Glycerol dehydrogenase plays a dual role in glycerol metabolism and 2,3-butanediol formation in *Klebsiella pneumoniae*

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Running title: Dual role of glycerol dehydrogenase in *Klebsiella pneumoniae*

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**Background:** Glycerol dehydrogenase catalyzes the initial step of glycerol oxidation.

**Results:** DhaD, a glycerol dehydrogenase from *Klebsiella pneumoniae*, plays a dual role in glycerol metabolism and 2,3-butanediol formation.

**Conclusion:** This dual role related to the enzyme promiscuity switches according to physiological requirements, suggesting a mechanism involved in evolution of the enzyme.

**Significance:** This work highlights the sophistication of enzyme evolution.

**ABSTRACT**

Glycerol dehydrogenase (GDH) is an important polyol dehydrogenase for glycerol metabolism in diverse microorganisms and for value-added utilization of glycerol in the industry. Two GDHs from *Klebsiella pneumoniae*, DhaD and GldA, were expressed in *Escherichia coli*, purified and characterized for substrate specificity and kinetic parameters. Both DhaD and GldA could catalyze the interconversion of (3R)-acetoin/(2R,3R)-2,3-butanediol or (3S)-acetoin/meso-2,3-butanediol, in addition to glycerol oxidation. Although purified GldA appeared more active than DhaD, in vivo inactivation and quantitation of their respective mRNAs indicate that *dhaD* is highly induced by glycerol and plays a dual role in glycerol metabolism and 2,3-butanediol formation. Complementation in *K. pneumoniae* further confirmed the dual role of DhaD. Promiscuity of DhaD may have vital physiological consequences for *K. pneumoniae* growing on glycerol, which include balancing the intracellular NADH/NAD⁺ ratio, preventing acidification, and storing carbon and energy. According to the kinetic response of DhaD to modified NADH concentrations, DhaD appears to show positive homotropic interaction with NADH, suggesting that the physiological role could be regulated by intracellular NADH levels. The co-existence of two functional GDH enzymes might be due to a gene duplication event. We propose that while DhaD is specialized for glycerol utilization, GldA plays a role in backup compensation and can turn into a more proficient catalyst to promote a survival advantage to the organism. Revelation of the dual role of DhaD could further the understanding of mechanisms responsible for enzyme evolution through promiscuity, and guide metabolic engineering methods of glycerol metabolism.

Polyol dehydrogenases consist of a large family of oxidoreductases with activity toward di- and polyhydroxylated species (1). Owing to their important physiological roles in organisms and applications in industry, studies on polyol dehydrogenases are of particular interest (1-4). Glycerol dehydrogenase (GDH), a typical polyol dehydrogenase, which catalyzes the initial step of glycerol oxidation, is responsible...
for glycerol utilization in diverse microorganisms (5). Reports show that some GDHs are involved in the pathogenicity of some human pathogens (6-7). GDHs are also crucial for the bioconversion of glycerol to produce useful biochemicals and biofuels such as 1,3-propanediol (1,3-PD) (a monomer for biomaterials), 2,3-butanediol (BD), and ethanol (8-11). During the past few decades, GDHs from different sources have been characterized (5,12-16). Recently, the glycerol surplus produced by the tremendous growth of the biodiesel industry stimulated intensive research efforts focused on glycerol metabolism and related enzymes and pathways, especially in efficient glycerol utilizers, such as Klebsiella and Clostridium (17-20). In K. pneumoniae, glycerol is metabolized in a dismutation process via two branches, reductive and oxidative (21) (Fig. 1a). NAD⁺-Dependent GDH initiates the oxidative branch by converting glycerol to dihydroxyacetone, which provides NADH for 1,3-PD production (21).

BD is a major product of the glycerol oxidative branch, as well as a promising bulk chemical with extensive industrial applications (22). There are three isomeric forms of BD: (2S,3S)-, (2R,3R)- and meso-forms. The optically active BD isomers are particularly valuable chemicals that provide chiral groups in pharmaceutical drugs or for liquid crystals (22-23). Another kind of polyol dehydrogenase, BD dehydrogenase (BDH), catalyzes the interconversion between acetoin (AC, 3-hydroxy-2-butanone) and BD (22). Stereospecific BDHs are responsible for the formation of different BD isomers. Several BDHs with different stereospecificities have been characterized in previous studies (24-32). The meso-BDHs from K. pneumoniae, Serratia marcescens, and Enterobacter cloacae belong to the short-chain dehydrogenase/reductase (SDR) family and catalyze the conversion of (3R)-AC to meso-BD and (3S)-AC to (2S,3S)-BD (24-28). (2R,3R)-BDHs from Paenibacillus polymyxa, Bacillus subtilis, and Saccharomyces cerevisiae belong to the medium-chain dehydrogenase/reductase family (MDR) and catalyze the conversion of (3R)-AC to (2R,3R)-BD, and (3S)-AC to meso-BD (29-32).

Generally, a mixture of meso- and (2S,3S)-BD is produced by K. pneumoniae due to the existence of a meso-BDH (23-24) (Fig. 1a and Fig. 1c). Recently, three isomers of BD were detected simultaneously in the fermentation broth, when K. pneumoniae strain ATCC 25955 utilized glycerol as a carbon source (33) (Fig. 1d). However, (2R,3R)-BD metabolism and the enzymes responsible for (2R,3R)-BD formation in K. pneumoniae have never been described. Analysis of the genome sequence of K. pneumoniae strain ATCC 25955 indicated the presence of a meso-BDH (budC encoding). However, no (2R,3R)-BDH encoding genes were identified (33). Therefore, it is likely that an oxidoreductase related to glycerol metabolism may be responsible for the formation of (2R,3R)-BD. It has been reported that there are two genes (dhaD and gldA) encoding GDH in K. pneumoniae, and the two GDHs both show activity toward BD (12). Considering that BD and AC have similar structures to glycerol and dihydroxyacetone, respectively, we hypothesized that GDHs catalyze the conversion between BD and AC, and are involved in (2R,3R)-BD formation.

