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Short-Chain-Length Polyhydroxyalkanoates: Synthesis in Metabolically Engineered *Escherichia coli* and Medical Applications

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Abstract Polyhydroxyalkanoates (PHAs) are homo or hetero polyesters of (*R*)-hydroxyalkanoates accumulated in various microorganisms under growth-limiting condition in the presence of excess carbon source. They have been suggested as biodegradable substitutes for chemically synthesized polymers. Recombinant *Escherichia coli* is one of the promising host strains for the economical production of PHAs, and has been extensively investigated for the process development. The heterologous PHA biosynthetic pathways have been established through the metabolic engineering and inherent metabolic pathways of *E. coli* have been redirected to supply PHA precursors. Fermentation strategies for cultivating these recombinant *E. coli* strains have also been developed for the efficient production of PHAs. Nowadays, short-chain-length (SCL) PHAs are being re-invited due to its improved mechanical properties and possible applications in the biomedical area. In this article, recent advances in the development of metabolically engineered *E. coli* strains for the enhanced production of SCL-PHAs are reviewed. Also, medical applications of SCL-PHAs are discussed.

Key words: Short-chain-length PHA, *E. coli*, metabolic engineering, medical application

Polyhydroxyalkanoates (PHAs) are polyesters of various (*R*)-hydroxyalkanoates (RHAs) that are accumulated in numerous bacteria as a carbon and/or energy storage material when the bacteria meet the unfavorable growth conditions with excess carbon sources [4, 14, 23, 30, 39, 54, 67]. More than 150 kinds of RHAs hydroxylated at the 3-, 4-, 5-, or 6-carbon position with various side groups have been found to be incorporated into PHAs [55]. Since PHAs exhibit biodegradable/biocompatible plastic and elastomeric properties, depending on the monomer constituents, their commercial applications have been extensively studied.

According to the number of carbon atoms constituting the monomer units, PHAs can be classified into two groups [30]: short-chain-length (SCL) PHA consisting of monomers having 3 to 5 carbon atoms, and medium-chain-length (MCL) PHA consisting of monomers having 6 to 14 carbon atoms. Most bacteria accumulate PHA granules of only one type, SCL or MCL. Recently, several bacteria that accumulate SCL-MCL-PHA copolymers have been isolated [6, 18, 22, 24, 29, 37].

The physical properties of PHAs are highly dependent on the type and composition of monomers. Poly(3-hydroxybutyrate) [P(3HB)] homopolymer is rather stiff and brittle, making commercialization of P(3HB) difficult. Since the random copolymer of 3HB and 3-hydroxyvalerate (3HV) has a decreased stiffness and an increased toughness compared with P(3HB), this copolymer among the group of PHAs was first commercialized (tradename Biopol™). In contrast to SCL-PHAs, MCL-PHAs have much lower crystallinity and higher elasticity, providing their applications as biodegradable rubber and elastomer. Random copolymers of 3HB and a little amount of MCL monomers are found to be flexible and tough materials comparable to low-density polyethylene (LDPE), which are suitable for commercial applications [16, 42, 43]. The material properties of PHAs consisting of different monomers are summarized in Table 1. Also, possible applications of PHA are shown in Fig. 1. Nowadays, development of novel SCL-PHAs with improved material properties and application studies in the biomedical area make SCL-PHAs to be watched again. P(3HB), having

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Table 1. Material properties of PHAs with different monomers.

	Tg[a] (°C)	Tm[b] (°C)	Tensile strength (MPa)	Crystallinity (%)	Elongation to break (%)
P(3HB)	4	177	40	60	5
UHMW P(3HB)	4	180	237	80	112
P(3HB-*co*-10 mol%3HV)	6	162	36	69	10
P(3HB-*co*-20 mol%3HV)	-1	145	32	53	-
P(3HB-*co*-10 mol%3HHx)	-1	127	21	34	400
P(3HB-*co*-15 mol%3HHx)	0	115	23	26	760
P(3HB-*co*-17 mol%3HHx)	-2	120	20	22	850
P(3HB-*co*-6 mol%3HA)	-8	133, 146	17	-	680
Commercial plastic (film)					
PP	-30	130-161	29.3	40	400
LDPE	-30	120	15.2	-	620

[a]Tg means glass transition temperature.
[b]Tm means melting temperature.

ultra-high-molecular weight and copolymers of 3HB and MCL monomers, have been developed [5, 16, 25, 26, 27].

