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Production of Poly(3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) from Plant Oil by Engineered *Ralstonia eutropha* Strains▽†

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The polyhydroxyalkanoate (PHA) copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(HB-*co*-HHx)] has been shown to have potential to serve as a commercial bioplastic. Synthesis of P(HB-*co*-HHx) from plant oil has been demonstrated with recombinant *Ralstonia eutropha* strains expressing heterologous PHA synthases capable of incorporating HB and HHx into the polymer. With these strains, however, short-chain-length fatty acids had to be included in the medium to generate PHA with high HHx content. Our group has engineered two *R. eutropha* strains that accumulate high levels of P(HB-*co*-HHx) with significant HHx content directly from palm oil, one of the world's most abundant plant oils. The strains express a newly characterized PHA synthase gene from the bacterium *Rhodococcus aetherivorans* I24. Expression of an enoyl coenzyme A (enoyl-CoA) hydratase gene (*phaJ*) from *Pseudomonas aeruginosa* was shown to increase PHA accumulation. Furthermore, varying the activity of acetoacetyl-CoA reductase (encoded by *phaB*) altered the level of HHx in the polymer. The strains with the highest PHA titers utilized plasmids for recombinant gene expression, so an *R. eutropha* plasmid stability system was developed. In this system, the essential pyrroline-5-carboxylate reductase gene *proC* was deleted from strain genomes and expressed from a plasmid, making the plasmid necessary for growth in minimal media. This study resulted in two engineered strains for production of P(HB-*co*-HHx) from palm oil. In palm oil fermentations, one strain accumulated 71% of its cell dry weight as PHA with 17 mol% HHx, while the other strain accumulated 66% of its cell dry weight as PHA with 30 mol% HHx.

Polyhydroxyalkanoates (PHAs) are polyesters synthesized by bacteria as carbon and energy storage compounds (2). The first PHA discovered was the homopolymer poly(3-hydroxybutyrate) (PHB) (22). It was established that other types of PHAs also exist in nature when Wallen and Rohwedder extracted PHA copolymers from sewer sludge (39) and when de Smet et al. observed that *Pseudomonas oleovorans* can synthesize poly(3-hydroxyoctanoate) (10). Today, PHAs are characterized as containing short-chain-length (SCL; C_3 to C_5) and/or medium-chain-length (MCL; C_6 and longer) monomers (30).

There has long been interest in using PHAs as biodegradable bioplastics that could serve as alternatives to petrochemical plastics. The commercial potential of PHB was first investigated by W. R. Grace and Company (3), who determined that this polymer has several issues that limit its value. PHB is a highly crystalline polymer that lacks toughness and begins to decompose near its melting temperature, making it difficult to process (21). Many studies were later conducted with poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)], but it was found that introduction of HV units into the polymer had limited impact on the material properties (17). This is due to the fact that HB and HV units are able to cocrystallize (4). Copolymerization of MCL monomers with HB leads to more dramatic changes to the properties of the plastic (27). The best-studied member of this class of PHA is poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(HB-*co*-HHx)]. P(HB-*co*-HHx) has a lower melting temperature, lower Young's modulus, and longer elongation to break than PHB (11, 27). This means that P(HB-*co*-HHx) is a tougher, more flexible plastic than PHB.

The model organism for studying PHA synthesis and accumulation is *Ralstonia eutropha* H16, because it accumulates large quantities of polymer when grown in nutrient-limited conditions (29). *R. eutropha* has also been shown to grow efficiently with plant oil as the sole carbon source (6). The wild type produces only SCL PHA, however, and thus is limited as an industrial PHA production organism. One of the first organisms identified that synthesizes P(HB-*co*-HHx) was *Aeromonas caviae* (33). This bacterium and related species store P(HB-*co*-HHx) when grown on plant oils and fatty acids but exhibit a low level of PHA accumulation (11). Investigations of *A. caviae* have revealed how this organism is able to synthesize P(HB-*co*-HHx). The PHA synthase from *A. caviae* ($PhaC_{Ac}$) efficiently polymerizes both HB-coenzyme A (HB-CoA) and HHx-CoA (12). *A. caviae* also has a gene encoding an (*R*)-specific enoyl-CoA hydratase ($phaJ_{Ac}$), which allows for conversion of fatty acid β-oxidation intermediates to PHA precursors (14, 16).

Plant oils and fatty acids are appealing feedstocks for industrial PHA production because of their high carbon contents

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and because metabolism of these compounds can influence the monomer composition of the resulting PHA (1). Several groups have produced P(HB-*co*-HHx) by using *Aeromonas* strains or recombinant *R. eutropha* expressing $phaC_{Ac}$ (9, 18, 23, 26). In these cases, however, production of PHA with high HHx content (>5 mol%) required feeding the cells short-chain-length fatty acids (≤12 carbons), which is undesirable because these compounds are more costly than raw plant oil. Mifune and coworkers recently reported engineered *R. eutropha* strains that expressed evolved $phaC_{Ac}$ and $phaJ_{Ac}$ (25). Strains from this study were able to accumulate high levels of PHA (>75% of the cell dry weight [CDW; i.e., wt%]) with up to 9.9 mol% HHx content when grown on soybean oil.

