Appl Microbiol Biotechnol
DOI 10.1007/s00253-014-5831-3

Enhancement of glycerol utilization ability of *Ralstonia eutropha* H16 for production of polyhydroxyalkanoates

Toshiaki Fukui · Masaharu Mukoyama · Izumi Orita · Satoshi Nakamura

Received: 15 April 2014 / Revised: 13 May 2014 / Accepted: 13 May 2014

Abstract *Ralstonia eutropha* H16 is a well-studied bacterium with respect to biosynthesis of polyhydroxyalkanoates (PHAs), which has attracted attentions as biodegradable bio-based plastics. However, this strain shows quite poor growth on glycerol of which bulk supply has been increasing as a major by-product of biodiesel industries. This study examined enhancement of glycerol assimilation ability of *R. eutropha* H16 by introduction of the genes of aquaglyceroporin (*glpF*) and glycerol kinase (*glpK*) from *Escherichia coli*. Although introduction of $glpFK_{Ec}$ into the strain H16 using a multi-copy vector was not successful, a recombinant strain possessing $glpFK_{Ec}$ within the chromosome showed much faster growth on glycerol than H16. Further analyses clarified that weak expression of $glpK_{Ec}$ alone allowed to establish efficient glycerol assimilation pathway, indicating that the poor growth of H16 on glycerol was caused by insufficient kination activity to glycerol, as well as this strain had a potential ability for uptake of extracellular glycerol. The engineered strains expressing $glpFK_{Ec}$ or $glpK_{Ec}$ produced large amounts of poly[(*R*)-3-hydroxybutyrate] [P(3HB)] from glycerol with much higher productivity than H16. Unlike other glycerol-utilizable wild strains of *R. eutropha*, the H16-derived engineered strains accumulated P(3HB) with no significant decrease in molecular weights on glycerol, and the polydispersity index of the glycerol-based P(3HB) synthesized by the strains expressing $glpFK_{Ec}$ was lower than those by the parent strains. The present study demonstrated possibility of *R. eutropha* H16-based platform for production of useful compounds from inexpensive glycerol.

Keywords Polyhydroxyalkanoate · *Ralstonia eutropha* H16 · Glycerol · Metabolic engineering

Introduction

Glycerol has become an attractive feedstock owing to its availability and low prices because it is accumulating in large quantities as a by-product of biodiesel, oleo-chemical, and bioethanol industries, thus biotechnology expanding microbial utilization of glycerol is increasingly important for production of bio-based fuels and materials (Dobson et al. 2012; Yang et al. 2012). One of the useful bio-based materials is polyhydroxyalkanoates (PHAs), produced by a wide variety of bacteria as intracellular carbon and energy storage materials from renewable carbon resources (Keshavarz and Roy 2010; Taguchi et al. 2012; Verlinden et al. 2007). Since many bacterial PHAs are applicable as biodegradable thermoplastics, they have attracted industrial attentions as possible alternatives to petroleum-based plastics (Pan and Inoue 2009; Taguchi et al. 2012). Given the growing surplus of raw glycerol, the use of glycerol as a feedstock of PHAs is a promising option for the low-cost microbial production (Nikodinovic-Runic et al. 2013). It is also considered that glycerol-converting ability may affect PHA productivity on vegetable oils, as they contain about 10 %(*w*/*v*) of glycerol moiety. Previous studies have also reported that several hydroxyl compounds, including glycerol, decreased molecular weight of PHAs by the chain transfer action during PHA synthase-catalyzed polymerization both in vivo and in vitro (Hiroe et al.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-5831-3) contains supplementary material, which is available to authorized users.

T. Fukui (✉) · I. Orita · S. Nakamura
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

M. Mukoyama
Strategic Technology Research Center, NIPPON SHOKUBAI,
1-25-12 Kannondai, Tsukuba 305-0856, Japan

Published online: 31 May 2014

2013; Madden et al. 1999; Taidi et al. 1994; Tomizawa et al. 2010, 2013). The relationship between the level of cellular ability for glycerol assimilation and molecular weights of the glycerol-based PHAs is also an interesting subject.

A chemolithoautotrophic β-proteobacterium *Ralstonia eutropha* (*Cupriavidus necator*) strain H16 has been well-known as an efficient producer of poly[(*R*)-3-hydroxybutyrate] [P(3HB)], the most abundant PHA in nature. There have been many studies focusing on PHA biosynthesis by this strain, including several omics analyses (Brigham et al. 2012; Fukui et al. 2014; Peplinski et al. 2010; Pohlmann et al. 2006; Raberg et al. 2011; Shimizu et al. 2013) and metabolic engineering aiming at biosynthesis of PHA copolymers such as flexible poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] [P(3HB-*co*-3HHx)] (Budde et al. 2011; Insomphun et al. 2013; Kawashima et al. 2012; Loo et al. 2005; Mifune et al. 2008, 2010; Riedel et al. 2011). Recent studies further demonstrated a potential of this strain for production of branched-chain alcohols (Lu et al. 2012). However, one drawback of this strain has been the rather narrow spectrum of utilizable carbon sources. This strain can well grow and produce PHAs on fructose, gluconate, fatty acids, and plant oils, whereas it cannot utilize glucose, xylose, and arabinose. Hence, extension of the substrate range of *R. eutropha* H16 has been considered to be necessary for the wider applications. So far, engineered strains of H16 capable of utilizing glucose were established by expressing glucose facilitator (*glf*) and glucokinase (*glk*) from *Zymomonas mobilis* (Orita et al. 2011; Sichwart et al. 2011), or by reconstituting mutations identified in *nag* operon within the glucose-utilizable mutants (Orita et al. 2011; Raberg et al. 2012). *R. eutropha* H16 also shows quite slow growth and low PHA productivity on glycerol, and Kaddor and Steinbuchel (2011) reported that PtsMHI, one of phosphoenolpyruvate-carbohydrate phosphotransferase systems (PEP-PTS) in H16, played some role in the slow growth and P(3HB) accumulation on glycerol. Interestingly, it has been reported that other wild strains of *R. eutropha* JMP134 and DSM 545 grew and accumulated PHAs on glycerol (Cavalheiro et al. 2009, 2012; Garcia et al. 2013; Mothes et al. 2007; Spoljaric et al. 2013a, b), and a glucose-utilizable strain NCIMB 40529 converted glycerol to P(3HB) in two-step cultivation (Taidi et al. 1994). A glycerol-utilizable mutant of ATCC 17699 (thought to be equivalent to H16) was isolated by adaptation in glycerol-rich medium (Tanadchangsaeng and Yu 2012). These previous studies observed decrease of molecular weight of P(3HB) synthesized from glycerol than that from sugars. So far, the genetic and metabolic properties responsible for glycerol utilization in the above strains have not been determined.