To confirm our hypothesis, we expressed the dhaD and gldA genes from K. pneumoniae in Escherichia coli and characterized the purified enzymes for their substrate specificity and kinetic properties. Additionally, deactivation and complementation of dhaD and gldA in K. pneumoniae were carried out to investigate their roles in BD isomer formation and glycerol utilization. This work provides insights into the dual role of DhaD in glycerol metabolism and BD production in K. pneumoniae. The physiological implications of the dual role and co-existence of two GDHs were also discussed.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids and growth conditions**—All bacterial strains and plasmids used in this study are listed in Table 1. K. pneumoniae (ATCC 25955) was obtained from American Type Culture Collection. E. coli DH5α and E. coli BL21 (DE3) were used for general cloning and expression procedures, respectively. E. coli S17-1 that is able to host pKR6K and its derivatives was used for conjugation with K. pneumoniae. The pEASY-Blunt cloning vector (Transgen, China) was used to subclone the gene, and pET-28a(+) was used for protein expression. pDK7 with a tac promoter was used for overexpression of protein in K. pneumoniae. R6K origin of replication was amplified by PCR from the plasmid pCAM140 by using primers oriR6K-F(BspHI) and oriR6K-R(BsaXI) (Table 2). We derived pKR6K from pK18mobSacB by
replacing its replicon with the oriR6K replicon through the BspHI and BsaXI sites. pKR6K was used to create gene disruption mutations by homologous recombination. *K. pneumoniae* was cultured in a medium described previously (34). Either 30 g liter$^{-1}$ of glucose or glycerol was used as the carbon source; 500-ml flasks with 100 ml of medium were incubated at a shaker speed of 180 rpm at 37°C. *E. coli* was cultured in Luria-Bertani (LB) broth or on LB agar at 37°C supplemented with ampicillin (100 μg ml$^{-1}$) or kanamycin (50 μg ml$^{-1}$) as required.

**Cloning, expression and purification of GDHs**–The cloning of *dhaD* was performed by PCR amplification of *K. pneumoniae* genomic DNA. A pair of primers that introduced the BamHI and XhoI restriction sites (*dhaD*-F(BamHI) and *dhaD*-R(XhoI)) (Table 2) was used. The amplified fragment of 1.1 kb was cloned into the pEASY-Blunt vector and sequenced. The 1.1 kb BamHI/XhoI fragment digested from the pEASY-Blunt-*dhaD* was ligated to pET-28a(+), yielding pET-28a-*dhaD*. To clone and construct the overexpression vector for *gldA*, the protocol described above was used with primers *gldA*-F(BamHI) and *gldA*-R(XhoI). The FastPfu DNA polymerase was acquired from Transgen Biotech (China). The restriction enzymes and T$_4$ DNA ligase were purchased from New England Bio-Labs (Beverly, MA). Genomic DNA of *K. pneumoniae* was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA).

*E. coli* BL21 (DE3) cells carrying the overexpression vector were cultivated in LB medium containing kanamycin (50 μg ml$^{-1}$) at 37°C. When the OD$_{420nm}$ reached 0.5-0.7, the expression of recombinant GDH was induced by addition of 1 mM IPTG at 16°C for approximately 10 h. The cells were harvested and then washed twice with 0.85% NaCl. The cell pellet was resuspended in binding buffer (20 mM potassium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4), and disrupted by sonication in an ice bath. The lysed cells were centrifuged at 18,000 x g for 30 min at 4°C, and the supernatant was used for further purification. The enzyme was purified from the crude extract by using a 5-ml HisTrap HP column (GE Healthcare, USA), which had been equilibrated with 25 ml binding buffer. GDH was eluted by using 20% and 100% elution buffer (20 mM potassium phosphate buffer, 500 mM NaCl, 500 mM imidazole, pH 7.4). The 100% fraction was collected, concentrated, and desalted by gel filtration on Sephadex G25 medium (Pharmacia). The expressed and purified enzyme was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stored at -80°C for further study.

**Enzyme activity assays–GDH activity** was assayed spectrophotometrically by measuring the initial velocity change in absorbance at 340 nm corresponding to the oxidation of NADH or reduction of NAD$^+$ ($ε_{340} = 6220$ M$^{-1}$ cm$^{-1}$) at 30°C. One unit of activity was defined as the amount of enzyme that consumed or produced 1 μmol of NADH per minute. The specific activity of GDH was defined as the enzyme unit (U) divided by the amount of enzyme protein (mg).

The reaction mixture contained 0.1 M potassium carbonate buffer, pH 9.0, 30 mM ammonium sulfate, 0.6 mM NAD$^+$ or 0.3 mM NADH, and 0.1 M substrate (35). To determine the kinetic constants, all parameters were kept constant, and only the substrate concentration was modified. NAD$^+$ was used for oxidation of glycerol and BD, while NADH was used for reduction of (3R)/(3S)-AC and diacetyl (DA). The Michaelis-Menten equation was used for determination of the kinetic parameters. To perform the Hill plots analyses, 100 mM of (3R)/(3S)-AC and modified NADH concentrations (0.025-0.5 mM) were used. Hill coefficient (h) was obtained from the Hill plots (36).

**Conversion of (3R)/(3S)-AC by purified enzyme**–To study the catalytic rate toward (3S)-AC and (3R)-AC, the conversion of (3R)/(3S)-AC by purified enzyme was carried out in a mixture containing 0.1 M potassium carbonate buffer, pH 9.0, 30 mM ammonium sulfate, 50 mM NADH, 100 mM (3R)/(3S)-AC, and 2 mg DhaD or GldA in a final volume of 1 ml. (3R)/(3S)-AC exists as a crystalline dimer and the two stereoisomers are presumed to have a ratio of 1:1. The conversion was stopped at 15 min by the addition of HCl and the products were detected by gas chromatography (GC).

**Quantitation of *dhaD* and *gldA* mRNAs**–*K. pneumoniae* cells cultured using glucose or glycerol as a carbon source in the middle of the exponential phase were collected for RNA isolation by using the RNAprep pure Cell/Bacteria Kit (Tiangen Biotech, Beijing, China). Total RNA was treated with DNase I (Fermentas) and then used as a template for cDNA synthesis by using random primers and SuperScript III Reverse Transcriptase.
Disruption of *budC* gene in *K. pneumoniae*—The *budC* gene disrupted mutant was designated as KPΔ*budC*. The *dhaD* and *gldA* disruption mutants were constructed by using the protocol described above and primers listed in Table 2.

