Appl Microbiol Biotechnol
DOI 10.1007/s00253-013-5490-9

Biosynthesis of polyhydroxyalkanoate copolymers from methanol by *Methylobacterium extorquens* AM1 and the engineered strains under cobalt-deficient conditions

Izumi Orita · Kouta Nishikawa · Satoshi Nakamura · Toshiaki Fukui

Received: 19 November 2013 / Accepted: 20 December 2013

Abstract *Methylobacterium extorquens* AM1 has been shown to accumulate polyhydroxyalkanoate (PHA) composed solely of (*R*)-3-hydroxybutyrate (3HB) during methylotrophic growth. The present study demonstrated that the wild-type strain AM1 grown under Co^{2+}-deficient conditions accumulated copolyesters of 3HB and a C_5-monomer, (*R*)-3-hydroxyvalerate (3HV), using methanol as the sole carbon source. The 3HV unit was supposed to be derived from propionyl-CoA, synthesized via the ethylmalonyl-CoA pathway impaired by Co^{2+} limitation. This assumption was strongly supported by the dominant incorporation of the 3HV unit into PHA when a strain lacking propionyl-CoA carboxylase was incubated with methanol. Further genetic engineering of *M. extorquens* AM1 was employed for the methylotrophic synthesis of PHA copolymers. A recombinant strain of *M. extorquens* AM1C_{Ac} in which the original PHA synthase gene $phaC_{Me}$ had been replaced by $phaC_{Ac}$, encoding an enzyme with broad substrate specificity from *Aeromonas caviae*, produced a PHA terpolymer composed of 3HB, 3HV, and a C_6-monomer, (*R*)-3-hydroxyhexanoate, from methanol. The cellular content and molecular weight of the PHA accumulated in the strain AM1C_{Ac} were higher than those of PHA in the wild-type strain. The triple deletion of three PHA depolymerase genes in *M. extorquens* AM1C_{Ac} showed no significant effects on growth and PHA biosynthesis properties. Overexpression of the genes encoding β-ketothiolase and NADPH-acetoacetyl-CoA reductase increased the cellular PHA content and 3HV composition in PHA, although the cell growth on methanol was decreased. This study opens up the possibility of producing practical PHA copolymers with methylotrophic bacteria using methanol as a feedstock.

Keywords Methanol · Polyhydroxyalkanoate · Ethylmalonyl-CoA pathway · *Methylobacterium extorquens* AM1 · Methylotroph

Introduction

Methanol is an important carbon feedstock in both the chemical and biological industries due to its low cost and sustainability (Schrader et al. 2009), because it can be produced not only from abundant natural gas but also from biomass and carbon dioxide (Olah 2005). Additionally, methanol is liquid under normal environmental conditions, which makes it easy to handle. Methylotrophic bacteria, which are capable of using reduced C_1 compounds such as methanol and methylamine as carbon and energy sources, have the potential to convert C_1 compounds into various useful chemicals and materials. Polyhydroxyalkanoate (PHA) is one such material and is produced by many microbes, including several methylotrophic bacteria. As PHAs are biodegradable polyesters that can be produced from renewable carbon sources (Madison and Huisman 1999; Verlinden et al. 2007), they have attracted attention as possible alternatives to conventional petrochemical-based plastics. Therefore, the application of methylotrophic bacteria is viewed as a powerful way for the microbial production of PHAs from methanol as an inexpensive, nonfood-competitive, and sustainable carbon feedstock.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-5490-9) contains supplementary material, which is available to authorized users.

I. Orita · K. Nishikawa · S. Nakamura · T. Fukui (✉)
Department of Bioengineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan
e-mail: tfukui@bio.titech.ac.jp

Published online: 16 January 2014

Methylotrophic bacteria employ one of two pathways, the serine cycle and the ribulose monophosphate (RuMP) pathway, for the assimilation of C_1 compounds. Several serine cycle-type methylotrophic bacteria have been shown to accumulate poly[(*R*)-3-hydroxybutyrate] [P(3HB)], the most representative natural PHA, from methanol (Follner et al. 1997; Korotkova and Lidstrom 2001). Interestingly, there has been no previous report of PHA production by wild strains of RuMP pathway-type methylotrophic bacteria so far (Follner et al. 1997; Korotkova and Lidstrom 2001). The best-studied serine cycle-type methylotrophic bacterium is *Methylobacterium extorquens* AM1, a pink-pigmented facultative methylotrophic α-proteobacterium whose complete genome sequence has been determined (Vuilleumier et al. 2009). Recent research has revealed that a newly identified ethylmalonyl-CoA (EMC) pathway functions in the essential regeneration of glyoxylate, an acceptor of the methylene group derived from the C_1 compounds in the serine cycle, from acetyl-CoA during the methylotrophic growth of *M. extorquens* (Peyraud et al. 2009). The EMC pathway contains many CoA-thioester intermediates, which are possible biologically relevant products (Alber 2011), and the initial two steps that convert two acetyl-CoA molecules to (*R*)-3HB-CoA are shared with the P(3HB) biosynthesis pathway (Fig. 1) (Korotkova and Lidstrom 2001; Alber et al. 2006; Alber 2011). In 2009, the importance of Co^{2+} to the methylotrophy of *M. extorquens* AM1 was reported by two groups (Chou et al. 2009; Kiefer et al. 2009). Chou et al. (2009) found that the improved cobalt uptake phenotype of *M. extorquens* mutants was caused by increased expression of *icuAB* encoding a novel cobalt transporter. It was suggested that the reduced growth ability of *M. extorquens* AM1 in metal-deficient media was attributed to shortage of adenosylcobalamin which was an essential cofactor for two vitamin B_{12}-dependent mutases, methylmalonyl-CoA mutase and ethylmalonyl-CoA mutase, in the EMC pathway. Kiefer et al. (2009) reported plasmid-induced cobalt limitation during the methylotrophic growth of *M. extorquens* of AM1. The metabolic profiling revealed significantly larger pools of ethylmalonyl-CoA and methylmalonyl-CoA in the Co^{2+}-deficient cells, probably reflecting the reduced function of the two B_{12}-dependent mutases in the EMC pathway. However, the effects of the Co^{2+} deficiency on PHA biosynthesis by *M. extorquens* have not been considered so far.

The range of the application for P(3HB) is very limited due to the stiffness and brittleness caused by the high crystallinity and low elongation before breaking. Many efforts have been made to overcome the poor mechanical properties of P(3HB) by the copolymerization of 3HB with different hydroxyalkanoate monomer(s). The initial strategy was the addition of precursor compounds that were structurally related to the desired comonomer units into the culture medium (Steinbuchel and Valentin 1995). For example, the biosynthesis of poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate] [P(3HB-*co*-3HV)] was achieved by the addition of propionate, pentaoate, or *n*-pentanol (Steinbuchel and Valentin 1995; Ueda et al. 1992). However, the addition of such precursors often leads to higher production costs, thereby biosynthesis of PHA copolymers from inexpensive biomass resources is obviously preferable. Several Gram-positive bacteria belonging to the genera *Nocardia*, *Corynebacterium*, and *Rhodococcus* are known to naturally synthesize P(3HB-*co*-3HV) from