Aerobic metabolism of glycerol has been well-studied in *Escherichia coli* (Durnin et al. 2009) and other bacteria. In *E. coli*, glycerol transported by aquaglyceroporin (GlpF) (Wang et al. 2005) was phosphorylated by glycerol kinase (GlpK) to glycerol 3-phosphate, which is oxidized by FAD-dependent glycerol 3-phosphate dehydrogenase (GlpD) to yield dihydroxyacetone phosphate for further metabolism through glycolysis (Austin and Larson 1991; Weissenborn et al. 1992). Mukoyama (2007) has reported that introduction of $glpFK_{Ec}$ into *Corynebacterium glutamicum*, *R. eutropha* ATCC 17697 (another wild strain distinct from H16), and *Alcaligenes faecalis* significantly enhanced the growth in glycerol-containing minimum media. However, the contribution of the individual gene in $glpFK_{Ec}$ to the glycerol assimilation ability and the effects of the metabolic modification on PHA production by the *R. eutropha* strain were not investigated. *C. glutamicum* was also engineered for glycerol utilization by introduction of $glpFK_{Ec}$ and $glpD_{Ec}$ genes (Meiswinkel et al. 2013; Rittmann et al. 2008). It was revealed that co-expression of $glpD_{Ec}$ along with $glpK_{Ec}$ was necessary for glycerol assimilation by *C. glutamicum*, and no growth of the strain expressing $glpFK_{Ec}$ without $glpD_{Ec}$ on glycerol was due to accumulation of sub-toxic glycerol 3-phosphate at very high concentration within the cells grown in the presence of glycerol (Rittmann et al. 2008).

This study dealt with engineering of *R. eutropha* H16 for efficient glycerol utilization. We found that weak expression of $glpK_{Ec}$ was a sufficient modification for much faster growth of the H16-derived strain on glycerol. The engineered *R. eutropha* strains harboring $glpK_{Ec}$ or $glpFK_{Ec}$ on the chromosome could highly accumulate P(3HB) on glycerol as well as on fructose, without significant decrease of molecular weights.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *R. eutropha* strains were cultivated at 30 °C in a nutrient-rich (NR) medium containing 10 g of meat extract, 10 g of polypeptone, and 2 g of yeast extract in 1 l of tap water. *E. coli* strains were grown at 37 °C on a lysogeny broth (LB) medium. Kanamycin (50 mg/l for *E. coli* and 200 mg/l for *R. eutropha* strains) or ampicillin (50 mg/l for *E. coli*) was added to the medium when necessary.

Construction of plasmids and strains

DNA manipulations were carried out according to standard procedures, and sequences of oligonucleotide primers used for PCR amplification are shown in Supplementary Table S1. pBBR-glpFK (described as glpFK/pBBR122 in the previous report (Mukoyama 2007)) was constructed by inserting $glpFK_{Ec}$ along with the adjacent promoter region, amplified

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant markers	Reference or source
Escherichia coli		
K-12	Wild strain	IFO 3301
DH5α	*deoR*, *endA1*, *gyrA96*, *hsdR17* (r_K^- m_K^+), *recA1*, *relA1*, *supE44*, *thi-1*, Δ(*lacZYA-argFV169*), φ80*lacZ*ΔM15, F^-, λ^-	Clontech
S17-1	*thi*, *pro*, *hsdR*, *recA*, *chromosomal RP4; Tra*$^+$*; Tmp*^{r} *Str/Spc*^{r}	Simon et al. (1983)
Ralstonia eutropha		
H16	Wild strain	DSM 428
H16_glpFK	H16 derivative; P_{A2858}-$glpFK_{Ec}$-*h16_A2858*	This study
H16_glpF	H16 derivative; P_{A2858}-$glpF_{Ec}$-*h16_A2858*	This study
H16_glpK	H16 derivative; P_{A2858}-$glpK_{Ec}$-*h16_A2858*	This study
NSDG	H16 derivative; Δ$phaC_{Re}$::$phaC_{NSDG}$	Mifune et al. (2010)
NSDG_glpFK	NSDG derivative; P_{A2858}-$glpFK_{Ec}$-*h16_A2858*	This study
TT013	Δ$phaCA_{Re}$::$phaC_{NSDG}$-$phaJ_{Ac}$-$phaJ4a_{Re}$	Kawashima et al. (2012)
TT013_glpFK	TT013 derivative; P_{A2858}-$glpFK_{Ec}$-*h16_A2858*	This study
Plasmids		
pBBR122	pBBR ori, Kan^r, Cm^r	MoBiTec
pBBR-glpFK	pBBR122 derivative; Cm^r::P_{glp}-$glpFK_{Ec}$	Mukoyama (2007)
pBBR-glpF	pBBR122 derivative; Cm^r::P_{glp}-$glpF_{Ec}$	This study
pBBR-glpK	pBBR122 derivative; Cm^r::P_{glp}-$glpK_{Ec}$	This study
pK18mobsacB	pMB1 ori, mob, Kan^r, *sacB*	Schafer et al. (1994)
pK18ms-glpFK-A2858	pK18mobsacB carrying P_{A2858}-$glpFK_{Ec}$-*h16_A2858*	This study
pK18ms-glpF-A2858	pK18mobsacB carrying P_{A2858}-$glpF_{Ec}$-*h16_A2858*	This study
pK18ms-glpK-A2858	pK18mobsacB carrying P_{A2858}-$glpK_{Ec}$-*h16_A2858*	This study

P_{glp} promoter region located at upstream of $glpF_{Ec}$ in *E. coli*, P_{A2858} promoter region located at upstream of *h16_A2858* in *R. eutropha* H16

from *E. coli* K-12 gDNA, at NcoI site in pBBR122 (MoBiTec). pBBR-glpF and pBBR-glpK were obtained by self-ligation of the linear fragment not containing the coding regions of $glpK_{Ec}$ and $glpF_{Ec}$ prepared by inverse PCR with primer sets of d-glpK-Inv3/d-glpK-Inv5 and d-glpF-Inv3/d-glpF-Inv5, respectively, and pBBR-glpFK as a template.

Transformation of *R. eutropha* strains with the pBBR122-based recombinant plasmids was performed by electroporation. The electrocompetent cells of *R. eutropha* were prepared by three times washing of the cells grown in 100 ml NR medium (30 °C, 5 h, OD_{600}≈0.6) with ice-cold glycerol solution (10 % (*v*/*v*)), and resuspended in 2 ml in the cold glycerol solution. The electroporation was done using Gene Pulser Xcell Electroporation System (Bio-Rad) and 2-mm cuvettes. The pulse conditions were 2.5 kV voltage, 25 μF capacity, and 200 Ω external resistance. The cells after the electroporation were diluted with 1 ml fresh NR medium, incubated at 30 °C for 3 h, and then plated onto an NR-agar medium containing 250 mg/l kanamycin.

pK18ms-glpFK-A2858 for insertion of $glpFK_{Ec}$ at upstream of *h16_A2858* was constructed as below. First, a 1.5-kbp fragment around the start codon of *h16_A2858* was amplified with *R. eutropha* H16 gDNA and an A2858-5/A2858-3 primer pair. The resulting fragment was digested by SalI and inserted into pK18mobSacB (Schafer et al. 1994) at the corresponding site. Inverse PCR was done to open the plasmid at the start codon of *h16_A2858* with a primer set A2858-Inv3/A2858-Inv5, and then ligated with the $glpFK_{Ec}$ fragment with the same direction as *h16_A2858*. The primers for the inverse PCR were designed to locate a 23-bp upstream region of *h16_A2858*, including the putative ribosome binding sequence, at the upstream of $glpF_{Ec}$ in the resulting plasmid. pK18ms-glpF-A2858 and pK18ms-glpK-A2858 were obtained by inverse PCR and subsequent self-ligation as the same way for construction of pBBR-glpF and pBBR-glpK, respectively.

