Synthesis Gas (Syngas)-Derived Medium-Chain-Length Polyhydroxyalkanoate Synthesis in Engineered \textit{Rhodospirillum rubrum}

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ABSTRACT

The purple nonsulfur alphaproteobacterium \textit{Rhodospirillum rubrum} S1 was genetically engineered to synthesize a heteropolymer of mainly 3-hydroxydecanoic acid and 3-hydroxyoctanoic acid \([P(3HD-co-3HO)]\) from CO- and CO2-containing artificial synthesis gas (syngas). For this, genes from \textit{Pseudomonas putida} KT2440 coding for a 3-hydroxyacetyl acyl carrier protein (ACP) thioesterase (\textit{phag}), a medium-chain-length (MCL) fatty acid coenzyme A (CoA) ligase (\textit{PP_0763}), and an MCL polyhydroxyalkanoate (PHA) synthase (\textit{phaC1}) were cloned and expressed under the control of the CO-inducible promoter \textit{P}_{cooF} from \textit{R. rubrum} S1 in a PHA-negative mutant of \textit{R. rubrum}. \textit{P(3HD-co-3HO)} was accumulated to up to 7.1\% (wt/wt) of the cell dry weight by a recombinant mutant strain utilizing exclusively the provided gaseous feedstock syngas. In addition to an increased synthesis of these medium-chain-length PHAs (PHA\textsubscript{MCL}), enhanced gene expression through the \textit{P}_{cooF} promoter also led to an increased molar fraction of 3HO in the synthesized copolymer compared with the \textit{P}_{lac} promoter, which regulated expression on the original vector. The recombinant strains were able to partially degrade the polymer, and the deletion of \textit{phaZ2}, which codes for a PHA depolymerase most likely involved in intracellular PHA degradation, did not reduce mobilization of the accumulated polymer significantly. However, an amino acid exchange in the active site of PhaZ2 led to a slight increase in PHA\textsubscript{MCL} accumulation. The accumulated polymer was isolated; it exhibited a molecular mass of 124.3 kDa and a melting point of 49.6°C. With the metabolically engineered strains presented in this proof-of-principle study, we demonstrated the synthesis of elastomeric second-generation biopolymers from renewable feedstocks not competing with human nutrition.

IMPORTANCE

Polyhydroxyalkanoates (PHAs) are natural biodegradable polymers (biopolymers) showing properties similar to those of commonly produced petroleum-based nondegradable polymers. The utilization of cheap substrates for the microbial production of PHAs is crucial to lower production costs. Feedstock not competing with human nutrition is highly favorable. Syngas, a mixture of carbon monoxide, carbon dioxide, and hydrogen, can be obtained by pyrolysis of organic waste and can be utilized for PHA synthesis by several kinds of bacteria. Up to now, the biosynthesis of PHAs from syngas has been limited to short-chain-length PHAs, which results in a stiff and brittle material. In this study, the syngas-utilizing bacterium \textit{Rhodospirillum rubrum} was genetically modified to synthesize a polymer which consisted of medium-chain-length constituents, resulting in a rubber-like material. This study reports the establishment of a microbial synthesis of these so-called medium-chain-length PHAs from syngas and therefore potentially extends the applications of syngas-derived PHAs.

Biodegradable and biocompatible polyhydroxyalkanoates (PHAs) are naturally occurring polyesters, which are synthesized by various bacteria. Depending on their composition, PHAs present different physical and mechanical properties that can resemble those of petrochemically produced conventional plastics (1). PHAs are divided into those consisting of short-chain-length (PHA\textsubscript{ACL}; 3 to 5 carbon atoms) and of medium-chain-length (PHA\textsubscript{MCL}; 6 to 14 carbon atoms) hydroxyalkanoic acids, with the two showing significantly different properties and thereby also being suitable for different applications. PHA\textsubscript{MCL}, which are mainly synthesized by pseudomonads, are elastomeric rubber-like polymers with a much lower melting point than the more-brittle and crystalline PHA\textsubscript{ACL} (2). Due to these attributes, PHA\textsubscript{MCL} expand the range of applications for PHAs to elastic materials, which are required in the food and pharmaceutical industries (3).

Generally, pseudomonads are able to synthesize PHA\textsubscript{MCL} from carbon sources that they can convert to acetyl coenzyme A (acetyl-CoA), which is then subjected to fatty acid \textit{de novo} synthesis. This pathway is linked to PHA synthesis via the 3-hydroxyacetyl acyl carrier protein (ACP) thioesterase PhaG, which in combination with a fatty acid CoA ligase transfers the hydroxyacyl moiety from ACP to CoA (4, 5). Alternatively, readily available fatty acids that are metabolized to 3-hydroxyacyl-CoA through \(\beta\)-oxidation can

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be converted to a substrate for the PHA polymerase PhaC. Many recent studies have focused on the efficient production of PHA\textsubscript{MCL} in wild-type and recombinant microorganisms from abundant and cheap carbon sources, such as carbohydrates (6), fatty acids (7), or even polyethylene and aromatic compounds (8, 9).