Since the PHA biosynthesis genes were first cloned from *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) [46, 49, 52], a variety of genes directly or indirectly involved in the biosynthesis of PHAs have been isolated from many bacteria and characterized at a molecular level [39]. The detailed information on the characteristics of the enzymes constituting PHA biosynthetic pathways has allowed us to construct metabolically engineered microorganisms for efficient PHA production. Among these, recombinant *Escherichia coli* has been extensively examined for the enhanced PHA production and was found to possess several advantages over other wild-type PHA producers, including a wide range of utilizable carbon sources, PHA accumulation to a high content [up to 90% of cell dry weight in the case of production of P(3HB)], fragility of cells enabling easy recovery of PHA, and no degradation of PHA due to the lack of PHA intracellular depolymerase [31].

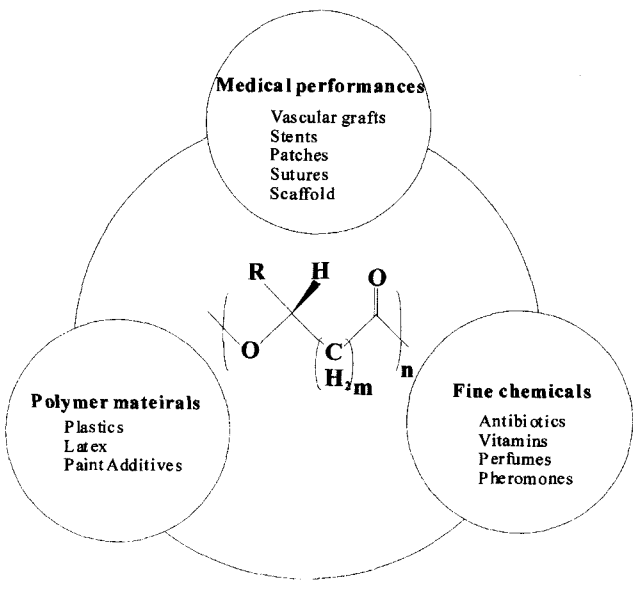

Fig. 1. Possible applications of PHA.

This article will review recent advances in the pathway and metabolic engineering of *E. coli* for the synthesis of SCL-PHAs. In addition, medical applications of SCL-PHAs will be discussed.

General Aspects of Metabolic Pathways for Short-Chain-Length PHA

The metabolic routes for SCL-PHA developed in recombinant *E. coli* are shown in Fig. 2. It has been shown that various metabolic pathways could be redirected to the PHA biosynthesis pathway in *E. coli* by metabolic engineering. A variety of metabolic intermediates of *E. coli* could be converted to SCL-(*R*)-hydroxyacyl-CoAs (HA-CoAs), the substrates for the PHA synthase, through the inherent or heterologous metabolic pathways from various structurally related or unrelated carbon sources, including glucose, SCL-fatty acids, SCL-fatty acid derivatives, and amino acids.

The 3-hydroxybutyryl-CoA (3HB-CoA) biosynthesis pathway uses acetyl-CoAs as starting metabolites. In this pathway, two acetyl-CoA molecules are condensed to form acetoacetyl-CoA by β-ketothiolase (PhaA). Acetoacetyl-CoA is then reduced to 3HB-CoA by acetoacetyl-CoA reductase (PhaB) [46, 48, 52]. The 3HV monomer is supplied through two different pathways: One is the direct conversion of valeric acid to 3-hydroxyvaleryl-CoA (3HV-CoA) by sequential reactions mediated by acyl-CoA dehydrogenase and enoyl-CoA hydratase in fatty acid β-oxidation pathway [44], and the other follows the same way as that of 3HB-CoA, except that propionyl-CoA and acetyl-CoA are used as substrates in the first condensation step by β-ketothiolase [51, 66]. Other SCL-HA-CoAs, such as 4-hydroxybutyryl-CoA (4HB-CoA) and 3-hydroxypropionyl-