Production of BD isomers by the wild-type and mutant strains—The wild-type and mutant strains of *K. pneumoniae* were cultured using glycerol as a carbon source for 12 h. The supernatant was used to assay for the BD isomers produced. The pellet of cells was washed twice with 0.85% NaCl and resuspended in 50 mM potassium phosphate buffer (pH 7.4). Whole-cell biocatalysis was carried out for 6 h with 10 ml of mixture in a 100 ml Erlenmeyer flask at 37°C, at 180 rpm. The cell concentration in the reaction mixture was 5.0 g DCW liter⁻¹. Substrate used was 10 g liter⁻¹ (3R)/(3S)-AC and the source of reducing equivalent was 20 g liter⁻¹ glucose. BD and AC isomers were analyzed by GC.

Complementation of *dhaD* and *gldA* in *K. pneumoniae* mutant—The *dhaD* fragment was digested with *BamHI* and *XhoI* from pET28a-*dhaD*, and was introduced into the *BamHI*-SalI site (*XhoI* and SalI were isocaudomers) of expression vector pDK7. The resultant recombinant plasmid pDK7-*dhaD* was transformed into KPΔ*dhaD*Δ*gldA* by standard transformation protocol by using a standard electroporation transformation protocol (38). The chloramphenicol-resistant transformants were selected, and the insert was confirmed by colony PCR and sequencing. The confirmed clone harboring pDK7-*dhaD* was designated as KPΔ*dhaD*Δ*gldA*(pDK7-*dhaD*). To complement the *gldA* disruption mutation, the protocol described above was used. The wild-type and mutant strains were cultured using glycerol as a carbon source at 37°C. When the OD₆₀₀nm reached 0.5-0.7, expression of the recombinant GDH was induced by addition 1 mM IPTG.

Analytical techniques—BD and AC isomers were analyzed by using a gas chromatograph equipped with a chiral column using a method described previously (32). Prior to GC analysis, the samples were withdrawn periodically and the biomass was determined by measuring the OD₆₀₀nm. Whole-cell biocatalysis was carried out for 6 h with 10 ml of mixture in a 100 ml Erlenmeyer flask at 37°C, at 180 rpm. The concentration of glycerol was measured by HPLC using a method described previously (26).

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column (Supelco Beta DEX™ 120, inside diameter, 0.25 mm; length, 30 m). Nitrogen was used as the carrier gas. The injector temperature and detector temperature were both 280°C. The following temperature program was used. The column oven was maintained at 40°C for 3 min and then programmed to increase to 80°C at a rate of 1.5°C min⁻¹. The temperature was then raised to 86°C at a rate of 0.5°C min⁻¹ and finally to 200°C at a rate of 30°C min⁻¹. The injection volume was 3 μl. A standard mixture of (3R)/(3S)-AC, (2S,3S)-BD, (2R,3R)-BD, and meso-BD was prepared by dissolving the pure compounds, and then analyzed by GC (Fig. 1b). Standard (2R,3R)-BD, (2S,3S)-BD, and meso-BD were purchased from ACROS (The Kingdom of Belgium). (3R)/(3S)-AC was obtained from Apple Flavor Group (Shanghai, China).

RESULTS

Cloning, expression, and purification of DhaD and GldA—K. pneumoniae ATCC 25955 has been sequenced, and two GDH-encoding genes (dhaD and gldA) were identified from the genome sequence (33). The dhaD gene is located in the dha regulon and is 1,089 bp long, with a deduced amino acid sequence that shares 58% identity with GDH of E. coli (13), 50% identity with GDH of Geobacillus stearothermophilus (14), and 46% identity with GDH of Clostridium butyricum (20). The gldA gene is 1,104 bp long, and its deduced amino acid sequence has 60% and 89% identities with DhaD of K. pneumoniae ATCC 25955 and GDH of E. coli, respectively (13). The dhaD and gldA genes were cloned into the pET-28a(+) vector and highly expressed in E. coli BL21 (DE3). Purified DhaD and GldA enzymes were detected by SDS-PAGE as shown in Fig. 2.

Substrate specificity and kinetic properties—Both DhaD and GldA could catalyze the oxidation of glycerol, (2R,3R)-BD and meso-BD, and the reduction of (3R)/(3S)-AC (a racemic mixture of (3R)- and (3S)-AC) (Table 3). (2S,3S)-BD and DA were not substrates for DhaD or GldA. A gas chromatograph equipped with a chiral column was used to further detect the products of the enzymatic reactions. With respect to the BD oxidation reactions, when meso-BD and NAD⁺ served as the substrates of DhaD or GldA, (3S)-AC was the only product detected, which was the result of the selective oxidation of the alcohol function at the R-carbon of meso-BD. Accordingly, (3R)-AC was the only product obtained from (2R,3R)-BD. When (3R)/(3S)-AC was incubated with NADH and DhaD or GldA, a complete enantioselective reduction of the carbonyl groups produced an R-alcohol. Consequently, a product mixture of (2R,3R)-BD (from (3R)-AC) and meso-BD (from (3S)-AC) was observed. The results indicated that the GDHs DhaD and GldA have similar substrate specificity and stereospecificity toward isomers of BD and AC with the experimentally verified (2R,3R)-BDHs from P. polymyxa and S. cerevisiae (30-31).

Kinetic parameters of DhaD and GldA for glycerol, (2R,3R)-BD, meso-BD, and (3R)/(3S)-AC were determined, and the results are shown in Table 3. The Km values of DhaD and GldA for glycerol are comparable with those of GDHs reported previously, such as GDHs from E. coli (38 mM) (13) and Candida valida (58 mM) (16), suggesting that the kinetic data are reasonable for this class of enzymes. The Km values of the two GDHs for different substrates were reduced by an order of magnitude from (2R,3R)-BD > glycerol > meso-BD > (3R)/(3S)-AC. Interestingly, both DhaD and GldA had much lower Km values for meso-BD and higher Km values for (2R,3R)-BD than (2R,3R)-BDHs from previous studies (30-31). The catalytic efficiency constant (kcat/Km) of DhaD was greater for the reduction of (3R)/(3S)-AC than for the oxidation of glycerol and BD. Considering that the Km value for (3R)/(3S)-AC was lower than that for other alcohols, the DhaD should preferentially function as a reductase rather than a dehydrogenase. With respect to GldA, the kcat/Km value for meso-BD was the highest, followed by (3R)/(3S)-AC, glycerol and (2R,3R)-BD. Although DhaD and GldA have similar catalytic properties, the much lower Km values and higher kcat/Km values for a specific substrate indicate that GldA is more active. The kinetic parameters combined with the results from the substrate specificity test suggest that DhaD and GldA could act as NAD⁺-dependent (2R,3R)-BDHs.