Transconjugation of the mobilizable pK18mobsacB-based plasmids from *E. coli* S17-1 (Simon et al. 1983) to *R. eutropha* strains and isolation of strains generated by pop in-pop out recombination were performed as described previously (Mifune et al. 2008, 2010).

Enzyme assay

R. eutropha cells in the late exponential growth phase on fructose or glycerol were harvested, washed with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, and resuspended in the same buffer. Crude extract was prepared from

the harvested cells as described previously (Orita et al. 2011). Glycerol kinase and FAD-dependent glycerol-3-phosphate dehydrogenase activities were assayed according to the procedure described by Rittmann et al. (2008).

PHA production by recombinant *R. eutropha* strains

PHA production by *R. eutropha* strains was carried out on a reciprocal shaker (115 strokes/min) at 30 °C in a 500 ml flask with a 100 ml of nitrogen-limited mineral salts (MB) medium (Kato et al. 1996). A filter-sterilized solution of fructose or glycerol was added to the medium at a final concentration of 0.5 % (*w/v*) as a sole carbon source. Soybean oil was directly added to the medium at 1.0 % (*v/v*). After the cultivation for appropriate time, the cells were harvested, washed once with cold deionized water, and then lyophilized. Cellular PHA content and composition were determined by gas chromatography (GC) after methanolysis of the dried cells in the presence of 15 % sulfuric acid as described previously (Kato et al. 1996).

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

Genes potentially related to glycerol metabolisms in *R. eutropha* H16 genome

In the whole genome of *R. eutropha* H16 (Pohlmann et al. 2006), two pairs of genes, *h16_A2507-A2508* and *h16_B1199-B1198*, have been identified for putative glycerol kinase (GK) and FAD-dependent glycerol 3-phosphate dehydrogenase (FAD-G3PDH) (Table 2). H16_A2507 shared higher identity (52 %) to $GlpK_{Ec}$ than H16_B1199 (28 %), while H16_A2508 and H16_B1198 both showed weak identities (27–30 %) to GlpD (aerobic FAD-G3PDH) and the *N*-terminal region of GlpA (large subunit of anaerobic FAD-G3PDH) from *E. coli*. Our recent RNA-seq analysis of *R. eutropha* H16 (Shimizu et al. 2013) indicated that these genes were weakly transcribed on fructose, where the reads per kilobase per million mapped reads (RPKM) values for *h16_A2507-A2508* were slightly larger than those for *h16_B1199-B1198*. Two genes *h16_B1197* and *h16_B1196*, lying downstream of *h16_B1198* with the same direction, encoded a homolog of alkyl-dihydroxyacetone phosphate synthase generally involved in long-chain ether lipid biosynthesis in animal cells, and a transcriptional regulator related to glycerol uptake operon antiterminator GlpP from *Bacillus subtilis*, respectively. The four proteins expressed from the gene cluster *h16_B1199-B1196* might play a role in some lipid metabolism in *R. eutropha*. *h16_A3690* was originally annotated as *glpF* encoding glycerol facilitator or related permease. However, taking into consideration the low and partial homology of H16_A3690 to $GlpF_{Ec}$ (25 % within 43 amino acids overlap), it was unclear whether the gene product has an ability to transport glycerol or not. *h16_A0336*, moderately transcribed on fructose, encoded a protein with 39 % identity to GpsA (NAD(P)H-dependent glycerol 3-phosphate dehydrogenase) from *E. coli*. The gene was estimated to participate in glycerophospholipid biosynthesis by catalyzing conversion of DHAP to *sn*-glycerol 3-phosphate. No gene related to other glycerol utilization pathways such as genes of glycerol dehydrogenase and dihydroxyacetone kinase, either genes of glycerol dehydratase, was identified in the genome of *R. eutropha* H16.

Introduction of glycerol assimilation genes from *E. coli* into *R. eutropha* H16

Based on the previous report conferring glycerol assimilation ability to *R. eutropha* ATCC 17697 (Mukoyama 2007), we first examined to introduce $glpFK_{Ec}$ along with the adjacent native promoter region into *R. eutropha* H16 by using a multicopy vector pBBR-glpFK. In addition, two derivative vectors pBBR-glpF and pBBR-glpK were constructed and introduced into H16 to investigate the effects of the individual gene expression on glycerol utilization. However, contrary to the previous result using the strain ATCC 17697, the H16-derived recombinant strain harboring pBBR-glpFK could not be obtained even after several trials. Although many kanamycin-resistant colonies appeared on the selective plate medium after electroporation of the cells of H16 with pBBR-glpFK, PCR amplification of the plasmid-borne $glpFK_{Ec}$ region was unsuccessful. The same phenomenon was also observed in transformation with pBBR-glpK, whereas the strain harboring pBBR-glpF could be isolated. Several plasmids extracted from the transformants with pBBR-glpFK or pBBR-glpK were mapped by PCR and restriction endonuclease digestion, and the results strongly suggested deletion of the *E. coli*-derived region from the plasmids in the isolates (data not shown).

The engineering of *R. eutropha* H16 was also attempted by inserting $glpFK_{Ec}$, $glpF_{Ec}$, or $glpK_{Ec}$ into the chromosome by homologous recombination, as shown in Fig. 1. We selected upstream of *h16_A2858*, a gene of hypothetical membrane protein containing transglycosylase SLT domain, as the target site for the gene insertion, because the gene was monocistronic and the transcriptional levels were high throughout the growth

Table 2 Genes potentially related to glycerol metabolisms in *R. eutropha* H16

Gene ID	Original annotation[a]	Length (aa)	Identity to *E. coli* protein (%/overlap)[b]	Transcription on fructose (RPKM)[c]		
				F16	F26	F36
H16_A3690	Glycerol facilitator or related permease	241	GlpF (25 %/43 aa)	209	168	951
H16_A2507	Glycerol kinase	523	GlpK (52 %/501 aa)	150	84	248
H16_A2508	Glycerol-3-phosphate dehydrogenase	534	GlpD (29 %/460 aa) GlpA (30 %/379 aa)	103	44	187
H16_B1196	Transcriptional regulator, GlpP-family	194	YgcP (23 %/185 aa)	544	449	1095
H16_B1197	Alkyldihydroxyacetone phosphate synthase	516	YgcU (29 %/436 aa)	181	148	140
H16_B1198	FAD-dependent glycerol-3-phosphate dehydrogenase	559	GlpD (27 %/464 aa) GlpA (30 %/395 aa)	65	25	24
H16_B1199	Glycerol kinase	507	GlpK (28 %/507 aa)	48	30	27
H16_A0336	Glycerol-3-P dehydrogenase [NAD(P)$^+$]	338	GpsA (39 %/338 aa)	916	243	835