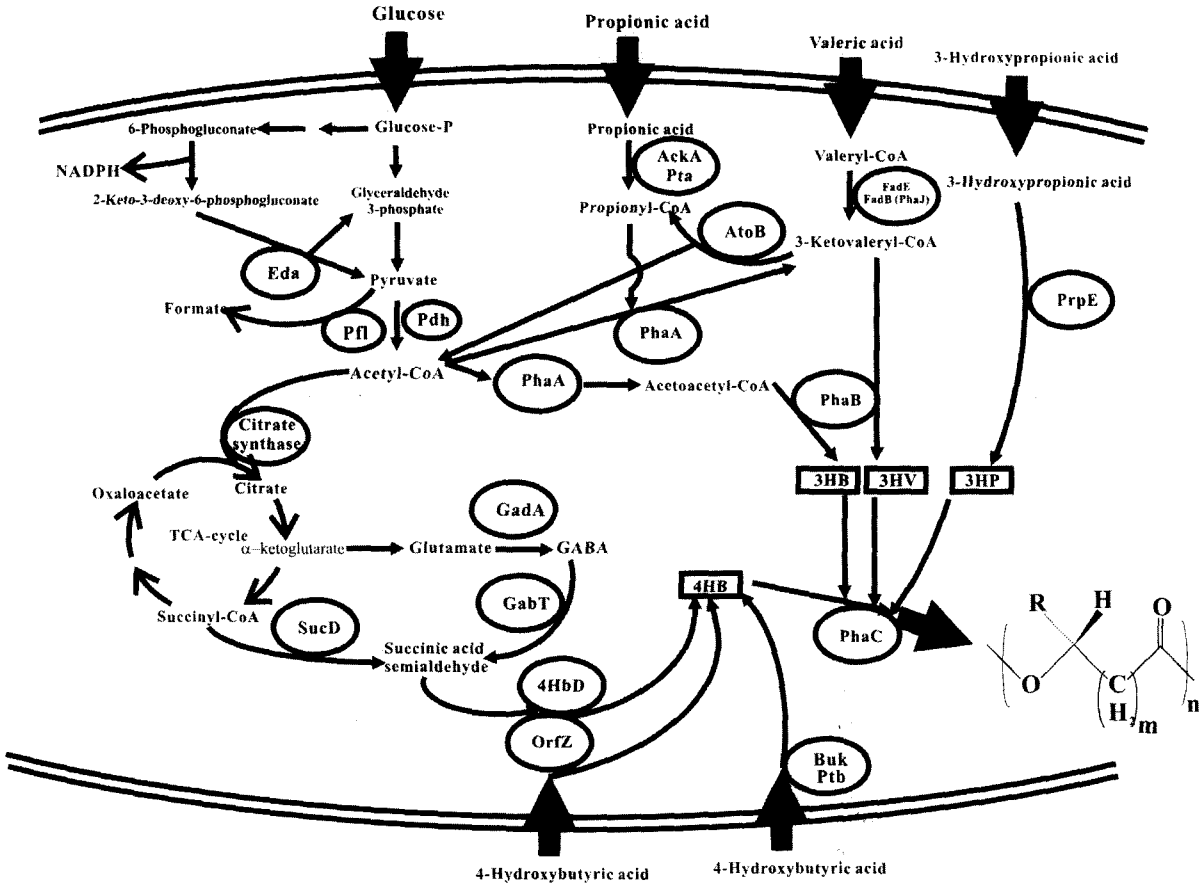

Fig. 2. Metabolic pathways engineered in *Escherichia coli* for the biosynthesis of short-chain-length PHAs.
Abbreviations: AckA, acetate kinase; AtoB, thiolase II; Buk, butyrate kinase from *Clostridium acetobutylicum*; Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; FadB, enoyl-CoA hydratase; FadE, acyl-CoA dehydrogenase; GabT, glutamate:succinic semialdehyde transaminase from *E. coli*; GadA, glutamate decarboxylase from *Escherichia coli*; 4HbD, hydroxybutyrate dehydrogenase from *Ralstonia eutropha*; SucD, succinic semialdehyde dehydrogenase; OrfZ, 4-hydroxybutyrate-CoA: CoA transferase from *Clostridium kluyveri*; Pdh, pyruvate dehydrogenase; Pfl, pyruvate formate lyase; PhaA, β-ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaJ, (*R*)-specific enoyl-CoA hydratase; PrpE, propionyl-CoA synthetase from *Salmonella enterica* serovar typhimurium; Pta, phosphotransacetylase; Ptb, phosphotransbutyrylase from *C. acetobutylicum*; 3HB, 3-hydroxybutyryl-CoA; 3HV, 3-hydroxyvaleryl-CoA; 3HP, 3-hydroxypropionyl-CoA; 4HB, 4-hydroxybutyryl-CoA.