Our group hypothesized that the high levels of HB-CoA produced by *R. eutropha* when it is grown on plant oil could limit incorporation of other monomers into the PHA, even if the strain expressed a PHA synthase that could polymerize both HB-CoA and HHx-CoA. Recently, we reported an *R. eutropha* strain in which genes encoding acetoacetyl-CoA reductases were deleted, and this strain makes significantly less PHB than the wild type (8). We planned to engineer this strain to produce P(HB-*co*-HHx). A series of strains was constructed in which PHA synthase genes from *A. caviae* and *Rhodococcus aetherivorans* I24 (7) were integrated into the genomes of *R. eutropha* strains with different levels of acetoacetyl-CoA reductase activity. *Rhodococcus* species have been shown to synthesize PHA copolymers (41), and analysis of a draft genome of *R. aetherivorans* revealed two putative PHA synthase genes that had not been described in the literature. The strains were further improved by incorporating *phaJ* genes and by increasing gene expression with a stable plasmid expression system. Our work resulted in the construction of two stable *R. eutropha* strains that accumulate high levels of P(HB-*co*-HHx) when grown on plant oil, in which the HHx contents of the PHAs from both strains are >12 mol%.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. All PHA production experiments in this study were conducted with *Ralstonia eutropha* H16 and mutants derived from this strain (Table 1). Strain genotypes are also illustrated in Fig. S1 in the supplemental material. The rich medium used for growth of *R. eutropha* was dextrose-free tryptic soy broth (TSB) medium (Becton Dickinson, Sparks, MD). The concentrations of salts in the *R. eutropha* minimal medium have been reported previously (8). Carbon and nitrogen sources were added to the minimal medium as described in the text. The carbon sources used in this study were fructose and palm oil (Wilderness Family Naturals, Silver Bay, MN). All media contained 10 μg/ml gentamicin sulfate. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. *R. eutropha* strains were always grown aerobically at 30°C. In shake flask experiments, 50 ml medium was used in 250-ml flasks. The shaker was set to 200 rpm.

Plasmid and strain construction. In this study, DNA was routinely amplified by using high-fidelity DNA polymerase (Qiagen, Valencia, CA) and digested using restriction enzymes from New England BioLabs (Ipswich, MA). Plasmids were transformed into *R. eutropha* via transconjugation with *Escherichia coli* S17-1. Markerless gene deletions and insertions in the *R. eutropha* genome were achieved following the protocol described in reference 8, which is based on the work of York et al. (42). The strains and plasmids used in this study are described in Table 1. The sequences of all oligonucleotide primers used in this study are provided in Table S1 in the supplemental material.

An *R. eutropha* strain with the *phaC1* gene deleted (Re1034) was previously constructed in our lab (42). This strain is unable to synthesize PHA. The plasmid used to make the *phaC1* deletion (pGY46) contained a section of DNA in which the region of the genome immediately upstream of *phaC1* was connected to the region of the genome downstream of *phaC1*. In order to insert new synthase genes at the *phaC1* locus, pGY46 was altered via site-directed mutagenesis using the Invitrogen GeneTailor kit (Carlsbad, CA). An SwaI site was inserted between the upstream and downstream sequences, allowing synthase genes to be cloned into this site in the mutated plasmid (pJV7). We investigated in this study two novel PHA synthase genes from *R. aetherivorans* I24, which were named $phaC1_{Ra}$ and $phaC2_{Ra}$. These genes were identified by analyzing a draft copy of the *R. aetherivorans* I24 genome, provided by John Archer (University of Cambridge, United Kingdom). When amplifying $phaC1_{Ra}$ from the *R. aetherivorans* I24 genome, a primer was used such that the start codon in the cloned gene was ATG, rather than the TTG found in the genome. A version of the *phaC* gene from *A. caviae* in which the DNA sequence was codon optimized for expression in *R. eutropha* was purchased from Codon Devices (Cambridge, MA). The optimized $phaC_{Ac}$ was designed with SwaI sites on both ends of the gene so that it could also be cloned into pJV7.

Many strains were constructed based on Re2115, an *R. eutropha* strain in which the three *phaB* genes in the *R. eutropha* genome had been deleted (8). New genes were inserted into the *phaB1* locus to alter production of PHA monomers by using plasmids based on pCB42. The gene inserted into this locus was *phaB2* from *R. eutropha* (8), *phaJ1* from *Pseudomonas aeruginosa* ($phaJ1_{Pa}$) (38), or *phaJ* from *A. caviae* (14). The $phaJ1_{Pa}$ gene was cloned via colony PCR from *P. aeruginosa* PAO1. The $phaJ_{Ac}$ gene was synthesized by Integrated DNA Technologies (Coralville, IA) and had EcoRV sites located at both ends of the gene, allowing it to be cloned into pCB42.

