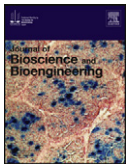

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Identification of mutation points in *Cupriavidus necator* NCIMB 11599 and genetic reconstitution of glucose-utilization ability in wild strain H16 for polyhydroxyalkanoate production

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Although the facultative chemolithoautotrophic *Cupriavidus necator* (formerly *Ralstonia eutropha*) wild strain H16 is potentially useful as a host for metabolic engineering aimed at polyhydroxyalkanoate production, this organism is deficient in assimilating glucose, a major sugar in non-edible cellulosic resources. Growth properties of *C. necator* H16 harboring heterologous *glf* (encoding glucose-facilitated diffusion transporter) and *glk* (encoding glucokinase) from *Zymomonas mobilis* strongly suggested that the lack of glucose-utilization ability of *C. necator* H16 was caused by deficiency of both glucose-uptake and phosphorylation abilities. Next examination focused on previously unknown mutation points in a glucose-utilizing mutant of *C. necator* NCIMB 11599. Direct sequencing of a region of genes for putative *N*-acetylglucosamine-specific phosphoenolpyruvate-dependent phosphotransferase system and its upstream region identified a missense mutation in *nagE* corresponding to Gly265Arg in the EIIC-EIIB component, and a nonsense mutation in *nagR* encoding a putative GntR-type transcriptional regulator. Further analyses demonstrated that the glucose-utilization ability of *C. necator* NCIMB 11599 is attributed to extended sugar specificity of the mutated NagE and derepression of *nagFE* expression by inactivation of NagR. The mutation in *nagE* and disruption of *nagR* were then introduced onto chromosome 1 of wild strain H16 by homologous recombination. The resulting engineered strain *C. necator* nagE_G265RΔnagR exhibited comparable growth and poly(3-hydroxybutyrate) accumulation on glucose to those of the wild strain on fructose, demonstrating successful reconstitution of functional glucose-uptake and phosphorylation system. This recombinant strain is expected to be useful in further engineering for efficient production of PHAs from inexpensive biomass resources.

[**Key words:** Biomass plastic; Biodegradable plastic; Polyhydroxyalkanoate; *Cupriavidus necator*; *Ralstonia eutropha*; Glucose-utilizing mutant; Homologous recombination]

With the aim of exploitation in alternative to the finite fossil resources such as petroleum for reduction of greenhouse gas emission, there is a growing concern about wider utilization of biomass, which represents an abundant carbon-neutral renewable resource for the production of biofuels and biomaterials (1). Recently, considerable progresses have been made in production of biofuels such as bioethanol from starch and biodiesel (fatty acid methyl esters) from plant oils, as well as biopolymers represented by polylactic acid from starch. However, the use of such edible biomass as feedstocks for them may cause an imbalance in global food supply and depletion of food sources. Therefore, much effort has been made to utilize a sugar mixture obtained by hydrolysis of non-edible cellulosic plant materials, to avoid competition between food supply and production of biofuels and biomaterials.

Polyhydroxyalkanoates (PHAs) are intracellular biopolyesters produced by various microorganisms as energy storage materials, and are attracting interest because they are biodegradable and biocompatible thermoplastics that can be applied in various ways (2,3). A chemolithoautotrophic bacterium *Cupriavidus necator* (formerly *Ralstonia eutropha*) strain H16 is one of the best-studied PHA producer (4). This bacterium can grow and synthesize poly(3-hydroxybutyrate) [P(3HB)], the most abundant PHA in nature, on fructose, gluconate, even-carbon number fatty acids, and vegetable oils as a carbon source. There have been many studies for this strain focusing on biochemistry and molecular biology of PHA biosynthesis, and fermentation and metabolic engineering for efficient production of PHAs. We have developed recombinant strains of *C. necator* H16 and its PHA-negative mutant PHB-4 for biosynthesis of PHA copolymers from biomass without addition of precursor compounds (5–9). Unfortunately, *C. necator* H16 and the closely related strains cannot grow on glucose, a major sugar in cellulosic materials, leading to limitation of these strains for production of PHAs from non–edible biomass.

