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Effects of mutation of 2,3-butanediol formation pathway on glycerol metabolism and 1,3-propanediol production by *Klebsiella pneumoniae* J2B

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HIGHLIGHTS

- 2,3-BDO synthetic pathway deletion mutants of *K. pneumoniae* Δ*ldhA* were studied.
- Δ*budO*-only completely eliminated 2,3-BDO production, but with lowered PDO production.
- Heavy carbon-metabolic traffic at the pyruvate node reduced glycerol assimilation and PDO production.
- DhaB activity was severely inhibited by glyceraldehyde-3-P, a glycolytic intermediate.

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ABSTRACT

The current study investigates the impact of mutation of 2,3-butanediol (BDO) formation pathway on glycerol metabolism and 1,3-propanediol (PDO) production by lactate dehydrogenase deficient mutant of *Klebsiella pneumoniae* J2B. To this end, BDO pathway genes, *budA*, *budB*, *budC* and *budO* (whole-*bud* operon), were deleted from *K. pneumoniae* J2B Δ*ldhA* and the mutants were studied for glycerol metabolism and alcohols (PDO, BDO) production. Δ*budO*-mutant-only could completely abolish BDO production, but with reductions in cell growth and PDO production. By modifying the culture medium, the Δ*budO* mutant could recover its performance on the flask scale. However, in bioreactor experiments, the Δ*budO* mutant accumulated a significant amount of pyruvate (>73 mM) in the late phase and PDO production stopped concomitantly. Glycolytic intermediates of glycerol, especially glyceraldehyde-3-phosphate (G3P) was highly inhibitory to glycerol dehydratase (GDHt); its accumulation, followed by pyruvate accumulation, was assumed to be responsible for the Δ*budO* mutant's low PDO production.

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1. Introduction

1,3-Propanediol (PDO) is an important platform chemical having a wide range of applications in the production of polymers, cosmetics, and lubricants, among others. The most important industrial use of PDO is as a monomer for synthesis of the new polyester polytrimethylene terephthalate (PTT) (Celińska, 2010; Maervoet et al., 2011; Saxena et al., 2009). Many organisms in the *Enterobacteriaceae* family, such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Enterobacter aerogenes* and *Enterobacter agglomerans*, naturally produce PDO from glycerol, among which *K. pneumoniae* is the most extensively studied (Celińska, 2010, 2012). *K. pneumoniae* dissimilates glycerol by two parallel "oxidative" and "reductive" pathways (Supplementary Fig. 1). In the oxidative pathway, glycerol is converted to dihydroxyacetone phosphate (DHAP) by a respiratory (aerobic or anaerobic, according to the electron acceptor type) and/or fermentative route, DHAP then being funneled into the glycolytic pathway. In the reductive pathway, glycerol is converted to PDO by a two-step process: first, it is dehydrated to 3-hydroxypropionaldehyde (3-HPA) by the coenzyme B_{12}-dependent glycerol dehydratase (GDHt), which is encoded by *dhaB*; then, 3-HPA, at the expense of NAD(P)H, is reduced to PDO by 1,3-propanediol oxidoreductases (PDORs) such as DhaT (NADH-PDOR) and/or NADPH-dependent

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oxidoreductase (Celińska, 2012; Kumar et al., 2012; Saxena et al., 2009). The reductive pathway regenerates NAD^+, which enables *K. pneumoniae* to grow on glycerol under limited-O_2 conditions.

During oxidative metabolism of glycerol, *K. pneumoniae* generates a number of metabolites including organic acids (lactic acid, succinic acid, acetic acid, formic acid) and alcohols [2,3-butanediol (BDO), ethanol] (Ashok et al., 2011; Kumar et al., 2013a). These glycerol-metabolic byproducts subdue the glycerol flux to PDO, thereby significantly reducing PDO production yields; additionally, at high concentrations, they are toxic to cell growth and PDO production. Lactic acid, particularly, is a major byproduct (Ashok et al., 2011; Durgapal et al., 2014; Huang et al., 2012), and much research efforts and resources have been devoted to the elimination of its formation (Kumar et al., 2013b; Oh et al., 2012; Xu et al., 2009). Another major byproduct, especially appearing when lactic acid production has been eliminated, is BDO. BDO production reduces PDO yield more significantly than does lactic acid, because synthesis of one molecule of BDO requires two molecules of glycerol. Moreover, due to their similar boiling points, the presence of BDO in the culture broth complicates PDO purification in downstream processing (Anand et al., 2011; Kaur et al., 2012; Zeng and Biebl, 2002). BDO synthesis begins with self-condensation of two molecules of pyruvate to one molecule of C5 intermediate α-acetolactate (Supplementary Fig. 1), which is catalyzed by α-acetolactate synthase (ALS, *budB*). In the next step, α-acetolactate is decarboxylated to acetoin (catalyzed by α-acetolactate decarboxylase (ALDC, *budA*)) and then reduced to BDO by 2,3-butanediol dehydrogenase/acetoin reductase (AR, *budC*) using NADH as the reductant. In the presence of oxygen, α-acetolactate is spontaneously decarboxylated to diacetyl, which is then reduced to acetoin by the action of diacetyl reductase, and acetoin in turn is reduced to BDO. When diacetyl is converted to acetoin or acetoin is converted to BDO, one NADH, at each step, is required (Celińska and Grajek, 2009; Ji et al., 2011). The genes coding for the three enzymes in the BDO-producing branch are located in one operon (*budO*) in the order *budA* (ALDC), *budB* (ALS) and *budC* (AR).