Conversion of (3R)/(3S)-AC by purified enzyme—Because the chiral (3S)-AC and (3R)-AC were not commercially available, it was difficult to determine the kinetic properties toward the two isomers. Conversions using purified enzymes were carried out to study their catalytic rate toward (3S)-AC and (3R)-AC. GC, described in the experimental procedures, was performed to quantify the concentration of BD.
isomers produced. As shown in Fig. 3, after 15 min reaction, higher concentrations of meso-BD than (2R,3R)-BD were achieved by both DhaD and GldA. This suggests a higher catalytic rate of (3S)-AC/meso-BD conversion. The two GDHs produced (2R,3R)-BD at similar concentrations, but GldA catalyzed the reaction from (3S)-AC to meso-BD more rapidly than DhaD, resulting a higher concentration of meso-BD. The results of GDHs were opposite to those of (2R,3R)-BDHs, which catalyzed the (3R)-AC/(2R,3R)-BD conversion more rapidly (30-31).

Homotropic interactions of GDHs with NADH–Kinetic responses of DhaD and GldA to modified NADH concentrations are shown in Fig. 4a. The enzyme activities of both DhaD and GldA increase at higher NADH concentrations, possibly suggesting a positive co-operativity between GDHs and NADH. Similar phenomena have been observed in a previously reported NADH/NAD+-dependent dehydrogenase, and a Hill plot with a Hill coefficient (h) was used to verify this co-operativity (36). Hill plots of the data obtained from plots of enzyme activity (v) versus concentration of NADH produced lines with maximal slopes (h value) greater than or close to 2 (2.32 for DhaD and 1.77 for GldA) (Fig. 4b). These findings support the existence of positive homotropic interactions between the two GDHs and NADH.

Quantitation of dhaD and gldA mRNAs–Both DhaD and GldA are able to catalyze the (3R)-AC/(2R,3R)-BD conversion and might be involved in (2R,3R)-BD formation when glycerol is utilized. Additionally, the production of (2R,3R)-BD occurred during glycerol metabolism, suggesting that GDH encoding genes might be glycerol-induced. Therefore, qPCR was performed to determine the transcription levels of dhaD and gldA when glucose or glycerol was utilized. When K. pneumoniae was cultured in glucose medium, the transcriptional levels of dhaD and gldA were rather low (Fig. 5). Nevertheless, when glycerol was used as a carbon source, the mRNA level of dhaD increased by approximately 130-fold, suggesting dhaD being highly induced by glycerol. Meanwhile, the transcription level of gldA was almost not affected by glycerol and remained rather low. Therefore, we suggest that dhaD is highly expressed when the strain utilizes glycerol and is crucial for the formation of (2R,3R)-BD in K. pneumoniae.

Construction of gene disruption mutations in K. pneumoniae–To investigate the roles of dhaD and gldA genes in the (2R,3R)-BD formation and glycerol metabolism in K. pneumoniae, dhaD and gldA genes disruption mutations were constructed. The meso-BDH encoding gene budC was also deactivated to eliminate its influence on the production of BD isomers. PCR followed by DNA sequencing was performed to confirm the disruption of the genes in K. pneumoniae. Four mutants of K. pneumoniae, KPΔbudC, KPΔbudCΔdhaD, KPΔbudCΔgldA and KPΔbudCΔdhaDΔgldA, were constructed.

Production of BD isomers by the wild-type and mutant strains–The mutants together with the wild-type K. pneumoniae (KPwild) were cultured using glycerol as a carbon source. Dramatic differences in the BD isomers produced by different strains were observed, and the results were shown in Fig. 6a. meso-, (2S,3S)-, and (2R,3R)-BD were detected simultaneously in the broth of KPwild, in an order of magnitude from meso-BD > (2R,3R)-BD > (2S,3S)-BD. Interestingly, deactivation of budC resulted in a large increase of (2R,3R)-BD and a large decrease of meso-BD, suggesting that there are other enzymes with different stereospecificities responsible for the formation of BD. (2S,3S)-BD was not a product of KPΔbudC strain. A double mutant was generated by disrupting the gldA gene in the budC mutant background. The resulting strain KPΔbudCΔgldA was still able to produce (2R,3R)-BD with a small amount of meso-BD. However, the final titer of BD decreased, indicating that gldA was not crucial for (2R,3R)-BD production.

To investigate the role of dhaD in (2R,3R)-BD formation, mutants deficient in dhaD were generated. Deactivation of dhaD eliminated the formation of all isomers of BD and weakened the glycerol oxidation pathway dramatically. This may be due to the fact that DhaD catalyzes the first step of glycerol oxidation (12). Besides the dihydroxyacetone pathway, glycerol can also be utilized through a sn-glycerol-3-phosphate pathway in several organisms, including K. pneumoniae (35,39-40). In the dhaD-disrupted mutants, KPΔbudCΔdhaD and KPΔbudCΔdhaDΔgldA, glycerol can be first phosphorylated by glycerol kinase to form sn-glycerol-3-phosphate. sn-Glycerol-3-phosphate can then be converted to dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase and further
fed into central metabolism (35). However, the sn-glycerol-3-phosphate pathway in K. pneumoniae is inefficient (40). Therefore, the dhaD-disrupted mutants were still able to grow on glycerol but little AC was accumulated, making the role of dhaD in formation of BD undetermined.

