ACS Sustainable Chemistry & Engineering

Research Article

pubs.acs.org/journal/ascecg

Engineering *Escherichia coli* for Improved Production of Short-Chain-Length-*co*-Medium-Chain-Length Poly[(*R*)-3-hydroxyalkanoate] (SCL-*co*-MCL PHA) Copolymers from Renewable Nonfatty Acid Feedstocks

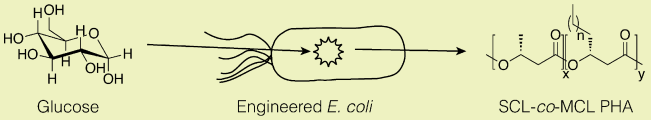
Ryan C. Tappel,[†] Wenyang Pan,[†] N. Scott Bergey,[†] Qin Wang,[†] Ivory L. Patterson,[†] Obiajulu A. Ozumba,[†] Ken'ichiro Matsumoto,[‡] Seiichi Taguchi,[‡] and Christopher T. Nomura*[,†,§]

[†]Department of Chemistry, College of Environmental Science and Forestry, State University of New York, 1 Forestry Drive, Syracuse, New York 13210, United States

[‡]Division of Biotechnology and Macromolecular Chemistry, Graduate School of Enbgineering, Hokkaido University, N13-28, Kita-ku, Sapporo 060-8638, Japan

[§]Center for Applied Microbiology, College of Environmental Science and Forestry, State University of New York, 1 Forestry Drive, Syracuse, New York 13210, United States

ABSTRACT: Polyhydroxyalkanoates (PHAs) are biorenewable and biodegradable polyesters that have garnered attention as alternatives to more common petroleum-based polymers. One of the current limitations for the widespread use of PHAs is the inability to produce PHA polymers with desired material properties. Previous studies have shown that PHA copolymers consisting primarily of one short-chain-length (SCL) repeating unit and a small concentration of medium-chain-length (MCL) repeating units have physical properties resembling the petroleum-based plastic polyethylene. In addition, these SCL-*co*-MCL PHA copolymers have been investigated for biomedical applications such as tissue engineering. However, bacterial production of these SCL-*co*-MCL PHA copolymers is often at a much lower yield compared to SCL PHA biosynthesis produced from simple sugars such as glucose. Here, we report the highest yield to date of SCL-*co*-MCL PHA copolymers produced from glucose. Two separate biosynthetic pathways for SCL and MCL PHAs were introduced into *Escherichia coli* LS5218, and copolymer production experiments were carried out in batch fermentations. The PHA copolymers produced consisted of repeating units with 4, 6, 8, 10, and 12 carbons at mol % concentrations similar to that of other SCL-*co*-MCL PHA copolymers reported to have desirable physical properties. The PHA repeating unit compositions, structures, and linkages between individual repeating unit types were analyzed by GC and NMR. The thermal properties of purified PHA copolymers were also examined. The engineered strain developed in this study (*E. coli* LS5218-STQKABGK) provides a platform to further increase PHA copolymer yields from unrelated carbon sources in a non-native PHA producing bacterial strain.



KEYWORDS: *Polyhydroxyalkanoates, Escherichia coli, Biodegradable polymer, PHA copolymer, Fatty acid biosynthesis, Biobased polymer*

INTRODUCTION

The production and utilization of petroleum-based plastic products presents multiple problems for the environment, including but not limited to contributions to greenhouse gas emissions and accumulation of plastic waste products. The United States alone generated 31 million tons of plastic waste in 2010.[1] Of this 31 million tons of plastic waste, only 8.2% was recovered for recycling, leaving the majority to accumulate in landfills or incineration. Of the petroleum-based plastics that end up in the environment, many are not biodegradable (i.e., mineralized to CO_2 and water) but instead physically degrade into small pieces that accumulate within the food chain.[2] In order to address the environmental issues associated with petroleum-based plastics, production of biobased and biodegradable alternatives are being actively explored.

Polyhydroxyalkanoates (PHAs) are bacterial polyesters that can be made from renewable plant-derived carbon feedstocks and can have physical properties similar to those of common petroleum-based plastics such as polyethylene.[3,4] The repeating unit composition of PHAs has a notable effect on the physical properties of the polymer. In particular, copolymers of PHAs containing mostly short-chain-length (SCL, C_3−C_5) and a fraction of medium-chain-length (MCL, C_6−C_{12}) repeating units have been shown to have a desirable set of physical properties, namely, they are flexible and elastic materials compared to rigid SCL homopolymers such as poly(3-hydroxybutyrate) [P(3HB)]. Therefore, PHA copolymers can be used in a wider range of applications including bulk commodity uses as well as in biomedical applications such as tissue engineering and drug delivery.[5] In fact, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] has been commercially produced under the trade name of

Received: March 26, 2014
Revised: May 5, 2014
Published: May 8, 2014

ACS Publications © 2014 American Chemical Society

Aonilex by Kaneka Corporation, and the production scale exceeded 1000 t/year in 2011. The metabolic pathways for these two classes of PHAs are typically distinct, with SCL PHAs being synthesized from acetyl-CoA and MCL PHAs synthesized either from fatty acid *de novo* biosynthesis or *β*-oxidation.[4,6] The highest yields have generally been achieved using structurally related carbon sources (oils, fatty acids, and fatty alcohols) via the fatty acid *β*-oxidation pathway.[7−12] As a typical example, the aforementioned Aonilex is produced from plant oils. However, currently, there is immense interest in utilizing lignocellulose-derived sugars as a nonedible and inexpensive feedstock to produce a wide array of chemicals including PHAs. This situation has prompted us to explore the production of SCL-*co*-MCL PHA copolymers from nonrelated carbon sources such as glucose.

It has been known that some *Pseudomonas* PHA producers produce MCL PHA from sugars. A key enzyme PhaG, which is essential to MCL PHA production from glucose, has been identified in *Pseudomonas putida*.[13] However, heterologous expression of the *phaG* gene in *Escherichia coli* failed to produce MCL PHA, indicating the presence of another factor contributing to the synthesis of MCL PHA.[6,14] We previously discovered a CoA ligase, AlkK, which is responsible for MCL PHA production together with PhaG in *P. putida*. The coexpression of PhaG, AlkK, and an engineered PHA synthase enabled *E. coli* to produce MCL PHA from glucose.[6] This finding allowed us to design a new versatile route for SCL-*co*-MCL PHA copolymer production from sugars (Figure 1). In this study, we have engineered *E. coli* to produce SCL-*co*-MCL PHA copolymers from glucose and achieved the highest levels to date of SCL-*co*-MCL PHA copolymer production from glucose. Because this pathway utilizes unrelated carbon sources for the production of substrates for SCL-*co*-MCL PHA production, it may be conveniently transferred to other organisms in future studies.