Although the currently decreasing oil price results in a competitive disadvantage for the production of bioplastics in comparison to petrochemically produced plastics, the rising awareness of the pollution of marine and terrestrial environments by nondegradable plastics and the emerging problem of the occurrence of microplastic particles lead to a growing demand for degradable biopolymers, such as PHAs (10). Additionally, the possibility of utilizing industrial exhaust gases and such gases arising from gasification and pyrolysis of organic waste, referred to as synthesis gas (syngas), could further promote environmentally friendly and cost-efficient production processes for these so-called second-generation biopolymers (11). The synthesis of PHAs from waste gases such as CO and CO\textsubscript{2}, which in addition to N\textsubscript{2} and H\textsubscript{2} are the major components of syngas, has gained increased attention over the last decade, as it is predicted to be economically feasible without competing with the human energy or food supply (12, 13). However, so far, only PHA\textsubscript{SCl} have been synthesized from these gaseous feedstocks, as there is no known microorganism capable of synthesizing PHA\textsubscript{MCL} from syngas.

The purple nonsulfur alphaproteobacterium Rhodospirillum rubrum is able to utilize CO and CO\textsubscript{2}, and has been a host and model organism for several studies focusing on the synthesis of PHA\textsubscript{SCl} from various feedstocks (14–16). Applying the water-gas shift reaction, \textit{R. rubrum} oxidizes CO with H\textsubscript{2}O to CO\textsubscript{2} and H\textsubscript{2}, employing a carbon monoxide dehydrogenase (CODH), thereby gaining energy and carbon that is channeled into the Calvin cycle via ribulose 1,5 bisphosphate carboxylase (17). Moreover, recent investigations have shown that other carboxylases, which are specifically active when acetate assimilation is required, play a major role in CO\textsubscript{2} fixation (16). These include carboxylases of the ethylmalonyl-CoA pathway and a ferredoxin-dependent pyruvate synthase (PFOR). The wild type of \textit{R. rubrum} naturally synthesizes PHA\textsubscript{SCl} with various amounts of incorporated 3-hydroxyvalerate.

The aim of this study was to genetically modify \textit{R. rubrum} S1 to synthesize PHA\textsubscript{MCL} from syngas-derived CO and CO\textsubscript{2}. For this, three genes from \textit{Pseudomonas putida} KT2440 coding for a 3-hydroxacyl-ACP thioesterase (phaG [4]), a medium-chain-length (MCL) fatty acid CoA ligase (PP\_0763 [5]), and a PHA synthase (phaC1 [18]) were chosen for overexpression in a \textit{ΔphaC1 ΔphaC2} mutant, which was unable to synthesize PHA\textsubscript{SCl} (Fig. 1). In order to evaluate syngas-mediated gene overexpression, the CO-inducible \textit{P\textsubscript{cop}} Promoter (19) was implemented. It was also investigated if cells of \textit{R. rubrum} would be able to degrade the synthesized PHA\textsubscript{SCl} and, consequently, if a mutation of the predicted intracellular PHA depolymerase PhaZ2 gene (20) would increase polymer accumulation.

**MATERIALS AND METHODS**

Microorganisms, plasmids, and oligonucleotides. All applied bacterial strains, plasmids, and oligonucleotides are listed in Table 1. For routine cloning, plasmids were introduced into and isolated from \textit{Escherichia coli} TOP10. \textit{E. coli} S17-1 was used for the transfer of derivatives of the suicide vector pQ200mp18 into \textit{R. rubrum} strains.

![Synthesis of PHA\textsubscript{MCL} from CO and CO\textsubscript{2} in recombinant \textit{R. rubrum}.](http://aem.asm.org/Downloaded from http://aem.asm.org)
This study

This study

Applying the protocol of Aneja et al. (24), the verified hybrid plasmids and digestion of the generated vectors with restriction endonucleases.

The resulting strains were verified by colony PCR as well as isolation of the generated constructs.

For this, the respective amount of nitrogen was restored after 3 days of cultivation. Precultures were grown for 24 h in 100-ml baffled Erlenmeyer flasks with a culture volume of 20 ml containing succinate as the sole carbon source in all cases.