Whole-cell biocatalysis using (3R)/(3S)-AC as substrate was performed to further investigate the role of dhaD in conversion of AC to BD. As shown in Fig. 6b, (2R,3R)-BD and meso-BD were detected in the broth of KPΔbudCΔgldA, while no BD was detected when budC, dhaD, and gldA were deactivated simultaneously. This result demonstrated that dhaD is crucial not only for glycerol metabolism but also for the formation of BD when glycerol was utilized. It should be noted that meso-BD became the main isomer of BD produced from (3R)/(3S)-AC by the KPΔbudCΔdhaD and KPΔbudCΔgldA strains. This result suggests DhaD and GldA have higher catalytic rate toward (3S)-AC/meso-BD conversion than (3R)-AC/(2R,3R)-BD conversion, which is consistent with the results of conversion of (3R)/(3S)-AC by the purified enzyme. The differences in the BD isomers produced from glycerol and (3R)/(3S)-AC were due to the amount of (3S)-AC in the two systems. (3R)-AC was the main isomer of AC produced from glycerol, and little (3S)-AC was formed. Therefore, (2R,3R)-BD was mainly produced with a small amount of meso-BD in the growth system, and meso-BD became the major product in whole-cell biocatalysis due to the high catalytic rate of (3S)-AC/meso-BD conversion of GDHs.

**Complementation of dhaD and gldA in K. pneumoniae mutants**—Since DhaD and GldA share similar catalytic properties, these two enzymes are likely to play the same physiological role in glycerol utilization. Unexpectedly, we found that dhaD was highly induced by glycerol while the expression level of gldA remained rather low. To confirm their roles in glycerol utilization and BD formation, dhaD and gldA were complemented in a dhaD and gldA disruption mutant (KPΔdhaDΔgldA) using protocols described in the experimental procedures. PCR was used to verify the disruption and complementation events of dhaD and gldA genes. The result in Fig. 7a shows that PCR generated the products of expected sizes. The resulting mutants, KPΔdhaDΔgldA(pDK7-dhaD) and KPΔdhaDΔgldA(pDK7-gldA), were evaluated in terms of cell growth and glycerol utilization. As controls, KPΔbudCΔdhaD and KPΔbudCΔgldA(pDK7-dhaD) were used.

As shown in Fig. 7b, KPΔdhaDΔgldA grew to a lower OD_{620nm} as compared to the wild-type strain, indicating that cell growth of K. pneumoniae was inhibited because of the disruption of dhaD and gldA. The glycerol uptake rate was also decreased (Fig. 7c). To induce the expression of dhaD and gldA in the plasmid, IPTG was added at 5 h when the OD_{620nm} reached approximately 0.7. At the stage of 6-10 h, KPΔdhaDΔgldA(pDK7-dhaD) and KPΔdhaDΔgldA(pDK7-gldA) grew more slowly than wild-type K. pneumoniae, possibly due to the heterologous expression of dhaD and gldA. At the stage of 10-16 h, cell growth and glycerol uptake recovered (Fig. 7b and Fig. 7c), indicating that DhaD and GldA are both usable for glycerol oxidation in K. pneumoniae. Introduction of dhaD and gldA also resumed the production of (2R,3R)-BD.

**DISCUSSION**

Enzymes are generally specific for their substrates and the reactions they catalyze. However, certain enzymes that do not show such specificity are sometimes called promiscuous (41). It has been suggested that enzyme promiscuity may have an important role in enzyme evolution and industrial applications (41-43). Here, we reported two promiscuous GDHs (DhaD and GldA) with relaxed substrate and reaction specificities. The promiscuity of DhaD and GldA allows them to act as (2R,3R)-BDHs and catalyze the interconversions of (3R)-AC/(2R,3R)-BD and (3S)-AC/meso-BD. However, further analysis of the kinetic data of DhaD and GldA showed that there are differences in the catalytic properties between GDHs and (2R,3R)-BDHs. First, DhaD and GldA show higher rates of both oxidation and reduction reactions towards vicinal alcohols of S-configuration, which is opposite to (2R,3R)-BDHs. Second, DA with two carbonyl groups does not serve as a substrate for DhaD or GldA, but is a good substrate for (2R,3R)-BDHs from P. polymyxa and S. cerevisiae (30-31), possibly indicating structural differences in substrate-binding or the catalytic sites between GDHs and (2R,3R)-BDHs. Both GDH and (2R,3R)-BDH belong to the MDR family (44), but it is possible that GDH has evolved separately from (2R,3R)-BDH, because GDHs...
from *K. pneumoniae* have a low amino acid sequence identity (9%) to (2R,3R)-BDH from *P. polymyxa* (31). To further investigate this, the amino acid sequences of GDHs and (2R,3R)-BDHs from different strains were used to construct a phylogenetic tree. The phylogenetic analysis clearly showed that these enzymes belong to different branches of the MDR family, suggesting that GDH has evolved separately from (2R,3R)-BDH (Fig. 8). The results also provide an explanation of why GDH does not have significant similarities with (2R,3R)-BDH. It is known that DhaD and GldA have high amino acid sequence identities and reserved substrate binding sites with GDHs from other strains (14), possibly indicating that enzyme promiscuity is common among GDHs.

Some scientists regard enzyme promiscuity as the starting point for enzyme divergent evolution (42-43). Based on the results obtained here, it is possible that GDH has been evolutionarily adapted to AC/BD conversion, facilitating a dual role of DhaD in glycerol utilization and BD production. We reasoned that the dual role of DhaD is favorable for the growth of *K. pneumoniae* on glycerol. Biosynthesis of BD has vital physiological relevance to the microbes, one of which is the regulation of the intracellular NADH/NAD+ ratio (22). Compared with glucose catabolism, an excess of NADH is generated by glycerol catabolism (45-46), making the NADH-consuming pathway necessary (Fig. 1a). The promiscuity of DhaD allows *K. pneumoniae* to use the AC/BD conversion for NADH disposal and recovery. The “spare NADH-regulation pathway” may be more important when *K. pneumoniae* grows anaerobically on glycerol (39). The promiscuity of DhaD may also contribute to the prevention of acidification by shifting glycerol metabolism from acid production to the formation of neutral compounds. This hypothesis is supported by the fact that BD production from glycerol is responsive to spontaneous pH drops during glycerol metabolism and can be increased by forced pH fluctuations (9,47). Additionally, as an intestinal bacterium, *K. pneumoniae* could utilize the glycerol that is decomposed from triglyceride in the intestinal tract. Considering that the intestines of animals are rich in nutrition, the dual role of DhaD may also include storing carbon and energy. Interestingly, DhaD has higher BD formation rates at higher NADH concentrations owing to the positive homotropic interaction between DhaD and NADH. This may allow DhaD to switch its role from glycerol utilization to BD formation according to the intracellular NADH level, which would allow DhaD to perform its physiological roles.