MATERIALS AND METHODS

Bacteria, Plasmids, and Media. All bacteria were grown in LB Broth (Lennox) purchased from Difco. Ampicillin (Amp, 100 μg mL^{-1}) and kanamycin (Km, 50 μg mL^{-1}) were added when necessary to maintain plasmids. Glucose from Acros Organics and isopropyl-*β*-D-1-thiogalactopyranoside (IPTG) from IBI Scientific were added to growth media when necessary for PHA production and induction of PHA biosynthetic pathway enzymes as detailed below. *E. coli* LS5218 (Coli Genetic Stock Center, Yale University) was transformed with pBBRSTQKAB[15] and pTrcGK[6] with an ECM 399 electroporator (BTX) following the manufacturer's protocols for transforming *E. coli*. pBBRSTQKAB contains *phaC1*(STQK), a broad-substrate PHA synthase, and the *phaA* and *phaB* genes, which are SCL PHA monomer supplying genes. The pTrcGK plasmid contains the *phaG* and PP0763 MCL PHA monomer supplying genes. Successful transformants (*E. coli* LS5218-STQKABGK) were grown in LB/Amp/Km overnight at 37 °C, and an aliquot of the culture was mixed with sterile 70% glycerol (v/v) and stored at −80 °C until needed.

Shake Flask Culture Conditions. *E. coli* LS5218-STQKABGK was recovered from −80 °C freezer stocks to LB/agar/Amp/Km plates and grown at 37 °C. For shake flask production experiments, individual colonies were used to inoculate 50 mL LB/Amp/Km liquid cultures, which were incubated in an orbital shaker (200 rpm) for 12−16 h at 30 °C. Aliquots of the culture (1 mL) were then used to inoculate 100 mL LB/Amp/Km cultures in 500 mL Delong style shake flasks with baffles (Pyrex), which were grown at 30 °C in an incubating orbital shaker at 200 rpm. When the optical density at 600 nm (OD_{600}) of the cultures reached ∼1.0, IPTG was added to varying concentrations, and glucose was added to a final concentration of 20 g L^{-1} either concurrently or post-IPTG induction (detailed in Results).

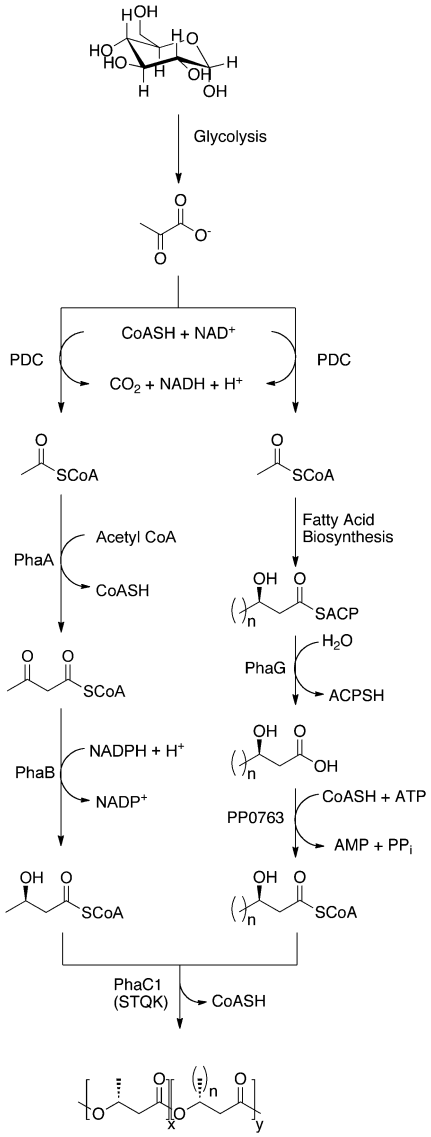


Figure 1. Proposed short-chain-length (SCL) and medium-chain-length (MCL) biosynthetic pathways introduced into *Escherichia coli* LS5218 for SCL-*co*-MCL PHA copolymer production. The substrate, glucose, is first metabolized via glycolysis and pyruvate dehydrogenase complex (PDC) to acetyl-CoA. Acetyl-CoA is made into SCL repeating units via a *β*-ketothiolase (PhaA), an acetoacetyl-CoA reductase (PhaB), and a PHA synthase [PhaC1(STQK)]. Acetyl-CoA is made into MCL repeating units after entering the fatty acid biosynthesis pathway. An (*R*)-3-hydroxyacyl-ACP intermediate of the fatty acid biosynthesis pathway is converted to an (*R*)-3-hydroxy fatty acid by PhaG. The (*R*)-3-hydroxy fatty acid has coenzyme A attached via PP0763, acting as an acyl-CoA synthetase. The MCL (*R*)-3-hydroxyacyl-CoA is polymerized by the PHA synthase, PhaC1-(STQK).

Shake flasks were incubated for a total of 25 h at 30 °C. After incubation, cells were harvested by centrifugation at 3716 × *g* for 15 min. Pelleted cells were then resuspended and washed with ultrafiltered water to remove residual media and collected again by centrifugation. Pellets were resuspended in water, frozen at −80 °C, and dried via lyophilization. Dried cells were stored in sealed vials at room temperature until analysis.

PHA Extraction. Extraction of PHAs from cells was based on a modified protocol of previously published work.[16] Dried cells were mixed with methanol (22 mL methanol per gram dried cells) at room temperature and stirred for 5 min. The cells were then collected by

Table 1. Production of SCL-*co*-MCL PHA Copolymers in *E. coli*[a]

sample (IPTG mM)	CDW[b] (g L^{-1})	PHA %	composition by GC (mol %)[c]			
			3HB	3HHx	3HO	3HD
0	6.8 ± 0.4	37.7 ± 5.0	100 ± 0	0	0	0
0.05	7.2 ± 0.3	47.0 ± 1.4	94.5 ± 0.5	0.2 ± 0.04	2.2 ± 0.2	3.1 ± 0.3
0.08	6.8 ± 0.3	43.9 ± 4.2	93.3 ± 0.2	0.2 ± 0.07	2.6 ± 0.1	3.8 ± 0.1
0.1	7.0 ± 0.4	53.2 ± 2.5	93.3 ± 0.5	0.2 ± 0.03	2.8 ± 0.2	3.8 ± 0.3
0.5	7.3 ± 0.9	50.0 ± 2.0	93.0 ± 0.2	0.2 ± 0.03	2.7 ± 0.2	4.2 ± 0.1
1	6.9 ± 0.2	45.3 ± 4.3	92.2 ± 0.5	0.2 ± 0.02	3.1 ± 0.2	4.5 ± 0.3

[a]Averages and standard deviations are the result of three independent replicates. [b]CDW, cell dry weight. [c]3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate, 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate.

centrifugation, resuspended in water to remove methanol, centrifuged again, collected, and dried via lyophilization. Polymer was extracted from the cells via Soxhlet extraction with 120 mL of chloroform. The solvent was refluxed for approximately 5 h after the first siphoning event. The extract was collected, concentrated to approximately 20 mL via rotary evaporation, and poured into an approximately 10× volume of cold methanol with stirring. After 5 min, the stirring was stopped; the solution was covered with DuraSeal laboratory stretch film (Diversified Biotech) and left undisturbed overnight to allow precipitants to settle. The supernatant was decanted through a 0.45 μm PTFE membrane under vacuum followed by the precipitated polymer. The sample was rinsed with methanol and dried under vacuum. Purified polymer was stored at room temperature.

