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Elimination of by-product formation during production of 1,3-propanediol in *Klebsiella pneumoniae* by inactivation of glycerol oxidative pathway

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Abstract The microbial production of 1,3-propanediol (1,3-PD) by *Klebsiella pneumoniae* involves the formation of various by-products, which are synthesized through the oxidative pathway. To eliminate the by-products synthesis, the oxidative branch of glycerol metabolism was inactivated by constructing two mutant strains. In one of the mutant strains, the structural genes encoding glycerol dehydrogenase and dihydroxyacetone kinase were deleted from the chromosomal DNA, whereas in the second mutant strain *dhaR*, which is a putative transcription factor that activates, gene expression was deleted from the chromosomal DNA. In the resultant mutant strains lacking the *dhaT* gene encoding 1,3-PD oxidoreductase, which was simultaneously deleted while replacing the native promoter with the *lacZ* promoter, the by-product formation except for acetate was eliminated, but it still produced 1,3-PD at a lower level, which might be due to a putative oxidoreductase that catalyzes the production of 1,3-PD. The recombinant strains in which the reductive pathway was recovered produced slightly lower amount of 1,3-PD as compared to the parent strain, which might be due to the reduced activity of DhaB caused by the substitution of the promoter. However, the production yield was higher in the recombinant strain (0.57 mol mol^{-1}) than the wild type Cu strain (0.47 mol mol^{-1}).

Keywords *Klebsiella pneumoniae* · 1,3-Propanediol · Glycerol metabolism · By-product

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Introduction

Currently, there is an ever-increasing demand for biodiesel production using animal fat or plant oil. However, a bulk amount of raw glycerol is formed as the main by-product during biodiesel production, which is as high as 10% (*w*/*w*) of biodiesel generated (Johnson and Taconi 2007). The surplus of raw glycerol has not only greatly disturbed the market of traditional glycerol in the preparation and price but also brought a significant environmental problem since it cannot be discharged direct into the environment without any treatment (da Silva et al. 2009). As a result of these issues, a large research effort has been devoted to developing methods to refine glycerol as a low-cost feedstock into industrially valuable materials such as fuels, building blocks, and bioactive substances.

1,3-Propanediol (1,3-PD) is a valuable chemical that is used mainly for the synthesis of polymethylene terephalates by polymerization with terephthalates (Pagliaro et al. 2007). Use of this polymer in the manufacturing of textile fiber, film, plastic, etc. is rapidly expanding. The 1,3-PD building block is currently produced by chemical processes such as hydroformylation of ethylene oxide and hydration of acrolein (Haas et al. 1994; Slaugh et al. 1995). Recently, a microbial fermentation process using a recombinant *Escherichia coli*

containing genes from *Klebsiella pneumoniae* and *Saccharomyces cerevisiae* was conducted, in which glucose was converted to 1,3-PD (Bhatia and Kurian 2008). However, the original route for the production of 1,3-PD was microbial fermentation using glycerol as the substrate. Due to the surplus of glycerol being produced as a major by-product of the biodiesel industry, the biological conversion of this chemical is being refocused.

K. pneumoniae is a typical microbial strain capable of producing 1,3-PD, in which the metabolic pathway responsible for the production of 1,3-PD has been well studied (Fig. 1; Skraly et al. 1998). Glycerol is first converted to 3-hydroxypropion aldehyde (3-HPA) by a coenzyme B_{12}-dependent glycerol dehyratase (DhaB), then which is reduced to 1,3-PD by a reduced nicotinamide adenine dinucleotide (NADH)-dependent 1,3-PD oxidoreductase (DhaT). In addition to the reductive pathway, glycerol is also metabolized by the oxidative pathway, where glycerol is dehydrogenated to dihydroxyacetone (DHA) by NAD+-dependent glycerol dehydrogenase (DhaD) and then DHA is phosphorylated to dihydroxyacetone phosphate (DHAP) by ATP-dependent DHA kinase (DhaK). Through the oxidative branch, glycerol is assimilated for an increase in biomass, which results in the generation of many different metabolites such as acetate, ethanol, lactate, succinate, and 2,3-butanediol (2,3-BD).

The genes encoding the functionally linked proteins, DhaB and DhaT for the reductive branch and DhaD and DhaK for the oxidative branch, are clustered in a region of the chromosomal DNA of *K. pneumoniae* called the *dha* regulon (Fig. 2a; Sun et al. 2003). Based on genetic information, many research groups have tried to enhance the production of 1,3-PD through metabolic engineering (Hao et al. 2008; Nakamura and Whited 2003; Tong et al. 1991; Zheng et al. 2006), thus largely increasing the enzyme activities (Yang et al. 2007; Zhang et al. 2006; Zhao et al. 2008) or NADH availability (Zhang et al. 2009).