CoA (3HP-CoA), can be generated by direct activation of hydroxyalkanoic acids through the incorporation of CoA by CoA transferase and CoA synthetase [38, 57]. All of the SCL-HA-CoAs are finally linked to the growing chain of SCL-PHAs by PHA synthase.

Biosynthesis of P(3HB)

P(3HB) is the most ubiquitous and well-known polyester accumulated in most of the PHA-producing bacteria. Since the discovery that P(3HB) could be accumulated in *E. coli* when the PHA biosynthesis genes of *R. eutropha* were introduced [48], recombinant *E. coli* has been extensively investigated for the high-level production of P(3HB). Because PHA is accumulated in the cytoplasm of bacteria, the final PHA productivity is highly dependent on the final cell density, PHA content (ratio of PHA concentration to cell concentration), and PHA yield on the carbon source [8, 9, 11]. From these points of view, recombinant *E. coli* strains have been proven to be suitable for the efficient production of P(3HB). Because there are several reviews dealing with the production of P(3HB) by high cell density cultivation of recombinant *E. coli* [32, 36], this section will focus on the metabolic pathways arranged to support the production of P(3HB) in recombinant *E. coli*.

P(3HB) biosynthesis by recombinant *E. coli* is known to be highly dependent upon the amount of available acetyl-CoA and NADPH [28, 60]. During the accumulation of P(3HB), recombinant *E. coli* was found to rearrange metabolic pathways to supply more acetyl-CoA and NADPH. Proteome analyses of recombinant *E. coli* producing high amount of P(3HB) revealed that the protein expression level of enzymes in the glycolytic pathway, including fructose-bisphosphate aldolase (FbaA) and triosephosphate isomerase (TpiA), was increased during P(3HB) accumulation [17]. Even though metabolic flux is not proportional to protein expression level, the reason for the increased expression of

FbaA and TpiA can be thought to compensate the pool of glyceraldehyde-3-phosphate, which is finally used up for P(3HB) synthesis. These hypotheses are supported by the recent report that enzyme activities of metabolic pathways in *E. coli* are highly proportional to the expression levels of each protein, as resolved by 2-D electrophoresis [45].

Oxygen limitation has been applied for enhanced P(3HB) biosynthesis during the cultivation of recombinant *E. coli* in fed-batch culture [32, 36]. It was proposed by metabolic flux analysis of recombinant *E. coli* producing P(3HB) that more acetyl-CoA was generated via pyruvate, which were generated by the additional flux of pyruvate formate lyase reaction without any decrease in the flux of pyruvate dehydrogenase reaction in oxygen limited condition (Fig. 2) [60, 64]. Because the P(3HB) biosynthesis pathway competes for acetyl-CoA with other inherent metabolic pathways such as TCA cycle, fatty acid biosynthesis, amino acid synthesis, and organic acid production, decreasing availability of acetyl-CoA for these pathways is beneficial for enhanced P(3HB) biosynthesis. For example, the activity of citrate synthase in P(3HB)-producing *E. coli*, which competes with β-ketothiolase (PhaA) for acetyl-CoA in the TCA cycle, was found to be reduced to redirect more acetyl-CoA to the P(3HB) biosynthesis pathway [28]. However, it was recently revealed that the changes of the activities of pyruvate formate lyase and citrate synthase are the responses to the oxygen limitation in *E. coli*, when the glucose is used as a carbon source [45]. Therefore, the modified metabolic pathways of recombinant *E. coli* producing P(3HB) should be carefully analyzed in order not to confuse what is the exact reason for the metabolic perturbation. Also, addition of a small amount of amino acid or oleic acid has been found to enhance P(3HB) accumulation during the culture of recombinant *E. coli* in defined medium [35]. Because amino acids and fatty acids are synthesized from intermediates of glycolytic pathway and acetyl-CoAs, addition of amino acids and oleic acid makes more acetyl-CoAs available for P(3HB) biosynthesis. Furthermore, a large amount of P(3HB) could be synthesized in mutant *E. coli* strains which are unable to produce acetic acid and/or lactic acid, compared with that in normal *E. coli* strain (Park SJ and Lee SY, unpublished results).