Gas Chromatography Analysis. PHA repeating unit compositions and yield were estimated by gas chromatography (GC). Approximately 15 mg of dried cells were dissolved in 2 mL of 15:85 sulfuric acid:methanol (v/v) and 2 mL of chloroform in 12 mL tubes with Teflon liner caps (Kimax) and heated at 100 °C for 140 min. Samples were allowed to cool to room temperature, and 1 mL of ultrafiltered Type I water was added to each sample. Samples were mixed by vortexing, and immiscible layers were separated by centrifugation at 485 × *g* for 3 min. The chloroform layer was removed and passed through a 0.45 μm PTFE syringe filter (Restek). An internal standard of 500 μL of caprylic acid methyl ester (1 g L^{-1}) was added to 500 μL of the filtered sample in a GC sample vial. Samples were injected and separated in a GC 2010 gas chromatograph with a flame ionization detector by an AOC-20i autoinjector (Shimadzu) as previously described.[17] Data were analyzed using Shimadzu's GCSolution software.

Nuclear Magnetic Resonance Spectroscopy. Nuclear magnetic resonance (NMR) spectroscopy was used to confirm the structure of PHAs. All spectra were acquired at 30 °C with a Bruker DPX 300 spectrometer (300 MHz ^{1}H frequency) equipped with a 5 mm BBFO z-gradient probe. Approximately 20 mg of extracted polymer samples were dissolved in 1 mL of deuterated chloroform (Cambridge Isotope Laboratories) and passed through a small plug of glass wool into sample tubes. Data were acquired and processed in TOPSPIN v1.3 from Bruker BioSpin. For ^{1}H, the recycle delay was 5.0 s, and the acquisition time was 3.4 s with a 45° pulse width. For ^{13}C, the recycle delay was 2.0 s, and the acquisition time was 1.0 s with a 90° pulse width. Spectral widths of 4800 and 19600 Hz were used for ^{1}H and ^{13}C spectra, respectively.

Gel Permeation Chromatography. Weight-average molecular weights (M_w), number-average molecular weights (M_n), and polydispersities for polymers generated were estimated by gel permeation chromatography (GPC). Polymer samples were dissolved in chloroform to a concentration of 0.7 g L^{-1} and passed through a 0.45 μm PTFE syringe filter. Samples were injected at a volume of 50 μL into a LC-20AD liquid chromatograph equipped with a SIL-20A autosampler, CTO-20A column oven, and RID-10A refractive index detector (Shimadzu). Samples were passed through an 8 mm × 50 mm styrenedivinylbenvene (SDV) guard column (5 μm particles; Polymer Standards Service) and an 8 mm × 300 mm SDV analytical column (5 μm particles; mixed bed porosity; max molecular weight 1E6 Da; Polymer Standards Service product sda08300Slim). The column oven was maintained at 40 °C, and chloroform was used as the mobile phase at 1 mL min^{-1}. M_w, M_n, and polydispersity values were determined using LCSolution software (Shimadzu) by comparing peak retention times to polystyrene standards of known molecular weights.

Thermal Analysis. The decomposition temperatures (T_d) of extracted PHA polymers were determined by thermogravimetric analysis (TGA). TGA experiments were performed on a TGA Q5000IR (TA Instruments). Approximately 10 mg of extracted polymer sample was heated under nitrogen atmosphere at 20 °C min^{-1} to 500 °C. The T_d was taken at the initiation of sample degradation.

The melting temperatures (T_m), crystallization temperatures (T_c), and glass transition temperatures (T_g) of the PHA polymers were determined by differential scanning calorimetry (DSC). DSC experiments were performed on a DSC 8500 (PerkinElmer) calibrated with a zinc standard. Approximately 10 mg of polymer sample were heated to 200 °C at 10 °C min^{-1}, quench cooled to −50 °C at 30 °C min^{-1}, and then heated back to 200 °C at 10 °C min^{-1}. All thermal values were taken from the final heating ramp. The T_m and T_c were taken at the peak maximum or peak minimum, respectively, and the T_g was taken at the inflection point of the transition. Both DSC and TGA data were analyzed using TA Instruments Universal Analysis 2000 software.

RESULTS

Production of SCL-*co*-MCL PHA Polymers in Recombinant *E. coli*. Effective incorporation of MCL repeating units into SCL-*co*-MCL PHA copolymers in non-native producers along with high yields has proven to be a challenging endeavor.[7−10] Studies that have successfully synthesized SCL-*co*-MCL PHAs from unrelated carbon sources in non-native PHA producers have done so at reduced yields compared to using related carbon sources in native PHA producers. Recently, we demonstrated the largest yield of MCL PHAs in *E. coli* from an unrelated carbon source.[6] We hypothesized that combining this MCL PHA biosynthetic pathway with an established P(3HB) biosynthetic pathway used to produce large amounts of P(3HB) in *E. coli* would result in improved productions of SCL-*co*-MCL PHA copolymers from an unrelated carbon source (glucose) in *E. coli*. To test this hypothesis, *E. coli* LS5218 was co-transformed with the pBBRSTQKAB and pTrcGK plasmids (hereafter written as *E. coli* LS5218-STQKABGK), which served to insert both SCL and MCL (*R*)-3-hydroxyacyl-CoA production pathways into *E. coli* (Figure 1).

Initially, different concentrations of IPTG, ranging from 0 to 1 mM, were used to induce the expression of the *phaG* and *alkK* genes, and the effect on SCL-*co*-MCL PHA copolymer composition was determined. Glucose was added 3 h after the induction with IPTG or at an OD600 of ∼1 in the case where there was no IPTG added. These results shown in Table 1 indicate that IPTG concentration, and thus expression level of the MCL PHA biosynthesis pathway, could change the ratio of

Table 2. Effect of Induction Time on SCL-*co*-MCL PHA Copolymers in *E. coli*[a]

sample (hours post IPTG[b] addition)	CDW[c] (g L^{-1})	PHA %	composition by GC (mol %)[d]			
			3HB	3HHx	3HO	3HD
0	6.0 ± 0.0	38.8 ± 1.4	96.2 ± 0.7	0.2 ± 0.01	1.3 ± 0.3	1.9 ± 0.4
6	6.8 ± 0.2	43.6 ± 2.3	97.2 ± 0.3	0.1 ± 0.04	1.1 ± 0.1	1.6 ± 0.2
9	7.6 ± 0.1	47.6 ± 1.6	97.5 ± 0.5	0.1 ± 0.02	0.9 ± 0.2	1.4 ± 0.3
12	7.6 ± 0.6	45.6 ± 6.4	97.7 ± 0.1	0.1 ± 0.02	0.8 ± 0.0	1.3 ± 0.1

[a]Averages and standard deviations are the result of 3 independent replicates. [b]IPTG added to 0.05 mM. [c]CDW, cell dry weight. [d]3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate, 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate.