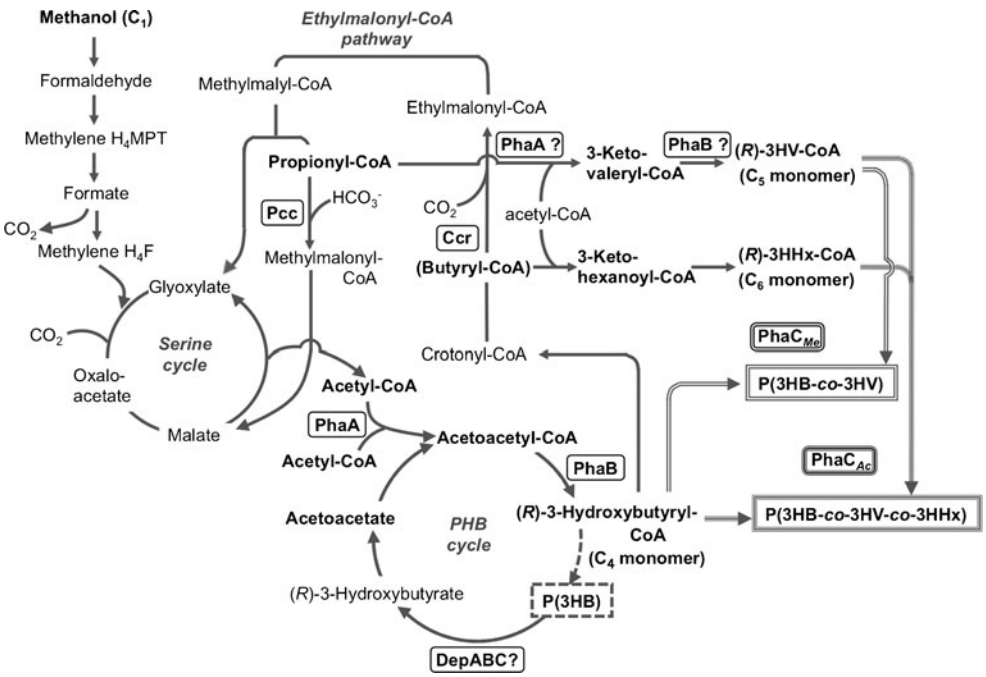

Fig. 1 Proposed pathways for PHA synthesis from methanol in *M. extorquens* AM1 and AM1C_{Ac} under Co^{2+}-deficient conditions. *Arrows with double* and *triple lines* represent polymerization by PHA synthase from *M. extorquens* AM1 (PhaC$_{Me}$) and that by PHA synthase from *A. caviae* (PhaC$_{Ac}$), respectively. *Dotted arrow* represents polymerization by PhaC$_{Me}$ under Co^{2+}-sufficient conditions. *PhaA*, β-ketothiolase; *PhaB*, NADPH-acetoacetyl-CoA reductase; *DepA*, *DepB*, and *DepC*, PHA depolymerases; *Pcc*, propionyl-CoA carboxylase; *Ccr*, crotonyl-CoA reductase/carboxylase

monosaccharides such as glucose (Valappil et al. 2007). In *Nocardia corallina*, propionyl-CoA produced through the methylmalonyl-CoA pathway is condensed with acetyl-CoA and subsequently reduced to (*R*)-3HV-CoA, which is then copolymerized with (*R*)-3HB-CoA by PHA synthase (Valentin and Dennis 1996). Alternatively, recent advances in metabolic engineering have allowed us to construct recombinant strains capable of PHA copolymer biosynthesis from single carbon sources; the synthesis of PHAs containing (*R*)-3HV, 4-hydroxybutyrate, medium chain length (*R*)-3-hydroxyalkanoates, or lactate from glucose by recombinant strains of *Escherichia coli* (Matsumoto and Taguchi 2013; Wang et al. 2013). We have previously engineered *Ralstonia eutropha*, a well-studied PHA producer, to convert plant oils into poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] [P(3HB-*co*-3HHx)] (Mifune et al. 2008; Mifune et al. 2010; Kawashima et al. 2012), as well as to convert fructose into P(3HB-*co*-3HHx) and poly((*R*)-3-hydroxybutyrate-*co*-3-hydroxypropionate) copolymers through artificial monomer supplying pathways (Fukui et al. 2002, 2009).

The present study focused on PHA copolymer biosynthesis by *M. extorquens* using methanol as the sole carbon source. We demonstrated that *M. extorquens* AM1 had a natural ability to synthesize P(3HB-*co*-3HV) copolymer during methylotrophic growth under Co^{2+}-deficient conditions and provided strong evidence that propionyl-CoA, the precursor of the 3HV unit, was supplied via the EMC pathway. Further engineering of *M. extorquens* AM1 constructed a strain capable of synthesizing P(3HB-*co*-3HV-*co*-3HHx) terpolymer from methanol. These results indicated the potential of methylotrophic bacteria in methanol biotechnology for the production of useful plastic materials.

Materials and methods

Bacterial strains and culture condition

E. coli DH5α (Clontech) was grown at 37 °C in a Luria-Bertani (LB) medium, and ampicillin (50 mg/l) or kanamycin (100 mg/l) was added to the medium when necessary. *M. extorquens* AM1 (a kind gift from Prof. M. E. Lidstrom) (Peel and Quayle 1961) and the derivative strains were grown on a hypho minimal medium (Okubo et al. 2007) in a 500-ml flask with reciprocal shaking (115 strokes/min). One milliliter of the trace element solution was added into the 1-l medium, and the standard composition of the trace element solution used in this study was 12.7 g of $Na_2EDTA{\cdot}2H_2O$, 4.4 g of $ZnSO_4{\cdot}7H_2O$, 1.47 g of $CaCl_2{\cdot}2H_2O$, 1.01 g of $MnCl_2{\cdot}4H_2O$, 0.998 g of $FeSO_4{\cdot}7H_2O$, 0.22 g of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.314 g of $CuSO_4{\cdot}5H_2O$, and 0.322 g of $CoCl_2{\cdot}6H_2O$ in 1 l of milli-Q water. The EDTA-free trace element solution was prepared at the time of use because metal precipitates were formed during the preservation at 4 °C. When necessary, the trace element solutions with different concentrations of $CoCl_2{\cdot}6H_2O$ were prepared and added into the medium. The cell growth was monitored with optical density (OD) at 600 nm. The standard cultivation on methanol was carried out as follows: *M. extorquens* strains were precultured in a 50-ml hypho minimal medium containing 0.5 % (*w*/*v*) succinate at 30 °C for 2 days. The cells were harvested by centrifugation at 8,000×*g* for 5 min at room temperature and resuspended in a fresh hypho minimal medium not containing carbon source nor trace element to adjust OD_{600} to 10. One milliliter of the resuspended cells was inoculated into a 100-ml hypho medium containing 0.5 % (*v*/*v*) methanol with the initial OD_{600} of 0.1, and the cultivation was carried out at 30 °C for 72 h. In the case of two-stage cultivation, *M. extorquens* strains were firstly cultivated in a 100-ml hypho medium containing 0.5 % (*w*/*v*) succinate at 30 °C for 48 h. The cells after the first-stage cultivation were harvested by centrifugation at 8,000×*g* for 10 min at room temperature and washed with 100 ml of a nitrogen-free fresh hypho minimal medium not containing carbon source. All of the cells were then transferred to a 100-ml nitrogen-free hypho minimal medium containing 0.5 % (*v*/*v*) methanol, and incubated at 30 °C for 48 h as the second-stage cultivation. Kanamycin (100 mg/l) was added to the medium for the strains harboring a vector.

Construction of *M. extorquens* mutants by homologous recombination

General genetic manipulations were performed according to the standard procedures. The bacterial strains and plasmids, and primers used in this study are shown in Tables S1 and S2, respectively. A vector pK18-ΔphaC_{Me}::phaC_{Ac}, used for replacement of original *phaC*$_{Me}$ on chromosome of *M. extorquens* AM1 by *phaC*$_{Ac}$ from *Aeromonas caviae*, was constructed as below. A DNA fragment containing *phaC*$_{Me}$ along with its flanking regions (approximately 800 bp each) was amplified from *M. extorquens* genomic DNA using a primer set phaC_{Me}_Eco-N/phaC_{Me}_Eco-C. The amplified fragment was inserted into pUC118 at *Hin*cII site. Inverse PCR was carried out using the resulting plasmid as a template and a primer set phaC_{Me}-Inv1/phaC_{Me}-Inv2 to amplify the flanking regions of *phaC*$_{Me}$ along with pUC118 backbone. The resulting fragment was ligated with a 5′-phosphorylated fragment of the *phaC*$_{Ac}$ coding region (without any upstream and downstream regions derived from *A. caviae*) which was amplified with pEE32d13 (Fukui and Doi 1997) and phaC_{Ac}-N/phaC_{Ac}-C as a template and a primer set, respectively. The fragment containing *phaC*$_{Ac}$ flanked to the upstream and downstream regions of *phaC*$_{Me}$ was excised by *Eco*RI and inserted into pK18mobsacB (Schafer et al. 1994) at the corresponding site to obtain pK18-ΔphaC_{Me}::phaC_{Ac}.

pK18-Δ$phaC_{Me}$::$phaC_{Ac}$ was introduced into *M. extorquens* AM1 by electroporation (Woodall 2003) using Gene Pulser Xcell™ Electroporation System (Bio-Rad). Kanamycin-resistant transformants formed by integration of the plasmid into the chromosome (pop-in strains) were selected on the plate of hypho minimal medium containing 0.5 % (*w*/*v*) succinate and 100 mg/l kanamycin. The selected transformants were then cultivated on a hypho minimal plate medium containing 5 % (*w*/*v*) sucrose, to obtain sucrose-resistant transformants formed by excision of the integrated plasmid region via the second recombination event (pop-out strains). The desired strain $AM1C_{Ac}$ harboring $phaC_{Ac}$ instead of $phaC_{Me}$ region was selected among the sucrose-resistant isolates by PCR analysis.