The current study investigated the role of the three enzymes of the BDO synthetic pathway in cell growth, glycerol metabolism and BDO production under different aeration conditions. Also, the possibility of producing PDO at an economically feasible level without BDO production was explored. The newly isolated *K. pneumoniae* J2B (KCCM11213P) strain was used, as it does not produce pathogenic and sticky lipopolysaccharides (Arasu et al., 2011), and also because it has better sedimentation properties and a higher sensitivity to antibiotics than other well-studied *K. pneumoniae* such as the DSMZ2026 strain. Using *K. pneumoniae* J2B Δ*ldhA* as the base strain, four mutant strains (Δ*budA*, Δ*budB*, Δ*budC* and Δ*budO*) were developed and their performances studied by shake-flask and bioreactor experiments.

2. Materials and methods

2.1. Materials

A genomic DNA isolation kit and pGEM-T vector was purchased from Promega (Madison, WI, USA). High-fidelity *pfx* polymerase was acquired from Invitrogen (Seoul, Korea). Restriction DNA-modifying enzymes were obtained from New England Bio-Labs (Beverly, MA, USA). The miniprep and DNA gel extraction kits were purchased from Qiagen (Mannheim, Germany). The primers were synthesized by Cosmotech Co. Ltd. (Seoul Korea).Yeast extract (Cat. 212750) was obtained from Difco (Becton Dickinson; Franklin Lakes, NJ, USA). Glycerol and all other chemicals and enzymes (unless indicated otherwise) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Construction of plasmids and mutant strains

All of the strains, plasmids and primers are listed in Table 1. For the design of the four mutant strains of *K. pneumoniae* J2B (Δ*budA*, Δ*budB*, Δ*budC* and Δ*budO*), an in-frame tagged deletion approach was employed according to a slightly modified version of the method introduced by Link et al. (1997). Briefly, PCR amplification of ~500 bp of the upstream (fragment A) and ~500 bp of the downstream (fragment B) regions of the above-noted genes was performed using the primers listed in Table 1. In the next step, the two amplified fragments A and B were ligated using overlapping PCR methods to synthesize the engineered fragment AB, which subsequently was cloned into the pGEMT vector. After confirming this sequence, the engineered fragment AB was sub-cloned into the pKOV vector between the restriction sites (shown in Table 1). These plasmids were used to knock out, by homologous recombination, the target genes from the chromosomal DNA of *K. pneumoniae* J2B Δ*ldhA*. The mutant strains were screened using PCR and confirmed by sequencing. The developed mutant strains of *K. pneumoniae* J2B Δ*ldhA*, Δ*ldhA*Δ*budA*, Δ*ldhA*Δ*budB*, Δ*ldhA*Δ*budC* and Δ*ldhA*Δ*budO*, were designated KpΔ*budA*, KpΔ*budB*, KpΔ*budC* and KpΔ*budO*, respectively. *K. pneumoniae* J2B Δ*ldhA* was designated as the *K. pneumoniae* control (Kp control).

2.3. Shake-flask cultivation

Shake-flask experiments were carried out at 37 °C with an initial pH of 7. Different strains used in gene-deletion studies were cultured in Luria–Bertani medium (LB medium) containing yeast extract (5 g/L), NaCl (10 g/L), and tryptone (10 g/L). For shake flask production experiments, a primary inoculum was prepared by culturing cells in LB medium from agar plates for 8 h. From the primary inoculum, the cells were transferred to fresh LB medium for 2 h in order to harvest active cells at the mid-log phase. The primary and the secondary inoculum cultures were grown under the same conditions (working volume: 50 mL in 250 mL Erlenmeyer flask; agitation speed: 200 rpm; initial pH: 7.0, temperature: 37 °C). The cell OD_{600} at the end of pre-cultures I and II were in the 5.0–6.0 and 1.5–2.0 ranges, respectively. The starting OD_{600} in all of the experiments was 0.1–0.2, and so the inoculum volume was dependent on the final OD_{600} achieved at the end of each stage. The main cultivation of the designed mutant strains of *K. pneumoniae* J2B (Kp control, KpΔ*budA*, KpΔ*budB*, KpΔ*budC* and KpΔ*budO*) was conducted in 250 mL flasks containing a medium of the following composition: glycerol – 20 g/L (220 mM), yeast extract – 1.0 g/L, $(NH_4)_2SO_4$ – 2.0 g/L, $MgSO_4{\cdot}7H_2O$ – 0.2 g/L, $CaCl_2.2H_2O$ – 0.02 g/L, K_2HPO_4 – 3.4 g/L, KH_2PO_4 – 1.3 g/L; Fe solution – 1 mL/L, and trace-element solution – 1 mL/L (Oh et al., 2012).

Shake-flask cultivation of the different mutant strains was performed under various aeration conditions: aerobic, microaerobic, and anaerobic. In the aerobic and microaerobic cultures, the flasks were plugged with an oxygen-permeable cotton stopper; in the anaerobic cultures, the flasks were closed with a screw cap, and the flask head space was replaced with argon gas prior to cultivation. The working volumes under aerobic, microaerobic, and anaerobic conditions were 25, 100 and 50 mL, respectively. The agitation speed under the aerobic condition was 250 rpm, and under both the microaerobic and anaerobic conditions, 100 rpm. The culture medium was supplemented with different components in order to investigate their effects on cell metabolism and PDO production. The KpΔ*budA* and KpΔ*budO* strains were supplemented with two different concentrations of branched-chain amino acids (leucine, isoleucine, and valine) (1.0 and 2.0 mM) as well as complex nitrogen sources (yeast extract – 1.0 g/L, peptone – 2.5 g/L, beef extract – 2.5 g/L, yeast extract – 2.5 g/L, peptone – 5.0 g/L, beef extract – 5.0 g/L). The shake-flask cultivation of KpΔ*budO* was additionally

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