[a] GenBank AM260479.1 (chromosome 1) and AM260480.1 (chromosome 2) (Pohlmann et al. 2006)

[b] Blastp search with SEG filtering

[c] RPKM values at growth (F16), PHA production (F26), and stationary (F36) phases on fructose (Shimizu et al. 2013)

on fructose in the recent RNA-seq analysis (Shimizu et al. 2013). pK18mobsacB-based vectors harboring the glp_{Ec} gene(s) between the upstream promoter region and coding region of *h16_A2858* were constructed and used to transform the strain H16. Unlike the case using the multi-copy vectors, the three strains harboring $glpFK_{Ec}$, $glpF_{Ec}$, and $glpK_{Ec}$ at the upstream of *h16_A2858*, designated as strains H16_glpFK, H16_glpF, and H16_glpK, respectively, were successfully obtained by the desired homologous recombination.

Growth and P(3HB) biosynthesis properties of the engineered *R. eutropha* strains

The growth behavior of the recombinant strains H16_glpFK, H16_glpF, and H16_glpK was investigated on a solid and liquid mineral salts media containing glycerol as the sole carbon source. On the solid medium, H16 and H16_glpF exhibited almost no growth on glycerol after 72 h cultivation (Fig. 2a) and slightly grew after 144 h cultivation (data not shown). In contrast, H16_glpFK and H16_glpK could well grow on glycerol for 72 h cultivation. The growth curves in a liquid medium containing 0.5 % (*w*/*v*) glycerol or 0.5 % (*w*/*v*) fructose are shown in Fig. 2b. All the strains examined here showed the same growth property on fructose, indicating that $glpFK_{Ec}$ or the individual gene did not affect the fructose metabolism in *R. eutropha* H16. The wild strain H16 grew quite slowly on glycerol and reached to maximum after 245 h cultivation. While H16_glpF showed very poor growth similar to H16, the strains H16_glpFK and H16_glpK, both harboring $glpK_{Ec}$, could grow on glycerol much faster than H16 although the specific growth rates on glycerol (0.15 h^{-1}) were lower compared to those on fructose (0.28 h^{-1}). These results clearly indicated that of $glpK_{Ec}$, but not $glpF_{Ec}$, was responsible for the enhanced glycerol utilization ability.

P(3HB) biosynthesis properties of the engineered strains on fructose were quite similar to that of wild strain as all the strains produced 1.3~1.4 g-P(3HB)/l from fructose (Table 3), again suggesting no effects of the glp_{Ec} gene(s) on the fructose

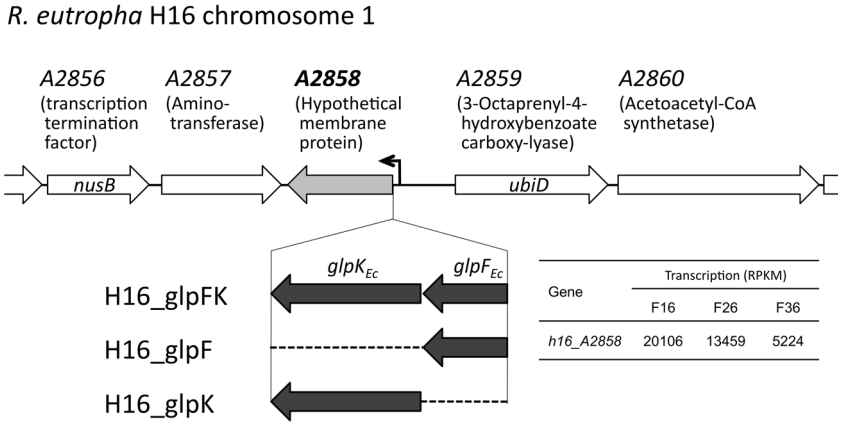

Fig. 1 Organization of $glpFK_{Ec}$, $glpF_{Ec}$, or $glpK_{Ec}$ inserted at upstream of *h16_A2858* on chromosome 1 of *R. eutropha* H16. *Inset table*, transcription levels of *h16_A2858* shown as RPKM values at growth (*F16*), PHA production (*F26*), and stationary (*F36*) phases on fructose (Shimizu et al. 2013)

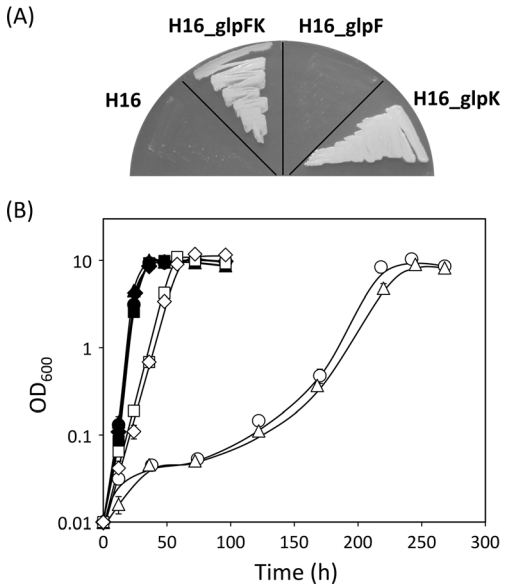

Fig. 2 Growth properties of the engineered strains of *R. eutropha* H16 on glycerol. **a** Growth of *R. eutropha H16*, *H16_glpFK*, *H16_glpF*, and *H16_glpK* on a nitrogen-limited MB solid medium containing 1 % (*w*/*v*) glycerol for 72 h at 30 °C. **b** Growth curves of *R. eutropha* H16 (*circles*), H16_glpFK (*diamonds*), H16_glpF (*triangles*), and H16_glpK (*squares*) in a nitrogen-limited MB liquid medium containing 0.5 % (*w*/*v*) fructose (*closed symbols*) or 0.5 % (*w*/*v*) glycerol (*open symbols*) at 30 °C

metabolisms in *R. eutropha*. Although H16 and H16_glpF grew very slowly on glycerol as described above, these strains produced 1.0~1.1 g/l of P(3HB) from glycerol (0.20~0.22 g/g) after 268-h cultivation. Notably, H16_glpFK and H16_glpK accumulated 1.3~1.4-fold more P(3HB) from glycerol (0.27~0.29 g/g) after much shorter cultivation time (72 h) compared to the strains not harboring $glpK_{Ec}$. When soybean oil was fed as a carbon source, the amount of P(3HB) produced by H16_glpFK (3.6 g/l, 0.39 g/g) was larger than those by the other three strains (2.9~3.1 g/l, 0.32~0.34 g/g).