In order to increase the gene copy number, and thus gene expression, the engineered PHA biosynthesis operon from strain Re2152 (Table 1) was amplified via PCR and cloned into plasmid pBBR1MCS-2, creating pCB81. This plasmid was maintained in *R. eutropha* by adding 200 μg/ml kanamycin to the growth media. In order to improve plasmid stability in the absence of kanamycin, *R. eutropha* strains in which the *proC* gene was deleted from their genomes were constructed. The gene *proC* (locus tag h16_A3106; GeneID number 4250351) encodes pyrroline-5-carboxylate reductase, which is part of the proline biosynthesis pathway. When constructing the Δ*proC* strains, 0.2% proline was added to all selection plates. The region of the *R. eutropha* genome containing *proC* and h16_A3105 was amplified via colony PCR and cloned into pCB81, creating plasmid pCB113. This plasmid was transformed into Δ*proC R. eutropha* strains.

Fermentation conditions. Strains Re2058/pCB113 and Re2160/pCB113 were grown to higher densities than is possible in shake flasks by using an Infors Sixfors multiple fermentor system (Bottmingen, Switzerland). Cultures were prepared by first growing the strains overnight in TSB containing 200 μg/ml kanamycin. These cultures were used to inoculate 50 ml minimal medium flask precultures containing 2% fructose and 0.1% NH_4Cl. The minimal medium precultures were used to inoculate the fermentors so that the initial optical density at 600 nm (OD_{600}) of each 400 ml culture was 0.1. Each fermentor contained either 4% (Re2160/pCB113) or 4.5% (Re2058/pCB113) palm oil and 0.4% NH_4Cl. Neither the fructose nor palm oil minimal medium cultures contained kanamycin.

The temperature of each fermentor was kept constant at 30°C. The pH of each culture was maintained at 6.8 ± 0.1 through controlled addition of 2 M sodium hydroxide. Stirring was provided by two six-blade Rushton impellers at speeds of 500 to 1,000 rpm. Air was supplied at 0.5 to 1 vol/vol/min, and the dissolved oxygen concentration was maintained above 40% through controlled addition of pure oxygen. Sterile silicone oil AR200 was used as an antifoam in these experiments and was added to cultures by hand as necessary.

Analytical methods. The cell dry weights (CDWs) of cultures were measured by taking 8- to 14-ml samples in preweighed plastic test tubes. The samples were centrifuged, and the pellets were washed with 5 ml cold water. For experiments using palm oil as the carbon source, 2 ml cold hexane was also included during the wash step to remove unused oil from the samples. Samples were then centrifuged again, resuspended in 1 ml cold water, frozen at −80°C, and lyophilized. The dried samples were weighed, and CDWs were determined. Residual cell dry weight (RCDW) values were calculated for each sample, and these were defined as the total CDW minus the mass of PHA. Ammonium concentrations in clarified culture supernatants were measured with an ammonium assay kit (catalog no. AA0100; Sigma-Aldrich) following the manufacturer's instructions.

The PHA contents and compositions from dried samples were determined using a methanolysis protocol adapted from reference 5. Dried cells were weighed in screw-top glass test tubes and reacted with methanol and sulfuric acid in the presence of chloroform for 2.5 h at 100°C. This reaction converts PHA monomers into their related methyl esters. The concentrations of methyl esters were determined via gas chromatography with an Agilent 6850 gas chromatograph (GC) (Santa Clara, CA) equipped with a DB-Wax column (30 m by 0.32 mm by 0.5 μm; Agilent) and a flame ionization detector. Two microliters of each sample was injected into the GC with a split ratio of 30:1 (vent:column). Hydro-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
R. eutropha strains		
H16	Wild-type strain, Gm resistant	ATCC 17699
Re1034	H16 Δ*phaC1*	42
Re2000	Re1034::$phaC1_{Ra}$, made with pINY3	This study
Re2001	Re1034::$phaC2_{Ra}$, made with pINY4	This study
Re2058	Re1034 Δ*proC*, made with pCB110	This study
Re2115	H16 Δ*phaB1* Δ*phaB2* Δ*phaB3*	8
Re2133	Re2115 Δ*phaC1*, made with pGY46	This study
Re2135	Re2133::$phaC2_{Ra}$, made with pINY4	This study
Re2136	Re2133::$phaC_{Ac}$, made with pLW484	This study
Re2151	Re2135::*phaB2*, made with pCB66	This study
Re2152	Re2135::$phaJ1_{Pa}$, made with pCB69	This study
Re2153	Re2135::$phaJ_{Ac}$, made with pCB72	This study
Re2154	Re2136::*phaB2*, made with pCB66	This study
Re2155	Re2136::$phaJ1_{Pa}$, made with pCB69	This study
Re2156	Re2136::$phaJ_{Ac}$, made with pCB72	This study
Re2160	Re2133 Δ*proC*, made with pCB110	This study
E. coli strains		
DH5α	General cloning strain	Invitrogen
S17-1	Strain for conjugative transfer of plasmids to *R. eutropha*	34
Other strains		
R. aetherivorans I24	Source of $phaC1_{Ra}$ and $phaC2_{Ra}$	7
P. aeruginosa PAO1	Source of $phaJ1_{Pa}$	36
Plasmids		
pGY46	Plasmid for deletion of *phaC1* from *R. eutropha* genome; the backbone was used to make other plasmids for gene deletion/insertion in *R. eutropha* genome; confers Km resistance	42
pJV7	pGY46 with SwaI site between regions of DNA upstream and downstream of *phaC1*, used to insert new genes at the *phaC1* locus	This study
pINY3	pJV7 with $phaC1_{Ra}$ cloned into SwaI site	This study
pINY4	pJV7 with $phaC2_{Ra}$ cloned into SwaI site	This study
pLW484	pJV7 with $phaC_{Ac}$ cloned into SwaI site	This study
pCB42	Plasmid for insertion of genes at the *phaB1* locus in the *R. eutropha* genome, confers Km resistance	8
pCB66	pCB42 with *R. eutropha phaB2* cloned into SwaI site	8
pCB69	pCB42 with $phaJ1_{Pa}$ cloned into SwaI site	This study
pCB72	pCB42 with $phaJ_{Ac}$ cloned into SwaI site	This study
pCB110	Plasmid for deletion of *R. eutropha proC*	This study
pBBR1MCS-2	Vector for plasmid-based gene expression in *R. eutropha*, confers Km resistance	19
pCB81	pBBR1MCS-2 with the PHA operon from Re2152 cloned between KpnI and HindIII sites	This study
pCB113	pCB81 with *R. eutropha proC* region cloned into AgeI site	This study