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As described by Sambrook et al. ((21), methods comprising the amplification, isolation, and manipulation of DNA were carried out as described by Sambrook et al. (21). Respective DNA fragments were amplified from the genomic DNA of R. rubrum S17-1, wild type, Kan, and phaZ2 deletion mutant, amino acid exchange in PhaZ2 (176C→A)

Site-directed mutagenesis of phaZ2 in R. rubrum. In order to exchange cysteine at position 176 of PhaZ2 of R. rubrum (phaZ2R_rubrum) with alanine, leading to an inactive PhaZ2, mismatched oligonucleotides (Table 1) causing a codon exchange in the respective gene were applied. DNA fragments hybridizing up- and downstream of the modified codon, which overlapped for 24 bp, were amplified from R. rubrum S1 genomic DNA. In a following fusion PCR, a single 1,206-bp phaZ2 fragment containing the altered codon was amplified from the two fragments. The product was cleaved with XbaI and ligated into a likewise-linearized plQ200mp18. The resulting plQ200mp18:phaZ2Cl76A was mobilized from E. coli S17-1 into R. rubrum ΔphaC1 ΔphaZ2 by conjugation via the spot agar mating technique (25). R. rubrum mutants were isolated on sucrose (10% [wt/vol])-containing agar plates with the medium composition described above but excluding yeast extract and fructose. R. rubrum

Table 1 Microorganisms, plasmids, and oligonucleotides used in this studya

<table>
<thead>
<tr>
<th>Organism, plasmid, or oligonucleotide</th>
<th>Relevant characteristic(s) or sequence (5′→3′)b</th>
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Plasmids

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<td>ATATCTAGATGCCGCGCCGCTCGCCCTCCAGAAGTGGG</td>
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a All heterologously overexpressed genes were amplified from genomic DNA of P. putida KT2440. The P∞α Promoter and the codon-exchanged phaZ2 were amplified from genomic DNA of R. rubrum S1. b Ste∞, streptomycin resistance; Km∞, kanamycin resistance; Gm∞, gentamicin resistance. Utilized restriction sites are underlined in the oligonucleotide sequences. Modifications in the resulting proteins, caused by the applied oligonucleotides, are in bold type.

Site-directed mutagenesis of phaZ2 in R. rubrum. In order to exchange cysteine at position 176 of PhaZ2 of R. rubrum (phaZ2R_rubrum) with alanine, leading to an inactive PhaZ2, mismatched oligonucleotides (Table 1) causing a codon exchange in the respective gene were applied. DNA fragments hybridizing up- and downstream of the modified codon, which overlapped for 24 bp, were amplified from R. rubrum S1 genomic DNA. In a following fusion PCR, a single 1,206-bp phaZ2 fragment containing the altered codon was amplified from the two fragments. The product was cleaved with XbaI and ligated into a likewise-linearized plQ200mp18. The resulting plQ200mp18:phaZ2Cl76A was mobilized from E. coli S17-1 into R. rubrum ΔphaC1 ΔphaZ2 by conjugation via the spot agar mating technique (25). R. rubrum mutants were isolated on sucrose (10% [wt/vol])-containing agar plates with the medium composition described above but excluding yeast extract and fructose. R. rubrum

concentration of 40%. For this, the respective amount of nitrogen was evacuated from flasks prior to the addition of CO. Gas atmospheres and kanamycin were restored after 3 days of cultivation. Precultures were grown for 24 h in 100-ml baffled Erlenmeyer flasks with a culture volume of 20 ml containing succinate as the sole carbon source in all cases.

Growth of R. rubrum strains was determined by measuring the optical density of the culture broth at 680 nm. Cells were harvested by centrifugation at 4,000 × g for 20 min. Cultivations were carried out in triplicate over the course of at least two separate experiments.

Construction of vectors and generation of recombinant strains of E. coli and R. rubrum. Methods comprising the amplification, isolation, and manipulation of DNA were carried out as described by Sambrook et al. (21). Respective DNA fragments were amplified from the genomic DNA of R. rubrum S1 and P. putida KT2440 using the Phusion high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA) and the oligonucleotides listed in Table 1. T4 DNA ligase (Thermo Scientific, Waltham, MA, USA) was applied for ligating amplified and digested DNA fragments. For validation, replication, and isolation of the generated constructs, chemically competent E. coli TOP10 cells were transformed (23), and the resulting strains were verified by colony PCR as well as isolation and digestion of the generated vectors with restriction endonucleases. Applying the protocol of Aneja et al. (24), the verified hybrid plasmids were transferred into strains of R. rubrum via electroporation.

In order to yield the expression vector pBBR1MCS-2::phaG::phaC::PP_0763, the empty pBBR1MCS-2 was initially digested with HindIII and BamHI and ligated with a likewise digested pchaC1 fragment. An Xhol/HindIII-digested pchaG fragment was then inserted into the correspond-
ΔphaC1 ΔphaC2 ΔphaZ2C176A mutants were confirmed by sequencing of phaZ2 genes with the same primers used for amplifying the fragments.