Despite the importance of enzyme promiscuity in physiology, it is not always advantageous for industrial applications because the side reactions negatively affect the biocatalytic efficiency and purity of products (48-49). BD is the main by-product of 1,3-PD production from glycerol by *K. pneumoniae*, and efforts have been made to eliminate BD production through inactivation of BDH (encoded by *budC*). However, BD was still detected in the fermentation broth (50-51). Our results show that the promiscuity of DhaD is responsible for the production of BD in *budC* inactivation mutations. Although disruption of *dhaD* eliminated the production of BD, it also dramatically lowered glycerol utilization rates (Fig. 7c), which further decreased 1,3-PD levels. Therefore, BD production in *K. pneumoniae* could not be eliminated simply by blocking the BD synthetic pathway. Combination of the approaches for enzymatic technology and metabolic engineering should help improve the specificities of key enzymes (48,52) and consequently eliminate by-products such as BD in 1,3-PD production.

The functional genes of prokaryotes usually exist as a single copy in the genome, including gldA, which exists widely in bacteria; however, a few species that can convert glycerol to 1,3-PD, such as *Citrobacter freundii*, *K. pneumoniae*, and *K. oxytoca*, possess a second GDH-encoding gene *dhaD*, which is located in the *dha* regulon (53). Our results suggest that DhaD is specialized for glycerol utilization and production of 1,3-PD and has evolved separately from GldA. This conclusion was supported by the following experimental data. First, the *K*~m~ value of DhaD toward a specific substrate was much higher than that of GldA, indicating that DhaD is less promiscuous than GldA (Table 3). Second, *dhaD* was highly induced by glycerol, whereas *gldA* was expressed constitutively at a low level (Fig. 5). Third, the topology of the phylogenetic tree (Fig. 8) suggests that DhaD and GldA belong to two different groups. The co-existence of *dhaD* and *gldA* in one strain might be due to a gene duplication event, which is of great importance and prevalence in biological evolution (54). This phenomenon might be an example of more rapid evolution
under the influence of functional specialization than under the influence of taxonomic divergence (12). Interestingly, when we compared DhaD and GldA with two archaeal GDHs from *Halopiger xanaduensis* (AEH39369) and *Methanosarcina barkeri* (YP_303855), we found that GldA had higher amino acid sequence identities (49% and 21%, respectively) than did DhaD (47% and 19%, respectively). This finding suggests that *gldA* is a more ancient gene and may serve as a backup that can turn into a more efficient catalyst under selective pressure, providing a survival benefit to the organism. A previous study provides considerable support for our hypotheses (55).

GDH (encoded by *gldA*) from *Bacillus coagulans* acquired D-lactate dehydrogenase activity through mutations during adaptive evolution. The expression level of *gldA* was also increased as a result of an upstream transposon insertion, facilitating production of D-lactic acid and restoring anaerobic growth (55).

In summary, the dual role of DhaD in glycerol metabolism and BD formation in *K. pneumoniae* was characterized. The dual role is able to switch according to physiological requirements, indicative of a mechanism of enzyme evolution though promiscuity. GldA, the other GDH of *K. pneumoniae*, may be a more ancient gene, which may serve as a backup for the organism.
REFERENCES


Dual role of glycerol dehydrogenase in Klebsiella pneumoniae


33. Wang, Y., Tao, F., Li, C., Li, L., and Xu, P. (2013) Genome Sequence of *Klebsiella pneumoniae* strain ATCC 25955, an oxygen-insensitive producer of 1,3-propanediol. *Genome Announc.* 1,
Dual role of glycerol dehydrogenase in Klebsiella pneumoniae

FOOTNOTES

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‡Co-first authors, both authors contributed equally to this work.

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The abbreviations used are: GDH, glycerol dehydrogenase; 1,3-PD, 1,3-propanediol; BD, 2,3-butanediol; BDH, 2,3-butanediol dehydrogenase; AC, acetoin; SDR, short-chain dehydrogenase/reductase; MDR, medium-chain dehydrogenase/reductase family; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; U, enzyme unit; DA, diacetyl; h, Hill coefficient; GC, gas chromatography; qPCR, quantitative PCR.

FIGURE LEGENDS

FIGURE 1. Formation of BD isomers in K. pneumoniae from glucose and glycerol. (a) BD isomers production pathway in K. pneumoniae from glucose and glycerol. Dashed lines represent the pathways that are active only in the glycerol medium. GDH(DhaD), glycerol dehydrogenase (encoded by dhaD); GDHt, glycerol dehydratase; 1,3-PD DH, 1,3-PD dehydrogenase; DHAK, dihydroxyacetone kinase; ALS, α-acetolactate synthase; ALDC, α-acetolactate decarboxylase; meso-BDH, meso-BD dehydrogenase. (b) GC analysis of BD and AC isomer standards. (c) GC analysis of BD isomers produced by K. pneumoniae from glucose. (d) GC analysis of BD isomers produced by K. pneumoniae from glycerol.

FIGURE 2. SDS-PAGE analysis of purified DhaD and GldA. Lane M, marker; lane 1, crude extract of E. coli BL21 (DE3); lane 2, crude extract of E. coli BL21 (DE3) (pET-28a-dhaD); lane 3, purified DhaD; lane 4, crude extract of E. coli BL21 (DE3) (pET-28a-gldA); lane 5, purified GldA.

FIGURE 3. Conversion of (3R)/(3S)-AC by purified DhaD and GldA. (a) BD isomers produced from (3R)/(3S)-AC by purified DhaD and GldA. (□) (2R,3R)-BD; (▨) meso-BD. Error bars indicate standard deviation (n = 3). (b) GC analysis of the products of (3R)/(3S)-AC conversion by DhaD. (c) GC analysis of the products of (3R)/(3S)-AC conversion by GldA.

FIGURE 4. Kinetic response of DhaD and GldA to different concentrations of NADH. (a) Enzyme activities toward (3R)/(3S)-AC at different concentrations of NADH. (b) Hill plot analyses of the data shown in Fig. 4a. The V_max values were obtained from Scatchard plots. h value (Hill coefficient) was the maximal slope of the lines given by Hill plot analyses. (■) DhaD; (○) GldA. Error bars indicate standard deviation (n = 3).