[a] Abbreviations: Gm, gentamicin; Km, kanamycin.

gen was used as the carrier gas at a flow rate of 3 ml/min. The oven was held at 80°C for 5 min, heated to 220°C at 20°C/min, and held at 220°C for 5 min. Pure standards of methyl 3-hydroxybutyrate and methyl 3-hydroxyhexanoate were used to generate calibration curves for the methanolysis assay.

PHA was extracted from dried cells by using chloroform for measurement of polymer molecular weights. Molecular weight measurements were made via gel permeation chromatography (GPC) relative to polystyrene standards as described previously (8). The number-average molecular weight (M_n), weight-average molecular weight (M_w), and polydispersity index (PDI) were measured for each sample.

Additional polymer characterization was performed with purified PHA recovered from samples of Re2058/pCB113 and Re2160/pCB113 taken at the end of palm oil fermentations (120 h of growth). PHA was isolated from lyophilized cells by extracting the polymer with methyl isobutyl ketone (MIBK). For each extraction, 100 ml of MIBK was added to 1.5 g of dried cells and stirred at 100°C for 4 h under reflux conditions. Cell debris was removed by centrifugation, and PHA was precipitated from solution by the addition of 3 volumes of hexane. The resulting precipitate was collected by centrifugation, washed with additional hexane, and dried. The monomer composition of the purified PHA was determined by proton nuclear magnetic resonance (NMR) spectroscopy. Polymer was dissolved in deuterated chloroform, and ^{1}H NMR spectra were collected with a Varian Mercury 300 MHz spectrometer.

Nucleotide sequence accession numbers. The sequences of $phaC1_{Ra}$ and $phaC2_{Ra}$ have been deposited in GenBank under accession numbers HQ130734 and HQ130735. The sequence of the optimized $phaC_{Ac}$ was deposited in GenBank under accession number HQ864571.

RESULTS

Characterization of *R. aetherivorans* I24 synthases. It was confirmed that the putative synthase genes from *R. aetherivorans* I24 encoded active enzymes by inserting the genes into the Re1034 genome at the *phaC1* locus. Insertion of either gene restored production of PHB from fructose (Table 2). Re2000 accumulated approximately the same amount of PHB as H16, while Re2001 made significantly less. It has been demonstrated that if a synthase capable of polymerizing MCL monomers is expressed in recombinant *R. eutropha*, the strain will accumulate MCL PHA when grown on fatty acids (24). The pathway through which MCL hydroxyacyl-CoA molecules are synthesized in wild-type *R. eutropha* has not yet been iden-

TABLE 2. Compositions of PHAs from strains grown on different carbon sources[b]

Carbon source	Strain	PHA (% of CDW)	PHA composition (mol%)[a]			
			HB	HV	HHx	HHp
Fructose	H16	75 ± 3	100			
	Re2000	79 ± 2	100			
	Re2001	39 ± 1	100			
Hexanoate	H16	49 ± 2	99.61 ± 0.01		0.39 ± 0.01	
	Re2000	51 ± 1	88.5 ± 0.2		11.5 ± 0.2	
	Re2001	48 ± 2	81.1 ± 0.4		18.9 ± 0.4	
Heptanoate	H16	52 ± 3	62.6 ± 0.5	37.4 ± 0.5		0
	Re2000	62 ± 1	40.4 ± 0.3	59.6 ± 0.3		tr
	Re2001	48 ± 6	25.2 ± 1.1	72.9 ± 1.6		1.9 ± 0.5
Octanoate	H16	66 ± 3	100		tr	
	Re2000	66 ± 2	93.44 ± 0.08		6.56 ± 0.08	
	Re2001	42 ± 4	89.6 ± 0.3		10.4 ± 0.3	