GK and FAD-G3PDH activities in recombinant *R. eutropha*

Enzymatic activities of GK and FAD-G3PDH were measured in *R. eutropha* H16 and H16_glpFK (Table 4). The cell extract of H16 grown on fructose contained no GK and FAD-G3PDH activities, while very low activities of GK (6.0 mU/mg) and FAD-G3PDH (1.4 mU/mg) were detected in the cells at the late exponential growth phase on glycerol (206 h). Interestingly, GK activity in H16_glpFK on glycerol at 48 h was 11.4 mU/mg, which was only slightly higher when compared to that in H16. These results indicated that the quite weak GK activity would cause the slow growth of the wild strain on glycerol, and the slight increase of GK activity by heterologous $glpK_{Ec}$ led to the faster growth of H16_glpFK on glycerol. FAD-G3PDH activity in H16_glpFK was subtly induced as well as in H16 when the cells were grown on glycerol.

Insertion of $glpFK_{Ec}$ into P(3HB-*co*-3HHx)-producing *R. eutropha* and examination of the PHA biosynthesis properties

R. eutropha strains NSDG and TT013 were previously constructed for P(3HB-*co*-3HHx) biosynthesis from vegetable oils by replacing the native $phaC_{Re}$ in H16 by $phaC_{NSDG}$ encoding N143S/D171G mutant of *Aeromonas caviae* PHA synthase (Mifune et al. 2010), and by replacing β-ketothiolase gene $phaA_{Re}$ in NSDG by a tandem of two (*R*)-enoyl-CoA hydratase genes $phaJ_{Ac}$ and $phaJ4a_{Re}$ (Kawashima et al. 2012), respectively. It has been reported that P(3HB-*co*-3HHx) composed of 3HHx fraction of 1.9 and 10.5 mol% could be produced from soybean oil by the strains NSDG and

Table 3 P(3HB) production by *R. eutropha* H16 and the derivatives harboring $glpFK_{Ec}$, $glpF_{Ec}$, or $glpK_{Ec}$

Strain	Carbon source (Cultivation time)	Dry cell mass (g/l)	PHA content (wt%)	PHA (g/l)	PHA comp. (mol%)	
					3HB	3HHx
H16	Fructose (72 h)	2.15±0.05	62.5±0.3	1.35±0.03	100	0
	Glycerol (268 h)	1.81±0.16	59.7±2.1	1.08±0.07	100	0
	Soybean oil (72 h)	3.82±0.04	81.8±0.6	3.12±0.05	100	0
H16_glpFK	Fructose (72 h)	2.12±0.04	61.3±0.9	1.30±0.08	100	0
	Glycerol (72 h)	2.25±0.06	63.9±1.7	1.44±0.08	100	0
	Soybean oil (72 h)	4.44±0.10	82.1±0.5	3.64±0.07	100	0
H16_glpF	Fructose (72 h)	2.12±0.03	61.6±0.2	1.31±0.02	100	0
	Glycerol (268 h)	1.66±0.07	60.1±3.4	1.00±0.10	100	0
	Soybean oil (72 h)	3.70±0.05	78.2±1.3	2.90±0.06	100	0
H16_glpK	Fructose (72 h)	2.19±0.01	61.4±1.0	1.35±0.02	100	0
	Glycerol (72 h)	2.10±0.01	63.3±1.0	1.33±0.03	100	0
	Soybean oil (72 h)	3.78±0.13	81.7±1.4	3.09±0.10	100	-

Cells were cultivated in a nitrogen-limited MB medium containing 0.5 % (*w*/*v*) fructose, 0.5 % (*w*/*v*) glycerol, or 1 % (*v*/*v*) soybean oil for 72 h at 30 °C. n=3~4

Table 4 Activities of glycerol kinase and FAD-glycerol 3-phosphate dehydrogenase in *R. eutropha* H16 and H16_glpFK

Strain	Carbon source (Cultivation time)	Specific activity (nmol min^{-1} $mg\text{-}protein^{-1}$)	
		Glycerol kinase	FAD-glycerol 3-phosphate dehydrogenase
H16	Fructose (24 h)	0	<0.1
	Glycerol (206 h)	6.0±1.3	2.2±0.6
H16_glpFK	Fructose (24 h)	2.0±0.4	0.14±0.06
	Glycerol (48 h)	11.4±1.6	2.8±0.7

Cells were cultivated in a nitrogen-limited MB medium containing 0.5 % (*w*/*v*) fructose or 0.5 % (*w*/*v*) glycerol at 30 °C. Data are the means of two determinations for two independent cultures

TT013, respectively (Kawashima et al. 2012; Mifune et al. 2010). The insertion of $glpFK_{Ec}$ at the upstream of *h16_A2858* was also conducted into these strains to investigate the effects of the metabolic modification on PHA copolymer biosynthesis from soybean oil mediated by the heterologous PHA synthase. The strain NSDG_glpFK showed fast growth and efficient P(3HB) production on glycerol, as seen for H16_glpFK. On soybean oil, NSDG_glpFK accumulated slightly larger amount of P(3HB-*co*-3HHx) than NSDG, whereas PHA production by TT013_glpFK was not increased from that by TT013. The 3HHx composition of PHAs on soybean oil was 1.7 and 9.9 mol% by NSDG_glpFK and TT013_glpFK, respectively. These values were almost the same as those by the corresponding parent strains, indicating little effects of the enhanced glycerol metabolism on composition of P(3HB-*co*-3HHx) synthesized from soybean oil.

Molecular weight of PHA synthesized from glycerol by recombinant *R. eutropha*

It is of interest whether the enhanced glycerol assimilation ability affected molecular weights of the glycerol-based PHAs, because the previous studies have demonstrated that supplement of glycerol into media for *R. eutropha* NCIMB 40529 decreased the molecular weights of P(3HB) possibly due to the chain transfer action of glycerol during PHA synthase-mediated polymerization (Madden et al. 1999; Taidi et al. 1994). In the present results, however, the number-average-molecular weights (M_n) and weight-average-molecular weights (M_w) of P(3HB) synthesized by H16 and H16_glpFK from glycerol were $6.8\sim9.2\times10^5$ and $21.5\sim22.5\times10^5$, respectively, which were slightly higher than those of P(3HB) on fructose and soybean oil (Table 6). It should be noted that the polydispersity index (PDI) of P(3HB) on glycerol synthesized by H16_glpFK was decreased to 2.4 from 3.2 of that synthesized by H16. Similarly, no significant decrease of molecular weights by glycerol was observed for the engineered strains expressing $phaC_{NSDG}$ derived from *A. caviae*. PDI of P(3HB) synthesized from glycerol by NSDG_glpFK was also lower than that by the parent strain NSDG, although the introduction of $glpFK_{Ec}$ in NSDG led to slight decrease of M_n and M_w of the glycerol-based P(3HB).

