han MJ, Jeong KJ, Yoo JS, Lee SY (2003) Engineering *Escherichia coli* for increased production of serine-rich proteins based on proteome profiling. Appl Environ Microbiol 69: 5772–5781

Han MJ, Park SJ, Park TJ, Lee SY (2004) Roles and applications of small heat shock proteins in the production of recombinant proteins in *Escherichia coli*. Biotechnol Bioeng 88:426–436

Han MJ, Park SJ, Lee JW, Min BH, Lee SY, Kim SJ, Yoo JS (2006) Analysis of poly(3-hydroxybutyrate) granule-associated proteome in Recombinant *Escherichia coli*. J Microbiol Biotechnol 16:901–910

Hangii UJ (1990) Pilot scale production of PHB with *Alcaligenes latus*. In: Dawes EA (ed) Novel biodegradable microbial polymers. Kluwer, Dordrecht, pp 65–70

Heinzle E, Lafferty RM (1980) A kinetic model for growth and synthesis of poly-β-hydroxybutyric acid (PHB) in *Alcaligenes eutrophus* H16. Eur J Appl Microbiol Biotechnol 11:8–16

Hoffmann N, Steinbüchel A, Rehm BHA (2000) Homologous functional expression of cryptic *phaG* from *Pseudomonas oleovorans* establishes the transacylase-mediated polyhydroxyalkanoate biosynthetic pathway. Appl Microbiol Biotechnol 54:665–670

Hong SH, Park SJ, Moon SY, Park JP, Lee SY (2003) *In silico* prediction and validation of the importance of Entner-Doudoroff pathway in poly(3-hydroxybutyrate) production by metabolically engineered *Escherichia coli*. Biotechnol Bioeng 83:854–863

Hrabak O (1992) Industrial production of poly-β-hydroxybutyrate. FEMS Microbiol Rev 103:251–256

Huisman GW, Wonink E, Meima R, Kazemier B, Terpstra P, Witholt B (1991) Metabolism of poly(3-hydroxyalkanoates) by *Pseudomonas oleovorans*: identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. J Biol Chem 266:2191–2198

Ishii N, Robert M, Nakayama Y, Kanai A, Tomita M (2004) Toward large-scale modeling of the microbial cell for computer simulation. J Biotechnol 113:281–294

Kabir MM, Shimizu K (2003) Fermentation characteristics and protein expression patterns in a recombinant *Escherichia coli* mutant lacking phosphoglucose isomerase for poly(3-hydroxybutyrate) production. Appl Microbiol Biotechnol 62:244–255

Kim BS, Lee SC, Lee SY, Chang HN, Chang YK, Woo SI (1994a) Production of poly(3-hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. Biotechnol Bioeng 43:892–898

Kim BS, Lee SC, Lee SY, Chang HN, Chang YK, Woo SI (1994b) Production of poly-(3-hydroxy-butyric-*co*-hydroxyvaleric acid) by fed-batch culture of *Alcaligenes eutrophus* with substrate feeding using on-line glucose analyzer. Enzyme Microb Technol 16:556–561

Klinke S, Ren Q, Witholt B, Kessler B (1999) Production of medium-chain-length poly(3-hydroxyalkanoates) from gluconate by recombinant *Escherichia coli*. Appl Environ Microbiol 65:540–548

Langenbach S, Rehm BHA, Steinbüchel A (1997) Functional expression of the PHA synthase gene *phaC1* from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis. FEMS Microbiol Lett 150:303–309

Lawrence AG, Schoenheit J, He A, Tian J, Liu P, Stubbe J, Sinskey AJ (2005) Transcriptional analysis of *Ralstonia eutropha* genes related to poly-(*R*)-3-hydroxybutyrate homeostasis during batch fermentation. Appl Microbiol Biotechnol 68:663–672

Lee SY (1996) Bacterial polyhydroxyalkanoates. Biotechnol Bioeng 49:1–14

Lee SY, Chang HN (1995) Production of poly(3-hydroxybutyric acid) by recombinant *Escherichia coli* strains: genetic and fermentation studies. Can J Microbiol 41:207–215

Lee SY, Papoutsakis ET (1999) Metabolic engineering. Dekker, New York

Lee SY, Choi JI, Wong HH (1999) Recent advances in polyhydroxyalkanoate production by bacterial fermentation: mini-review. Int J Biol Macromol 25:31–36

Lee SH, Oh DH, Ahn WS, Lee Y, Choi J, Lee SY (2000) Production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) by high cell density cultivation of *Aeromonas hydrophila*. Biotechnol Bioeng 67:240–244