For deletion of *pccA* (Mex_1p3203) from the chromosome of *M. extorquens*, a fragment of the coding region along with its flanking regions (approximately 1,000 bp each) was amplified from *M. extorquens* genomic DNA with a primer set pccA_Xba-N/pccA_Xba-C. The amplified fragment was inserted into pUC118 at *Hin*cII site. The flanking regions along with pUC118 backbone, not containing the *pccA* coding region, were then amplified by inverse PCR with a primer set pccA-Inv1/pccA-Inv2. The amplified fragment was self-ligated and then digested with *Xba*I. The excised fragment was inserted into pK18mobsacB at the corresponding site. *M. extorquens* $AM1C_{Ac}$ΔpccA was obtained by pop-in and pop-out recombination by using the resulting plasmid pK18-ΔpccA and a host strain $AM1C_{Ac}$, according to the procedure mentioned above.

Recombinant vectors for deletion of *depA* (Mex_1p0419), *depB* (Mex_1p4597), or *depC* (Mex_1p4148) were also constructed in a similar way. A fragment of the coding region along with the flanking regions (approximately 1,000 bp each), amplified from *M. extorquens* genomic DNA, was digested using restriction enzymes of which recognition site had been added to the primer, and then inserted into pK18mobsacB at the corresponding sites. A vector not containing the coding region was prepared by inverse PCR and subsequent self-ligation. The primer sets for the first PCR were depA_Hind-N/depA_Xba-C, depB_Xba-N/depB_Eco-C, and depC_Hind-N/depC_Xba-C, and those for the inverse PCR were depA-Inv1/depA-Inv2, depB-Inv1/depB-Inv2, and depC-Inv1/depC-Inv2. The pop-in and pop-out recombination were performed with the resulting plasmids, pK18-ΔdepA, pK18-ΔdepB, or pK18-ΔdepC, for construction of three single deletants. Double deletants and a triple deletion strain ΔdepABC were constructed by sequential treatments with the vectors.

Construction of *phaA-phaB* expression vector

A broad host range expression vector pCM80Km, containing methanol dehydrogenase promoter from *M. extorquens* AM1, was constructed by replacement of antibiotic resistance gene in pCM80 (a kind gift from Prof. M. E. Lidstrom) (Marx and Lidstrom 2001). A linear fragment of plasmid backbone not containing the tetracycline resistance genes *tetA-tetR* was prepared by inverse PCR using pCM80 as a template and a primer set pCM80-Inv1/pCM80-Inv2. A kanamycin resistance gene cassette was amplified from pK18mobsacB using a primer set Km-Fw/Km-Rv and ligated with the inverse PCR product, to obtain pCM80Km. The coding regions for *phaA-phaB* (Mex_1p3700-1p3701) were amplified from *M. extorquens* genomic DNA using a primer set $phaA_{Me}$_Hind-N/$phaB_{Me}$_Xba-C. The amplified fragment was digested with *Hin*dIII and *Xba*I and inserted into pCM80Km at the corresponding sites, and the resulting vector was designated pCM80Km-phaAB.

Enzyme assay

M. extorquens AM1 and AMC_{Ac} cells grown on methanol at 30 °C for 72 h were washed and resuspended in 50 mM Tris-HCl (pH 7.5). The cells were disrupted by high pressure homogenization (20 kpsi) using an One Shot Model of Constant Cell Disruption System (Constant Systems, Northants, UK) and then centrifuged with low speed (1,500×*g*, 5 min, 4 °C) to obtain whole-cell extracts containing PHA granules. PHA synthase activity in the whole-cell extracts was spectrophotometrically determined with (*R*)-3HB-CoA and 5,5′-dithiobis(2-nitrobenzoic acid), as described previously (Valentin and Steinbuchel 1994).

PHA analyses

The cells after the cultivation were harvested and washed with distilled water, followed by lyophilization. The dried cells were then subjected to methanolysis in the presence of 15 % (*v*/*v*) sulfuric acid in methanol as described previously (Kato et al. 1996; Fukui and Doi 1997). Cellular PHA content and composition were determined by GC analysis with a GC-17A (Shimadzu, Kyoto, Japan) equipped with an InertCap 1 capillary column (30 m by 0.23 mm; GL Sciences, Tokyo, Japan) and a flame ionization detector. The methyl esters of 3-hydroxyalkanoate (3HA-Me) were also analyzed by gas chromatography-mass spectrometry with a GCMS-QC 2010 (Shimadzu, Kyoto, Japan) equipped with an InertCap 1 column.

The intracellular polyester was extracted from the dried cells by using chloroform with stirring for 72 h at room temperature, and the extract was filtrated with a PTFE membrane filter to remove the cell debris. The polymer fraction was then purified by precipitation in methanol and collected on a PTFE membrane filter. The ^{1}H NMR spectra of the purified polymers were recorded using a Varian 400-MR spectrometer in $CDCl_3$. The molecular weight and polydispersity were determined by gel permeation chromatography (GPC) using a Shimadzu 10A GPC system and a 10A

refractive index detector equipped with a serial of Shodex K-806 M and K-802 columns at 40 °C. Chloroform was used as the eluent at a flow rate of 0.8 ml/min. The calibration curve was generated using polystyrene standards with a low polydispersity (STANDARD SM-105, Shodex).

Results

Incorporation of 3HV unit into PHA synthesized from methanol by *M. extorquens* AM1 under cobalt-deficient conditions

In our examination of PHA biosynthesis by *M. extorquens* AM1 from methanol, we noticed that the Co^{2+} concentration in the medium markedly affected not only the methylotrophic growth rate as reported previously (Chou et al. 2009; Kiefer et al. 2009), but also the composition of PHA accumulated within the cells; very interestingly, the 3HV unit was detected in PHA synthesized from methanol by *M. extorquens* AM1 under cobalt-deficient conditions. Figure 2 shows the effects of Co^{2+} concentration on the growth rate of *M. extorquens* AM1 on methanol and the 3HV fraction in the accumulated PHA. As the over-chelation of Co^{2+} by EDTA, which was added into the trace element solution to prevent metal precipitation, has been reported to inhibit the methylotrophic growth of *M. extorquens* AM1 (Chou et al. 2009), we also investigated the effects of Co^{2+} concentration under an EDTA-free condition. The specific growth rate was increased markedly with the increase of Co^{2+} concentration within the range of 1.35–5.4 μM in the presence of EDTA and was saturated at higher concentration (Fig. 2a). As reported previously (Chou et al. 2009), poor growth was observed at much lower concentrations of Co^{2+} (1.35 pM–1.35 nM) in the absence of EDTA (Fig. 2b). The specific growth rates at the saturated Co^{2+} concentrations, both with and without EDTA, were comparable to each other (0.135 and 0.134 h^{-1}, respectively), although the rates obtained in this study were slightly lower than those reported previously (approximately 0.18 h^{-1}) (Chou et al. 2009; Kiefer et al. 2009).