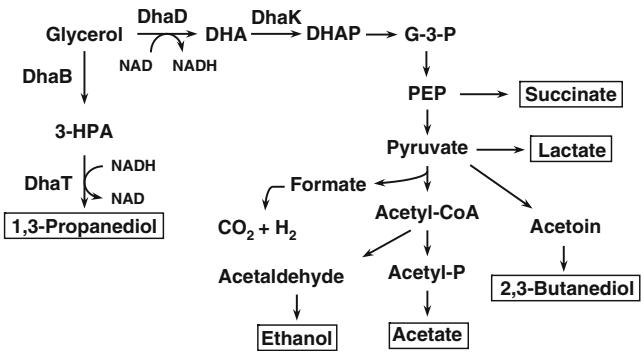

Fig. 1 Glycerol metabolic pathway in *K. pneumoniae*. *DHA* dehydroxyacetone, *DHAP* dehydroxyacetone phosphate, *G-3-P* glycerol-3 phosphate, *PEP* phosphoenol pyruvate, *3-HPA* 3-hydroxypropion aldehyde, *DhaD* glycerol dehydrogenase, *DhaK* DHA kinase, *DhaB* glycerol dehydratase, *DhaT* 1,3-propanediol oxidoreductase

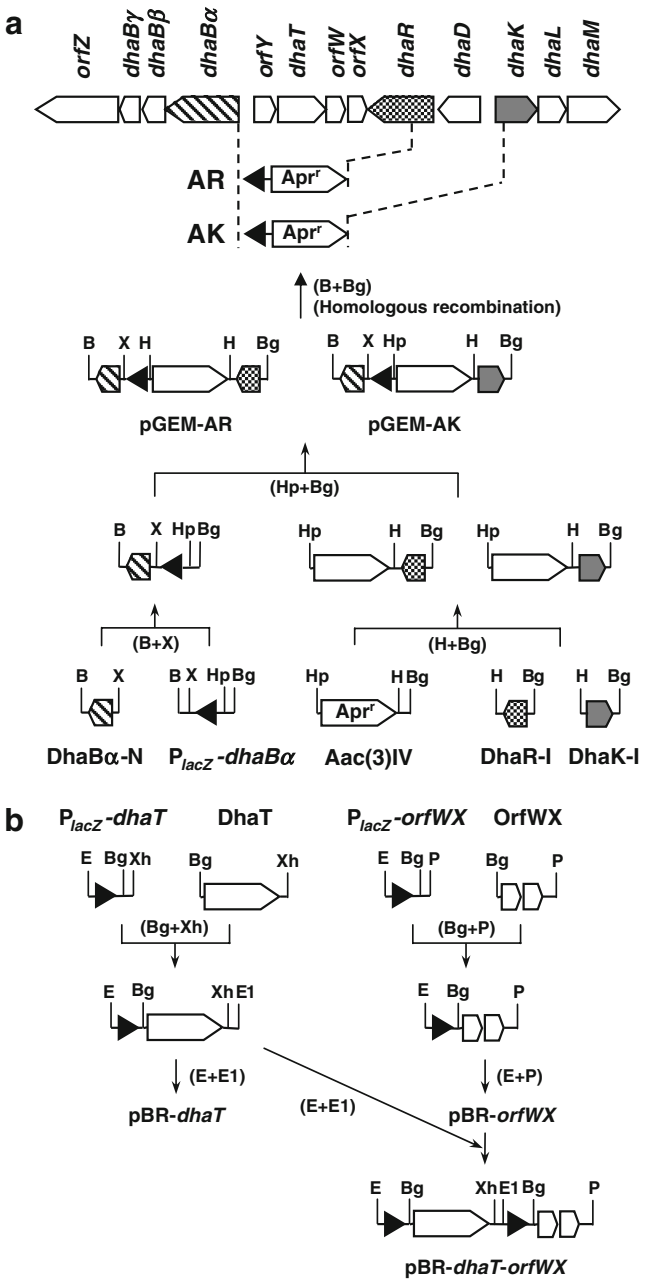

Fig. 2 Schematic representation of the strategies for construction of pGEM-AK and pGEM-AR (**a**) that were used to create AK and AR mutant strains by homologous recombination, respectively, and recombinant plasmids, pBR-dhaT, pBR-orfWX, and pBR-dhaT-orfWX (**b**). The DNA sequences encoding DhaBα-N (amino-terminal 165-amino acids of DhaBα [from start codon to Val-165]), DhaK-I (internal 185-amino acids of DhaK [from Leu-49 to Gln-233]), DhaR-I (internal 182-amino acids of DhaR; from Leu-74 to Val-255), DhaT (complete open reading frame [1.16-kb]), OrfWX (complete open reading frame [0.88-kb]), Aac(3)IV (1.19-kb DNA sequence, including apramycin resistance gene and the upstream promoter), and *lacZ* promoter sequences (P_{lacZ}-dhaBα for *dhaBα*, P_{lacZ}-dhaT for *dhaT*, and P_{lacZ}-orfWX for *orfWX*) were amplified using a set of primers (Table 1). Abbreviations for restriction enzymes: *B Bam*HI, *Bg Bgl*II, *E Eco*RI, *E1 Eco*RI (on pGEM-T Easy), *H Hin*dIII, *Hp Hpa*I, *P Pst*I, *X Xba*I, and *Xh Xho*I