In an alternate approach, phaZ2_R. rubrum was deleted. For this, flanking regions of 403 and 635 bp up- and downstream of phaZ2 were amplified by PCR, adding XbaI and PstI sites to the fragments. Upon cleavage with PstI, ligation, and amplification of the fused ΔphaZ2 fragment, the fragment was digested with XbaI and ligated into a similarly linearized pQ2000mp18. Then, deletion of phaZ2 in R. rubrum ΔphaC1 ΔphaC2 through homologous recombination was carried out as described above. Markerless gene deletion was confirmed by PCR analysis of the phaZ2 region, using internal and flanking primers of phaZ2 (Table 1).

Analysis of protein patterns. Cells were lysed by sonication, and the cell extract was separated from the cell debris by centrifugation (10 min, 13,000 × g, 4°C). Upon measuring the protein concentration (26), 40 µg of protein was mixed with denaturing buffer, incubated for 10 min at 95°C, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli (27), and stained with Coomassie brilliant blue R-250.

Isolation and determination of PHA. In order to determine the PHA content of cells, 5 to 10 mg of freeze-dried cell matter was methanolyzed, and the resulting methylesters were identified and quantified by gas chromatography (GC) and GC-mass spectrometry, as described previously (28). PHA was isolated from cells with chloroform in a Soxhlet apparatus. Upon the evaporation of excess chloroform, PHA was precipitated in 20 volumes of ice-cold methanol. The precipitate was dissolved in chloroform and once more precipitated in methanol. The isolate was again dissolved in chloroform and cast in a glass petri dish to dry. Hydroxyalkanoic acids were identified and quantified with standards provided by Bioplas-tech (Dublin, Ireland). For the observation of PHA granules under the fluorescence microscope, cells were stained with Nile red (29).

Characterization of PHA. The molecular mass and the molecular mass/number-average molar mass (Mn) of isolated PHA were determined by gel permeation chromatography (GPC) with a PLgel 5-µm Mixed-C plus PLgel column (PerkinElmer, Waltham, MA, USA) and a PELV 290 UV-Vis detector operating at 254 nm. Samples were injected at a concentration of 1% (wt/vol) in volumes of 500 µL. Spectroscopy-grade chloroform was used as the mobile phase at a flow rate of 1.0 ml min⁻¹. Molecular weights were calculated using a standard curve, which was prepared with polystyrene samples of low polydispersity.

Differential scanning calorimetry (DSC) was carried out to determine the melting temperature (Tm) and glass transition temperature (Tg) of isolated PHA. For this, a PerkinElmer (Waltham, MA, USA) Pyris-Diamond calorimeter calibrated to Indium standards was used. Samples were heated in hermetically sealed aluminum pans from −90 to 100°C at 10°C · min⁻¹. The Tm was obtained by holding the sample at 100°C for 1 min and rapidly lowering the temperature to −90°C. To determine the Tg, samples were then reheated from −90 to 100°C.

RESULTS

PHA accumulation in cells of engineered R. rubrum strains from different feedstocks. As R. rubrum is capable of converting carbon monoxide and carbon dioxide to acetyl-CoA, the goal was to establish a synthetic pathway from this central metabolite to PHA (Fig. 1). Furthermore, the natural synthesis of PHA in R. rubrum had to be inhibited in order to synthesize solely PHA in a recombinant R. rubrum strain. For this, the phaC1 and phaC2 genes coding for the two PHA synthases in this bacterium, which are essential for the polymerization of 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA, were deleted. This resulted in a R. rubrum ΔphaC1 ΔphaC2 strain incapable of synthesizing PHA (30, 31). Applying the findings of previous studies, three genes of the known PHA producer P. putida KT2440 were cloned into the broad-host-range expression vector pBRR1MCS-2 and overexpressed in R. rubrum ΔphaC1 ΔphaC2: (i) phaG, whose product was originally described as a 3-hydroxyacyl-ACP-CoA transferase (4) but recently identified as a 3-hydroxyacyl-ACP thioesterase (49); (ii) the PP_0763 gene, predicted to code for an MCL fatty acid-CoA ligase (5); and (iii) phaC1, which codes for a PHA synthase with a high specificity for C₆ to C₁₄ substrates (18). Additionally, a CO-inducible promoter from R. rubrum itself (19) was applied to potentially establish a CO-mediated induction of gene expression of vector-bond heterologous genes and PHA synthesis. Upon cloning of the respective DNA fragments into the expression vector, R. rubrum ΔphaC1 ΔphaC2 was transformed with the two generated plasmids and, as a control, with the empty pBRR1MCS-2 vector. With the medium described above, the recombinant strains were cultivated in (i) an artificial syngas atmosphere resembling a possible composition of actual synthesis gas (by volume) 40% CO, 40% H₂, 10% CO₂, and 10% N₂ (black) or of 40% CO and 60% N₂ (gray) for 5 days. Optical densities of samples, whose standard deviations are represented by error bars, were measured at 680 nm. d, days.