Whole genome information of *C. necator* wild strain H16 (10) has allowed us to estimate carbohydrate metabolisms in this bacterium. Extracellular fructose is possibly transported into the cells by fructose-specific ATP-binding cassette (ABC)-type transporter (H16_B1498-B1500), and subsequently converted to glucose-6-phosphate

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(G6P) by catalytic reactions with fructokinase (H16_B1503) and glucose-6-phosphate isomerase (H16_B1502). G6P then enters into Entner-Doudoroff pathway mediated by glucose-6-phosphate (G6P) 1-dehydrogenases (H16_A0316, H16_B1501, and H16_B2566), 6-phosphogluconolactonase (H16_B2565), phosphogluconate dehydratase (H16_B2567), and 2-keto-3-deoxy-6-phosphogluconate aldolase (H16_B1213), to generate pyruvate and glyceraldehyde-3-phosphate. With respect to glucose uptake, *C. necator* H16 possesses no protein highly homologous to known specific transport systems which are energy-dependent specific carriers belonging either to phospho-enolpyruvate-dependent phosphotransferase system (PTS) transporters, cation-linked permeases, or ABC-type transporters, or energy-independent facilitator-type transporters (11,12). Glucokinase (Glk) or hexokinase then acts on formation of G6P from the imported glucose besides transportation by PTS mediating 6-phosphorylation-associated transportation. One gene (H16_B2564) was estimated to encode a putative Glk on choromosome 2, although the deduced amino acid sequence has only 33% identities towards Glk from *Escherichia coli* and *Zymomonas mobilis*, and expression of this gene and function of the translated product in sugar metabolisms have not been elucidated. Considering the shared metabolisms between fructose and glucose after the formation of G6P, it is feasible that *C. necator* H16 may have deficiency in generation of G6P from extracellular glucose. Indeed, Sichwart et al. recently constructed a recombinant strain of *C. necator* H16 having glucose utilization ability by expression of genes for glucose-facilitated diffusion transporter (Glf) from *Z. mobilis* and Glk from *E. coli* (13).

On the other hand, it is of interest that several glucose-utilizing mutants were obtained from *C. necator* wild strains by UV and spontaneous mutagenesis (14–16). Such mutants had been applied for the pioneer pilot-scale industrial production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] from mixed carbon sources of glucose and propionate in the late of 1980s (17), and one mutant *C. necator* NCIMB 11599 was frequently used for studies on biosynthesis of P(3HB) and P(3HB-*co*-3HV) from glucose in high-cell density fed-batch cultures (18–20). Nevertheless, the genetic background responsible for the glucose assimilation in these mutants has been little considered except for recent report regarding one mutant *C. necator* G^+1 (21), and reconstitution of the glucose-utilization ability in wild strain has not yet been achieved. Reconstitution of useful mutations is important in rational engineering of *C. necator* for biosynthesis of practical PHA copolymers from cellulosic materials.

This study aimed to establish a recombinant strain derived from *C. nacator* H16 capable of utilizing glucose for the growth and PHA production. First, the deficiency of both uptake and successive phosphorylation of glucose in wild strain H16 was confirmed by heterologous expression of genes for glucose transport and assimilation system from *Z. mobilis*. Then, we identified previously unknown mutation points in the glucose-utilizing mutant *C. necator* NCIMB 11599, and examined to reconstitute the glucose-utilization ability by introducing the identified mutations onto the chromosome 1 of wild strain H16. This effort successfully resulted in development of an engineered strain of *C. necator* that can well utilize glucose as a sole carbon source for PHA production.