However, along with an increased production of 1,3-PD, minimization of by-product biosynthesis (approximately 70% of 1,3-PD) should be considered as part of the metabolic engineering process (Oh et al. 2008). 2,3-BD is a major by-product during the synthesis of 1,3-PD, and it may serve as an obstacle for obtaining a high purity of 1,3-PD in downstream processes because of its similar boiling point (Xiu and Zeng 2008). In this study, we constructed and analyzed mutant strains that eliminated the formation of by-products during 1,3-PD synthesis by inactivating the oxidative branch of the glycerol metabolic pathway in *K. pneumoniae*.

Materials and methods

Bacterial strains and plasmids and media

The *K. pneumoniae* parent strain Cu was a derivative of ATCC 200721 obtained by curing of the cryptic plasmids using ethidium bromide (15 μg mL^{-1}; Nath and Deb 1997). *E. coli* DH5α was used for DNA manipulation. pGEM-T Easy (Promega) was used for cloning and sequencing the amplified DNA fragment. pBR322 was used to construct recombinant strains of *K. pneumoniae*. pIJ773 (Gust et al. 2002) and pBluescript KS (Stratagene) were used as template DNA to amplify *aac(3)IV* and *lacZ* promoter, respectively. For the construction of recombinant strains, the microbial cells were grown in LB medium (yeast extract [Difco], 0.5%; Bacto-trypton [Difco], 1.0%; and NaCl, 1.0%) supplemented with the appropriate antibiotics [ampicillin (50 μg mL^{-1}), apramycin (50 μg mL^{-1}) or tetracycline (20 μg mL^{-1} for *E. coli* and 50 μg mL^{-1} for *K. pneumoniae*)].

Construction of AK and AR strains

Schematic representation of the strategies for construction of AK and AR mutant strains are indicated in Fig. 2a. For disruption of chromosomal DNA, which includes *orfY-dhaK*, the amino-terminal region of *dhaBα* and internal region of *dhaK* were amplified by PCR. Oligonucleotides used in this study are shown in Table 1. The 0.5-kb amino-terminal region of *dhaBα* was amplified using primers PdhaBα-N-F and PdhaBα-N-R. The 0.5-kb internal region of *dhaK* was amplified with PdhaK-I-F and PdhaK-I-R. The *lacZ* promoter sequences were amplified using primers P_{lacZ}-dhaBα-F and P_{lacZ}-dhaBα-R. The 1.2-kb *aac(3)IV* gene was amplified with primers Paac(3)IV-F and Paac(3) IV-R. The DNA fragments were cloned into pGEM-T Easy (Promega) followed by nucleotide sequencing to confirm the absence of any errors in the DNA polymerization reaction. The *Bam*HI–*Xba*I fragment, including the amino-terminal region of *dhaBα*, and *Hin*dIII-*Bgl*II fragment, including the internal region of *dhaK*, were ligated into the corresponding restriction sites adjacent to the *lacZ* promoter sequences and *aac(3)IV*, respectively. The *Hpa*I–*Bgl*II fragment, including the internal region of *dhaK* linked to *aac(3)IV*, was then inserted into the corresponding sites of the pGEM-T Easy vector with the amino-terminal region of *dhaBα* under the control of the *lacZ* promoter sequences. The resultant plasmid was linearized by *Bam*HI and *Bgl*II digestion and introduced into *K. pneumoniae* Cu by