Besides acetyl-CoA, the availability of NADPH also affects the P(3HB) biosynthesis, because the second enzyme acetoacetyl-CoA reductase (PhaB) requires NADPH as a cofactor to reduce the acetoacetyl-CoA to 3HB-CoA. It was found that the NADPH level in recombinant *E. coli*, producing a large amount of P(3HB), was higher than that in *E. coli* producing less P(3HB) [28]. This phenomenon can be elucidated by the proteome analysis of P(3HB)-producing recombinant *E. coli* cells [17]. The expression level of 2-keto-3-deoxy-6-phosphogluconate aldolase (Eda), which catalyzes the final step of the Entner-Doudoroff (ED) pathway, was found to be elevated in P(3HB)-producing *E. coli*. This means that the increased demand for NADPH and acetyl-CoA during P(3HB) production makes the ED pathway in operation in order to adjust the cellular metabolic network to be more suitable for P(3HB) biosynthesis.

The importance of Eda in P(3HB) biosynthesis was also confirmed by the metabolic flux analysis (MFA) and experiment using *eda* mutant *E. coli* [20]. MFA to compare the metabolic flux in wild-type *E. coli* and recombinant *E. coli* producing P(3HB) suggested significant increase of ED pathway flux under P(3HB) accumulating condition. These MFA results were supported by comparison of P(3HB) biosynthesis in normal and *eda* mutant *E. coli* strains. When the chromosomal *eda* gene was deleted in recombinant *E. coli*, the P(3HB) concentration and content were decreased, compared with those of its parent *E. coli*. However, as previously reported by our group, metabolic capacities of different recombinant *E. coli* strains might be different for the production of P(3HB) [34]: Even though the same PHA biosynthesis genes and culture conditions were applied, P(3HB) was accumulated with different efficiency, depending on the employed *E. coli* strains. Based on these results, we suggest that, when the recombinant *E. coli* strains are used for the production of PHA, different metabolic engineering strategies should be designed and employed by considering metabolic activities of *E. coli* strains for the enhanced PHA biosynthesis. Strategies suitable for some *E. coli* strains might not work in other strains.

The metabolic flux to the biosynthesis of P(3HB) turned out to be highly sensitive to the ratio of both acetyl-CoA/CoA and NADPH/NADP [60]. Also, the activity of acetoacetyl-CoA reductase was found to exert a large influence on the enhancement of P(3HB) productivity, in agreement with the finding that P(3HB) biosynthesis is more sensitive to the NADPH/NADP ratio than to the acetyl-CoA/CoA ratio [60].

Biosynthesis of Ultra-High-Molecular-Weight P(3HB)

Material properties of polymers are significantly affected by their molecular weight. From the *in vivo* and *in vitro* polymerization studies, the major factors regulating the molecular weight of P(3HB) have been suggested to be the activity of PHA synthase and a hypothetical chain transfer agent. As the activity of PHA synthase increased, the molecular weight of synthesized P(3HB) decreased [50]. Also, the chain transfer reaction was found to occur during the course of P(3HB) synthesis, resulting in a decrease of molecular weight of P(3HB) with time [25]. However, under a specific condition where no chain transfer is generated, extremely high molecular weight of P(3HB) could be produced [25]. Recombinant *E. coli* harboring *R. eutropha* PHA biosynthesis genes can produce P(3HB), having very high molecular weights (M_w) of 3-20×10^6 Da, which exceeds the M_w of P(3HB) typically produced by *R. eutropha* in an

order of magnitude, when the culture pH is maintained in the range of 6.0–6.5 [25–27]. Recently, stretched film of ultra-high-molecular-weight (UHMW) P(3HB) has been prepared and shown to have improved material properties compared with those of unstretched films [5, 21]. Remarkable increase of tensile strength and elongation to break up to 237 MPa and 112%, respectively, was achieved by uniaxially cold-drawn UHMW P(3HB) films [21]. Also, annealing treatment of stretched films was found to further improve the material properties of UHMW P(3HB) films [5, 21]. These results suggest that UHMW P(3HB) produced by metabolically engineered *E. coli* can be a possible candidate for commercial applications.