Discussion

R. eutropha H16 was able to produce P(3HB) from glycerol, although it required very long cultivation time due to the poor growth ability on glycerol. In the present study, we demonstrated that the H16-based engineered strains possessing $glpFK_{Ec}$ or $glpK_{Ec}$ produced large amount of P(3HB) from glycerol with much higher productivity than the wild strain H16. A previous report described that glycerol utilization ability was conferred to *R. eutropha* strain ATCC 17697 by introducing $glpFK_{Ec}$ (Mukoyama 2007), but the participation of the individual gene in the enhanced glycerol utilization has not been considered. At least for *R. eutropha* H16, the sole expression of $glpK_{Ec}$ established the pathway for more efficient assimilation of glycerol, and the expression of $glpF_{Ec}$ was not essential. Namely, insufficient kination activity to glycerol was a major reason for the poor growth of *R. eutropha* H16 on glycerol. The function of the two putative GKs in H16 genome, H16_A2507 and H16_B1199, appeared to be none or too weak to support the growth on glycerol. The results also indicated that *R. eutropha* H16 had a potential ability for uptake of extracellular glycerol by native transporters such as PEP-PTS encoded by *ptsMHI* as reported previously (Kaddor and Steinbuchel 2011), H16_A3690 originally annotated as GlpF sharing partial and weak identity to $GlpF_{Ec}$, or other unidentified transporters. However, we observed that production of P(3HB) by H16_glpFK from soybean oil was increased up to 117 % when compared to H16_glpK not harboring $glpF_{Ec}$ (Table 3). This would raise a possibility that the glycerol facilitator encoded by $glpF_{Ec}$ may contribute to uptake and utilization of low concentration of glycerol generated through hydrolysis of soybean oil by lipase (Lu et al. 2013). NSDG_glpFK also produced slightly larger P(3HB-*co*-3HHx) than the parent strain NSDG from soybean oil, whereas no such increase of P(3HB-*co*-3HHx) production by $glpFK_{Ec}$ was observed for TT013_glpFK (Table 5). This was estimated to be due to the lack of *phaA* encoding β-ketothiolase in TT013, as previously reported that *phaA* deletion in H16-derived strains resulted in reduction of (*R*)-3HB-CoA formation from acetyl-CoA during the growth on soybean oil (Mifune et al. 2010).

When $glpFK_{Ec}$ or $glpK_{Ec}$ alone was introduced using multi-copy vectors into *R. eutropha* H16, occurrence of unexpected deletion probably in the $glpK_{Ec}$ region was strongly

Table 5 P(3HB) and P(3HB-*co*-3HHx) production by *R. eutropha* NSDG ($\Delta phaC_{Re}$::$phaC_{NSDG}$) and the derivatives harboring $glpFK_{Ec}$

Strain	Carbon source (Cultivation time)	Dry cell mass (g/l)	PHA content (wt%)	PHA (g/l)	PHA comp. (mol%)	
					3HB	3HHx
NSDG	Fructose (72 h)	2.16±0.01	61.0±0.6	1.32±0.01	100	0
	Glycerol (268 h)	1.95±0.12	62.2±2.9	1.22±0.13	100	0
	Soybean oil (72 h)	6.13±0.18	84.6±1.2	5.18±0.04	98.2	1.8
NSDG_glpFK	Fructose (72 h)	2.17±0.06	67.8±0.8	1.47±0.04	100	0
	Glycerol (72 h)	2.26±0.02	68.0±0.9	1.53±0.02	100	0
	Soybean oil (72 h)	6.30±0.17	86.5±1.2	5.45±0.20	98.3	1.7
TT013	Soybean oil (72 h)	4.73±0.08	82.8±0.8	3.92±0.09	89.9	10.1
TT013_glpFK	Soybean oil (72 h)	4.52±0.24	80.3±0.4	3.62±0.18	90.1	9.9

Cells were cultivated in a nitrogen-limited MB medium containing 0.5 % (*w*/*v*) fructose, 0.5 % (*w*/*v*) glycerol, or 1 % (*v*/*v*) soybean oil for 72 h at 30 °C. n=3~4

suggested. This phenomenon was not seen in *R. eutropha* ATCC 17697 (Mukoyama 2007), implying a different metabolic background between H16 and another wild strain ATCC 17697. The deletion was thought to be due to some toxic effects of overexpression of $glpK_{Ec}$ specifically in H16. It has been reported that bacterial glycerol metabolisms sometimes accompanied by-production of toxic compounds, such as methylglyoxal formed in *E. coli* strains having unregulated glycerol metabolism (Freedberg et al. 1971) or an engineered pathway for 1,3-propanediol production (Zhu et al. 2001), and glycerol 3-phosphate highly accumulated in *C. glutamicum* overexpressing $glpFK_{Ec}$ (Rittmann et al. 2008). However, unlike these cases, the probable gene deletion in the transformants of *R. eutropha* H16 occurred during the growth on nutrient-rich plate medium without supplement of glycerol. Nevertheless, we could not rule out the possibility that formation of certain toxic compound(s) from glycerol-related metabolites may be induced in the cells of H16 by overexpression of $glpK_{Ec}$, and the reason for this unanticipated deletion has remained to be cleared.

The enhancement of glycerol utilization ability of *R. eutropha* H16 was achieved by insertion of $glpFK_{Ec}$ or $glpK_{Ec}$ at upstream of *h16_A2858* on chromosome 1. We chose the targeted insertion site with the aim of constitutive and strong expression of the heterologous gene(s) in *R. eutropha*, based on the results of RNA-seq-based transcriptional analysis (Shimizu et al. 2013). However, contrary to the expectation, the expression level of the inserted $glpK_{Ec}$ was not high as shown by the enzyme assay (Table 4). This was consequently favorable, because strong expression of the inserted $glpK_{Ec}$ in excess was predicted to be toxic as well as observed with the plasmid-borne copy of the gene; i.e., the weak expression of $glpK_{Ec}$ would be a key factor for the successful enhancement of glycerol utilization ability of *R. eutropha* H16. The results of enzyme assay also clarified that the slight increase of GK activity drastically improved the growth on glycerol. Similar significant increase of growth rate of H16-based strains was also observed by slightly higher glucokinase activity when glucose was used as the sole carbon source (Orita et al. 2011). It was further revealed that FAD-G3PDH activity in *R. eutropha* H16_glpFK was also very low, but appeared to be sufficient to support the fairly fast growth on glycerol. At the present state, the growth rates of *R. eutropha* H16_glpFK and H16_glpK on glycerol were still lower when compared to that on fructose. Subtly higher but not excess expression of *glpK*, overexpression of *glpD*, or the combination of them may allow to obtain H16-derived strains capable of growing on glycerol as fast as on fructose.