[a] Abbreviations: HB, 3-hydroxybutyrate; HV, 3-hydroxyvalerate; HHx, 3-hydroxyhexanoate; HHp, 3-hydroxyheptanoate; tr, trace amounts.
[b] PHA produced by H16 and recombinant *R. eutropha* strains expressing $phaC1_{Ra}$ and $phaC2_{Ra}$ was analyzed after the strains were grown for 60 h on 2% fructose or 0.4% fatty acids. All media contained 0.05% NH_4Cl. The values reported are averages from triplicate cultures ± SDs.

tified. Re2000 and Re2001 were therefore grown on a series of fatty acids in order to characterize the substrate specificities of the *R. aetherivorans* PHA synthases (Table 2). The cultures contained 0.05% NH_4Cl and an initial fatty acid concentration of 0.2%. An additional 0.2% fatty acid was added to the cultures after 24 h of growth. It was found that both recombinant strains were able to incorporate more HHx into PHA than H16 and that PHA from the strain harboring $phaC2_{Ra}$ also included 3-hydroxyheptanoate (HHp) when heptanoate was used as the carbon source. No PHA monomers longer than HHp were detected in any of the samples.

Analysis of *R. eutropha* strains with engineered genomes. The goal of this study was to produce SCL/MCL PHA copolymers by using palm oil as the carbon source. Palm oil is an important agricultural product in Southeast Asia, with a high yield of oil per acre of land (40). Re2000 and Re2001 were therefore grown in minimal medium with palm oil as the sole carbon source. While these strains accumulated P(HB-*co*-HHx) with significant HHx content when grown on hexanoate and octanoate, the PHA made from palm oil consisted of <2 mol% HHx (Table 3). We hypothesized that high intracellular concentrations of HB-CoA may limit HHx incorporation into the PHA made by the recombinant strains. Our group previously constructed a strain with low acetoacetyl-CoA reductase activity that accumulates significantly less PHB than H16 (Re2115). The *phaC1* gene from the genome of Re2115 was deleted, and $phaC2_{Ra}$ (Re2135) or $phaC_{Ac}$ (Re2136) was inserted in its place. The synthase gene $phaC2_{Ra}$ was chosen because the primary focus was to produce PHA with high HHx content, and the strain containing $phaC2_{Ra}$ synthesized PHA with the highest levels of HHx during fatty acid growth (Table 2, compare Re2001 to Re2000 and H16). The gene $phaC_{Ac}$ was

TABLE 3. Cell dry weights and levels of PHA and HHx[a]

Strain	48 h			72 h		
	CDW (g/liter)	PHA (% of CDW)	HHx (mol%)	CDW (g/liter)	PHA (% of CDW)	HHx (mol%)
H16	5.3 ± 0.4	71 ± 1	0	6.0 ± 0.2	79.2 ± 0.9	0
Re2000	6.1 ± 0.1	75.3 ± 0.3	1.5 ± 0.1	7.3 ± 0.1	82 ± 4	1.1 ± 0.3
Re2001	1.89 ± 0.04	49 ± 2	1.6 ± 0.2	2.19 ± 0.09	50 ± 3	1.5 ± 0.2
Re2115	0.78 ± 0.04	16.9 ± 0.2	1.68 ± 0.01	1.13 ± 0.06	22 ± 3	1.7 ± 0.3
Re2135	1.0 ± 0.1	22.3 ± 0.2	31.4 ± 0.2	1.22 ± 0.08	26 ± 2	31.4 ± 0.8
Re2136	0.72 ± 0.04	21.3 ± 0.2	15.01 ± 0.02	1.05 ± 0.01	25.5 ± 0.7	13.9 ± 0.5
Re2151	0.83 ± 0.01	28.63 ± 0.01	15.04 ± 0.01	1.01 ± 0.07	33 ± 3	12 ± 1
Re2152	1.15 ± 0.07	35.27 ± 0.07	23.29 ± 0.02	1.40 ± 0.02	40.4 ± 0.4	22.44 ± 0.08
Re2153	1.1 ± 0.1	31.5 ± 0.8	22.29 ± 0.01	1.32 ± 0.09	37 ± 2	22.29 ± 0.07
Re2154	1.26 ± 0.08	45.8 ± 0.8	5.8 ± 0.2	1.92 ± 0.04	53 ± 3	4.83 ± 0.01
Re2155	1.87 ± 0.01	54.9 ± 0.5	3.85 ± 0.07	2.55 ± 0.06	63 ± 3	4.00 ± 0.04
Re2156	2.2 ± 0.2	53 ± 3	3.8 ± 0.3	2.45 ± 0.09	57 ± 2	2.8 ± 0.4
Re1034/pCB81	3.3 ± 0.2	68.8 ± 0.8	13.6 ± 0.2	4.0 ± 0.2	73.0 ± 0.9	11.6 ± 0.2
Re2058/pCB113	3.24 ± 0.03	68 ± 2	15.3 ± 0.4	3.6 ± 0.3	73.1 ± 0.2	12.7 ± 0.3
Re2133/pCB81	2.3 ± 0.1	60 ± 4	24.3 ± 0.8	2.9 ± 0.1	67.0 ± 0.3	23.3 ± 0.2
Re2160/pCB113	2.00 ± 0.01	56.0 ± 0.5	25.32 ± 0.09	2.74 ± 0.06	63.99 ± 0.03	24.13 ± 0.02

[a] *R. eutropha* strains were grown in minimal medium with 1% palm oil and 0.05% NH_4Cl. Samples were harvested after 48 and 72 h of growth to analyze CDW and P(HB-*co*-HHx) content. Re1034/pCB81 and Re2133/pCB81 cultures contained kanamycin. All values represent means from duplicate or triplicate cultures, with the uncertainties indicating the range of observed values.

also investigated because it has been used in most of the P(HB-*co*-HHx) production studies in the literature. Both Re2135 and Re2136 made PHA with high HHx content from palm oil (Table 3), but these strains did not accumulate significant polymer (~25% of the CDW after 72 h).