MATERIALS AND METHODS

Bacterial strains and cultivation condition Strains and plasmids used in this study are shown in Table 1. *E. coli* DH5α and S17-1 (22) were grown at 37°C in Luria-Bertani medium. When necessary, ampicillin (50 mg/l), kanamycin (50 mg/l), or tetracycline (12.5 mg/l) was added to the medium. *C. necator* H16 (wild-type, DSM428) and their derivatives, and *C. necator* NCIMB 11599 were pre-cultivated at 30°C in a nutrient-rich medium (23). The cells in 1 ml broth of the pre-culture were harvested by centrifugation at 8000 ×*g*, 5 min at 4°C, washed within the same volume of fresh mineral salt (MB) medium (8) not containing carbon source, and then inoculated into a 100 ml MB medium in 500 ml flask. A filter-sterilized solution of fructose or glucose was added to the medium as a sole carbon source at a final concentration of 0.5% or 2%, and the cultivation was carried out on a reciprocal shaker (115 strokes/min) at 30°C. In the case of two-stage cultivation, the cells grown in a 100 ml MB medium containing 0.5% fructose for 24 h at 30°C were harvested and washed as above, and then transferred into a fresh 100 ml MB medium containing 0.5% fructose or glucose followed by incubation for further 24 h at 30°C. Kanamycin

TABLE 1. Strains and plasmids used in this study.

Strains or plasmids	Genotype	Source or reference
Escherichia coli		
DH5α	*deoR endA1 gyrA96 hsdR17* (r_K^- m_K^+) *recA1 relA1 supE44 thi-l* Δ(*lacZYA-argFV169*) ϕ80Δ*lacZ*dM15 F^- λ^-	Clontech
S17-1	*thi pro hsdR recA; chromosomal RP4; Tra*$^+$*; Tmp*^{r}*Str/Spc*^{r}	22
Cupriavidus necator		
H16	Wild-type	DSM428
NCIMB 11599	H16 derivative; glucose-utilizable	NCIMB 11599
ΔnagR	H16 derivative; ΔnagR	This study
nagE_G265R	H16 derivative; nagE (G793C)	This study
nagE_G265RΔnagR	nagE_G265R derivative; ΔnagR	This study
Zymomonas mobilis		
DSM424	Wild-type	DSM424
Plasmids		
pUC18, pUC118	P_{lac}, *lacZ*α Amp^r	Takara
pBBR1-MCS3	Broad-host-range vector, *mob*, P_{lac}, *lacZ*α, Tet^r,	24
pUTmini-Tn5*gfp*	Mini-Tn5, *gfp*, *mob*, Amp^r, Tet^r	25
pBAD24	P_{BAD}, *araC*, T_{rrnB}, Amp^r	26
pJAK14	Broad-host-range vector, *mob*, P_{tac}, *lacI* Kan^r,	ATCC77289
pK18mobsacB	*mob*, modified *sacB*, *lacZ*α, Kan^r	28
pEE32	pUC18 derivative, *phaPCJ* with native promoter region ($P_{pha\text{-}Ac}$) from *A. caviae*	23
pBBR3Tn5TetA	pBBR1-MCS3 derivative; replacement of original tetA by tetA from pUTmini-Tn5*gfp*	This study
pBPHAc	pBBR3Tn5TetA derivative; $P_{pha\text{-}Ac}$	This study
pBBR3-glf	pBBR1-MCS3 derivative; *glf* from *Z. mobolis*	This study
pBBR3-glf_zeg	pBBR1-MCS3 derivative; *glf-zwf-edd-glk* from *Z. mobolis*	This study
pBBR3-glf_glk	pBBR1-MCS3 derivative; *glf*, P_{tac}-*glk* from *Z. mobolis*.	This study
pBPHAc-nagFEH16	pBPHAc derivative; *nagFE* from *C. necator* H16.	This study
pBPHAc-nagFE11599	pBPHAc derivative; *nagFE* from *C. necator* NCIMB 11599	This study
pBPHAc-nagEH16	pBPHAc derivative; *nagE* from *C. necator* H16	This study
pBPHAc-nagE11599	pBPHAc derivative; *nagE* from *C. necator* NCIMB 11599	This study
pK18-nagE_G265R	pK18mobsacB derivative; *nagE* (G793C)	This study
pK18-ΔnagR	pK18mobsacB derivative; Δ*nagR*	This study