The results also demonstrated that, regardless of the presence or absence of EDTA, 1–6 mol% of the 3HV unit was incorporated in the PHA on methanol when the growth rates were limited by the low Co^{2+} concentrations, although many previous studies have reported that this methylotroph accumulated only P(3HB) homopolymer when methanol was a sole carbon source (Barnard and Sanders 1989; Korotkova and Lidstrom 2001; Korotkova et al. 2002b). The 3HV fraction was larger at lower Co^{2+} concentrations that led to lower growth rates, but not detected under Co^{2+}-sufficient conditions with the saturated growth rate, indicating an inverse correlation between the 3HV composition and the growth rate.

PHA copolymer biosynthesis from methanol by a recombinant strain of *M. extorquens* harboring $phaC_{Ac}$ from *A. caviae*

A previous study demonstrated that *R. eutropha* PHB^{-}4 expressing the PHA synthase gene from *A. caviae* ($phaC_{Ac}$) and the crotonyl-CoA reductase/carboxylase (formerly crotonyl-CoA reductase) gene from *Streptomyces cinnamonensis* (ccr_{Sc}) synthesized PHA containing a small amount of 3HHx unit from structurally unrelated fructose (Fukui et al. 2002). In this recombinant strain, (*R*)-3HHx-CoA was supposed to be formed by the condensation of acetyl-CoA with the butyryl-CoA generated from crotonyl-CoA by Ccr_{Sc}, followed by (*R*)-specific reduction. Because Ccr is present in the EMC pathway as a key enzyme (Erb et al. 2007), the functional expression of a gene encoding PHA synthase with broad substrate specificity was expected to lead to incorporation of the 3HHx unit into

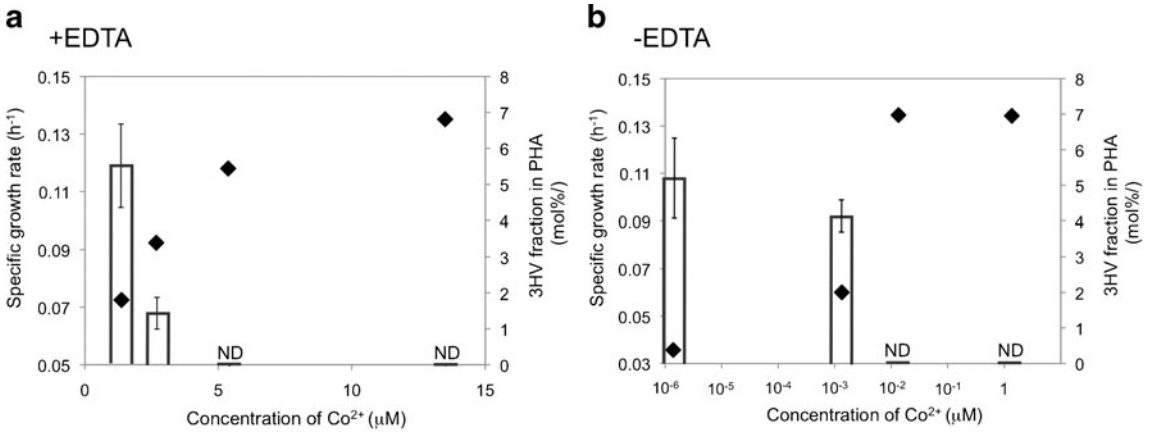

Fig. 2 Effects of Co^{2+} concentration on growth of *M. extorquens* AM1 on methanol and composition of PHA accumulated during the methylotrophic growth in the presence (**a**) and absence (**b**) of EDTA. Each of the Co^{2+} concentration was adjusted by the addition of the trace element solution containing the corresponding amount of $CoCl_2{\cdot}6H_2O$. *Closed diamonds*, maximum specific growth rate; *bars*, 3HV fraction in PHA. *ND*, not detected

PHA in methanol-grown *M. extorquens*. We therefore replaced the original *phaC* on the chromosome of AM1 ($phaC_{Me}$) with $phaC_{Ac}$ by homologous recombination. The resulting transformant, designated $AM1C_{Ac}$, was cultivated on methanol under a Co^{2+}-deficient condition (1.35 μM Co^{2+} in the presence of EDTA). The strain $AM1C_{Ac}$ accumulated a terpolymer composed of trace 3HHx units along with 3HB and 3HV units, [P(3HB-*co*-3HV-*co*-3HHx)], with a 4-fold greater cellular PHA content than the wild-type (WT) strain (Table 1). Although the 3HV composition in the terpolymer (0.71 mol%) was lower than 3.92 mol% in the P(3HB-*co*-3HV) copolymer synthesized by the WT strain, the amounts of 3HV incorporated into the polymer fractions were only slightly different from each other (0.023 and 0.016 mmol/l culture by the strains AM1 and $AM1C_{Ac}$, respectively). This fact indicated that the larger PHA content in *M. extorquens* $AM1C_{Ac}$ could primarily be attributed to the incorporation of more 3HB units into PHA, as the incorporation of 3HV during the methylotrophic growth was rather constant. This is the first report of a significant enhancement of methanol-dependent PHA production by the genetic engineering of a methylotroph, as well as of the biosynthesis of P(3HB-*co*-3HV-*co*-3HHx) terpolymer from methanol as the sole carbon source.

To investigate relationship between the PHA content and the expression level of *phaC* in *M. extorquens* grown on methanol, PHA synthase assay was examined for the strains AM1 and $AM1C_{Ac}$. Unexpectedly, the extract of $AM1C_{Ac}$ contained less than 3 mU/mg, lower than 30 mU/mg in the WT strain, indicating that the larger accumulation of PHA in $AM1C_{Ac}$ was not attributed to higher intracellular activity of PHA synthase. When compared to PHA synthase activity of about 200 mU/mg in fructose-grown *R. eutropha* H16 (Valentin and Steinbuchel 1994; Mifune et al. 2008), the activity in *M. extorquens* was much lower.

Characterization of PHAs synthesized by *M. extorquens* strains

The structures of the comonomers in the PHAs synthesized by *M. extorquens* AM1 and $AM1C_{Ac}$ from methanol were verified by GC-MS analysis of 3HA-Me after methanolysis of the intracellular PHAs. Figure 3 shows ion chromatograms of *m/z* 103, corresponding to $CH_3–O–CO–CH_2–CH=OH^+$ commonly formed by fragmentation of 3HA-Me. In addition to the major peak observed for 3HB-Me at 1.9 min, a minor peak at 2.6 min and two minor peaks at 2.6 and 3.7 min were detected for the PHAs from *M. extorquens* AM1 and $AM1C_{Ac}$, respectively. The retention times and mass spectra of the minor peaks at 2.6 and 3.7 min were identical to those of 3HV-Me and 3HHx-Me derived from authentic samples of P(3HB-*co*-3HV) and P(3HB-*co*-3HHx), respectively. The polymer fractions in the cells of *M. extorquens* AM1 and $AM1C_{Ac}$ grown on methanol were extracted and purified for further analyses. In a 400-MHz ^{1}H-NMR spectrum of the PHA from *M. extorquens* AM1, a triplet peak was observed with a chemical shift at 0.9 ppm, corresponding to the methyl proton resonance of 3HV (Fig. 4a). The signals at 0.9 ppm in the PHA from the strain $AM1C_{Ac}$ were detected as overlapped triplet peaks, which was consistent with the presence of the methyl groups of 3HV and 3HHx (Fig. 4b). These results confirmed the biosynthesis of P(3HB-*co*-3HV) copolymer and P(3HB-*co*-3HV-*co*-3HHx) terpolymer from methanol by *M. extorquens* AM1 and $AM1C_{Ac}$, respectively.

The molecular weights of the extracted polymers were determined by GPC. The number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI, M_w/M_n) of the P(3HB-*co*-3HV) obtained from *M. extorquens* AM1 were 0.66×10^5, 2.7×10^5, and 4.1, respectively. The same values for the of P(3HB-*co*-3HV-*co*-3HHx) obtained from the strain $AM1C_{Ac}$ were 1.2×10^5, 3.8×10^5, and 3.1, respectively, indicating that $PhaC_{Ac}$ synthesized PHA with a slightly higher molecular weight than did $PhaC_{Me}$ in the cells grown on methanol.