FIG 2 Cultivation of strains of R. rubrum ΔphaC1 ΔphaC2, harboring pBRR1MCS-2 (empty vector, triangles), pBRR1MCS-2::phaG::phaC1::PP_0763 (circles), or pBRR1MCS-2::P_coe::phaG::phaC1::PP_0763 (squares). Cells were cultivated in medium modified from Bose et al. (22), with an artificial syngas atmosphere of (by volume) 40% CO, 40% H₂, 10% CO₂, and 10% N₂ (black) or of 40% CO and 60% N₂ (gray) for 5 days. Optical densities of samples, whose standard deviations are represented by error bars, were measured at 680 nm. d, days.
under the control of the pBBR1MCS-2 standard P\textsubscript{lac} promoter, which cannot be induced in R. rubrum but shows basal activity without induction of isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) or lactose that cannot enter the cell. This was reflected in the specific growth rates of the strain carrying pBBR1MCS-2-P\textsubscript{cooF}::phag::phaC1::PP\textsubscript{0763} (syngas, 0.024 \(\text{h}^{-1} \pm 0.003 \text{ h}^{-1}\); CO, 0.020 \(\text{h}^{-1} \pm 0.004 \text{ h}^{-1}\), which were decreased by approximately 20% compared to R. rubrum \(\Delta\text{phaC1} \Delta\text{phaC2}\) harboring the empty pBBR1MCS-2 harboring the empty pBBR1MCS-2 (syngas, 0.029 \(\text{h}^{-1} \pm 0.005 \text{ h}^{-1}\); CO, 0.027 \(\text{h}^{-1} \pm 0.006 \text{ h}^{-1}\)) and 25% compared to R. rubrum \(\Delta\text{phaC1} \Delta\text{phaC2}\) harboring the empty pBBR1MCS-2 harboring the empty pBBR1MCS-2 (syngas, 0.029 \(\text{h}^{-1} \pm 0.003 \text{ h}^{-1}\); CO, 0.030 \(\text{h}^{-1} \pm 0.004 \text{ h}^{-1}\)). By means of an unpaired \(t\) test, the difference in impacts on growth between pBBR1MCS-2-PP\textsubscript{cooF}::phag::phaC1::PP\textsubscript{0763} and pBBR1MCS-2 could be regarded as significant (\(P < 0.05\)), at least when pure CO was applied in the gas phase of flasks. In contrast, all three strains grew similarly when cultivated with fructose as the main source of carbon (data not shown). Gas chromatographic analysis revealed that the recombinant strains of R. rubrum harboring the three genes of P. putida KT440 synthesized a polymer, which solely consisted of MCL 3-hydroxyalkanoic acids, from all of the given unrelated carbon sources: syngas, carbon monoxide, and fructose (Table 2). In contrast, R. rubrum \(\Delta\text{phaC1} \Delta\text{phaC2}\) harboring pBBR1MCS-2 harboring the empty pBBR1MCS-2 harboring the empty pBBR1MCS-2 remained PHA\textsubscript{SCL} and PHA\textsubscript{MCL} negative. The predominant constituents of the synthesized polymer [poly(3HD-co-3HO)] were 3-hydroxyoctanoic acid (3HO) and 3-hydroxydecanoic acid (3HD). When cultivated with fructose or artificial syngas, both of the strains harboring phaG, phaC1, and PP\textsubscript{0763} from P. putida incorporated also traces of 3-hydroxyhexanoate (3HH) into the polymer, which, however, accounted for less than 0.2 mol%. Although the P\textsubscript{cooF} promoter had a negative effect on the optical density, indicating slower growth when cultivated in the presence of CO, the strain carrying pBBR1MCS-2-PP\textsubscript{cooF}::phag::phaC1::PP\textsubscript{0763} showed the highest production of PHA\textsubscript{SCL}, under these conditions, synthesizing the heteropolymer to 6.7% (wt/wt) of the cell dry weight when cultivated in a syngas atmosphere. This was a significant increase compared to cultivations of this strain with fructose or to the other strains cultivated in the presence of any carbon source (Table 2). Moreover, the applied P\textsubscript{cooF} affected the composition of the polymer synthesized by the respective strain, as the 3HO fraction of the accumulated poly(3HD-co-3HO) was significantly increased to 42.8 mol% (Syngas) and 48.2 mol% (CO) compared to the polymer synthesized upon heterologous gene expression via the pBBR1MCS-2 standard P\textsubscript{lac} promoter (syngas, 31.8 mol% 3HO; CO, 33.5 mol% 3HO). An SDS-PAGE of samples taken from syngas cultures after 2 days of growth showed a distinct protein spot at 29 kDa for the P\textsubscript{cooF}–harboring recombinant R. rubrum strain, similar to the previously purified PhaG (4). This suggested a strong overexpression of phaG in this strain compared to the strain expressing the genes under the control of the P\textsubscript{lac} promoter and the strain carrying the empty vector (Fig. 3).