Another interesting subject was the effect of the metabolic engineering enhancing glycerol assimilation on molecular weight of PHAs synthesized from glycerol. It has been

Table 6 Molecular weight of PHAs produced by *R. eutropha* H16, NSDG, and the recombinant strains harboring $glpFK_{Ec}$

Strain	Carbon source	3HHx comp. (mol%)	Mn ($\times 10^5$)	Mw ($\times 10^5$)	PDI
H16	Fructose	0	5.4±0.4	14.9±2.3	2.8
	Glycerol	0	6.8±0.2	21.5±1.4	3.2
	Soybean oil	0	4.3±0.4	16.2±1.2	3.7
H16_glpFK	Fructose	0	7.4±0.3	20.8±0.7	2.8
	Glycerol	0	9.2±1.4	22.5±2.8	2.4
	Soybean oil	0	5.6±0.3	19.7±1.1	3.5
NSDG	Fructose	0	9.0±0.8	19.4±0.9	2.2
	Glycerol	0	8.4±1.2	26.5±2.5	3.2
	Soybean oil	1.8	7.1±1.5	17.2±1.3	2.5
NSDG_glpFK	Fructose	0	8.0±0.3	18.0±2.3	2.3
	Glycerol	0	7.2±0.8	17.6±2.3	2.4
	Soybean oil	1.7	5.9±0.3	14.6±0.4	2.5

Determined by GPC. n=3~4

reported that, with respect to *R. eutropha* strains, M_w of P(3HB) produced from glycerol by JMP134, DSM 545, and NCIMB 40529 were in the range of $5.5 \sim 10 \times 10^5$, which were about half of $12 \sim 18 \times 10^5$ of P(3HB) from fructose or glucose, probably caused by the chain transfer action of glycerol during the polymerization (Cavalheiro et al. 2009; Mothes et al. 2007; Taidi et al. 1994). As well, rather low M_w of 5.5×10^5 was reported for P(3HB) synthesized from glycerol by a glycerol-adapted mutant of *R. eutropha* ATCC 17699 (Tanadchangsaeng and Yu 2012). In this study, significant decrease of molecular weights by glycerol was not observed for *R. eutropha* H16 (Table 6). It might be due to less frequent transfer of growing PHA chains to intracellular glycerol caused by possibly lower glycerol-uptake ability of H16 than the other *R. eutropha* strains. However, when molecular weights were compared between P(3HB) synthesized from glycerol by H16 and that by H16_glpFK, M_n was increased and PDI was consequently decreased by the introduction of $glpFK_{Ec}$. The predicted low concentration of intracellular glycerol might be further decreased through the enhanced glycerol assimilation in H16_glpFK, thus leading to infrequent chain transfer reaction with glycerol. While, PDI and molecular weights of glycerol-based P(3HB) synthesized by NSDG_glpFK was lower than those by the parent strain NSDG, both expressing a mutant of PHA synthase derived from *A. caviae* (Tsuge et al. 2007). The decrease of PDI observed with NSDG_glpFK was also assumed to be due to the enhanced glycerol assimilation ability, but the reason for the decrease of molecular weights were not clear. This may be due to specific property of PHA synthase derived from *A. caviae*, as demonstrated by unique change of molecular weights of P(3HB) polymerized by *A. caviae* PHA synthase in *E. coli* under the presence of poly(ethylene glycol) (Tomizawa et al. 2010).

In conclusion, the engineered strains of *R. eutropha* harboring $glpFK_{Ec}$ were applicable for biosynthesis of PHAs with high molecular weight from glycerol, and the present results would open the door for utilization of the *R. eutropha* H16-based platform for production of other value-added materials from inexpensive and renewable glycerol.

Acknowledgments The authors thank Assoc. Prof. Takeharu Tsuge and Dr. Ayaka Hiroe (Department of Innovative and Engineered Materials, Tokyo Institute of Technology, Japan) for GPC analysis. This work was supported by JSPS KAKENHI grant number 25292058.

References

Austin D, Larson TJ (1991) Nucleotide sequence of the *glpD* gene encoding aerobic *sn*-glycerol 3-phosphate dehydrogenase of *Escherichia coli* K-12. J Bacteriol 173:101–107

Brigham CJ, Speth DR, Rha C, Sinskey AJ (2012) Whole-genome microarray and gene deletion studies reveal regulation of the polyhydroxyalkanoate production cycle by the stringent response in *Ralstonia eutropha* H16. Appl Environ Microbiol 78:8033–8044

Budde CF, Riedel SL, Willis LB, Rha C, Sinskey AJ (2011) Production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from plant oil by engineered *Ralstonia eutropha* strains. Appl Environ Microbiol 77: 2847–2854

Cavalheiro JMBT, de Almeida MCMD, Grandfils C, da Fonseca MMR (2009) Poly(3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. Process Biochem 44:509–515

Cavalheiro JM, Raposo RS, de Almeida MC, Cesario MT, Sevrin C, Grandfils C, da Fonseca MM (2012) Effect of cultivation parameters on the production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) and poly(3-hydroxybutyrate-4-hydroxybutyrate-3-hydroxyvalerate) by *Cupriavidus necator* using waste glycerol. Bioresour Technol 111:391–397

Dobson R, Gray V, Rumbold K (2012) Microbial utilization of crude glycerol for the production of value-added products. J Ind Microbiol Biotechnol 39:217–226

Durnin G, Clomburg J, Yeates Z, Alvarez PJ, Zygourakis K, Campbell P, Gonzalez R (2009) Understanding and harnessing the microaerobic metabolism of glycerol in *Escherichia coli*. Biotechnol Bioeng 103: 148–161

Freedberg WB, Kistler WS, Lin EC (1971) Lethal synthesis of methylglyoxal by *Escherichia coli* during unregulated glycerol metabolism. J Bacteriol 108:137–144

Fukui T, Chou K, Harada K, Orita I, Nakayama Y, Bamba T, Nakamura S, Fukusaki E (2014) Metabolite profiles of polyhydroxyalkanoate-producing *Ralstonia eutropha* H16. Metabolomics 10:190–202

Garcia IL, Lopez JA, Dorado MP, Kopsahelis N, Alexandri M, Papanikolaou S, Villar MA, Koutinas AA (2013) Evaluation of by-products from the biodiesel industry as fermentation feedstock for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) production by *Cupriavidus necator*. Bioresour Technol 130:16–22

Hiroe A, Hyakutake M, Thomson NM, Sivaniah E, Tsuge T (2013) Endogenous ethanol affects biopolyester molecular weight in recombinant *Escherichia coli*. ACS Chem Biol 8:2568–2576

Insomphun C, Mifune J, Orita I, Numata K, Nakamura S, Fukui T (2013) Modification of β-oxidation pathway in *Ralstonia eutropha* for production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from soybean oil. J Biosci Bioeng 117:184–190

Kaddor C, Steinbuchel A (2011) Implications of various phosphoenolpyruvate-carbohydrate phosphotransferase system mutations on glycerol utilization and poly(3-hydroxybutyrate) accumulation in *Ralstonia eutropha* H16. AMB Express 1:16

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

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

Keshavarz T, Roy I (2010) Polyhydroxyalkanoates: bioplastics with a green agenda. Curr Opin Microbiol 13:321–326

Loo CY, Lee WH, Tsuge T, Doi Y, Sudesh K (2005) Biosynthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from palm oil products in a *Wautersia eutropha* mutant. Biotechnol Lett 27:1405–1410