Additional genes were therefore inserted into the genomes of these strains at the *phaB1* locus, with the goal of increasing total polymer accumulation. One of these genes was *phaB2*, which encodes a low-activity acetoacetyl-CoA reductase (8). It was hypothesized that expression of this gene would increase HB-CoA production but not to the level of H16. We also inserted the *phaJ* genes from *P. aeruginosa* and *A. caviae*, which would allow the strains to convert intermediates of fatty acid β-oxidation into 3-hydroxyacyl-CoA molecules. All of these strains exhibited greater PHA production than Re2135 and Re2136 when grown on palm oil (Table 3). The strains containing $phaC_{Ac}$ (Re2154, Re2155, and Re2156) made the most polymer, but the HHx content of the PHA was reduced to 4 to 5 mol% at 72 h. The strains containing $phaC2_{Ra}$ (Re2151, Re2152, and Re2153) made more PHA than Re2135, and the polymer still contained significant HHx. Of these strains, Re2152 was the most promising, as it accumulated 40 wt% P(HB-*co*-HHx) with 22 mol% HHx.

Analysis of engineered *R. eutropha* strains harboring plasmids. It was hypothesized that polymer accumulation could be increased in the engineered *R. eutropha* strains by increasing expression of the PHA biosynthesis genes. To accomplish this, the engineered PHA operon from Re2152 ($phaC2_{Ra}$-*phaA*-$phaJ1_{Pa}$) was amplified and cloned into pBBR1MCS-2. The cloned region included 460 bp from the genome upstream of the start codon of $phaC2_{Ra}$ so that the operon in the plasmid would be expressed from the native *R. eutropha* promoter. The resulting plasmid (pCB81) was transformed into Re1034 and Re2133 to determine how the different acetoacetyl-CoA reductase activity levels of the two host strains would influence PHA synthesis. When these strains were grown in palm oil minimal medium containing kanamycin, both accumulated >65 wt% P(HB-*co*-HHx) (Table 3) at 72 h. At this time point, the PHA from Re1034/pCB81 contained 12 mol% HHx, while the PHA from Re2133/pCB81 contained 23 mol% HHx.

While both strains harboring pCB81 accumulated significant P(HB-*co*-HHx) with high HHx content, these strains are not suitable for industrial PHA production from palm oil. The use of plasmid pCB81 would require the addition of expensive antibiotics to fermentations, which would add excessive cost at the industrial scale. A common strategy for maintaining plasmid stability without the use of antibiotics is to create an auxotrophic mutant through a genome mutation and then to complement the mutation with a plasmid containing the deleted gene (20). We deleted the *proC* gene from Re1034 and Re2133 to create Re2058 and Re2160, respectively. These strains were unable to grow in minimal medium that did not contain proline (data not shown). Plasmid pCB113 was created by cloning the *proC* region of the *R. eutropha* genome into pCB81. When pCB113 was transformed into Re2058 and Re2160, the ability of these strains to grow in minimal medium without proline was restored. PHA production from palm oil in kanamycin-free medium by Re2058/pCB113 and Re2160/pCB113 closely matched the results observed for Re1034/pCB81 and Re2133/pCB81 (Table 3). It was also found that these strains made the desired PHA copolymers only when oil or fatty acids were provided as carbon sources. When Re2058/pCB113 and Re2160/pCB113 were grown in fructose minimal medium, the strains accumulated only 40 wt% and 17 wt% PHA, respectively, and no HHx was detectable in the polymer.