Microscopic observation of strain R. rubrum \(\Delta\text{phaC1} \Delta\text{phaC2}\) (pBBR1MCS-2-PP\textsubscript{cooF}::phag::phaC1::PP\textsubscript{0763}) revealed the presence of 2 to 4 granules in many of the cells (Fig. 4). They appeared to be smaller than the 6 to 8 granules which were displayed by the PHA\textsubscript{SCL}–accumulating wild-type strain R. rubrum S1 harboring

### Table 2 CDW and PHA accumulation of recombinant strains of R. rubrum \(\Delta\text{phaC1} \Delta\text{phaC2}\) cultivated with different carbon sources

<table>
<thead>
<tr>
<th>R. rubrum (\Delta\text{phaC1} \Delta\text{phaC2}) recombinant strain with:</th>
<th>Carbon source</th>
<th>CDW (g/liter)</th>
<th>PHA (wt%)</th>
<th>3HO (mol%)</th>
<th>3HD (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phaG::phaC1::PP\textsubscript{0763}</td>
<td>Syngas mixture</td>
<td>1.1 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>31.8 ± 1.1</td>
<td>68.1 ± 1.1</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>33.5 ± 1.5</td>
<td>66.5 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>3.6 ± 0.2</td>
<td>2.4 ± 0.4</td>
<td>30.1 ± 0.9</td>
<td>69.8 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>P\textsubscript{cooF}::phag::phaC1::PP\textsubscript{0763}</td>
<td>Syngas mixture</td>
<td>1.0 ± 0.0</td>
<td>6.7 ± 0.8</td>
<td>42.8 ± 1.5</td>
<td>57.0 ± 1.5</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>0.8 ± 0.1</td>
<td>4.9 ± 0.3</td>
<td>48.2 ± 2.8</td>
<td>51.8 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>3.5 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>31.3 ± 1.7</td>
<td>68.6 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Empty vector</td>
<td>Syngas mixture</td>
<td>1.2 ± 0.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>1.1 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>3.8 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*Cells were cultivated in medium modified from the formulation of Bose et al. (22) with an artificial syngas atmosphere (40% CO, 40% H\textsubscript{2}, 10% CO\textsubscript{2}, 10% N\textsubscript{2}), carbon monoxide (40% CO, 60% N\textsubscript{2}), or 1% (wt/vol) fructose. 3HO and 3HD contents were measured by GC analysis of dried cell matter from samples taken after 5 days of gas cultivation or after 2 days when cultivated with fructose. Standard deviations of measurements are indicated. ND, not detected.
the empty vector. As expected, cells of the PHA<sub>SL</sub>-negative strain <i>R. rubrum</i> ΔphaC1 ΔphaC2 did not contain any granules. Additionally, strains of <i>R. rubrum</i> ΔphaC1 ΔphaC2 appeared shorter than the spiral-shaped wild-type cells, measuring a length of approximately 4 μm in comparison to the length of 6 to 8 μm of the wild-type cells.

**Analysis of PHA<sub>MCL</sub> degradation in engineered <i>R. rubrum</i> strains.** As polymer degradation by producing strains is crucial for the efficiency of PHA production processes, the ability of a recombinant strain of <i>R. rubrum</i> to degrade the PHA<sub>MCL</sub>, that it was genetically engineered to synthesize was examined. Furthermore, mutagenesis experiments of the <i>R. eutropha</i> gene in <i>R. rubrum</i> were carried out to study the effect of a putatively intracellular PHA<sub>SL</sub> depolymerase (PhaZ2) on PHA<sub>MCL</sub> accumulation (PhaZ2) on PHA<sub>MCL</sub> accumulation.