Biosynthesis of Poly(3-Hydroxybutyrate-*co*-3-Hydroxyvalerate)

The process for the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] was first developed using the natural PHA-producing bacterium, *R. eutropha*. In this process, propionic acid was added to supply the precursors for 3HV monomer (Fig. 2). The same strategy was applied to produce P(3HB-*co*-3HV) by recombinant *E. coli*. Because the assimilation of propionic acid in normal *E. coli* strain is rather inefficient, the *fadR atoC* mutant *E. coli* LS5218, which can constitutively express the enzymes involved in fatty acid metabolism, has often been employed to improve the uptake and utilization of propionic acid [51]. Also, adaptation of cells by first growing in acetic acid has been shown to facilitate propionate metabolism [51]. By this strategy, recombinant *E. coli* LS5218 harboring the *R. eutropha* PHA biosynthesis genes could accumulate P(3HB-*co*-3HV), in which the 3HV monomer fraction was proportional to the concentration of propionic acid in the medium. Alternatively, by providing the environment that has similar effects on *fadR* mutation, normal *E. coli* strains could produce the copolyester of 3HB and 3HV from glucose and propionic acid [66]. This was achieved by the addition of oleic acid and acetic acid to the medium to induce the enzymes involved in the generation of 3HV monomers, especially the enzymes in the metabolism of short, medium, and long chain fatty acids. However, the exact reason of why the *fadR atoC* mutation allows efficient generation of 3HV monomers from propionic acid could not be explained, because metabolic perturbation by this mutation is generated without any specific variation in the activities of enzymes directly involved in the fatty acid metabolism. For example, the increased acetic acid uptake was not due to the increased activities of acetate kinase (AckA) and phosphotransacetylase (Pta) in the *fadR* mutant *E. coli* [40]. In the absence of either of these enzymes, however, the 3HV incorporation was reduced significantly. The overexpression of AckA in the *ackA* mutant strain restored the 3HV monomer composition to that obtained in *ackA*$^+$ *E. coli* strain, but not in the *pta* mutant strain [47]. These results led to the conclusion that *E. coli* generates propionyl-CoA by AckA and Pta [47].

Based on these biosynthetic studies on 3HV monomer, a fermentation strategy for the production of P(3HB-*co*-3HV) was developed using recombinant *E. coli* XL1-Blue harboring the *Alcaligenes latus* PHA biosynthesis genes [10]. When an optimized feeding strategy along with acetic acid induction and oleic acid supplementation were used, P(3HB-*co*-3HV) having various 3HV monomer compositions could be produced to a high concentration of 158.8 g/l with a high productivity of 2.88 g/l-h [10].

The material properties of PHA copolymers are known to be highly dependent on the monomer composition. Monomer fraction of 3HV in copolymer PHA has been mainly modulated by varying concentration of substrates for the 3HV such as propionic acid and valeric acid. Generally, an increase of the concentration of propionic acid or valeric acid results in the increase of the monomer fraction of 3HV in the copolymer. But, the concentration of these SCL-fatty acids should be maintained at an optimal level, which can support desirable monomer composition and good cell growth, because they exert toxic effect on the cell growth, when present at excess concentration.

Different aspect on the modulation of monomer fraction of 3HV was suggested by using recombinant *Salmonella enterica* Serovar Typhimurium [3]. The first step of generating 3HV from propionic acid is the activation of propionic acid to propionyl-CoA. When the expression level of *prpE* gene encoding propionyl-CoA synthetase which mediates this reaction was increased, the monomer fraction of 3HV in P(3HB-*co*-3HV) was found to be concomitantly increased [3]. This strategy can be employed, when the substrate concentration for the production of PHA copolymer containing 3HV monomer can not be adjustable.

Biosynthesis of Other SCL-PHAs

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] shows some improved flexibility as biodegradable thermoplastics, compared with P(3HB) [15]. P(3HB-*co*-4HB) has been produced in *R. eutropha* from 4-hydroxybutyrate, 1,4-butanediol, or γ-butyrolactone. Similar to the strategy for the construction of recombinant *E. coli* strain producing P(3HB-*co*-3HV), P(3HB-*co*-4HB)-producing recombinant *E. coli* was developed by following the *R. eutropha* metabolic pathway to generate 4HB. 4HB is produced from succinic semialdehyde and succinate in *R. eutropha*, which are mediated by 4-hydroxybutyrate dehydrogenase (4HbD) and succinic semialdehyde dehydrogenase (SucD), respectively [59]. 4HB monomers could be produced from succinyl-CoA in recombinant *E. coli* by three enzymes, including succinic semialdehyde dehydrogenase, 4-hydroxybutyrate dehydrogenase, and 4-hydroxybutyryl-CoA: CoA transferase from *Clostridium kluyveri* (Fig. 2) [59]. By employing this pathway, 4HB could be assimilated from glucose to P(3HB-

co-4HB) by up to 2.8 mol%. Furthermore, the co-expression of 4-hydroxybutyryl-CoA:CoA transferase gene from *C. kluyveri* and the *R. eutropha* PHA synthase gene in *E. coli* allowed production of P(4HB) homopolymer from glucose and 4-hydroxybutyric acid [19]. In the absence of glucose, P(3HB-*co*-4HB) containing 72 mol% of 3HB was accumulated. These results suggest that *E. coli* possesses hither-to-unknown pathways to convert 4HB to 3HB, which is suppressed in the presence of glucose [19].