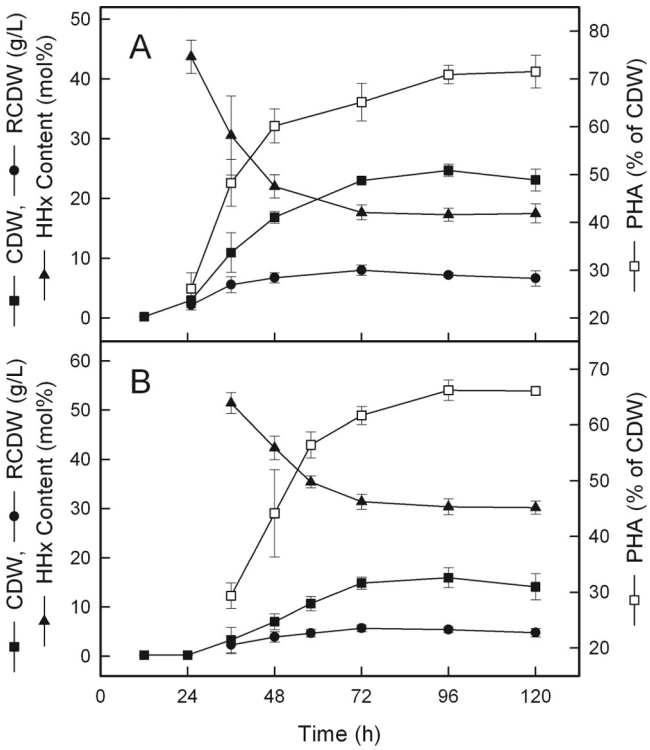

FIG. 1. Re2058/pCB113 (A) and Re2160/pCB113 (B) fermentations were carried out using palm oil as the sole carbon source. Plasmid pCB113 was retained by the cells without the use of kanamycin. Data points are means from triplicate fermentations, and error bars indicate standard deviations (SDs). Note that different scales are used for the *y* axes in panels A and B.

The performances of Re2058/pCB113 and Re2160/pCB113 in higher-density palm oil cultures were evaluated by growing these strains in fermentors, using medium with an NH_4Cl concentration eight times that of the medium in the flask cultures (Fig. 1). No kanamycin was added to the fermentation medium or the minimal medium precultures in these experiments. Both strains grew in the high-nitrogen medium, although Re2160/pCB113 exhibited a lag phase of 24 h. By the end of the fermentations, Re2058/pCB113 accumulated 71 wt% PHA with 17 mol% HHx, while Re2160/pCB113 accumulated 66 wt% PHA with 30 mol% HHx. The PHA contents of the cells in both fermentations closely matched the values measured in the low-density flask cultures, suggesting that plasmid loss did not occur at the higher cell densities. When samples taken from similar fermentations were diluted and plated onto solid TSB with and without kanamycin, equal numbers of colonies were observed (data not shown), further indicating that plasmid loss does not occur with these strains.

Several interesting observations were made when analyzing the PHA made in these experiments. In both fermentations, the HHx content of the polymer was extremely high (>40 mol%) early in the cultures. Over time, the HHx contents

TABLE 4. Molecular weights and polydispersities[a]

Strain	Time point (h)	M_n (10^3)	M_w (10^3)	PDI
Re2058/pCB113	48	191 ± 27	362 ± 38	1.9
	96	105 ± 40	260 ± 52	2.5
Re2160/pCB113	48	192 ± 15	350 ± 17	1.8
	96	108 ± 12	276 ± 19	2.6

[a] PHA was extracted from Re2058/pCB113 and Re2160/pCB113 samples with chloroform, and the molecular weights were measured by GPC relative to polystyrene standards. Values reported represent means from three independent samples ± SDs.

decreased and then remained stable over the final 48 h of each experiment. The final HHx content in the PHA in the fermentor cultures was higher than that in the low-density flask cultures (Table 3). When analyzing the gas chromatograms of the methanolysis samples from both fermentations, small peaks with the same retention time as methyl 3-hydroxyoctanoate were observed (data not shown). These peaks were also present when polymer purified from dried cells of both strains was subjected to the methanolysis assay. This suggests that the PHA produced in these fermentations contained trace amounts of 3-hydroxyoctanoate in addition to HB and HHx.

In order to confirm the HHx content of the PHA produced by these strains, polymer was extracted from dried cells harvested at the end of the fermentations. PHA was dissolved using MIBK and precipitated by the addition of hexane. Proton NMR spectra were taken for PHA from each strain (see Fig. S2 in the supplemental material). The NMR spectroscopy data indicated that PHA from Re2058/pCB113 contained 21 mol% HHx, while the PHA from Re2160/pCB113 contained 28 mol% HHx. These values agree well with the methanolysis results.

PHA was extracted from lyophilized fermentation samples taken after 48 h and 96 h of growth, and the number-average (M_n) and weight-average (M_w) molecular weights were measured relative to polystyrene standards (Table 4). PHAs from both Re2058/pCB113 and Re2160/pCB113 had similar molecular weights at each time point. In both cases, the polymers had significantly shorter chain lengths than PHB made by wild-type *R. eutropha* H16, which has an M_w of $\sim 3 \times 10^6$ (8). For both Re2058/pCB113 and Re2160/pCB113, the average PHA molecular weight decreased and the polydispersity increased from 48 to 96 h. This agrees with previous work that showed that PHA is continuously turned over by *R. eutropha*, even under PHA storage conditions, and that this turnover is accompanied by a decrease in average polymer molecular weight (37). This means that it is important to harvest the biomass from *R. eutropha* fermentations as soon as maximum PHA accumulation has been reached, as additional time will lead to a decrease in average polymer chain length.