FIGURE 5. Determination of relative transcriptional levels of dhaD and gldA in glucose and glycerol mediums using qPCR. (□) Relative transcriptional levels of dhaD; (▨) relative transcriptional levels of gldA. Error bars indicate standard deviation (n = 3).

FIGURE 6. Production of BD isomers by the wild-type and mutant strains. (a) Growth system using glycerol as a carbon source. (b) Whole-cell biocatalysis using (3R)/(3S)-AC as a substrate. (□) (2S,3S)-BD; (▨) (2R,3R)-BD; (■) meso-BD.

FIGURE 7. Complementation of dhaD and gldA in the K. pneumoniae mutant. (a) Analysis of PCR fragments to confirm disruption and complementation of dhaD and gldA. Lane M: marker; lane 1-3,
dhaD products amplified with KPwild, KPΔdhaDΔgldA, and KPΔdhaDΔgldA(pDK7-dhaD) genomic DNAs as the template, respectively; lane 4-6, gldA products amplified with KPwild, KPΔdhaDΔgldA, and KPΔdhaDΔgldA(pDK7-gldA) genomic DNA as the template, respectively. PCR of dhaD and gldA was performed with primers dhaD-F(BamHI)/dhaD-R(XhoI) and gldA-F(BamHI)/gldA-R(XhoI), respectively. (b) Effect of disruption and complementation of dhaD and gldA on cell growth. (c) Effect of disruption and complementation of dhaD and gldA on glycerol consumption. Error bars indicate standard deviation (n = 3).

FIGURE 8. Phylogenetic analysis of amino acid sequences of GDH and (2R,3R)-BDH from different strains. The tree was constructed using the MEGA 5 program using the neighbor-joining method (56). The sequences compared include GDHs from *Escherichia coli* (EcGDH, P0A9S6); *Shigella sonnei* (SsGDH, YP_005459262); *Citrobacter freundii* (CfGDH(GldA), WP_003847842 and CfGDH(DhaD), P45511); *Salmonella enterica* (SeGDH, YP_006888481); *Enterobacter cloacae* (EclGDH, AFM62175); *Klebsiella pneumoniae* (KpGDH(GldA) and KpGDH(DhaD), this study); *Klebsiella oxytoca* (KoGDH(GldA), YP_005017437 and KoGDH(DhaD), YP_005016612); *Geobacillus stearothermophilus* (GsGDH, P32816); *Clostridium butyricum* (CbGDH, AAN17729); and (2R,3R)-BDHs from *Bacillus subtilis* (Bs(2R,3R)-BDH, NP_388505); *Paenibacillus polymyxa* (Pp(2R,3R)-BDH, ADV15558); *Thermoanaerobacter brockii* (Tb(2R,3R)-BDH, CAA46053); and *Clostridium beijerinckii* (Cbe(2R,3R)-BDH, AAA23199).
TABLE 1 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Reference or source</th>
</tr>
</thead>
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<td><strong>Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em> ATCC 25955</td>
<td>Wild-type</td>
<td>ATCC 25955</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>supE44ΔlacU169 (Φ80 lacZΔM15)hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Novagen</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>F'ompT hsdSB (rB mB') gal (λ c 1 857 ind1 Sam7 nin5 lacUV5 T7gene1) dcm (DE3)</td>
<td>Novagen</td>
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<td><em>E. coli</em> S17-1</td>
<td>thi pro hsdR recA Tra* , conjugative strain able to host λ-pir-dependent plasmids</td>
<td>(57)</td>
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<td>KPΔbudC</td>
<td><em>K. pneumoniae</em> ATCC 25955 budC disruption mutant strain</td>
<td>This study</td>
</tr>
<tr>
<td>KPΔbudCΔdhaD</td>
<td><em>K. pneumoniae</em> ATCC 25955 budC and dhaD disruption mutant strain</td>
<td>This study</td>
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<tr>
<td>KPΔbudCΔgldA</td>
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<tr>
<td>KPΔdhaDΔgldA</td>
<td><em>K. pneumoniae</em> ATCC 25955 dhaD and gldA disruption mutant strain</td>
<td>This study</td>
</tr>
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<td><em>K. pneumoniae</em> ATCC 25955 dhaD and gldA disruption mutant strain harboring pDK7-dhaD</td>
<td>This study</td>
</tr>
<tr>
<td>KPΔdhaDΔgldA(pDK7-gldA)</td>
<td><em>K. pneumoniae</em> ATCC 25955 dhaD and gldA disruption mutant strain harboring pDK7-gldA</td>
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<td><strong>Plasmid</strong></td>
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<tr>
<td>pEASY-Blunt</td>
<td>Ap′, cloning vector</td>
<td>Transgene</td>
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<td>pET-28a(+)</td>
<td>Km′, overexpression vector, P_T7</td>
<td>Novagen</td>
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<tr>
<td>pCAM140</td>
<td>Sm′, Sp′, Ap′, R6K origin, mTn5SSgusA40</td>
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<td>pK18mobsacB</td>
<td>Km′, gene replacement vector derived from plasmid pK18, Mob′ sacB</td>
<td>(59)</td>
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<td>pDK7</td>
<td>Cm′, expressing vector, P_tac</td>
<td>(60)</td>
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<tr>
<td>pKR6K</td>
<td>Km′, gene replacement vector derived from plasmid pK18mobsacB, R6K origin, Mob′ sacB</td>
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<td>pET-28a-dhaD</td>
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<td>pET-28a-gldA</td>
<td>gldA in pET-28a</td>
<td>This study</td>
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<tr>
<td>pKR6K-budC::BamHI</td>
<td>pKR6K derivative, carries a 354-bp deletion of budC</td>
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<td>pKR6K-dhaD::BamHI</td>
<td>pKR6K derivative, carries a 904-bp deletion of dhaD</td>
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<td>pKR6K-gldA::HindIII</td>
<td>pKR6K derivative, carries a 770-bp deletion of gldA</td>
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<td>pDK7-dhaD</td>
<td>dhaD in pDK7</td>
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<tr>
<td>pDK7-gldA</td>
<td>gldA in pDK7</td>
<td>This study</td>
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*Ap′, Sm′, Sp′, Km′ and Cm′; resistance to ampicillin, streptomycin, spectinomycin, kanamycin and chloramphenicol respectively.
### TABLE 2 Sequences of primers used in this study