Table 3. Production of SCL-MCL PHA in Recombinant *E. coli*

strain	SCL-MCL PHA produced		related vs unrelated carbon source	MCL PHA content (mol %)	ref
	(g L^{-1})	(% CDW)			
Escherichia coli LS5218	3.72	53.2	unrelated (LB/glucose)	7.1	this study
Escherichia coli LS5218	NA	38	related (dodecanoate)	17	33
Escherichia coli LS5218	0.95	36	related (dodecanoate)	92	34
Escherichia coli LS5218	0.406	12.3	unrelated (LB/glucose)	0.5	35
Escherichia coli LS5218	NA	50	related (dodecanoate)	2	36
Escherichia coli LS5218	0.29	31	related (dodecanoate)	90	37
Escherichia coli LS5218	0.156	4.18	both (gluconate/dodecanoate)	47.6	38
Escherichia coli LS5218	21.5	27.2	related (dodecanoate)	10.8	39
Escherichia coli BL21	NA	8.4	related (dodecanoate)	24.8	40
Escherichia coli DH5α	0.358	7.3	both (glucose/decanoate)	16.6	41
Escherichia coli JC7623ABC1J1	1.03	34	both (glucose/decanoate)	82	42
Escherichia coli JM109	0.008	2.6	unrelated (LB/glucose)	6.4	43
Escherichia coli JM109	1.44	36.8	unrelated (LB/glucose)	0.2	15
Escherichia coli JM109	0.041	4.5	unrelated (LB/glucose)	5.9	44
Escherichia coli K-12 MG1655	0.017	1	unrelated (LB/glucose)	2.3	45
Escherichia coli LSBJ	0.83	51.8	related (LB/fatty acids)	38.0	19

the SCL to MCL repeating unit incorporated into the PHA copolymers. With increasing IPTG concentrations, increasing levels of MCL PHA repeating units incorporated into the copolymer were observed.

An additional experiment was performed to assess the effect of the timing of the induction of the SCL and MCL PHA biosynthetic genes on SCL-*co*-MCL PHA copolymer production and composition. In this experiment, the concentration of the inducer, IPTG, was held constant at 0.05 mM, and glucose was added at different times post-induction. The results are shown in Table 2. The cell dry weight increased dependent on the time of glucose addition with slight increases in overall SCL-*co*-MCL PHA copolymer production when glucose was added 9−12 h after induction

For all SCL-*co*-MCL PHA copolymer products produced in this study, the mol % concentration of (*R*)-3-hydroxybutyrate (3HB) varied between 92% and 100% with the remainder of the polymer composed of MCL repeat units with sizes dependent on both IPTG concentration and timing of glucose addition (Tables 1 and 2). PHA polymers with a mol ratio of 90−95% SCL to 5−10% MCL repeat units in a PHA copolymer have been previously shown to have highly desirable physical properties similar to low density polyethylene.[3] The amount of SCL-*co*-MCL PHAs produced in this study was greater than any previous report for a non-native PHA producer and also greater than any previous SCL-*co*-MCL PHA production from an unrelated carbon source as shown in Table 3.

Structural Evaluation of PHA Polymers by NMR. In order to more accurately verify the composition of the SCL-*co*-MCL PHA copolymers, structural analysis via ^{1}H and ^{13}C NMR was performed. Figures 2 and 3 depict the spectra from a sample that was synthesized in *E. coli* LS5218-STQKABGK grown in shake flasks with glucose added 3 h after IPTG induction, and they are representative of the spectra for other samples. The ^{13}C spectra were used to confirm the presence of repeating units found by GC (Table 2) based on previously determined peak assignments for SCL-*co*-MCL PHAs.[18] The GC estimations for the (*R*)-3-hydroxyhexanoate (3HHx) and (*R*)-3-hydroxydodecanoate (3HDD) repeating units were quite low for all samples (≤0.2 mol %), and their presence could not be confirmed in the ^{13}C NMR spectrum (Figure 2). For this reason, the remaining discussion on the composition and structure of the PHAs made in this study will focus on the 3HB, (*R*)-3-hydroxyoctanoate (3HO), and (*R*)-3-hydroxydecanoate (3HD) repeating units, which were in relatively large quantities compared to 3HHx and 3HDD based on GC and had readily identifiable unique chemical shifts in the ^{13}C NMR spectra (Figure 2B).

In addition to confirming the presence of 3HB, 3HO, and 3HD, the ^{13}C NMR spectra confirmed the linkages between SCL and MCL repeating units, indicating the random distribution of repeating units and copolymeric nature of the PHA samples. Figure 2C displays the region of the ^{13}C spectrum in which the signal for the carbonyl carbons of the PHAs is observed. Between 169.0 and 169.5 ppm, there are two peaks corresponding to the different environments a carbonyl carbon may be found in for these SCL-*co*-MCL PHA copolymers. The larger peak represents a carbonyl of a 3HB repeating unit bound to another 3HB repeating unit. The smaller peak represents a carbonyl of a 3HB repeating unit bound to a MCL PHA repeating unit (e.g., 3HO and 3HD) and