Enhancement of 3HV incorporation into PHA by the disruption of *pccA*

(*R*)-3HV-CoA is generally formed from propionyl-CoA and acetyl-CoA, and propionyl-CoA is one of the CoA-thioester intermediates in the EMC pathway essential for methanol

Table 1 PHA biosynthesis from methanol by *M. extorquens* AM1 and the recombinant strains under Co^{2+}-deficient condition (cells were cultivated in a hypho medium containing 0.5 % (*v/v*) methanol and 1.35 μM Co^{2+} for 72 h)

Strain	Dry cell mass (g/l)	Residual cell mass (g/l)	PHA content (wt%)	PHA monomer composition (mol%)		
				3HB (C_4)	3HV (C_5)	3HHx (C_6)
AM1	0.70±0.10	0.64±0.08	8.4±2.3	95.7±1.8	3.92±1.8	0
$AM1C_{Ac}$	0.87±0.06	0.58±0.07	32.6±4.0	99.2±0.4	0.71±0.31	0.28±0.12
$AM1C_{Ac}$ΔdepABC	0.90±0.06	0.59±0.05	34.7±0.8	99.1±0.1	0.64±0.09	0.25±0.05
$AM1C_{Ac}$/pCM80Km	0.83±0.03	0.57±0.07	30.7±5.8	99.2±0.1	0.46±0.12	0.34±0.03
$AM1C_{Ac}$/pCM80Km-phaAB	0.42±0.03	0.24±0.01	43.6±3.3	96.6±1.3	3.23±1.3	0.20±0.02

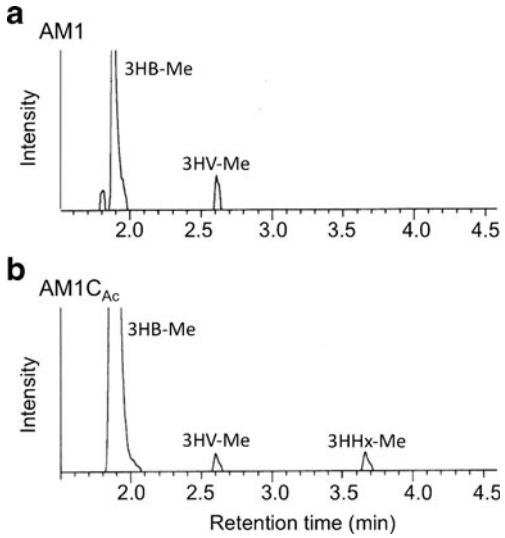

Fig. 3 GC-MS analysis of PHA copolymers synthesized from methanol by *M. extorquens* AM1 (**a**) and AM1C$_{Ac}$ (**b**) under the Co^{2+}-deficient condition. The *m*/*z* 103 ion chromatograms of methyl 3-hydroxyalkanoates obtained by methanolysis of PHA are shown

assimilation by *M. extorquens* AM1. When the propionyl-CoA for (*R*)-3HV-CoA formation was supplied via the EMC pathway, a lack of propionyl-CoA carboxylase was expected to increase the accumulation of propionyl-CoA and consequently enhance the 3HV fraction in the PHA copolymers. In order to confirm this, the *pccA* gene, which encodes the α-subunit of propionyl-CoA carboxylase, was deleted in *M. extorquens* AM1C$_{Ac}$ by homologous recombination. We confirmed that the resulting strain, AM1C$_{Ac}$Δpcc, lost its ability to grow on methanol due to blockage of the EMC pathway, as reported previously (Korotkova et al. 2002a). Therefore, the deletion mutant was first cultivated on succinate, then the grown cells were transferred into a nitrogen-free methanol medium to induce PHA biosynthesis by methanol. The wild strain AM1 and the parent strain AM1C$_{Ac}$ were also cultivated by the same procedure for comparison.

After the first-stage cultivation on succinate, *M. extorquens* AM1 accumulated a small amount (0.6 wt%) of PHA, whereas AM1C$_{Ac}$ and AM1C$_{Ac}$Δpcc unexpectedly accumulated 15–20 wt% of PHA (Table 2). Notably, these strains accumulated P(3HB-*co*-3HV) copolymer during the first-stage cultivation, indicating that *M. extorquens* could produce propionyl-CoA from succinate. This result was consistent with the previous work, which reported a weak flux of the EMC pathway on succinate with an unknown physiological role (Smejkalova et al. 2010). A more significant difference in PHA composition was observed in the PHA accumulated after the second-stage cultivation on methanol: the 3HV fraction was increased from 0.5 up to 6.6 mol% by the deletion of *pccA*. We further estimated the composition of the PHA

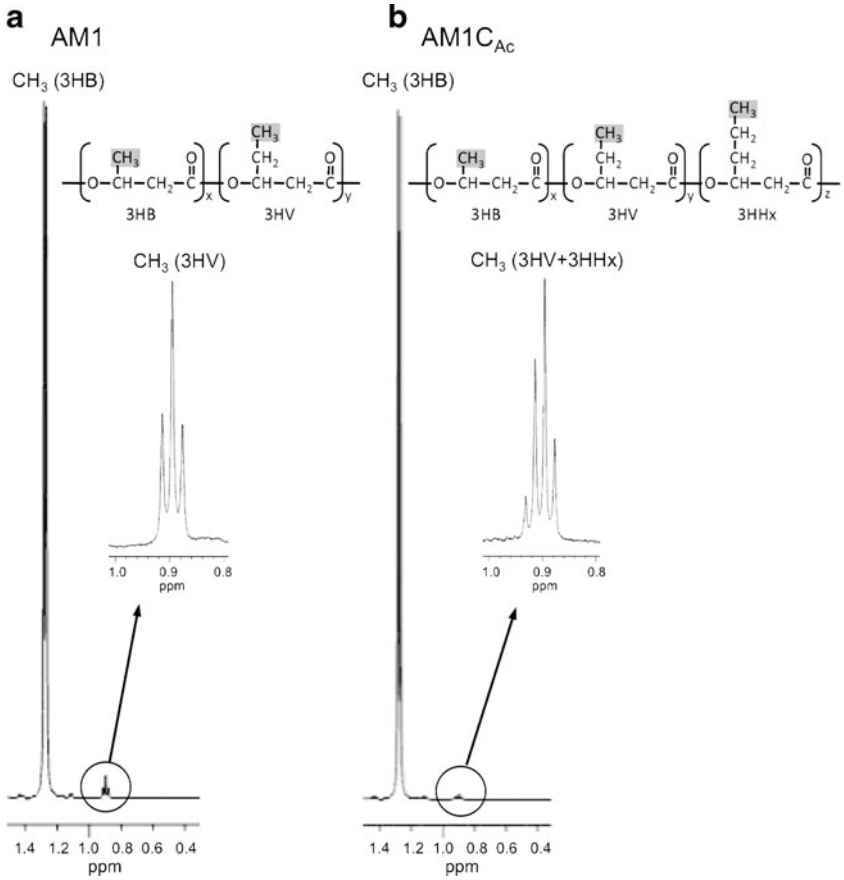

Fig. 4 ^{1}H NMR spectra of terminal methyl protons of PHAs synthesized from methanol by *M. extorquens* AM1 (**a**) and AM1C$_{Ac}$ (**b**) under the Co^{2+}-deficient condition

Table 2 Effect of *pccA* disruption on PHA biosynthesis by two-stage cultivation of *M. extorquens* strains

Strain	Cultivation[a]	Dry cell mass (g/l)	Residual cell mass (g/l)	PHA content (wt%)	PHA monomer composition (mol%)[b]		
					3HB (C_4)	3HV (C_5)	3HHx (C_6)
AM1	First stage	0.62±0.03	0.62±0.03	0.60±0.11	65.6±1.8	34.4±1.8	0
$AM1C_{Ac}$		0.68±0.02	0.57±0.03	15.7±1.29	99.0±0.07	1.03±0.07	0
$AM1C_{Ac}$ΔpccA		0.65±0.01	0.52±0.01	20.0±0.29	97.8±0.05	2.17±0.05	0
AM1	Second stage	0.87±0.03	0.62±0.03	27.2±1.65	99.5±0.16 (100)	0.54±0.16 (0)	0 (0)
$AM1C_{Ac}$		0.94±0.04	0.57±0.02	35.1±4.36	99.1±0.18 (99.1)	0.51±0.07 (0.27)	0.39±0.11 (0.58)
$AM1C_{Ac}$ΔpccA		0.64±0.03	0.48±0.03	24.7±1.60	93.1±0.79 (21.7)	6.57±0.77 (77.7)	0.33±0.06 (0.52)