Table 1 Primers used in this study

Primers	Nucleotide sequence (5′ to 3′)	Properties	
PdhaBα-N-F	*TCTAGA***ATG**AAAAGATCAAAACGATTT	*Xba*I	Start codon
PdhaBα-N-R	*GGATCC***GTC**AGCGGCAATCTGCAC	*Bam*HI	Val-165
PdhaK-I-F	*AAGCTT*CATG**CTC**TCCGGCG	*Hin*dIII	Leu-49
PdhaK-I-R	*AGATCT*AT**TTG**GTCCAGCGAGCTGAAGC	*Bgl*II	Gln-233
PdhaR-I-F	*AGATCTC***CTG**GGATTTCGCGACGGCA	*Bgl*II	Leu-74
PdhaR-I-R	*AAGCTT*TC**GAC**AATCGGTTTTAAGGTG	*Hin*dIII	Val-255
PdhaT-F	*AGATCT***ATG**AGCTATCGTATGTTTGA	*Bgl*II	Start codon
PdhaT-R	*CTCGAG*AAGCT**TCA**GAATGCCTGGCGG	*Xho*I	Stop codon
PorfWX-F	*AGATCT***ATG**TCGCTTTCACCG	*Bgl*II	Start codon
PorfWX-R	*CTGCAG***TCA**AGCGCAAGCATCAGG	*Pst*I	Stop codon
Paac(3)IV-F	*GTTAAC***G**CTGACGCCGTTGGA	*Hpa*I	−336 bp
Paac(3)IV-R	*AGATCT*AAAAGCTT**TTA**TGAGCTCAG	*Bgl*II–*Hin*dIII	Stop codon
P_{lacZ}-dhaBα-F	*AGATCT*GTTAAC**G**CGCAACGCAAT	*Bgl*II–*Hpa*I	-122 bp
P_{lacZ}-dhaBα-R	*GGATCC*TCTAGA**A**GCTGTTTCCTGTGT	*Bam*HI–*Xba*I	−1 bp
P_{lacZ}-dhaT-F	*GAATTC***G**CGCAACGCAATTAATG	*Eco*RI	−122 bp
P_{lacZ}-dhaT-R	*CTCGAG*AGATCT**A**GCTGTTTCCTGTGT	*Xho*I–*Bgl*II	−1 bp
P_{lacZ}-orfWX-F	*GAATTC***G**CGCAACGCAATTAATG	*Eco*RI	−122 bp
P_{lacZ}-orfWX-R	*CTGCAG*TCTAGA**A**GCTGTTTCCTGTGT	*Pst*I–*Bgl*II	−1 bp

Italic and underlined letters indicate restriction enzyme sites. Bold letters indicate amino acids that co-inside with the template sequences. Numbers of base pair (bp) indicate nucleotide sequences with respect to the start codon of *aac(3)IV* and *lacZ*, respectively

electrophoration (Fournet-Fayard et al. 1995). Conjugants showing resistance against apramycin by integration of the linearized DNA fragment, including apramycin resistant gene into the chromosome, were isolated. Correct integration of the DNA fragment by homologous recombination was confirmed by Southern hybridization with the amino-terminal fragment of *dhaBα* and *aac(3)IV* as probes against the *Eco*RV-digested chromosomal DNA. The mutant strain was named AK (KCTC11419BP).

Mutant strain AR (KCTC11420BP), in which the *orfY-dhaR* region was replaced with the *lacZ* promoter and *aac (3)IV*, was created using the same procedure described above, except the internal region of *dhaR* was used instead of the internal region of *dhaK*. The 0.5-kb internal region of *dhaR* was amplified with PdhaR-I-F and PdhaR-I-R.

Construction of recombinant plasmids

To recover the reductive pathway in the AK and AR mutant strains, recombinant plasmids containing *dhaT* and/or *orfWX* were constructed. Schematic representation of the strategies for construction of pBR-dhaT, pBR-orfWX, and pBR-dhaT-orfWX are indicated in Fig. 2a. The 1.16-kb open reading frame (*orf*) of *dhaT* was amplified with PdhaT-F and PdhaT-R. The DNA fragment, including the 0.9-kb *orfWX*, was amplified with PorfWX-F and PorfWX-R. To introduce the *lacZ* promoter upstream of *dhaT* and *orfWX*, the *lacZ* promoter sequences were also amplified by PCR with the following set of primers: for *dhaT*, P_{lacZ}-dhaT-F and P_{lacZ}-dhaT-R, and for *orfWX*, P_{lacZ}-orfWX-F and P_{lacZ}-orfWX-R. The DNA fragments were cloned into the pGEM TEasy (Promega) vector followed by nucleotide sequencing to confirm the absence of any error in the DNA polymerization reaction. The *Bgl*II–*Xho*I fragment, including the *dhaT*, and *Bgl*II–*Pst*I fragment, including *orfWX*, was inserted into the corresponding restriction sites downstream of *lacZ* promoter sequences, respectively. The *Eco*RI–*Eco*RI (on pGEM-T Easy) fragment, including the *dhaT*, and *Eco*RI–*Pst*I fragment and *orfWX* were transferred to the pBR322 vector, creating pBR-dhaT and pBR-orfWX, respectively. Finally, the *Eco*RI fragment, including *dhaT*, was inserted into the corresponding site of pBR-orfWX to create pBR-dhaT-orfWX. The resultant plasmids were transformed into *K. pneumoniae* Cu, AK, and AR strain by electrophoration.