Most probably, there are three PHA depolymerases (PhaZ1, PhaZ2, and PhaZ3) encoded in the genome of <i>R. rubrum</i>. PhaZ1 was shown to be located in the cell’s periplasm and is unlikely physically involved in <i>in vivo</i> polyhydroxybutyrate (PHB) degradation (20). In <i>in vitro</i> studies on the gene product of phaZ3 showed that this PHA depolymerase was specific for amorphous PHA<sub>SL</sub> but was predicted to be inactive at cytoplasm-typical concentrations of divalent cations, such as Mg<sup>2+</sup> (32). In contrast, PhaZ2 shows significant homologies to the well-characterized intracellular PHA<sub>SL</sub> depolymerase of <i>Ralstonia eutropha</i> (PhaZ1) and was therefore predicted to be responsible for intracellular PHA degradation in <i>R. rubrum</i> (20, 32, 33). Thus, phaZ2 (locus tag Rru_A3356) was chosen for (i) deletion in <i>R. rubrum</i> ΔphaC1 ΔphaC2, as well as (ii) site-directed mutagenesis leading to an amino acid exchange in the putative active site of PhaZ2. The putative active site of PhaZ2<sub>R. rubrum</sub> was identified by a sequence alignment with the known PHA depolymerase PhaZ1<sub>R. eutropha</sub> whose catalytic triad was identified by Kobayashi and Saito (34). Using the BLAST algorithm (35), a catalytic triad of cysteine (C176), aspartate (D349), and histidine (H382) was identified for PhaZ2<sub>R. rubrum</sub> (see Fig. S2 in the supplemental material). Therefore, an exchange of cysteine for alanine at amino acid position 176 would possibly inhibit the thiolyzing activity of PhaZ2 and disturb the binding of potential PhaZ2 isoenzymes to the PHA granule surface but still allow putative protein-protein interactions.

Upon generation of the mutants <i>R. rubrum</i> ΔphaC1 ΔphaC2 ΔphaZ2 and <i>R. rubrum</i> ΔphaC1 ΔphaC2 ΔphaZ2<sub>C176A</sub>, these strains were transformed with pBBR1MCS-2-<i>phaG</i>:<i>phaC1</i>:<i>PP_0763</i> as this construct led to the largest amounts of synthesized PHA<sub>MCL</sub> in the foregoing cultivations. To compare the PHA accumulation and degradation of the generated strains with the phaZ2 wild-type genotype, the three strains were cultivated for 5 days in artificial syngas atmosphere, which proved to be the most suitable feedstock (Table 2), as described above. In order to stimulate the potential degradation of synthesized polymer by limiting available carbon, the syngas was replaced by a nitrogen atmosphere, and the cultures were incubated for two additional days. The PHA contents were measured from 20-ml samples, which were obtained prior to and after the gas exchange. The three cultivated engineered strains of <i>R. rubrum</i> grew equally and showed optical densities which were similar to those of the foregoing cultivations with artificial syngas (Fig. 2). GC analyses of cell material obtained from the three cultivated strains revealed that the PHA<sub>MCL</sub> contents of the recombinant strains of <i>R. rubrum</i> had decreased over 2 days of carbon-limited cultivation, suggesting partial polymer degradation. In the remaining polymer, the fractions of 3HO and 2 days of carbon-limited cultivation, suggesting partial polymer degradation. In the remaining polymer, the fractions of 3HO and 3HD occurred in a similar ratio as in the sample taken prior to carbon starvation. The deletion of phaZ2<sub>R. rubrum</sub> did not have a positive effect on the accumulation of PHA<sub>MCL</sub>, and did not result in a reduced degradation of the polymer, compared to the strain carrying the wild-type phaZ2. Although the strain <i>R. rubrum</i> ΔphaC1 ΔphaC2 ΔphaZ2<sub>ΔcooF</sub> carrying pBBR1MCS-2-<i>phaG</i>:<i>phaC1</i>:<i>PP_0763</i> accumulated slightly more PHA (7.1% ± 0.5% [wt/wt] cell dry weight [CDW]) than the strain carrying the wild-type phaZ2 (6.5% ± 0.3%), significantly reduced degradation of the polymer was not observed either, as approximately 21% of the accumulated polymer was degraded within 2 days without carbon supply (Fig. 5). This was slightly less than that measured for the two other tested strains harboring the plasmid (R. rubrum ΔphaC1 ΔphaC2, 24% degradation; R. rubrum ΔphaC1 ΔphaC2 ΔphaZ2, 25% degradation).

**Isolation and analysis of PHA<sub>MCL</sub> derived from syngas cultivations.** In order to isolate PHA<sub>MCL</sub>, which is synthesized from CO<sub>2</sub> and CO<sub>2</sub>-containing syngas, cells of the strain accumulating the largest amount of PHA<sub>MCL</sub> over the course of the conducted cultivation experiments were chosen. This strain was <i>R. rubrum</i> ΔphaC1 ΔphaC2 ΔphaZ2<sub>ΔcooF</sub> carrying pBBR1MCS-2-<i>phaG</i>:<i>phaC1</i>:<i>PP_0763</i>. To obtain a sufficient amount of cell mass,
Moreover, calorimetric analysis determined a melting point (10% CO2, and 10% N2 for 5 days and for a further 2 days in pure N2. Optical of 49.6°C and a glass transition temperature (91 mg of synthesized polymer was isolated from 2.9 g of dried cells that were harvested from a total of 3 liters of culture broth. The precipitated and dried polymer appeared as a gluey rubber-like material (see Fig. S3 in the supplemental material). GC analysis confirmed the composition that was previously determined from dried cell mass, as the polymer-isolate fractions were argued that any polymer synthesized in these cultivations resulted from CO and CO2 dissolved in the medium, as the recombinant strains did not synthesize any PHA from the applied medium when syngas or pure CO was omitted.