[a] Cells were cultivated in a hypho minimal medium containing 0.5 % (*w*/*v*) succinate for 48 h (for first-stage cultivation) and then transferred into the nitrogen-free medium containing 0.5 % (*v*/*v*) methanol for 48 h (for second-stage cultivation). Co^{2+} concentration was 1.35 μM in both stages

[b] Monomer compositions of PHA accumulated during the second-stage cultivation, calculated by subtracting PHA accumulated after the first-stage cultivation from those after the second-stage cultivation, are shown in parentheses

synthesized from methanol by subtracting the PHAs present before the second stage. As shown in parentheses in Table 2, the 3HV fraction in the PHA accumulated by *M. extorquens* $AM1C_{Ac}$Δpcc during the second stage reached to 77.7 mol%, whereas that accumulated by $AM1C_{Ac}$ was only 0.27 mol%. These results strongly supported the assumption that the propionyl-CoA in the EMC pathway was a precursor of the 3HV unit synthesized during the methylotrophic growth.

The $phaC_{Ac}$-expressing strains were also confirmed to synthesize P(3HB-*co*-3HV-*co*-3HHx) terpolymer during the second-stage cultivation. The 3HHx compositions were calculated to be 0.5–0.6 mol% by these strains during the second stage and were not changed by the deletion of *pccA*, likely because the gene deletion had no appreciable effect on the reactions in the EMC pathway other than the formation of methylmalonyl-CoA from propionyl-CoA.

Effects of deletion of the *dep* genes

M. extorquens AM1 has been reported to possess two intracellular PHA depolymerase genes, *depA* and *depB*, which are potentially involved in PHA mobilization; the single deletion of each the gene yielded no significant changes in growth and P(3HB) biosynthesis on methanol (Korotkova and Lidstrom 2001). In the *M. extorquens* AM1 genome, we found the third putative intracellular PHA depolymerase gene, Mex_1p4148, designated as *depC* in this study. DepC was deduced to be a 45-kDa protein containing a PHB_depo_C domain (pfam06850) and shared 43 and 45 % identity with DepA and DepB, respectively. As we assumed the possibility that the lack of *depA* or *depB* may be compensated by the remaining two *dep* genes for PHA mobilization in the previous single deletants, the three *dep* genes were all disrupted in *M. extorquens* $AM1C_{Ac}$ with the aim of blocking PHA degradation and consequently increasing cellular PHA content. The resulting strain, $AM1C_{Ac}$ΔdepABC, was cultivated on methanol under the Co^{2+}-deficient condition. Contrary to our expectations, the growth and PHA biosynthesis properties of *M. extorquens* $AM1C_{Ac}$ΔdepABC were very similar to those of the parent strain $AM1C_{Ac}$ (Table 1). Single and double deletions of the *dep* genes also showed no significant difference in the properties (data not shown). These results raised the possibility that the intracellular mobilization of PHA was mediated by other PHA depolymerases or did not occur under the examined cultivation condition.

Effects of the overexpression of *phaA* and *phaB*

The *M. extorquens* AM1 genes encoding the 3HB monomer-supplying enzymes, *phaA* and *phaB*, were overexpressed in the strain $AM1C_{Ac}$ for further enhancement of PHA biosynthesis on methanol and regulation of the PHA composition. The strain $AM1C_{Ac}$ was transformed with pCM80Km-phaAB, constructed for the expression of $phaAB_{Me}$ under the control of the methanol dehydrogenase promoter from *M. extorquens* AM1, and then cultivated on methanol under the Co^{2+}-deficient condition. The cellular content and 3HV composition of the PHA synthesized by *M. extorquens* $AM1C_{Ac}$/pCM80Km-phaAB were approximately 1.4- and 7.0-fold higher than those of the control strain $AM1C_{Ac}$/pCM80Km, respectively (Table 1). However, the cell growth (residual cell mass) of the strain harboring pCM80Km-phaAB was decreased to approximately half of that of the control strain.

Discussion

The present study confirmed that Co^{2+} is a critical trace element for the methylotrophic growth of *M. extorquens* (Chou et al. 2009; Kiefer et al. 2009) and, moreover, found that the Co^{2+} concentration markedly affected the properties of PHA biosynthesis in this bacterium on methanol. The growth rate on

methanol was impaired at a very low Co^{2+} concentration, approximately 1.35 nM, and was saturated at 13.5 nM in the absence of EDTA (Fig. 2b), as reported previously (Chou et al. 2009). When EDTA was added to the trace element solution to avoid metal precipitation, the cobalt limitation was observed at a much higher Co^{2+} concentration (1.35 μM) probably due to chelation of the metal ions by EDTA (Fig. 2a). The Co^{2+} concentration of 1.35 μM in the presence of EDTA, which has often been adopted as the regular dose for this methylotroph, appeared to be insufficient for supporting its maximum growth rate on methanol. The reduced growth ability of *M. extorquens* AM1 under the limited Co^{2+} concentrations was possibly caused by a decrease of the activities of two vitamin B_{12}-dependent mutases in the EMC pathway, as suggested previously (Chou et al. 2009; Kiefer et al. 2009).

We further clarified that *M. extorquens* AM1 is able to synthesize P(3HB-*co*-3HV) copolymers from methanol as a sole carbon source under the Co^{2+}-deficient conditions. Considering the previous observation that a shortage of Co^{2+} induced accumulation of propionyl-CoA as well as ethylmalonyl-CoA and methylmalonyl-CoA (Kiefer et al. 2009), the accumulation of propionyl-CoA was supposed to promote condensation with acetyl-CoA and subsequent reduction to form (*R*)-3HV-CoA by β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB), respectively; and the resulting (*R*)-3HV-CoA was then copolymerized with (*R*)-3HB-CoA by PHA synthase (PhaC). This presumption was strongly supported by two observations. First, the 3HV composition in the copolyester showed an inverse correlation with the growth rates on methanol. Copolyester biosynthesis was observed only when the methylotrophic growth was impaired due to the shortage of Co^{2+} accompanied by the accumulation of propionyl-CoA, whereas no 3HV was incorporated into PHA during faster growth with less accumulated propionyl-CoA under the sufficient Co^{2+} concentrations (Fig. 2). Second, the blockage of the EMC pathway by the disruption of *pccA* resulted in preferential incorporation of the 3HV unit into the polymer fraction during incubation with methanol (Table 2). It seemed feasible that propionyl-CoA was further accumulated within the cells due to the lack of PccA in the EMC pathway. Although the reason for the growth inhibition observed for the strain AM1/pCM80Km-phaAB was unclear, the increases in the PHA content and 3HV fraction induced by the overexpression of $phaAB_{Me}$ may suggest that the excess conversion of acetyl-CoA and propionyl-CoA to PHA had a negative effect on the methylotrophic growth of *M. extorquens*. Alternatively, protein overproduction may have caused the growth inhibition, as reported previously (Chou and Marx 2012; Kurland and Dong 1996), because the methanol dehydrogenase promoter used for the overexpression of $phaAB_{Me}$ is known to be among the strongest promoters in *M. extorquens* AM1 (Marx and Lidstrom 2001).