Cultivation of *K. pneumoniae* strains

For the fermentation experiments, *K. pneumoniae* strains were cultivated in 250-mL round flask with 50 mL of a defined medium containing glycerol (20 g L^{-1} glycerol, 3.4 g L^{-1} K_2HPO_4, 1.3 g L^{-1} KH_2PO_4, 0.2 g L^{-1} $MgSO_4$, 0.002 g L^{-1} $CaCl_2$ $2H_2O$, 1 g L^{-1} yeast extract), 1 mL Fe solution [5 g L^{-1} $FeSO_4$ $7H_2O$ and 4 mL HCl (37%, *w*/*v*)], and 1 mL trace element solution [70 mg L^{-1} $ZnCl_2$, 100 mg L^{-1} $MnCl_2$ $4H_2O$, 60 mg L^{-1} H_3BO_3, 200 mg L^{-1} $CoCl_2$ $4H_2O$, 20 mg L^{-1} $CuCl_2$ $2H_2O$, 25 mg L^{-1} $NiCl_2$ $6H_2O$, 35 mg L^{-1} Na_2MoO_4 $2H_2O$, 4 mL HCl (37%, *w*/*v*)] at 37°C and 120 rpm for 30 h. Isopropyl-D-thiogalactoside (IPTG; 0.5 mM) was added to induce gene expression. Growth of the cells was monitored by removing aliquots of the culture at different time intervals and determining the OD_{600}. The cells were used for the enzyme activity assay, and the culture broth was subjected to metabolite analysis.

Enzyme activity assay

The activities of DhaB and DhaT were measured using the modified method of Toraya (Toraya et al. 1979) and of Johnson and Lin (1987). One unit of enzyme activity was defined as the amount of enzymes that consumed 1 μmol substrate per minute at 37°C. The amount of protein was determined with a protein assay kit (Bio-Rad), where bovine serum albumin was used as the standard (Sigma).

Metabolite analysis

Concentrations of glycerol, 1,3-PD, ethanol, lactate, succinate, and 2,3-BD in the culture broth were determined using a high-performance liquid chromatography system (1200, Agilent, CA, USA) that was equipped with a refractive index detector and an organic acid analysis column (300 mm× 78 mm, Aminex HPX-87H, Bio-Rad, USA; Oh et al. 2008). Mobile phase consisted of 0.005 mol L^{-1} H_2SO_4 solution, and the flow rate of 0.8 mL per minute was maintained. The column temperature was maintained at 65°C.

Results

Construction of *K. pneumoniae* mutant strains defective in oxidative pathway of glycerol metabolism

A large amount of metabolites such as ethanol, lactate, succinate, and 2,3-butanediol (2,3-BD) are co-produced with 1,3-propanediol (1,3-PD) from glycerol as by-products via oxidative metabolism (Fig. 1). Among these by-products, especially 2,3-BD may serve as a severe obstacle in the purification of 1,3-PD because of their similar boiling temperatures. The production of by-products starts with the action of DhaD followed by DhaK (Fig. 1). A couple of schemes were adopted to inactivate the enzymes involved in the synthesis of by-products. First, a region of the chromosome, containing *dhaD* and *dhaK*, was replaced with the *lacZ* promoter-apramycin resistance gene [*aac(3) IV*] cassette by homologous recombination, such that the *lacZ* promoter was inserted in front of *dhaB* (Fig. 2a). This

mutant strain was named AK. The second mutant strain was created by deleting the *dhaR* gene, which encodes the transcription factor that activates the expression of genes in the *dha* regulon and replacing it with the *lacZ* promoter-*aac (3)IV*. This mutant strain was named AR. The mutant strains, AK and AR, were not only defective in the oxidative branch but were also likely to be defective in the reductive branch due to the loss of the *dhaT* and *orfWX*, which leads to the synthesis of 1,3-PD. A set of plasmids, including *dhaT* and/or *orfWX*, were prepared (Fig. 2b) and introduced into the AK and AR strains to recover the reductive pathway.

Physiological analysis of the AK and AR mutant strains

The physiological properties of the AK and AR strains were studied as described in the "Materials and methods" section. The mutant strains showed nearly the same growth pattern as the parent strain, named Cu, in LB medium (Fig. 3a, top). However, when the cells were cultivated in a defined medium that contained glycerol as the sole carbon source, the AK and AR strains had lower cell growth than the parent strain (Fig. 3a, bottom). The lack of normal growth for the mutant strains in the glycerol medium was expected since these strains were engineered such that they were defective in glycerol catabolic pathways employing DhaD and DhaK; as a result, the pathway employing glycerol kinase was the only one that was functional, which can be utilized by the cells. HPLC was used to analyze the culture broth of cells grown in medium containing glycerol. Based on this analysis, 100% of the glycerol was found to be utilized by the parent strain, which produced a variety of metabolites, including 1,3-PD, whereas only 30% of the glycerol was utilized by the AK and AR mutant strains (Fig. 3b). Interestingly, 1,3-PD was still produced at lower level in the mutant strains, even though *dhaT* was removed. This result suggests that a putative oxidoreductase might catalyze the production of 1,3-PD at lower level compared to DhaT. However, no by-products were produced in the mutant strains, except for acetate (Fig. 3b).