Lu J, Brigham CJ, Gai CS, Sinskey AJ (2012) Studies on the production of branched-chain alcohols in engineered *Ralstonia eutropha*. Appl Microbiol Biotechnol 96:283–297

Lu J, Brigham CJ, Rha C, Sinskey AJ (2013) Characterization of an extracellular lipase and its chaperone from *Ralstonia eutropha* H16. Appl Microbiol Biotechnol 97:2443–2454

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

Meiswinkel TM, Rittmann D, Lindner SN, Wendisch VF (2013) Crude glycerol-based production of amino acids and putrescine by *Corynebacterium glutamicum*. Bioresour Technol 145:254–258

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

Mothes G, Schnorpfeil C, Ackermann JU (2007) Production of PHB from crude glycerol. Eng Life Sci 7:475–479

Mukoyama M (2007) Method of imparting glycerol-assimilation ability to bacterium. International Patent Publication WO2007/013695

Nikodinovic-Runic J, Guzik M, Kenny ST, Babu R, Werker A, O'Connor KE (2013) Carbon-rich wastes as feedstocks for biodegradable polymer (polyhydroxyalkanoate) production using bacteria. Adv Appl Microbiol 84:139–200

Orita I, Iwazawa R, Nakamura S, Fukui T (2011) Identification of mutation points in *Cupriavidus necator* NCIMB 11599 and genetic reconstitution of glucose-utilization ability in wild strain H16 for polyhydroxyalkanoate production. J Biosci Bioeng 113:63–69

Pan P, Inoue Y (2009) Polymorphism and isomorphism in biodegradable polyesters. Prog Polym Sci 34:605–640

Peplinski K, Ehrenreich A, Doring C, Bomeke M, Reinecke F, Hutmacher C, Steinbuchel A (2010) Genome-wide transcriptome analyses of the 'Knallgas' bacterium *Ralstonia eutropha* H16 with regard to polyhydroxyalkanoate metabolism. Microbiology 156: 2136–2152

Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, Eitinger T, Ewering C, Potter M, Schwartz E, Strittmatter A, Voss I, Gottschalk G, Steinbuchel A, Friedrich B, Bowien B (2006) Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. Nat Biotechnol 24:1257–1262

Raberg M, Peplinski K, Heiss S, Ehrenreich A, Voigt B, Doring C, Bomeke M, Hecker M, Steinbuchel A (2011) Proteomic and transcriptomic elucidation of the mutant *Ralstonia eutropha* $G^{+}1$ with regard to glucose utilization. Appl Environ Microbiol 77:2058–2070

Raberg M, Kaddor C, Kusian B, Stahlhut G, Budinova R, Kolev N, Bowien B, Steinbuchel A (2012) Impact of each individual component of the mutated PTS^{Nag} on glucose uptake and phosphorylation in *Ralstonia eutropha* $G^{+}1$. Appl Microbiol Biotechnol 95:735–744

Riedel SL, Bader J, Brigham CJ, Budde CF, Yusof ZA, Rha C, Sinskey AJ (2011) Production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) by *Ralstonia eutropha* in high cell density palm oil fermentations. Biotechnol Bioeng 109:74–83

Rittmann D, Lindner SN, Wendisch VF (2008) Engineering of a glycerol utilization pathway for amino acid production by *Corynebacterium glutamicum*. Appl Environ Microbiol 74:6216–6222

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

Shimizu R, Chou K, Orita I, Suzuki Y, Nakamura S, Fukui T (2013) Detection of phase-dependent transcriptomic changes and Rubisco-mediated CO_2 fixation into poly(3-hydroxybutyrate) under heterotrophic condition in *Ralstonia eutropha* H16 based on RNA-seq and gene deletion analyses. BMC Microbiol 13:169

Sichwart S, Hetzler S, Broker D, Steinbuchel A (2011) Extension of the substrate utilization range of *Ralstonia eutropha* strain H16 by metabolic engineering to include mannose and glucose. Appl Environ Microbiol 77:1325–1334

Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering. Transposon mutagenesis in Gram negative bacteria. Bio/Technology 1:784–791

Spoljaric IV, Lopar M, Koller M, Muhr A, Salerno A, Reiterer A, Horvat P (2013a) In silico optimization and low structured kinetic model of poly[(*R*)-3-hydroxybutyrate] synthesis by *Cupriavidus necator* DSM 545 by fed-batch cultivation on glycerol. J Biotechnol 168: 625–635

Spoljaric IV, Lopar M, Koller M, Muhr A, Salerno A, Reiterer A, Malli K, Angerer H, Strohmeier K, Schober S, Mittelbach M, Horvat P (2013b) Mathematical modeling of poly[(*R*)-3-hydroxyalkanoate] synthesis by *Cupriavidus necator* DSM 545 on substrates stemming from biodiesel production. Bioresour Technol 133:482–494

Taguchi S, Iwata T, Abe H, Doi Y (2012) Poly(hydroxyalkanoate)s. In: Matyjaszewski K, Möller M (eds) Polymer science: a comprehensive reference. Elsevier, Amsterdam, pp 157–182

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

Tanadchangsaeng N, Yu J (2012) Microbial synthesis of polyhydroxybutyrate from glycerol: gluconeogenesis, molecular weight and material properties of biopolyester. Biotechnol Bioeng 109:2808–2818

Tomizawa S, Saito Y, Hyakutake M, Nakamura Y, Abe H, Tsuge T (2010) Chain transfer reaction catalyzed by various polyhydroxyalkanoate synthases with poly(ethylene glycol) as an exogenous chain transfer agent. Appl Microbiol Biotechnol 87:1427–1435

Tomizawa S, Sato S, Lan JC, Nakamura Y, Abe H, Tsuge T (2013) In vitro evidence of chain transfer to tetraethylene glycols in enzymatic polymerization of polyhydroxyalkanoate. Appl Microbiol Biotechnol 97:4821–4829

Tsuge T, Watanabe S, Shimada D, Abe H, Doi Y, Taguchi S (2007) Combination of N149S and D171G mutations in *Aeromonas caviae* polyhydroxyalkanoate synthase and impact on polyhydroxyalkanoate biosynthesis. FEMS Microbiol Lett 277: 217–222

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

Wang Y, Schulten K, Tajkhorshid E (2005) What makes an aquaporin a glycerol channel? A comparative study of AqpZ and GlpF. Structure 13:1107–1118

Weissenborn DL, Wittekindt N, Larson TJ (1992) Structure and regulation of the *glpFK* operon encoding glycerol diffusion facilitator and glycerol kinase of *Escherichia coli* K-12. J Biol Chem 267:6122–6131

Yang F, Hanna MA, Sun R (2012) Value-added uses for crude glycerol—a byproduct of biodiesel production. Biotechnol Biofuels 5:13

Zhu MM, Skraly FA, Cameron DC (2001) Accumulation of methylglyoxal in anaerobically grown *Escherichia coli* and its detoxification by expression of the *Pseudomonas putida* glyoxalase I gene. Metab Eng 3:218–225