Another interesting linkage between the EMC and PHA biosynthesis pathways appeared to be mediated by crotonyl-CoA reductase (Ccr). Ccr was first identified from *Streptomyces collinus* (Wallace et al. 1995), and its role in providing butyryl-CoA for monensin A biosynthesis was reported for *S. cinnamonensis* (Liu and Reynolds 1999). We have previously employed the *ccr* gene from *S. cinnamonensis* for the generation of butyryl-CoA as a precursor of (*R*)-3HHx-CoA in recombinant *R. eutropha* (Fukui et al. 2002). Ccr has since been revealed to possess dual activities of reduction and reductive carboxylation of crotonyl-CoA (Erb et al. 2007); the enzyme is now thought to mediate the direct formation of ethylmalonyl-CoA from crotonyl-CoA in the EMC pathway. Nevertheless, butyryl-CoA has been detected at a significant level with LC-MS analysis in the cell extracts of *M. extorquens* AM1 grown on methanol (Peyraud et al. 2009), suggesting that the reductase activity of Ccr was not negligible in the methanol-grown cells. Indeed, P(3HB-*co*-3HV-*co*-3HHx) terpolymers were synthesized from methanol by the recombinant strains of *M. extorquens* in which $PhaC_{Ac}$ with broad substrate specificity was functional in the polymerization. Although the 3HHx compositions of the terpolymers were somewhat low at the present state (Table 1), the results suggested the possible utilization of the EMC pathway to supply not only propionyl-CoA but also butyryl-CoA for biosynthesis of PHA copolyesters (Fig. 1).

The present results also demonstrated that PHA production was improved by the replacement of the original $phaC_{Me}$ gene by $phaC_{Ac}$ on the chromosome (Table 1). This observation was not due to higher PHA synthase activity in the engineered strain; in fact, lower PHA synthase activity was detected in this strain. We further observed that PHA biosynthesis in *M. extorquens* AM1 was repressed on succinate, whereas this repression did not occur in the strains expressing $phaC_{Ac}$ (Table 2). Although the reasons for these phenomena require further exploration, the specific properties of $PhaC_{Ac}$, such as its catalytic characteristics or affinity to the PHA granule surface, may be favorable for the synthesis and accumulation of PHA in *M. extorquens*.

The M_n and PDI of the P(3HB-*co*-3HV) produced by *M. extorquens* AM1 were determined to be 0.66×10^5 and 4.1, respectively; these values were comparable to those of P(3HB) synthesized from methanol by the genus *Methylobacterium* ($0.24–1.43 \times 10^5$ and 4.0–11.1, respectively) (Daniel et al. 1992; Taidi et al. 1994; Yezza et al. 2006). When compared with PHAs produced by *R. eutropha* from sugars and organic acids (M_n $5.1–7.5 \times 10^5$, PDI 2.4–3.2) (Taidi et al. 1994), the PHAs produced from methanol showed lower M_n with higher PDI. This result was likely due to the use of methanol as the carbon source, because it has been previously reported that several hydroxyl compounds, including methanol, can act as chain transfer agents during polymerization by PHA synthase (Kawaguchi and Doi 1992;

Taidi et al. 1994; Madden et al. 1999). The role of methanol as the chain transfer agent has been assessed by NMR-based end-group analysis, and a methyl end-group was actually detected in the P(3HB) produced from methanol by *M. extorquens* strain NCIMB 9133 (Madden et al. 1999). In the present study, *M. extorquens* AM1C$_{Ac}$ harboring *phaC*$_{Ac}$ also produced PHA with low molecular weight from methanol, although PhaC$_{Ac}$ has the ability to synthesize PHAs with higher molecular weight (M_n, 8.9×10^5) from fructose in recombinant strains of *R. eutropha* (Mifune et al. 2008). This observation was in agreement with the chain transfer function of methanol. Nevertheless, a 1.8-fold increase of M_n and a decrease in the PDI of the accumulated PHA were observed due to the replacement of the original *phaC*$_{Me}$ by *phaC*$_{Ac}$ in *M. extorquens* AM1. The low polymerizing activity in the strain AM1C$_{Ac}$, as shown by the enzyme assay, may be responsible for the slightly higher molecular weight of PHA (Sim et al. 1997); alternatively, PhaC$_{Ac}$ may possess a lower affinity for the chain-terminating methanol than PhaC$_{Me}$. The engineering of *M. extorquens* for production of PHAs with high molecular weight from methanol will be an interesting project.

The utilization of methylotrophic bacteria is a feasible method for PHA production from methanol, a feedstock with significant potential, and several groups have therefore optimized the fed-batch cultivation of *M. extorquens* for P(3HB) production (Suzuki et al. 1986; Bourque et al. 1995; Kim et al. 2003). However, P(3HB) is unsuitable for practical applications due to its hard and brittle properties. Recently, *M. extorquens* ATCC 55366 expressing *phaC* from *Pseudomonas fluorescens* GK13 was reported to accumulate PHA copolymers containing C–C double bonds in the side chains, when unsaturated carboxylic acids were added to the medium along with methanol (Hofer et al. 2010). To our knowledge, there has been no previous report of the production of PHA copolymer using methanol as the sole carbon source. The present study revealed that *M. extorquens* was capable of synthesizing PHA copolymers without the addition of expensive precursor compounds and that the EMC pathway was potentially utilizable to supply CoA-thioester precursors for PHA biosynthesis. Further investigation into the metabolic engineering of methylotrophic bacteria will allow efficient production of PHA copolymers with desirable physical and mechanical properties from methanol.

Acknowledgments We thank Assoc. Prof. T. Tsuge (Tokyo Institute of Technology, Japan) for supporting GC-MS and GPC analyses and Assoc. Prof. T. Hata (Tokyo Institute of Technology, Japan) for supporting NMR analysis. We also thank Prof. M. E. Lidstrom (Washington University, USA) and Prof. Y. Sakai (Kyoto University, Japan) for the generous gift of *Methylobacterium extorquens* AM1 and pCM80 vector. This work was supported by MEXT KAKENHI grant numbers 21780066 and 24580103 and NISR (Noda Institute for Scientific Research) Young Investigator Research Grant.

References

Alber BE (2011) Biotechnological potential of the ethylmalonyl-CoA pathway. Appl Microbiol Biotechnol 89:17–25

Alber BE, Spanheimer R, Ebenau-Jehle C, Fuchs G (2006) Study of an alternate glyoxylate cycle for acetate assimilation by *Rhodobacter sphaeroides*. Mol Microbiol 61:297–309

Barnard GN, Sanders JK (1989) The poly-β-hydroxybutyrate granule in vivo. A new insight based on NMR spectroscopy of whole cells. J Biol Chem 264:3286–3291

Bourque D, Pomerleau Y, Groleau D (1995) High cell density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: production of high-molecular-mass PHB. Appl Microbiol Biotechnol 44:367–376

Chou HH, Berthet J, Marx CJ (2009) Fast growth increases the selective advantage of a mutation arising recurrently during evolution under metal limitation. PLoS Genet 5:e1000652

Chou HH, Marx CJ (2012) Optimization of gene expression through divergent mutational paths. Cell Reports 1:133–140

Daniel M, Choi JH, Kim JH, Lebeault JM (1992) Effect of nutrient deficiency on accumulation and relative molecular weight of poly-β-hydroxybutyric acid by methylotrophic bacterium, *Pseudomonas* 135. Appl Microbiol Biotechnol 37:702–706

Erb TJ, Berg IA, Brecht V, Muller M, Fuchs G, Alber BE (2007) Synthesis of C_5-dicarboxylic acids from C_2-units involving crotonyl-CoA carboxylase/reductase: the ethylmalonyl-CoA pathway. Proc Natl Acad Sci U S A 104:10631–10636

Follner CG, Madkour M, Mayer F, Babel W, Steinbuchel A (1997) Analysis of the PHA granule-associated proteins GA20 and GA11 in *Methylobacterium extorquens* and *Methylobacterium rhodesianum*. J Basic Microbiol 37:11–21

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

Fukui T, Doi Y (1997) Cloning and analysis of the poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. J Bacteriol 179:4821–4830

Fukui T, Suzuki M, Tsuge T, Nakamura S (2009) Microbial synthesis of poly((*R*)-3-hydroxybutyrate-*co*-3-hydroxypropionate) from unrelated carbon sources by engineered *Cupriavidus necator*. Biomacromolecules 10:700–706

Höfer P, Choi YJ, Osborne MJ, Miguez CB, Vermette P, Groleau D (2010) Production of functionalized polyhydroxyalkanoates by genetically modified *Methylobacterium extorquens* strains. Microb Cell Fact 9:70