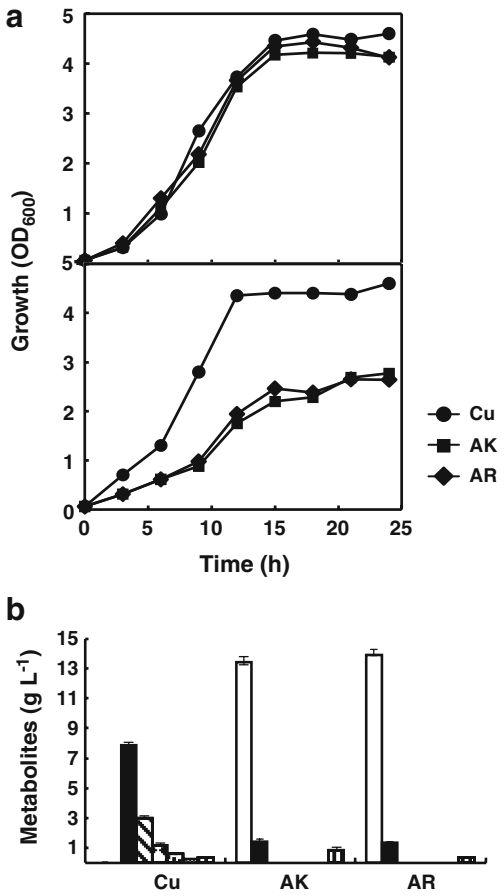

Fig. 3 Cell growth of *K. pneumoniae* strains Cu, AK, and AR in LB (*top*) and media containing glycerol (*bottom*; **a**) and metabolite analysis (**b**). The maximum values of metabolites, acetate (▥), ethanol (◘), glycerol (□), lactate (▩), 1,3-propanediol (■), succinate (▦), and 2,3-butanediol (◪), obtained from the culture broth grown to the stationary phase are presented

Recovery of glycerol reductive pathway in the AK and AR strains

Although the reductive pathway was recovered by introducing *dhaT* and/or *orfWX* on episomal plasmids, as expected, cell growth was not affected when glycerol was used as the sole carbon source; the level of growth was still lower than the corresponding parent Cu strain (data not shown). Consistent with the previous results, HPLC analysis of the culture broth from the recombinant strains revealed no production of metabolites such as ethanol, lactate, succinate, and 2,3-BD (Fig. 4), indicating that the oxidative metabolic pathway was still not functional. However, a dramatic increase in the production of 1,3-PD was found in the recombinant strains harboring the plasmid pBR-dhaT or pBR-dhaT-orfWX, which contained *dhaT* (Fig. 4). The amount of 1,3-PD produced was lower in the AK and AR strain, which was harboring pBR-dhaT-orfWX (5.2 g L^{-1}), than the corresponding Cu, which was harboring pBR-dhaT-orfWX (7.7 g L^{-1}). In the fermentation conditions used in this study, the production yield was higher in the AK strain (0.57 mol mol^{-1}) than the Cu strain (0.47 mol mol^{-1}) harboring pBR-dhaT-orfWX.

Enzyme activity analysis of the reductive pathway

To better understand the reason why the production level of 1,3-PD was lower in the AK or AR strains compared to the Cu strain, we examined the enzymatic activities of DhaB and DhaT, which catalyze the biosynthetic reaction of 1,3-PD from glycerol. As shown in Fig. 5, the DhaB activity of the AK and AR mutant strains (pBR322) was reduced by