Another pathway for supplying 4HB was constructed in *E. coli* by the simultaneous expression of butyrate kinase (*buk*) and phosphotransbutyrylase (*ptb*) genes of *Clostridium acetobutylicum* [38]. Supplementation in the growth medium of 3-hydroxybutyric acid, 4-hydroxybutyric acid, and 4-hydroxyvaleric acid resulted in the formation of homopolymers of 3HB, 4HB, and 4HV, respectively.

To construct a metabolic pathway in *E. coli* to generate 4HB from glutamate, glutamate decarboxylases from *Arabidopsis thaliana* or *E. coli*, glutamate:succinic semialdehyde transaminase from *E. coli*, 4-hydroxybutyrate dehydrogenase from *R. eutropha*, and 4-hydroxybutyrate-CoA: CoA transferase from *C. kluyveri* have been employed (Fig. 2) [60]. When the *R. eutropha* PHA biosynthesis genes were co-expressed with these engineered pathways, the copolymer of 3HB and 4HB was produced. However, the extent of 4HB assimilation into the copolymer was low (up to 3.5 mol%) [58].

Recently, a metabolically engineered *E. coli* strain was developed to produce the copolymer of 3-hydroxypropionate (3HP) and 3HB, which was found to have reduced crystallinity, compared with P(3HB) homopolymer [57]. The propionyl-CoA synthetase gene (*prpE*) from *Salmonella enterica* Serovar Typhimurium was overexpressed in recombinant *E. coli* to activate 3-hydroxypropionic acid to 3-HP-CoA, the substrate of *R. eutropha* PHA synthase. When 3-hydroxypropionic acid was present in the medium, recombinant *E. coli*, expressing the *prpE* gene and PHA synthase gene, could produce the copolymer of 3HP and 3HB [57].

Medical Applications of SCL-PHAs

Biocompatibility is essential for the materials to be used in humans by implantation and drug carriers. Since the traditional materials such as silicone and synthetic polymer are considered to cause lethal effects on the human tissue, new materials having a good biocompatibility have been searched. From these points of view, biocompatible and biodegradable PHA has the potential for medical applications. Medical applications of PHA can be found in vascular grafts, stents, patches, sutures, and scaffolds in tissue engineering and drug carriers [41].

Since (*R*)-3-hydroxybutyric acid is the naturally occurring metabolite in human blood at concentrations between 0.3 and 1.3 mM [1], P(3HB) was first employed for medical applications. Applications of P(3HB) as drug carrier can be found in several reports, in which microspheres of P(3HB) were employed for sustained drug release [2, 7]. The higher the molecular weight of P(3HB) used in the microsphere, the higher the rate of drug release was achieved, which can be attributable to uneven drug distribution at higher polymer molecular weight [7]. However, P(3HB) is degraded at a slower rate in the body than commercial polymers used in medical applications such as polyglycolide (PGA), because of its high crystallinity. Therefore, P(3HB-*co*-3HV) copolymer with less crystallinity has been studied for medical applications [49]. P(3HB-*co*-3HV) has been used as drug carrier in microspheres and implants: Tetracycline (Tc) was encapsulated in a microsphere of P(3HB-*co*-3HV) in acidic and neutral forms, both of which resulted in the rapid release of Tc [49]. The efficiency of encapsulation was higher when the neutral form was used compared to acidic form (increased from 18% to 65%) [49]. Also, implantation of antibiotic-loaded rods of P(3HB-*co*-3HV) for treatment of chronic osteomyelitis was examined in rabbit tibia artificially infected by *Staphylococcus aureus*, and complete curing was observed in 30 days [65]. The applications of P(3HB-*co*-3HV) as sutures were also examined in test animal, Wistar rats [61]: Compared with the silk and catgut sutures, P(3HB-*co*-3HV) suture was found to have no adverse effect on physiological, biochemical, and functional parameters of the animals during the post-surgery period.