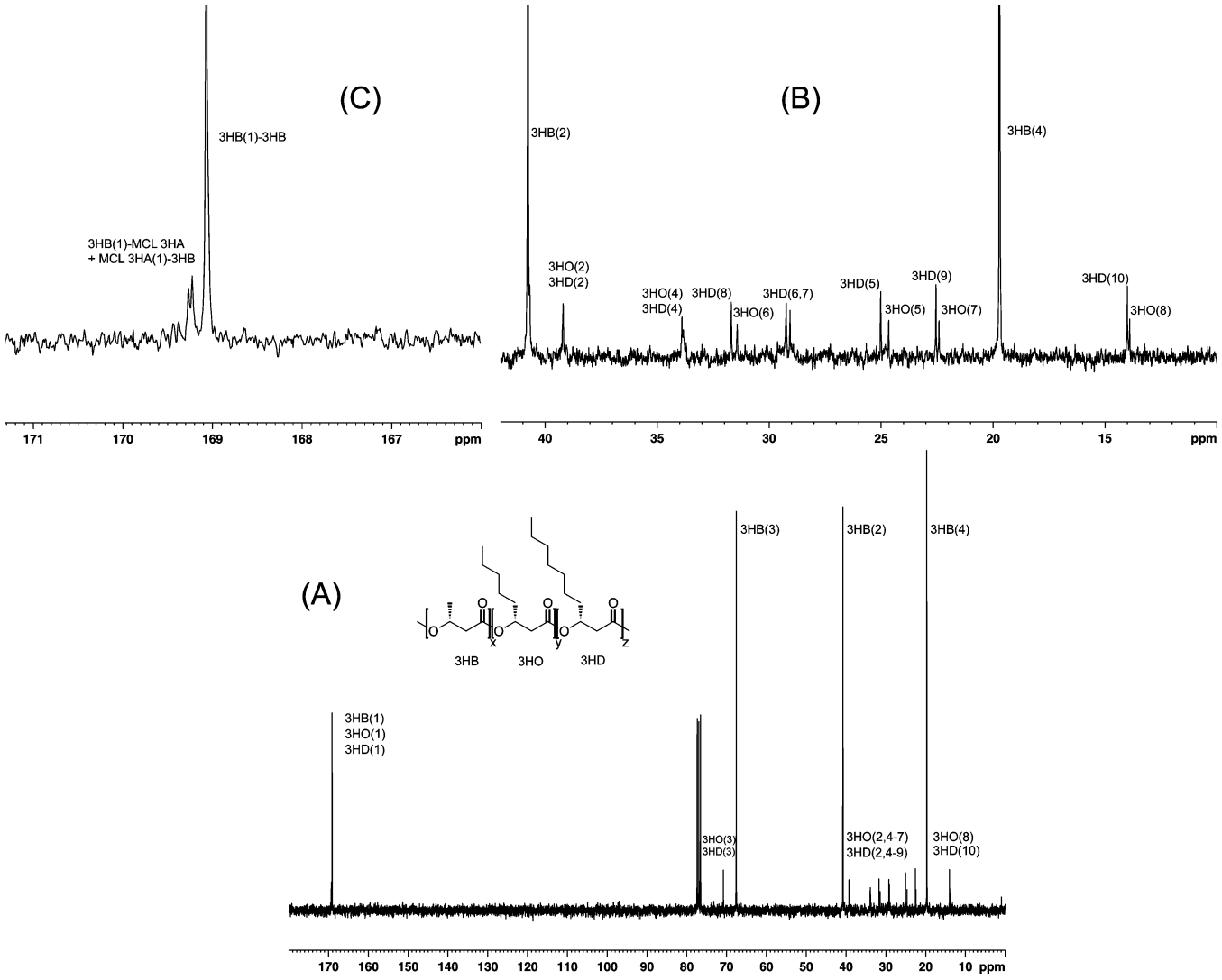


Figure 2. ^{13}C NMR spectrum of PHA synthesized in *E. coli* LS5218-STQKABGK. Bacteria were grown in a bioreactor at 30 °C for 25 h with pH held at 7. MCL PHA synthesis genes expression was induced by IPTG, and the substrate glucose was added 3 h post-induction. (A) Full ^{13}C NMR spectrum containing all detected peaks. (B) Expanded 10−42 ppm portion of full spectrum. (C) Expanded 166−171.3 ppm portion of full spectrum. Numbers in parentheses indicate what carbon atom the protons of various repeating units are connected to for the labeled peaks, with the carbonyl carbon being position 1. 3HB, (*R*)-3-hydroxybutyrate; 3HO, (*R*)-3-hydroxyoctanoate; 3HD, (*R*)-3-hydroxydecanoate.

a carbonyl of a MCL PHA repeating unit bound to 3HB. This indicates that the samples generated are SCL-*co*-MCL PHA copolymers and not simply mixtures of SCL and MCL PHAs. A peak for a MCL PHA carbonyl connected to another MCL PHA would be at a slightly higher chemical shift compared to that of the second peak in Figure 2C.[18] The concentration of MCL PHA repeating units in the polymer samples is likely too low to observe this signal.

The ^{1}H spectra of the chloroform extracts from *E. coli* LS5218-STQKABGK were also used to confirm the identity of the polymers. The chemical shifts seen in Figure 3 align with those seen previously for SCL-*co*-MCL PHAs.[19] The protons of the terminal methyl groups of the MCL PHAs separate from the methyl group of the 3HB repeating unit (0.9 and 1.25 ppm, respectively). Also shown is a signal for the methylene protons closest to the side chain of MCL repeating units at 1.6 ppm, confirming the presence of both SCL and MCL repeating units in the extracted polymers. Overall, these results confirm the production of SCL-*co*-MCL PHA copolymers by the recombinant *E. coli* system developed in this study.

Molecular Weights and Thermal Properties of PHA Polymers. GPC was employed for estimation of molecular weights and polydispersities of PHA samples generated in *E. coli* LS5218-STQKABGK. Samples from experiments used to generate PHAs from varying concentrations of IPTG were examined (Table 4). All PHA copolymers had number-average molecular weight (M_n) values between 92×10^3 and 111×10^3 and weight-average molecular weight (M_w) values between 163×10^3 and 211×10^3. The polymers generated when glucose was added after induction with IPTG generally had lower molecular weights than the P(3HB) homopolymer produced without the addition of IPTG. PHA polymers with higher SCL repeating unit content also had larger polydispersities (≥2 on average) compared to PHA polymers with higher MCL repeating unit content (1.7 to 1.8).

In order to measure the thermal properties of the PHA polymers, the samples were subjected to thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). The decomposition temperatures (T_d) of the PHA polymers

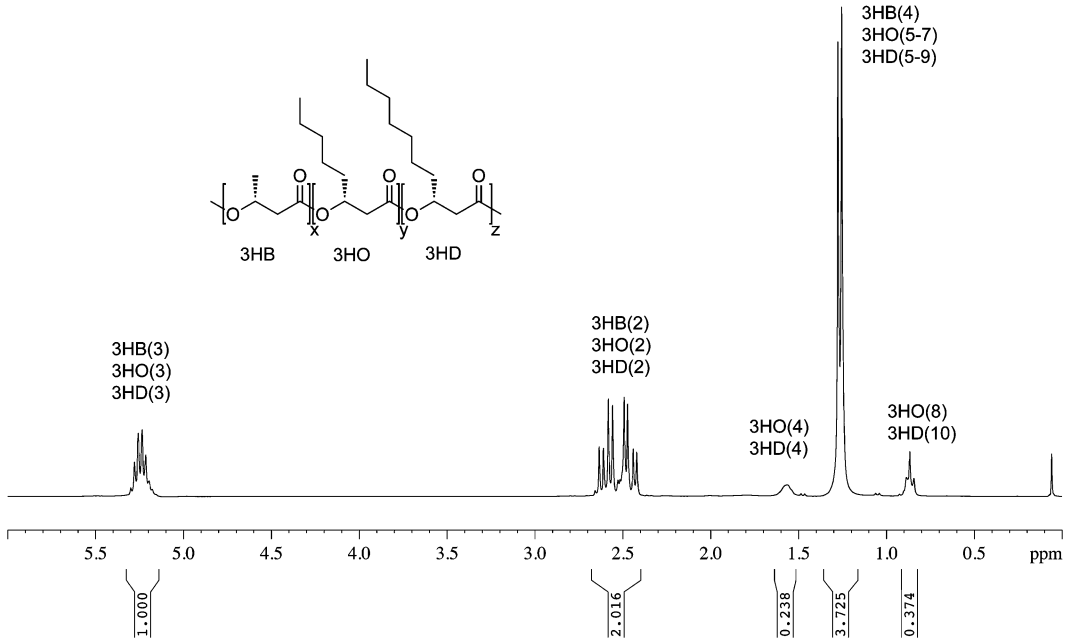


Figure 3. [1]H NMR spectrum of PHA synthesized in *E. coli* LS5218-STQKABGK. Bacteria were grown in a bioreactor at 30 °C for 25 h with pH held at 7. MCL PHA synthesis genes expression was induced by IPTG, and the substrate glucose was added 3 h post-induction. Numbers in parentheses indicate what carbon atom the protons of various repeating units are connected to for the labeled peaks, with the carbonyl carbon being position 1. The numbers below the peaks represent integration values relative to the signal of the proton at the chiral carbon of the repeating units. 3HB, (*R*)-3-hydroxybutyrate; 3HO, (*R*)-3-hydroxyoctanoate; 3HD, (*R*)-3-hydroxydecanoate.