Lee JH, Lee DE, Lee BU, Kim HS (2003) Global analyses of transcriptomes and proteomes of a parent strain and an L-threonine-overproducing mutant strain. J Bacteriol 185:5442–5451

Lee SY, Lee DY, Kim TY (2005) Systems biotechnology for strain improvement. Trends Biotechnol 23:349–358

Lee KH, Park JH, Kim TY, Kim HU, Lee SY (2007) Systems metabolic engineering of *Escherichia coli* for L-threonine production. Mol Syst Biol 3:1–8

Lim SJ, Jung YM, Shin HD, Lee YH (2002) Amplification of the NADPH-related genes *zwf* and *gnd* for the oddball biosynthesis of PHB in an *E. coli* transformant harboring a cloned *phbCAB* Operon. J Biosci Bioeng 93:543–549

Liu W, Chen GQ (2007) Production and characterization of medium-chain-length polyhydroxyalkanoate with high 3-hydroxytetradecanoate monomer content by *fadB* and *fadA* knockout mutant of *Pseudomonas putida* KT2442. Appl Microbiol Biotechnol 76:1153–1159

Loo CY, Lee WH, Tsuge T, Doi Y, Sudesh K (2005) Biosynthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from palm oil products in a *Wautersia eutropha* mutant. Biotechnol Lett 27:1405–1410

Madison LL, Huisman GS (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53

McInerney MJ, Amos DA, Kealy KS, Palmer J (1992) Synthesis and function of polyhydroxyalkanoates in anaerobic syntrophic bacteria. FEMS Microbiol Rev 103:195–206

Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VAP, Fouts DE, Gill SR, Pop M, Holmes M, Brinkac L, Beanan M, DeBoy RT, Daugherty S, Kolonay J, Madupu R, Nelson W, White O, Peterson J, Khouri H, Hance I, Chris Lee P, Holtzapple E, Scanlan D, Tran K, Moazzez A, Utterback T, Rizzo M, Lee K, Kosack D, Moestl D, Wedler H, Lauber J, Stjepandic D, Hoheisel J, Straetz M, Heim S, Kiewitz C, Eisen J, Timmis KN, Düsterhöft A, Tümmler B, Fraser CM (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol 4:799–808

Nomura CT, Taguchi K, Taguchi S, Doi Y (2004) Coexpression of genetically engineered 3-ketoacyl-ACP synthase III (*fabH*) and polyhydroxyalkanoate synthase (*phaC*) genes leads to short-chain-length-medium-chain-length polyhydroxyalkanoate copolymer production from glucose in *Escherichia coli* JM109. Appl Environ Microbiol 70:999–1007

O’Leary ND, O’Connor KE, Duetz W, Dobson ADW (2001) Transcriptional regulation of styrene degradation in *Pseudomonas putida* CA-3. Microbiology 147:973–979

O'Leary ND, O'Connor KE, Ward P, Goff M, Dobson ADW (2005) Genetic characterisation of accumulation of polyhydroxyalkanoate from styrene in *Pseudomonas putida* CA-3. Appl Environ Microbiol 71:4380–4387

Ohnishi J, Mitsuhashi S, Hayashi M, Ando S, Yokoi H, Ochiai K, Ikeda M (2002) A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. Appl Microbiol Biotechnol 58:217–223

Ohnishi J, Hayashi M, Mitsuhashi S, Ikeda M (2003) Efficient 40 degrees C fermentation of L-lysine by a new *Corynebacterium glutamicum* mutant developed by genome breeding. Appl Microbiol Biotechnol 62:69–75

Ouyang SP, Luo RC, Chen SS, Liu Q, Chung A, Wu Q, Chen GQ (2007) Production of polyhydroxyalkanoates with high 3-hydroxydodecanoate monomer content by *fadB* and *fadA* knockout mutant of *Pseudomonas putida* KT2442. Biomacromolecules 8:2504–2511

Palmeros B, Wild J, Szybalski W, Le Borgne S, Hernández-Chávez G, Gosset G, Valle F, Bolivar F (2000) A family of removable cassettes designed to obtain antibiotic-resistance-free genomic modifications of *Escherichia coli* and other bacteria. Gene 247:255–264

Park SJ, Lee SY (2003) Identification and characterization of a new enoyl coenzyme a hydratase involved in biosynthesis of medium-chain-length polyhydroxyalkanoates in recombinant *Escherichia coli*. J Bacteriol 185:5391–5397