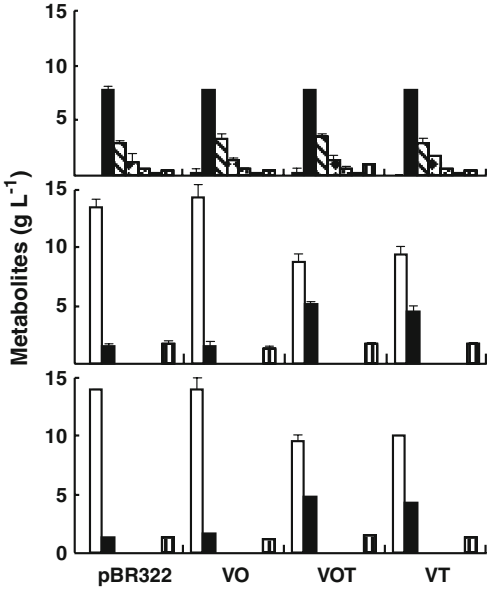

Fig. 4 Metabolite analysis of *K. pneumoniae* Cu (*top*), AK (*center*), and AR (*bottom*) strains harboring recombinant plasmid pBR322 (control), pBR-orfWX (*VO*), pBR-dhaT (*VT*), or pBR-dhaT-orfWX (*VOT*). The maximum values of metabolites, acetate (▥), ethanol (▤), glycerol (□), lactate (▩), 1,3-propanediol (■), succinate (▦), and 2,3-butanediol (▨), obtained from the culture broth grown to the stationary phase are presented

46% and 60%, respectively, compared to that of the Cu strain (pBR322). Although the enzyme activity was slightly recovered by the introduction of *orfW* (on pBR-orfWX or pBR-dhaT-orfWX), which encodes a reactivation factor of the protein (Tobimatsu et al. 2000), the DhaB activity was still lower than the corresponding Cu strain. In contrast, the DhaT activity was barely detected in the AK and AR strains (pBR322 or pBR-orfWX), which was expected to occur due to the deletion of the *dhaT* gene. However, the DhaT activity in the AK and AR strains harboring pBR-dhaT-orfWX or pBR-dhaT, which contained the *dhaT* gene, dramatically recovered up to the same level of the Cu strains (Fig. 5). These results indicate that the difference in the production level of 1,3-PD may be related to the activity of DhaB. To control the activity of the reductive pathway, we replaced the native promoter of *dhaB* with the IPTG-inducible *lacZ* promoter during the construction of the mutant AK and AR strain. However, substituting the promoter sequences resulted in a decrease in DhaB activity, although the introduction of multiple copies of *dhaT* into these strains eliminated this negative effect.

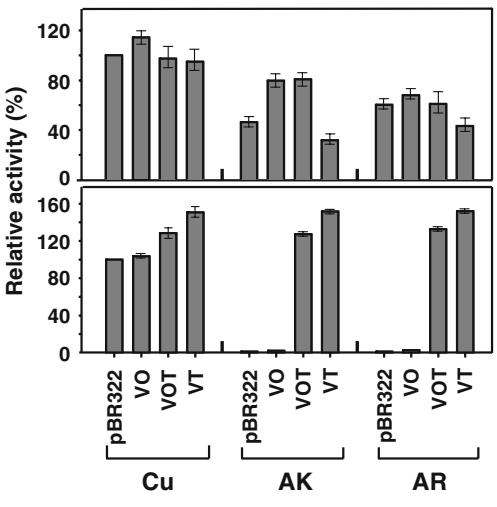

Fig. 5 Enzyme activity analysis of DhaB (*top*) and DhaT (*bottom*) in *K. pneumoniae* Cu, AK, and AR strains harboring recombinant plasmid pBR322 (control), pBR-orfWX (*VO*), pBR-dhaT (*VT*), or pBR-dhaT-orfWX (*VOT*)

Effect of IPTG concentration

To assess whether high concentration of the inducer could recover the activity of DhaB, the enzyme activity and production level of 1,3-PD was examined from the culture broth of the AK strain harboring pBR-dhaT-orfWX grown with various concentrations of IPTG. Unfortunately, the DhaB activity was almost constant regardless of IPTG concentration. In contrast, the DhaT activity remarkably increased in the presence of IPTG, although the activity was not proportional to the concentration of the inducer (Fig. 6). However, the production level of 1,3-PD was affected by the enzyme activity of DhaT. This was probably due to the low activity of DhaB or limited availability of NADH as discussed below.

Discussion

The elimination of by-products co-produced with 1,3-PD is likely to be a starting point for the development of this compound in industrial settings. This is especially true for the by-product, 2,3-BD, which could act as a major obstacle in the downstream process of purifying 1,3-PD because of its similar boiling point. By disrupting chromosomal genes