Table 4. Molecular Weight Distributions and Thermal Properties of SCL-*co*-MCL PHA Copolymers Produced in *E. coli*[a]

repeat unit composition (mol %)		molecular weight		thermal properties				
3HB	MCL 3HAs	M_w (× 10^3)	PDI	T_m (°C)	T_c (°C)	T_g (°C)	ΔH_m (J/g)	ΔH_c
100	0	652.6 ± 9.0	2.4 ± 0.1	168.7 ± 1.0	53.7 ± 4.1	1.3 ± 0.5	74.4 ± 0.9	38.3 ± 1.5
95	5	211.2 ± 34.5	2.0 ± 0.3	163.7 ± 0.3	63.4 ± 6.4	−3.0 ± 0.1	37.1 ± 0.9	35.6 ± 3.5
93	7	163.8 ± 29.5	1.7 ± 0.2	163.8 ± 0.1	56.5 ± 0.4	−3.3 ± 0.3	36.1 ± 0.5	33.2 ± 0.6
92	8	168.3 ± 15.5	1.8 ± 0.2	163.9 ± 0.1	64.3 ± 1.8	−2.9 ± 0.2	31.7 ± 0.4	29.7 ± 1.0

[a]Averages and standard deviations are the result of three independent samples. P(93 mol % 3HB-*co*-3HA) was synthesized using 0.08 mM IPTG induction.

were determined by TGA. The T_d values were similar for all the samples (between 238 and 240 °C).

Samples from cells treated with varying IPTG concentration led to variations in the repeat unit composition, and the results are shown in Table 2. This variation in repeat unit composition resulted in changes in the thermal properties of the polymers as shown in Table 4. The average T_m and T_g for the 3HB homopolymer were 168.7 and 1.3 °C, respectively, while the average T_m and T_g for the copolymer samples ranged from 163.7 to 168.7 °C and −3.3 to 1.3 °C, respectively. The average T_c values for the copolymer samples were in general about 3 to 11 °C greater than that of the 3HB homopolymer. The enthalpy of melting was much greater for the homopolymer (74.4 J/g) than the copolymers (31.7 to 37.1 J/g), and the enthalpy of crystallization was slightly greater for the homopolymer (38.3 J/g) than the copolymers (29.7 J/g to 35.6 J/g). In general, the enthalpy from both transitions decreased with increasing fractions of MCL repeating units incorporated into the PHA copolymers.

DISCUSSION

There is growing interest in the replacement of petroleum-based products with biobased and biodegradable alternatives. This has spawned widespread efforts to produce fermentable sugars such as glucose from lignocellulosic biomass for the production of these petroleum product alternatives. Biobased polymers such as PHAs are one class of molecules that can potentially replace petroleum-based materials. Of particular importance is the ability to produce and control ratios of SCL to MCL PHA repeating units in the polymer in order to produce materials with desirable physical properties. SCL-*co*-MCL PHA copolymers consisting predominantly of the SCL 3HB repeating unit and a small mol % of MCL repeating units have been described previously as having desirable physical properties compared to the homopolymer P(3HB), namely decreased crystallinity, decreased melting temperature, and increased elongation to break.[4,20] These changes in repeating unit composition to a SCL-*co*-MCL PHA copolymer from the homopolymer P(3HB) yield samples that have physical properties similar to those of some common, non-biodegradable, and petroleum-based polymers like low-density polyethylene.[4] The goal of this work was to improve SCL-*co*-MCL PHA copolymer bacterial production from glucose in a non-native PHA producer by engineering a strain of *E. coli*. Because these engineered pathways utilize common metabolites, they can be transferred to any recombinant organism.

There are several important features of the engineered *E. coli* LS5218-STQKABGK strain developed in this study that led to high-level production of SCL-*co*-MCL PHA copolymers. The first is the engineered PhaC1(STQK) PHA synthase, which, unlike native PHA synthases, has broad substrate specificity for both SCL and MCL PHA substrates.[21,22] This engineered *E. coli* system also utilizes two separate synthetic pathways for generating SCL and MCL substrates for the PhaC1(STQK) to polymerize into the SCL-*co*-MCL PHA copolymer (Figure 1). The SCL (*R*)-3-hydroxybutyryl-CoA is generated from acetyl-CoA by a β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB). MCL (*R*)-3-hydroxyacyl-CoA substrates are generated from various sizes of the fatty acid biosynthesis intermediates, (*R*)-3-hydroxyacyl-ACPs. The ACP is cleaved by PhaG, acting as a 3-hydroxyacyl-ACP thioesterase, and coenzyme A (CoA) is added to the (*R*)-3-hydroxy fatty acid by the AlkK-like PP0763. The SCL PHA production pathway using PhaA and PhaB to produce P(3HB) is arguably the most well understood PHA biosynthesis pathway and has been used to synthesize P(3HB) homopolymer to very high yields in *E. coli*.[23,24] We recently utilized the MCL PHA production pathway shown in Figure 1 to generate the highest published yields of MCL PHAs in *E. coli* from an unrelated carbon source.[6] Combining these two PHA biosynthetic pathways in *E. coli* resulted in the highest yield of SCL-*co*-MCL PHAs in a non-native PHA producer published to date (Table 1).

E. coli LS5218 was selected as a host strain for PHA production due to its differences in fatty acid uptake and metabolism compared to wild-type *E. coli* strains.[25] Specifically, *E. coli* LS5218 constitutively expresses the *fad* regulon due to the *fadR* mutation and the *ato* regulon due to the consitutive expression of the response regulator, AtoC.[26] Growth of *E. coli* on glucose can lead to large amounts of acetate, which negatively affects growth and potentially yields of desired products.[27] The *atoC*(Con) mutation for constitutive expression of the *ato* operon and the transgenic PHA biosynthesis pathways may aid in reducing the burden of acetate production from glucose. The *ato* operon consists of the genes *atoD*, *atoA*, *atoE*, and *atoB*, which are responsible for uptake and metabolism of acetoacetate and short-chain fatty acids.[28−31] AtoE is a predicted short-chain fatty acid and acetoacetate transporter, which could aid in bringing acetate leaked into the media back into the cell. AtoB is a β-ketothiolase. Although the preferred reaction catalyzed is acetoacetyl-CoA conversion into two acetyl-CoA moieties, AtoB can perform the reverse and form acetoacetyl-CoA, which is the first step of forming P(3HB) from acetyl-CoA (Figure 1, catalyzed by PhaA). AtoD and AtoA combine to form the active acetoacetyl-CoA transferase, AtoDA. AtoDA can catalyze the transfer of CoA to short-chain fatty acids from acetyl-CoA or to acetate from acetoacetyl-CoA.

The compositions of the SCL-*co*-MCL PHA polymers produced in this study were determined by GC (Tables 1 and 2) and NMR spectroscopy (Figures 2 and 3). ^{13}C NMR was used to determine that the PHAs were, in fact, copolymers as opposed to blends of SCL PHAs and MCL PHAs. The SCL and MCL substrates were generated by separate engineered pathways (Figure 1), and both pathways have been utilized in the past to generate SCL and MCL PHAs separately.[6,21] Although the synthesis of all PHAs went through the same PHA synthase, it was suspected that the polymers might be blends of the two different PHAs as opposed to true copolymers. As detailed above and shown in Figure 2, the links of 3HB to MCL repeating units' carbonyls could be seen at slightly higher chemical shifts compared to those of the 3HB carbonyl connections. These results show that *E. coli* LS5218-STQKABGK is a true SCL-*co*-MCL PHA biosynthesis system.