Park SJ, Lee SY (2004) New fadB homologous enzymes and their use in enhanced biosynthesis of medium-chain-length polyhydroxyalkanoates in *fadb* mutant *Escherichia coli*. Biotechnol Bioeng 86:682–686

Park SJ, Lee SY (2005) Systems biological approach for the production of various polyhydroxyalkanoates by metabolically engineered *Escherichia coli*. Macromol Symp 224:1–9

Park SJ, Park JP, Lee SY (2002) Metabolic engineering of *Escherichia coli* for the production of medium-chain-length polyhydroxyalkanoates rich in specific monomers. FEMS Microbiol Lett 214:217–222

Park SJ, Park JP, Lee SY, Doi Y (2003) Enrichment of specific monomer in medium-chain-length poly(3-hydroxyalkanoates) by amplification of *fadD* and *fadE* genes in recombinant *Escherichia coli*. Enzyme Microb Technol 33:62–70

Park SJ, Choi JI, Lee SY (2004) Engineering of *Escherichia coli* fatty acid metabolism for the production of polyhydroxyalkanoates. Enzyme Microb Technol 36:579–588

Park JH, Lee KH, Kim TY, Lee SY (2007) Metabolic engineering of *Escherichia coli* for the production of L-valine based on transcriptome analysis and *in silico* gene knockout simulation. Proc Natl Acad Sci U S A 104:7797–7802

Park JH, Lee SY, Kim TY, Kim HU (2008) Application of systems biology for bioprocess development. Trends Biotechnol 26:404–412

Peoples OP, Sinskey AJ (1989) Poly-β-hydroxybutyrate bio-synthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the P(3HB) polymerase gene (*phbC*). J Biol Chem 264:15298–15303

Pieper U, Steinbüchel A (1992) Identification, cloning and molecular characterization of the poly(3-hydroxyalkanoic acid) synthase structural gene of the Gram-positive *Rhodococcus ruber*. FEMS Microbiol Lett 96:73–80

Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, Eitinger T, Ewering C, Potter M, Schwartz E, Strittmatter A, Voss I, Gottschalk G, Steinbüchel A, Friedrich B, Bowien B (2006) Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. Nat Biotechnol 24:1257–1262

Potter M, Madkour MH, Mayer F, Steinbüchel A (2002) Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. Microbiology 148:2413–2426

Raberg M, Reinecke F, Reichelt F, Malkus U, Konig S, Potter M, Fricke WF, Pohlmann A, Voigt B, Hecker M, Friedrich B, Bowien B, Steinbüchel A (2008) *Ralstonia eutropha* H16 flagellation changes according to nutrient supply and state of poly(3-hydroxybutyrate) accumulation. Appl Environ Microbiol 74:4477–4490

Rehm BHA (2003) Polyester synthases: natural catalysts for plastics. Biochem J 376:15–33

Rehm BHA, Kruger N, Steinbüchel A (1998) A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis: the *phaG* gene from *Pseudomonas putida* KT2440 encodes a 3-hydroxyacyl-acyl carrier protein-coenzyme a transferase. J Biol Chem 273:24044–24051

Rehm BHA, Mitsky TA, Steinbüchel A (2001) Role of fatty acid de novo biosynthesis in polyhydroxyalkanoic acid (PHA) and rhamnolipid synthesis by *Pseudomonads*: establishment of the transacylase (PhaG)-mediated pathway for PHA biosynthesis in *Escherichia coli*. Appl Environ Microbiol 67:3102–3109

Ren Q, Sierro N, Witholt B, Kessler B (2000) FabG, an NADPH-dependent 3-ketoacyl reductase of *Pseudomonas aeruginosa*, provides precursors for medium-chain-length poly-3-hydroxyalkanoate biosynthesis in *Escherichia coli*. J Bacteriol 182:2978–2981

Repaske R, Mayer R (1976) Dense autotrophic culture of *Alcaligenes eutrophus*. Appl Environ Microbiol 32:592–597

Rückert C, Pühler A, Kalinowski J (2003) Genome-wide analysis of the L-methionine biosynthetic pathway in *Corynebacterium glutamicum* by targeted gene deletion and homologous complementation. J Biotechnol 104:213–228

Schubert P, Steinbüchel A, Schlegel HG (1988) Cloning of the *Alcaligenes eutrophus* poly-β-hydroxybutyrate synthetic pathway and synthesis of P(3HB) in *Escherichia coli*. J Bacteriol 170:5837–5847

Slater S, Voige WH, Dennis DE (1988) Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J Bacteriol 170:4431–4436

Slater S, Gallaher T, Dennis D (1992) Production of poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in a recombinant *Escherichia coli* strain. Appl Environ Microbiol 58:1089–1094