Application of the P_{pahG} promoter showed that the level of heterologous expression, especially of pahG, as indicated by the protein patterns of strains (Fig. 3), was crucial in terms of the amount of PHA precursors that was subtracted from fatty acid synthesis for PHA formation (Table 2). Interestingly, this also had an impact on the constituent fractions of the synthesized polymer (Table 2), as higher quantities of PhaG molecules presumably resulted in an earlier withdrawal of 3-hydroxyacyl-ACP from the fatty acid de novo synthesis pathway. This may have caused the increased incorporation of 3H0. The regulation of heterologous gene expression, other than RubisCO, which play a major role in the fixation of CO2 in R. rubrum when acetate is present in the cultivation medium but not with malate. Therefore, the use of another citric acid cycle intermediate, such as succinate, as an auxiliary carbon source should likewise not lead to an upregulation of the acetate assimilation route ethylmalonyl-CoA pathway or acetyl-CoA carboxylation via PFOR (Fig. 1). Thus, available CO2 is likely to be assimilated via the Calvin cycle or, alternatively, by a 2-oxoglutarate synthase via the reductive citric acid cycle (Fig. 1) (16).

Further cultivations with recombinant PHA_{MCL}-Producing strains of R. rubrum showed that the strains were able to partially degrade the synthesized polymer (Fig. 5), regardless of a mutated pahZ2R. rubrum PhaZ2, described as an intracellular PHA depolymerase (20), therefore likely acts specifically on PHA_{MCL} which are synthesized by wild-type R. rubrum. The partial degradation of the accumulated polymer might be attributed to the activity of a lipase present in R. rubrum, as these enzymes have been shown to degrade the synthesized polymer (Fig. 5), regardless of a mutated pahZ2R. rubrum PhaZ2, described as an intracellular PHA depolymerase (20), therefore likely acts specifically on PHA_{MCL} which are synthesized by wild-type R. rubrum.
degrade PHAs of various chain lengths as well (44). Nevertheless, the phaZZC
176A mutation, leading to an amino acid exchange in the therefore-inactive catalytic site of the enzyme, caused a slight increase in accumulated PHAMCL, possibly due to hitherto-unknown interactions on the PHA granule surface.

The isolated poly(3HD-co-3HO) exhibited properties similar to those of PHA produced from P. putida. The detected molecular mass of 124.3 kDa was in the range of that of PHAMCL (co-SCL) produced by different recombinant strains in previous studies (3, 45–47), but was lower than PHAMCL commonly produced by the Pseudomonas wild-type strains (2, 48). The quantities of PhaC molecules, which catalyze polymerization, have been shown to be related to the number of polymer chains and consequently influence the individual molecular weight of each chain. Therefore, further genetic engineering leading to a reduced expression of heterologous phaC1, or even a complete absence of PhaC, could increase the molecular mass of the synthesized polymer (49).

Additional metabolic engineering, leading, e.g., to an enhanced flux of acetyl-CoA, derived from CO and CO2 assimilation, to fatty acid synthesis, and foremost, a scale-up that optimizes the cultivation conditions and increases gas-liquid mass transfer, would most likely result in a higher productivity of the process. Moreover, the approach of activating gene expression in the recombinant R. rubrum strain with a CO-inducible promoter could be applied in a potential two-step process, in which an aerobic and heterotrophic cultivation phase to gain high cell density could be followed by an anaerobic syngas-mediated PHAMCL production phase. By this, the relatively slow cell growth with CO or CO2, as sources of energy and carbon would be countered. A further alternative process modification might be cultivation in the presence of light, which has been shown to promote growth of the phototrophic R. rubrum under various conditions (50). However, due to the intracellular accumulation of PHA, high cell density fermentation in a large vessel volume is required for productivity. This, along with the need for high hydrostatic pressure to maximize gas solubility, would make the use of, e.g., a loop reactor unpractical in most potential scale-up approaches.

This study has demonstrated in principle the possibility of synthesizing PHA from the renewable syngas feedstock synthesis pathway. The results presented here contribute to the goal of extending the potential applications of second-generation biofuels from toxic (CO) and greenhouse (CO2) waste gases, which do not compete with human nutrition, by completely altering the monomer composition of PHA produced by the industrially promising bacterium Rhodospirillum rubrum.

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ulinar” poly(3-hydroxybutyrate (PHB) depolymerase of Rhodospirillum