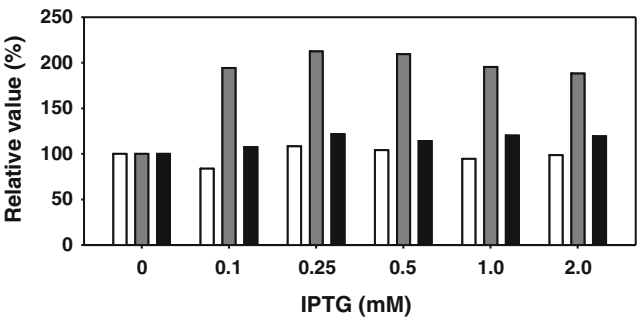

Fig. 6 Effect of IPTG concentration on the activity of DhaB (*open bar*) and DhaT (*gray bar*) and 1,3-PD production (*closed bar*) in AK strain harboring pBR-dhaT-orfWX

encoding enzymes that catalyze the oxidative branch of the glycerol metabolic pathway or transcription factors that induce gene expression, engineered strains that successfully abolished the synthesis of by-products, with the exception of acetate, were prepared. The metabolic flux of glycerol in the engineered strains of *K. pneumoniae* seemed to be similar to that of the recombinant strain of *E. coli*, in which the reductive branch of the glycerol metabolic pathway of *K. pneumoniae* was settled by introducing genes for DhaB and DhaT (Nakamura and Whited 2003). With further engineering of the recombinant strain, for example, the substitution of DhaT with another 1,3-PD oxidoreductase (YqhD) unrelated to the original metabolic pathway, the production of 1,3-PD was dramatically improved (Nakamura and Whited 2003), and the microbial strain was actually used for the industrial manufacturing of 1,3-PD (Bio-PDO, DuPont; Bhatia and Kurian 2008). The previous report inspired the idea for the constructed strains developed in this study, which could also be used as a platform for the industrial production of 1,3-PD, although productivity in this strain is relatively low. Moreover, industrial strains derived from *K. pneumoniae* could be superior to those derived from *E. coli* based on its metabolic ability to synthesize coenzyme B_{12}, which is critical for the reactivation of the enzymatic activity of DhaB (Nakamura and Whited 2003).

In this study, two strategies were adopted to block the oxidative branch of glycerol metabolism. First, the enzymes, DhaD and DhaK, which lead to the oxidative branch, were inactivated by deletion of genes on the *K. pneumoniae* chromosomal DNA (AK strain). Second, the putative transcription factor (DhaR), which induces gene expression, was targeted by genetic engineering (AR strain). The primary structure of DhaR consists of GAF (52–199), which is found in phytochromes and cGMP-specific phosphodiesterase, PAS (203–267). These proteins are found in a two-component sensor protein that recognizes oxygen, redox potential, energy charge, light, sigma 54 factor interaction (327–544), and histidine HTH_8 (588–628) domains (Raynaud et al. 2003). DhaR had 70% homology to that of *E. coli*, which regulates the expression of DHA kinase (Bächler et al. 2005). This suggests that DhaR also controls the expression of genes in the *dha* regulon of *K. pneumonia*, such as *dhaD* and *dhaK*, in response to cellular physiological parameters such as redox potentials or energy charge. This hypothesis was supported by the similar physiological properties observed in the AK and AR strains, which did not contain the oxidative branch of glycerol metabolism. To the best of our knowledge, this is the first report where DhaR was found to play a direct role in the *dha* regulon of *K. pneumoniae*.

The oxidative branch of glycerol metabolism was also simultaneously disrupted in the AK and AR mutants by gene deletion of the *dhaT* gene, which encodes the 1,3-PD oxidoreductase. As a result of this deletion, enzymatic activity of DhaT was barely detected in the mutant strains (Fig. 5). However, 1,3-PD was still produced in the mutants although the level was low (at 18.4%) compared to the parent (Cu). This may be due to the activity of an unknown 1,3-PD oxidoreductase (PDOR) beside DhaT. As mentioned above, *E. coli* YqhD catalyzes the conversion of 3-hydroxypropion aldehyde (3-HPA) to 1,3-PD (Nakamura and Whited 2003). A homolog of YqhD with 88% homology in amino acid sequence was also found in the genomic DNA of *K. pneumoniae* (Zhao et al. 2009), suggesting that the YqhD homolog is a PDOR. Recently, YqhD was reported to prefer NADPH as a cofactor instead of NADH, which is preferred by DhaT (Marçal et al. 2009).

The amount of 1,3-PD produced was slightly lower in the AK and AR mutant strains harboring pBR-dhaT-orfWX than the parent strain (Fig. 4). A possible reason for this phenomenon was the lower activity of DhaB in the mutant strains (Fig. 5). In order to intentionally manipulate the reductive branch, we substituted the native promoter of *dhaB*, which was driven by the regulator DhaR, with the IPTG-inducible *lacZ* promoter. Experimental results indicated that the native promoter was superior to the *lacZ* promoter.

Another possibility is an imbalance of the cellular ratio of NAD/NADH in the mutant strains, e.g., shortage of NADH for the action of DhaT catalyzing transition of 3-HPA to 1,3-PD by blocking of the oxidative branch. Therefore, further engineering to increase the production level of 1,3-PD in the mutant strains will be required to solve the problems discussed above.

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