Snell KD, Feng F, Zhong L, Martin D, Madison LL (2002) Yfcx enables medium-chain-length poly(3-hydroxyalkanoate) formation from fatty acids in recombinant *Escherichia coli fadB* strains. J Bacteriol 184:5696–5705

Steinbüchel A, Valentin HE (1995) Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Lett 128:219–228

Stephanopoulos G (2004) Exploiting biological complexity for strain improvement through systems biology. Nat Biotechnol 22:1261–1267

Taguchi K, Aoyagi Y, Matsusaki H, Fukui T, Doi Y (1999) Over-expression of 3-ketoacyl-ACP synthase III or malonyl-CoA-ACP transacylase gene induces monomer supply for the polyhydroxybutyrate production in *Escherichia coli* HB101. Biotechnol Lett 21:579–584

Timm A, Steinbüchel A (1992) Cloning and molecular analysis of the polyhydroxyalkanoic acid gene locus of *Pseudomonas aeruginosa* PAOl. Eur J Biochem 209:15–30

Timm A, Wiese S, Steinbüchel A (1994) A general method for identification of polyhydroxyalkanoic acid synthase genes from *Pseudomonads* belonging to the rRNA homology group I. Appl Microbiol Biotechnol 40:669–675

Tsuge T, Fukui T, Matsusaki H, Taguchi S, Kobayashi G, Ishizaki A, Doi Y (2000) Molecular cloning of two (*R*)-specific enoyl-CoA hydratase genes from *Pseudomonas aeruginosa* and their use for polyhydroxyalkanoate synthesis. FEMS Microbiol Lett 184:193–198

Tsuge T, Saito Y, Kikkawa Y, Hiraishi T, Doi Y (2004) Biosynthesis and compositional regulation of poly[(3-hydroxybutyrate)-*co*-(3-hydroxyhexanoate)] in recombinant *Ralstonia eutropha* expressing mutated polyhydroxyalkanoate synthase genes. Macromol Biosci 4:238–242

Tummala SB, Junne SG, Paredes CJ, Papoutsakis ET (2003) Transcriptional analysis of product-concentration driven changes in cellular programs of recombinant *Clostridium acetobutylicum* strains. Biotechnol Bioeng 84:842–854

Ward PG, O'Connor KE (2005) Induction and quantification of phenylacyl-CoA ligase enzyme activities in *Pseudomonas putida* CA-3 grown on aromatic carboxylic acids. FEMS Microbiol Lett 251:227–232

Wiechert W (2002) Modeling and simulation: tools for metabolic engineering. J Biotechnol 94:37–63

Witholt B, Kessler B (1999) Perspectives of medium chain length poly(hydroxyalkanoates), a versatile set of bacterial bioplastics. Curr Opin Biotechnol 10:279–285

Wittmann C, Heinzle E (2001) Modeling and experimental design for metabolic flux analysis of lysine-producing *Corynebacteria* by mass spectrometry. Metab Eng 3:173–191

Wong MS, Causey TB, Mantzaris N, Bennett GN, San KY (2007) Engineering poly(3-hydroxy-butyrate-*co*-3-hydroxyvalerate) copolymer composition in *E. coli*. Biotechnol Bioeng 99:919–928

Yamane T, Fukunaga M, Lee YW (1996) Increased PHB productivity by high-cell-density fed-batch culture of *Alcaligenes latus*, a growth-associated PHB producer. Biotechnol Bioeng 50:197–202

Yim KS, Lee SY, Chang HN (1996) Synthesis of poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by recombinant *Escherichia coli*. Biotechnol Bioeng 49:495–503

Yoon SH, Han MJ, Lee SY, Jeong KJ, Yoo JS (2003) Combined transcriptome and proteome analysis of *Escherichia coli* during high cell density culture. Biotechnol Bioeng 8:753–767

York GM, Junker BH, Stubbe J, Sinskey AJ (2001) Accumulation of the PhaP phasin of *Ralstonia eutropha* is dependent on production of polyhydroxybutyrate in cells. J Bacteriol 183:4217–4226

York GM, Stubbe J, Sinskey AJ (2002) The *Ralstonia eutropha* PhaR protein couples synthesis of the PhaP phasin to the presence of polyhydroxybutyrate in cells and promotes polyhydroxybutyrate production. J Bacteriol 184:59–66

Yuan LZ, Rouviere PE, Larossa RA, Suh W (2006) Chromosomal promoter replacement of the isoprenoid pathway for enhancing carotenoid production in *E. coli*. Metab Eng 8:79–90