Recently, the applications of PHA in tissue engineering have been suggested [41, 63, 70]. Five key properties should be satisfied to be used in tissue engineering: biocompatibility, supporting cell growth, guiding and organizing the cells, supporting ingrowth of tissue, and production of nontoxic compounds after degradation in the body [63, 70]. Also, the surface structure of the scaffold is an important factor for successful tissue engineering. To improve the surface wettability of PHA, gas plasma has been typically employed to introduce new functional groups, resulting in covalent modification of the surface of PHA [63, 68]. Usually, a salt-leaching method is employed to fabricate three-dimensional scaffolds. Because P(3HB) has poor mechanical properties, blend P(3HB) with more flexible PHA copolymer, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)], has been investigated for improved proliferation of cells [13, 69]. Cells grew better in scaffold consisting of blend polymer of P(3HB)/P(3HB-*co*-3HHx) in the ratio of 2:1 and 1:2, compared with P(3HB)/P(3HB-*co*-3HHx) of 1:1. This was due to the surface morphology of the PHA scaffold. The P(3HB) scaffold has a coralloid surface with large pores, whereas the blend of P(3HB) and P(3HB-*co*-3HHx) has a continuous pore-free surface [13]. Treatment of the P(3HB) scaffold with lipase converted the coralloid surface to a regular one, resulting in enhanced cell growth by 40-folds [69].

Recently, Tepha Inc. (Cambridge, MA, U.S.A.) developed the fermentation process to produce P(4HB) for medical

applications by employing recombinant *E. coli* as the host strain [41]. They have examined P(4HB) as medical applications in scaffold for preparing autologous cardiovascular tissue for congenital cardiovascular defects, heart valve, vascular graft, sutures, and medical textile products, all of which turned out to be superior to traditional polymer such as PGA [41].

To use bacterial PHA in medical applications, impurities of PHA should be removed to meet regulatory approval in medical devices. These include residual protein, surfactant, and endotoxin, which induce immune response and fever, when introduced into human bodies [41, 70]. Specifically, the content of endotoxin in medical applications is carefully regulated by the US Food and Drug Administration (FDA) not to exceed 20 US Pharmacopeia (USP) endotoxin units per medical device [41, 70]. From this point of view, recombinant *E. coli* is the promising host strain for PHA production, because the endotoxin level of PHA recovered from recombinant *E. coli* was low enough to be suitable for medical applications [33].

The intensive studies during the past decade on the metabolic pathways involved in PHA biosynthesis allowed construction of metabolically engineered *E. coli* strains which produce various SCL-PHAs. For efficient PHA production, it is most important to properly channel PHA precursors to the PHA biosynthetic pathway, using inherent or newly constructed metabolic pathways. This has been accomplished by metabolic engineering through the integration of our knowledge on the physiology, biochemistry, and molecular genetics of PHA biosynthesis. The results on the production of SCL-PHAs obtained by the fed-batch fermentation of recombinant *E. coli* are summarized in Table 2. So far, only P(3HB) and P(3HB-*co*-3HV) have been produced by recombinant *E. coli* with high productivity. However, it is expected that other SCL-PHA copolymers, the candidates for a wide range of commercial applications, will also be produced with high productivity. Obviously, the first step is the development of a superior strain by further optimizing the metabolic pathways through metabolic engineering. Once a superior strain is constructed, an optimized fermentation strategy needs to be developed to accomplish the goal of efficient SCL-PHA production. These upstream to downstream optimization strategies will make recombinant *E. coli* a strong candidate for SCL-PHA production suitable for medical and non-medical applications.

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Table 2. Summary of the production of SCL-PHA by fed-batch culture of recombinant *E. coli*.

PHA	Substrate	Time (h)	Cell conc. (g/l)	PHA conc. (g/l)	PHA content (%)	Productivity (g/l/h)	Reference
P(3HB)	Glucose	49	204.3	157.1	77	3.2	61
P(3HB)	Glucose	30.6	194.1	141.6	73	4.63	12
P(3HB)	Glucose	36	153.7	101.3	65.9	2.8	61[a]
P(3HB-*co*-3HV)	Glucose+Propionic acid	55.1	203.1	158.8	78.2	2.88	10
P(4HB)	Glucose+4HB	60	12.6	4.4	36	0.07	52[b]

[a]Fermentation was carried out in a 50-l stirred tank fermentor.
[b]Fermentation was carried out in a 27-l stirred tank fermentor.

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