That these products are copolymers is further supported by the changes in temperature profiles for the polymers observed via DSC (Table 4). The glass transition temperatures for the SCL-*co*-MCL PHA copolymers produced here are between −2.9 and −3.3 °C on average (Table 4), which is similar to the values observed for other SCL-*co*-MCL PHA copolymers of similar compositions.[3] The crystallization temperatures and melting temperatures were similar to those of other PHA copolymers with low mol % concentrations of MCL repeating units to 3HB.[19] The observed shift to lower temperatures in the T_m and T_g values for all of the copolymers with respect to the values observed for the 3HB homopolymer is consistent with the confirmation of copolymer formation because the incorporation of the MCL units within the polymer chain will disrupt crystallinity while adding lower-melting elastomeric components. Additionally, these values are similar to those commonly observed for common petroleum-based polymers polypropylene and polyethylene.[3,4,19,32]

This study has resulted in the production of an engineered *E. coli* strain, *E. coli* LS5218-STQKABGK, that has produced the highest yield of SCL-*co*-MCL PHA copolymers in a non-native PHA-producing organism and the highest yield of SCL-*co*-MCL PHA copolymers form an unrelated carbon source (LB and glucose) to date. The use of an engineered *E. coli* strain capable of producing SCL-*co*-MCL PHA polymers from glucose offers some significant advantages over the use of fatty acids as a substrate given the interest in using lignocellulose-derived sugars as feedstocks for producing value-added bioproducts. This study has generated a strain capable of using these inexpensive sugars as a feedstock to generate SCL-*co*-MCL PHA copolymers that have been demonstrated to possess material properties similar to polypropylene or polyethylene.[3,4,19,32]

AUTHOR INFORMATION

Corresponding Author

*E-mail: ctnomura@esf.edu. Phone: 315-470-6854.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge support from NSF CBET 1263905 and NYSERDA 25924 awarded to C.T. Nomura. I.L. Patterson was supported by NSF REU 1156942 awarded to J.M. Hasenwinkel and P.T. Mather of the Syracuse Biomaterials Institute. O. Ozumba was supported by the Louis Stokes Alliance for Minority Participation (LSAMP) and Syracuse Biomaterials Institute at Syracuse University.

REFERENCES

(1) Municipal Solid Waste Generation, Recycling, and Disposal in the United States: Facts and Figures for 2010; U.S. Environmental Protection Agency: Washington, DC, 2012; pp 1−12.

(2) Boerger, C. M.; Lattin, G. L.; Moore, S. L.; Moore, C. J. Plastic ingestion by planktivorous fishes in the North Pacific Central Gyre. *Mar. Pollut. Bull.* **2010**, *60*, 2275−2278.

(3) Sudesh, K.; Abe, H.; Doi, Y. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.* **2000**, *25*, 1503−1555.