DISCUSSION

Two novel PHA synthases from the bacterium *R. aetherivorans* I24 were identified and characterized. When these PHA synthase genes were integrated into the Re1034 genome, the recombinant strains accumulated P(HB-*co*-HHx) when grown on even-chain-length fatty acids, with the strain containing $phaC2_{Ra}$ synthesizing polymer with the highest HHx content (Table 2). These strains also accumulated P(HB-*co*-HHx) when grown on palm oil, but the HHx content of the PHA was significantly lower. For example, the PHA from Re2001 contained 10 mol% HHx when the strain was grown on octanoate but only 1.5 mol% HHx when the strain was grown on palm oil. It has previously been demonstrated that HHx content in PHA decreases as the lengths of the fatty acids fed to recombinant *R. eutropha* increase (26). As the most abundant fatty acids in palm oil are palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) (31), our results agree with this observation.

In order to increase the HHx content of the PHA, *R. eutropha* strains that expressed recombinant PHA synthases and had low acetoacetyl-CoA reductase activity were constructed. It was previously discovered that *R. eutropha* strains in which the acetoacetyl-CoA reductase genes (*phaB*) had been deleted made significantly less PHB than the wild type, presumably because the HB-CoA synthesis pathway had been disrupted (8). The PHA made by the *phaB* deletion strains with recombinant synthases had high HHx contents, but the strains stored little polymer (Table 3). Notably, the strain containing $phaC2_{Ra}$ (Re2135) made PHA with an HHx content much higher than that of the analogous strain containing $phaC_{Ac}$ (Re2136). The $PhaC_{Ac}$ synthase has been the most widely studied enzyme for synthesis of P(HB-*co*-HHx) (12, 13, 23, 25, 26).

In order to increase synthesis of HB-CoA and HHx-CoA from fatty acid β-oxidation intermediates, *phaJ* genes were inserted into the genomes of the recombinant strains. PhaJ enzymes from *A. caviae* and *P. aeruginosa* have been shown to hydrate crotonyl-CoA and 2-hexenoyl-CoA at similar rates, leading to synthesis of both HB and HHx monomers (14, 38). It was found that insertion of either $phaJ_{Ac}$ or $phaJ1_{Pa}$ into our recombinant strains led to increased PHA accumulation, with the strains expressing $phaJ1_{Pa}$ generating polymer with slightly higher HHx contents (Table 3).

Expression of the PHA biosynthesis genes was increased using a plasmid-based expression system. Plasmid stability issues have been reported in high-density *R. eutropha* cultures, even in the presence of antibiotics (35). In order to ensure that our strains would produce PHA in high-density cultures without the need for kanamycin, we adapted a plasmid stability system that has been used successfully with other species of bacteria (32). The *proC* gene was deleted from the genomes of *R. eutropha* strains and expressed from plasmid pCB113. One scenario that could potentially lead to plasmid loss with this system is if some cells produced excess proline and excreted it into the medium, which would allow other cells to grow and replicate without pCB113. Plasmid loss was not observed in Re2058/pCB113 or Re2160/pCB113 cultures, suggesting that proline excretion does not occur under the conditions tested.

The data presented in Fig. 1 show that the polymers produced by Re2058/pCB113 and Re2160/pCB113 varied over time in the fermentations. The HHx content in the PHA is very high early in the cultures, then it decreases, and eventually it stabilizes. This means that late in the cultures, newly synthesized polymer has an HHx content lower than the overall average. For example, Re2058/pCB113 produced 10.1 g/liter PHA with 22.0 mol% HHx by the 48-h time point. By the 96-h time point, this strain had produced 17.5 g/liter PHA with 17.3 mol% HHx. Therefore, from 48 to 96 h, 7.4 g/liter PHA accumulated with an average HHx content of 10.9 mol%. The

reason for higher HHx contents in the PHA early in cultures is not completely understood. Some of the HB-CoA made by the strains is produced from acetyl-CoA through the actions of a β-ketothiolase (PhaA) and an acetoacetyl-CoA reductase (PhaB1 in Re2058/pCB113 and unknown reductases in Re2160/pCB113). It has been shown that during the *R. eutropha* growth phase, the high intracellular concentration of free CoA inhibits PhaA, decreasing the rate of HB-CoA synthesis (28). This suggests that early in the cultures, the ratio of HHx-CoA to HB-CoA is high, causing more HHx to be incorporated into the PHA. This could also explain the HHx content observed in the fermentor cultures being higher than that in the flask cultures. The fermentation medium contained more NH_4Cl than the flask medium, leading to a longer growth phase in which more HHx was included in the PHA.

Interesting questions concerning MCL PHA formation in *R. eutropha* remain. Strains Re2000 and Re2001 expressing *R. aetherivorans* PHA synthases produced P(HB-*co*-HHx). As PhaB1 can reduce 3-ketohexanoyl-CoA in addition to acetoacetyl-CoA (15), this enzyme likely contributed to some of the HHx-CoA formation in these strains. It is unclear, however, how HHx-CoA is synthesized in Re2135 and Re2136, as these two strains lack both *phaB* and *phaJ* genes.

Moving forward, our group will scale up the size and density of Re2058/pCB113 and Re2160/pCB113 palm oil cultures in order to characterize the PHA made under these conditions. High-density fermentations will likely require an oil feeding strategy to prevent excess substrate in the bioreactors. We are also exploring routes to further increase the amount of bioplastic accumulated by our engineered strains and the average molecular weights of the polymers.

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