Kato M, Bao HJ, Kang CK, Fukui T, Doi Y (1996) Production of a novel copolyester of 3-hydroxybutyric acid and medium chain length 3-hydroxyalkanaic acids by *Pseudomonas* sp 61-3 from sugars. Appl Microbiol Biotechnol 45:363–370

Kawaguchi Y, Doi Y (1992) Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in *Alcaligenes eutrophus*. Macromolecules 25:2324–2329

Kawashima Y, Cheng W, Mifune J, Orita I, Nakamura S, Fukui T (2012) Characterization and functional analyses of *R*-specific enoyl coenzyme A hydratases in polyhydroxyalkanoate-producing *Ralstonia eutropha*. Appl Environ Microbiol 78:493–502

Kiefer P, Buchhaupt M, Christen P, Kaup B, Schrader J, Vorholt JA (2009) Metabolite profiling uncovers plasmid-induced cobalt limitation under methylotrophic growth conditions. PLoS One 4:e7831

Kim P, Kim JH, Oh DK (2003) Improvement in cell yield of *Methylobacterium* sp. by reducing the inhibition of medium components for poly-β-hydroxybutyrate production. World J Microbiol Biotechnol 19:357–361

Korotkova N, Chistoserdova L, Kuksa V, Lidstrom ME (2002a) Glyoxylate regeneration pathway in the methylotroph *Methylobacterium extorquens* AM1. J Bacteriol 184:1750–1758

Korotkova N, Chistoserdova L, Lidstrom ME (2002b) Poly-β-hydroxybutyrate biosynthesis in the facultative methylotroph *Methylobacterium extorquens* AM1: identification and mutation of *gap11*, *gap20*, and *phaR*. J Bacteriol 184:6174–6181

Korotkova N, Lidstrom ME (2001) Connection between poly-β-hydroxybutyrate biosynthesis and growth on C_1 and C_2 compounds in the methylotroph *Methylobacterium extorquens* AM1. J Bacteriol 183:1038–1046

Kurland CG, Dong HJ (1996) Bacterial growth inhibition by overproduction of protein. Mol Microbiol 21:1–4

Liu HB, Reynolds KA (1999) Role of crotonyl coenzyme A reductase in determining the ratio of polyketides monensin A and monensin B produced by *Streptomyces cinnamonensis*. J Bacteriol 181:6806–6813

Madden LA, Anderson AJ, Shah DT, Asrar J (1999) Chain termination in polyhydroxyalkanoate synthesis: involvement of exogenous hydroxy-compounds as chain transfer agents. Int J Biol Macromol 25:43–53

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

Matsumoto K, Taguchi S (2013) Enzyme and metabolic engineering for the production of novel biopolymers: crossover of biological and chemical processes. Curr Opin Chem Biol 24:1–7

Marx CJ, Lidstrom ME (2001) Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. Microbiology 147:2065–2075

Mifune J, Nakamura S, Fukui T (2008) Targeted engineering of *Cupriavidus necator* chromosome for biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from vegetable oil. Can J Chem 86:621–627

Mifune J, Nakamura S, Fukui T (2010) Engineering of *pha* operon on *Cupriavidus necator* chromosome for efficient biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from vegetable oil. Polym Degrad Stab 95:1305–1312

Okubo Y, Skovran E, Guo XF, Sivam D, Lidstrom ME (2007) Implementation of microarrays for *Methylobacterium extorquens* AM1. Omics 11:325–340

Olah GA (2005) Beyond oil and gas: the methanol economy. Angew Chem Int Ed Engl 44:2636–2639

Peel D, Quayle JR (1961) Microbial growth on C_1 compounds. 1. Isolation and characterization of *Pseudomonas* AM 1. Biochem J 81:465–469

Peyraud R, Kiefer P, Christen P, Massou S, Portais JC, Vorholt JA (2009) Demonstration of the ethylmalonyl-CoA pathway by using ^{13}C metabolomics. Proc Natl Acad Sci U S A 106:4846–4851

Schafer A, Tauch A, Jager W, Kalinowski J, Thierbach G, Puhler A (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69–73

Schrader J, Schilling M, Holtmann D, Sell D, Villela M, Marx A, Vorholt JA (2009) Methanol-based industrial biotechnology: current status and future perspectives of methylotrophic bacteria. Trends Biotechnol 27:107–115

Sim SJ, Snell KD, Hogan SA, Stubbe J, Rha CK, Sinskey AJ (1997) PHA synthase activity controls the molecular weight and polydispersity of polyhydroxybutyrate *in vivo*. Nat Biotechnol 15:63–67

Smejkalova H, Erb TJ, Fuchs G (2010) Methanol assimilation in *Methylobacterium extorquens* AM1: demonstration of all enzymes and their regulation. PLoS One 5:e13001

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

Suzuki T, Yamane T, Shimizu S (1986) Mass production of poly-β-hydroxybutyric acid by fully automatic fed-batch culture of methylotroph. Appl Microbiol Biotechnol 23:322–329

Taidi B, Anderson AJ, Dawes EA, Byrom D (1994) Effect of carbon source and concentration on the molecular-mass of poly(3-hydroxybutyrate) produced by *Methylobacterium extorquens* and *Alcaligenes eutrophus*. Appl Microbiol Biotechnol 40:786–790

Ueda S, Matsumoto S, Takagi A, Yamane T (1992) Synthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from methanol and *n*-amyl alcohol by the methylotrophic bacteria *Paracoccus denitrificans* and *Methylobacterium extorquens*. Appl Environ Microbiol 58:3574–3579

Valappil SP, Boccaccini AR, Bucke C, Roy I (2007) Polyhydroxyalkanoates in Gram-positive bacteria: insights from the genera *Bacillus* and *Streptomyces*. Antonie Van Leeuwenhoek 91:1–17

Valentin HF, Dennis D (1996) Metabolic pathway for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) formation in *Nocardia corallina*: inactivation of *mutB* by chromosomal integration of a kanamycin resistance gene. Appl Environ Microbiol 62:372–379

Valentin HE, Steinbuchel A (1994) Application of enzymatically synthesized short-chain-length hydroxy fatty-acid coenzyme A thioesters for assay of polyhydroxyalkanoic acid synthases. Appl Microbiol Biotechnol 40:699–709

Verlinden RA, Hill DJ, Kenward MA, Williams CD, Radecka I (2007) Bacterial synthesis of biodegradable polyhydroxyalkanoates. J Appl Microbiol 102:1437–1449

Vuilleumier S, Chistoserdova L, Lee MC, Bringel F, Lajus A, Zhou Y, Gourion B, Barbe V, Chang J, Cruveiller S, Dossat C, Gillett W, Gruffaz C, Haugen E, Hourcade E, Levy R, Mangenot S, Muller E, Nadalig T, Pagni M, Penny C, Peyraud R, Robinson DG, Roche D, Rouy Z, Saenampechek C, Salvignol G, Vallenet D, Wu Z, Marx CJ, Vorholt JA, Olson MV, Kaul R, Weissenbach J, Medigue C, Lidstrom ME (2009) *Methylobacterium* genome sequences: a reference blueprint to investigate microbial metabolism of C1 compounds from natural and industrial sources. PLoS One 4:e5584

Wallace KK, Bao ZY, Dai H, Digate R, Schuler G, Speedie MK, Reynolds KA (1995) Purification of crotonyl-CoA reductase from *Streptomyces callinus* and cloning, sequencing and expression of the corresponding gene in *Escherichia coli*. Eur J Biochem 233:954–962

Wang Q, Zhuang Q, Liang Q, Qi Q (2013) Polyhydroxyalkanoic acids from structurally-unrelated carbon sources in *Escherichia coli*. Appl Microbiol Biotechnol 97:3301–3307

Woodall CA (2003) Electroporation of *E. coli*. Methods Mol Biol 235:55–69

Yezza A, Fournier D, Halasz A, Hawari J (2006) Production of polyhydroxyalkanoates from methanol by a new methylotrophic bacterium *Methylobacterium* sp. GW2. Appl Microbiol Biotechnol 73:211–218