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<td>oriR6K-F(BspHI)</td>
<td>TCATGACAGTTCAACCTGTGATAGTAC</td>
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<tr>
<td>oriR6K-R(BsaXI)</td>
<td>GGAAGGCCGCTAGAGAGAGAATTGTCAGCCGTTAAGTGTTC</td>
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<tr>
<td>ΔbudC-F(EcoRI)</td>
<td>ATCGGAAATTCCGCTGGCTGGTAATCTCTG</td>
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<tr>
<td>ΔbudC-F1(BamHI)</td>
<td>ATCGGATCCGGCCGTAACAAAGTGCGAC</td>
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<td>ΔbudC-R1(BamHI)</td>
<td>ATTAGGATCAGAGGTCACGCGGGAAGAAAA</td>
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<tr>
<td>ΔbudC-Rai(PsiI)</td>
<td>CCGCCTGCAGTTAATACCAATCCCGGCA</td>
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<tr>
<td>ΔdhaD-F(EcoRI)</td>
<td>ATAGGAATTCTCAGGATGCGAGCGGACTCA</td>
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<td>ΔdhaD-F1(BamHI)</td>
<td>ATTAGGATCCCTACCGCCGATCTGTGAG</td>
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<tr>
<td>ΔdhaD-R1(BamHI)</td>
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<tr>
<td>ΔdhaD-Rai(PsiI)</td>
<td>ATGGCGTGCAATTGTAAGGCGATTACGCG</td>
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<td>ΔgldA-F(EcoRI)</td>
<td>ATCGGAAATTCTGGATTTTGCTGGGTG</td>
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<td>ΔgldA-F1(HindIII)</td>
<td>ATGCAGGCGTTTTTGCGGTATCCAGGGTTT</td>
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<td>ΔgldA-R1(HindIII)</td>
<td>ATTAAAGGTTTGGTCAGCGCTCTGCTGCAG</td>
</tr>
<tr>
<td>ΔgldA-Rai(XmaI)</td>
<td>TCTACCAGGGGTTGATTTTGCTGGGCATAACGA</td>
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<td>dhaD-F(BamHI)</td>
<td>GCGGGATCCATGCTAAAAGTTATTCAT</td>
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<td>dhaD-Rai(XhoI)</td>
<td>AATACTCGAGTTAACCGCGCCGCACTGC</td>
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<tr>
<td>gldA-F(BamHI)</td>
<td>GCAAGGATCCATGGATGCGATTATTTCAATTC</td>
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<td>gldA-Rai(XhoI)</td>
<td>TTTATGGGCTGGATATTCCCATTCATTCAG</td>
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<td>dhaD-RT-R</td>
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TABLE 3 Kinetic parameters of GDHs and BDHs for glycerol, (2R,3R)-BD, meso-BD, (2S,3S)-BD, (3R)/(3S)-AC, and DA

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<tr>
<th>Substrate</th>
<th>DhaD</th>
<th>GldA</th>
<th>EcGDH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>(2R,3R)-BDH&lt;sup&gt;b&lt;/sup&gt;</th>
<th>meso-BDH&lt;sup&gt;c&lt;/sup&gt;</th>
<th>DhaD</th>
<th>GldA</th>
<th>DhaD</th>
<th>GldA</th>
<th>DhaD</th>
<th>GldA</th>
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<tr>
<td>Glycerol</td>
<td>93.62 ± 3.17</td>
<td>14.49 ± 0.10</td>
<td>38.00</td>
<td>ND</td>
<td>16.13 ± 0.61</td>
<td>29.07 ± 0.11</td>
<td>10.51 ± 0.40</td>
<td>18.88 ± 0.18</td>
<td>0.11 ± 0.00</td>
<td>1.30 ± 0.00</td>
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<td>(2R,3R)-BD</td>
<td>236.36 ± 6.17</td>
<td>119.25 ± 14.55</td>
<td>14.00 ± 5.00</td>
<td>ND</td>
<td>8.89 ± 0.18</td>
<td>5.89 ± 0.64</td>
<td>5.80 ± 0.12</td>
<td>3.82 ± 0.40</td>
<td>0.03 ± 0.00</td>
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</tr>
<tr>
<td>meso-BD</td>
<td>37.39 ± 6.48</td>
<td>4.94 ± 0.19</td>
<td>65.00 ± 9.00</td>
<td>13.00 ± 0.32</td>
<td>7.17 ± 0.83</td>
<td>23.62 ± 0.12</td>
<td>4.67 ± 0.54</td>
<td>15.33 ± 0.01</td>
<td>0.13 ± 0.01</td>
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<td>(2S,3S)-BD</td>
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<tr>
<td>(3R)/(3S)-AC</td>
<td>12.06 ± 0.06</td>
<td>4.15 ± 0.28</td>
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<tr>
<td>DA</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
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</tr>
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</table>

<sup>a</sup>Data for EcGDH (GDH from *E. coli*) are from reference 13. No average ± standard deviation was available.

<sup>b</sup>Data for (2R,3R)-BDH of *S. cerevisiae* are from reference 30.

<sup>c</sup>Data for meso-BDH of *K. pneumoniae* are from reference 24.

ND; the kinetic parameters were not determined because the enzymes showed no or extremely low activity towards the substrates.
FIGURE 2
FIGURE 3

(a) Concentration of BD (g liter\(^{-1}\)) in DhuD and GldA.

(b) Chromatograms showing the separation of BD isomers.

(c) Additional chromatograms with different conditions.
FIGURE 5

![Graph showing relative transcriptional levels of dhaD and gldA in glucose and glycerol mediums.](http://www.jbc.org/)

*Dual role of glycerol dehydrogenase in Klebsiella pneumoniae*
FIGURE 6
FIGURE 7

a) Gel electrophoresis showing DNA fragments.

b) Growth curves of different strains over time.

c) Glycerol concentration over time for different strains.
FIGURE 8
Glycerol dehydrogenase plays a dual role in glycerol metabolism and 2,3-butanediol formation in Klebsiella pneumoniae
Yu Wang, Fei Tao and Ping Xu

J. Biol. Chem. published online January 15, 2014

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