(4) Lu, J.; Tappel, R. C.; Nomura, C. T. Mini-review: Biosynthesis of poly(hydroxyalkanoates). *Polym. Rev.* **2009**, *49*, 226−248.
(5) Rai, R.; Keshavarz, T.; Roether, J. A.; Boccaccini, A. R.; Roy, I. Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future. *Mater. Sci. Eng., R* **2011**, *72*, 29−47.
(6) Wang, Q.; Tappel, R. C.; Zhu, C.; Nomura, C. T. Development of a new strategy for production of medium-chain-length polyhydroxyalkanoates by recombinant *Escherichia coli* via inexpensive non-fatty acid feedstocks. *Appl. Environ. Microbiol.* **2012**, *78*, 519−527.
(7) Kang, H. O.; Chung, C. W.; Kim, H. W.; Kim, Y. B.; Rhee, Y. H. Cometabolic biosynthesis of copolyesters consisting of 3-hydroxyvalerate and medium-chain-length 3-hydroxyalkanoates by *Pseudomonas* sp. DSY-82. *Antonie van Leeuwenhoek* **2001**, *80*, 185−191.
(8) Budde, C. F.; Riedel, S. L.; Willis, L. B.; Rha, C.; Sinskey, A. J. Production of poly(3-Hydroxybutyrate-*co*-3-hydroxyhexanoate) from plant oil by engineered *Ralstonia eutropha* strains. *Appl. Environ. Microbiol.* **2011**, *77*, 2847−2854.
(9) Chee, J. Y.; Lau, N. S.; Samian, M. R.; Tsuge, T.; Sudesh, K. Expression of *Aeromonas caviae* polyhydroxyalkanoate synthase gene in *Burkholderia* sp. USM (JCM15050) enables the biosynthesis of SCL-MCL PHA from palm oil products. *J. Appl. Microbiol.* **2012**, *112*, 45−54.
(10) Chen, G.-Q.; Zhang, G.; Park, S. J.; Lee, S. Y. Industrial scale production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). *Appl. Microbiol. Biotechnol.* **2001**, *57*, 50−55.
(11) Lee, S. H.; Oh, D. H.; Ahn, W. S.; Lee, Y.; Choi, J.; Lee, S. Y. Production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) by high-cell-density cultivation of *Aeromonas hydrophila*. *Biotechnol. Bioeng.* **2000**, *67*, 240−244.
(12) Mifune, J.; Nakamura, S.; Fukui, T. Engineering of *pha* operon on *Cupriavidus necator* chromosome for efficient biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from vegetable oil. *Polym. Degrad. Stab.* **2010**, *95*, 1305−1312.
(13) Rehm, B. H.; Krüger, N.; Steinbüchel, A. A new metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid synthesis. *J. Biol. Chem.* **1998**, *273*, 24044−24051.
(14) Zheng, Z.; Zhang, M.; Zhang, G.; Chen, G. Production of 3-hydroxydecanoic acid by recombinant *Escherichia coli* HB101 harboring *phaG* gene. *Antonie van Leeuwenhoek* **2004**, *85*, 93−101.
(15) Nomura, C. T.; Tanaka, T.; Gan, Z.; Kuwabara, K.; Abe, H.; Takase, K.; Taguchi, K.; Doi, Y. Effective enhancement of short-chain-length-medium-chain-length polyhydroxyalkanoate copolymer production by coexpression of genetically engineered 3-ketoacyl-acyl-carrier-protein synthase III (*fabH*) and polyhydroxyalkanoate synthesis genes. *Biomacromolecules* **2004**, *5*, 1457−1464.
(16) Jiang, X.; Ramsay, J. A.; Ramsay, B. A. Acetone extraction of mcl-PHA from *Pseudomonas putida* KT2440. *J. Microbiol. Methods* **2006**, *67*, 212−219.
(17) Tappel, R. C.; Wang, Q.; Nomura, C. T. Precise control of repeating unit composition in biodegradable poly(3-hydroxyalkanoate) polymers synthesized by *Escherichia coli*. *J. Biosci. Bioeng.* **2012**, *113*, 480−486.
(18) Matsusaki, H.; Abe, H.; Taguchi, K.; Fukui, T.; Doi, Y. Biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthase gene *phaC1* from *Pseudomonas* sp. 61−3. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 401−409.
(19) Tappel, R. C.; Kucharski, J. M.; Mastroianni, J. M.; Stipanovic, A. J.; Nomura, C. T. Biosynthesis of poly[(R)-3-hydroxyalkanoate] copolymers with controlled repeating unit compositions and physical properties. *Biomacromolecules* **2012**, *13*, 2964−2972.
(20) Doi, Y.; Kitamura, S.; Abe, H. Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). *Macromolecules* **1995**, *28*, 4822−4828.
(21) Takase, K.; Taguchi, S.; Doi, Y. Enhanced synthesis of poly(3-hydroxybutyrate) in recombinant *Escherichia coli* by means of error-prone PCR mutagenesis, saturation mutagenesis, and *in vitro* recombination of the type II polyhydroxyalkanoate synthase gene. *J. Biochem.* **2003**, *133*, 139−145.
(22) Takase, K.; Matsumoto, K.; Taguchi, S.; Doi, Y. Alteration of Substrate Chain-Length Specificity of Type II Synthase for Polyhydroxyalkanoate Biosynthesis by in Vitro Evolution: in Vivo and in Vitro Enzyme Assays. *Biomacromolecules* **2004**, *5*, 480−485.
(23) Wang, F.; Lee, S. Y. Production of poly(3-hydroxybutyrate) by fed-batch culture of filamentation-suppressed recombinant *Escherichia coli*. *Appl. Environ. Microbiol.* **1997**, *63*, 4765−4769.
(24) Ahn, W. S.; Park, S. J.; Lee, S. Y. Production of poly(3-hydroxybutyrate) by fed-batch culture of recombinant *Escherichia coli* with a highly concentrated whey solution. *Appl. Environ. Microbiol.* **2000**, *66*, 3624−3627.
(25) Spratt, S. K.; Ginsburgh, C. L.; Nunn, W. D. Isolation and genetic characterization of *Escherichia coli* mutants defective in propionate metabolism. *J. Bacteriol.* **1981**, *146*, 1166−1169.
(26) Rhie, H. G.; Dennis, D. Role of *fadR* and *atoC*(Con) mutations in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) synthesis in recombinant *pha*$^+$ *Escherichia coli*. *Appl. Environ. Microbiol.* **1995**, *61*, 2487−2492.
(27) Luli, G. W.; Strohl, W. R. Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *Appl. Environ. Microbiol.* **1990**, *56*, 1004−1011.
(28) Pauli, G.; Overath, P. *ato* Operon: A highly inducible system for acetoacetate and butyrate degradation in *Escherichia coli*. *Eur. J. Biochem.* **1972**, *29*, 553−562.
(29) Jenkins, L. S.; Nunn, W. D. Genetic and molecular characterization of the genes involved in short-chain fatty acid degradation in *Escherichia coli*: The *ato* system. *J. Bacteriol.* **1987**, *169*, 42−52.
(30) Jenkins, L. S.; Nunn, W. D. Regulation of the *ato* operon by the *atoC* gene in *Escherichia coli*. *J. Bacteriol.* **1987**, *169*, 2096−2102.
(31) Duncombe, G. R.; Frerman, F. E. Molecular and catalytic properties of the acetoacetyl-coenzyme A thiolase of *Escherichia coli*. *Arch. Biochem. Biophys.* **1976**, *176*, 159−170.
(32) Doi, Y. *Microbial Polyesters*; VCH Publishers: New York, 1990.
(33) Fukui, T.; Yokomizo, S.; Kobayashi, G.; Doi, Y. Co-expression of polyhydroxyalkanoate synthase and (*R*)-enoyl-CoA hydratase genes of *Aeromonas caviae* establishes copolyester biosynthesis pathway in *Escherichia coli*. *FEMS Microbiol. Lett.* **1999**, *170*, 69−75.
(34) Tsuge, T.; Taguchi, K.; Taguchi, S.; Doi, Y. Molecular characterization and properties of (*R*)-specific enoyl-CoA hydratases from *Pseudomonas aeruginosa*: metabolic tools for synthesis of polyhydroxyalkanoates via fatty acid β-oxidation. *Int. J. Biol. Macromol.* **2003**, *31*, 195−205.
(35) Nomura, C. T.; Tanaka, T.; Eguen, T. E.; Appah, A. S.; Matsumoto, K.; Taguchi, S.; Ortiz, C. L.; Doi, Y. FabG mediates polyhydroxyalkanoate production from both related and nonrelated carbon sources in recombinant *Escherichia coli* LS5218. *Biotechnol. Prog.* **2008**, *24*, 342−351.
(36) Matsumoto, K.; Takase, K.; Yamamoto, Y.; Doi, Y.; Taguchi, S. Chimeric enzyme composed of polyhydroxyalkanoate (PHA) synthases from *Ralstonia eutropha* and *Aeromonas caviae* enhances production of PHAs in recombinant *Escherichia coli*. *Biomacromolecules* **2009**, *10*, 682−685.
(37) Sato, S.; Kanazawa, H.; Tsuge, T. Expression and characterization of (*R*)-specific enoyl coenzyme A hydratases making a channeling route to polyhydroxyalkanoate biosynthesis in *Pseudomonas putida*. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 951−959.
(38) Gao, X.; Yuan, X.-X.; Shi, Z.-Y.; Shen, X.-W.; Chen, J.-C.; Wu, Q.; Chen, G.-Q. Production of copolyesters of 3-hydroxybutyrate and medium-chain-length 3-hydroxyalkanoates by *E. coli* containing an optimized PHA synthase gene. *Microb. Cell Fact.* **2012**, *11*, 130.
(39) Park, S. J.; Ahn, W. S.; Green, P. R.; Lee, S. Y. Production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) by metabolically engineered *Escherichia coli* strains. *Biomacromolecules* **2001**, *2*, 248−254.
(40) Hu, F.; Cao, Y.; Xiao, F.; Zhang, J.; Li, H. Site-directed mutagenesis of *Aeromonas hydrophila* enoyl coenzyme A hydratase enhancing 3-hydroxyhexanoate fractions of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). *Curr. Microbiol.* **2007**, *55*, 20−24.

(41) Li, Q.; Chen, Q.; Li, M.-J.; Wang, F.-S.; Qi, Q.-S. Pathway engineering results the altered polyhydroxyalkanoates composition in recombinant *Escherichia coli*. *New Biotechnol.* **2011**, *28*, 92−95.
(42) Davis, R.; Anilkumar, P. K.; Chandrashekar, A.; Shamala, T. R. Biosynthesis of polyhydroxyalkanoates co-polymer in *E. coli* using genes from *Pseudomonas* and *Bacillus*. *Antonie van Leeuwenhoek* **2008**, *94*, 207−216.
(43) Nomura, C. T.; Taguchi, K.; Taguchi, S.; Doi, Y. 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.* **2004**, *70*, 999−1007.
(44) Nomura, C. T.; Taguchi, K.; Gan, Z.; Kuwabara, K.; Tanaka, T.; Takase, K.; Doi, Y. Expression of 3-ketoacyl-acyl carrier protein reductase (*fabG*) genes enhances production of polyhydroxyalkanoate copolymer from glucose in recombinant *Escherichia coli* JM109. *Appl. Environ. Microbiol.* **2005**, *71*, 4297−4306.
(45) Mueller, A. P.; Nomura, C. T. Mutations to the active site of 3-ketoacyl-ACP synthase III (FabH) increase polyhydroxyalkanoate biosynthesis in transgenic *Escherichia coli*. *J. Biosci. Bioeng.* **2011**, *113*, 300−306.