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RESEARCH PAPER

The role of *bud*ABC on 1,3-propanediol and 2,3-butanediol production from glycerol in *Klebsiella pneumoniae* CICIM B0057

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ABSTRACT
1,3-propanediol (1,3-PD) is an important compound from which many others can be synthesized. 2,3-butanediol (BDO) is the key by-product in the biosynthesis of 1,3-PD from glycerol, but it impedes its downstream purification. In *Klebsiella*, the *bud*A, *bud*B and *bud*C genes encode enzymes that are responsible for the synthesis of BDO. In this study, 3 individual antisense RNAs were designed to repress the expression and hence activity of BudA-C. Compared with the parent strains, the activities of BudB and BudC were reduced by 60.5% and 70.5%, respectively, and the mRNA level of *bud*A was reduced by 70%. Decreased BudC activity had no effect on cell growth or carbon distribution. However, reduced BudA and BudB activity decreased the BDO concentration by 35% and led to a 10% increase in the yield of 1,3-PD. This result suggests the activities of BudA and BudB could be key factors in the production of BDO from glycerol in *Klebsiella*. This study provides a deeper understanding of the role of *bud*ABC in glycerol metabolism in *Klebsiella*.

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Introduction

The rapid expansion of biodiesel production has led to the abundance of glycerol waste, and the conversion of glycerol to high value-added chemicals is receiving increasing attention. Among the glycerol derivatives, 1,3-propanediol (1,3-PD) is a particularly high value product,[1] with considerable commercial value in the synthesis of polytrimethylene terephthalate.[2] *Klebsiella pneumoniae* is one of the most efficient organisms identified to date for the biosynthesis of 1,3-PD. In *Klebsiella*, glycerol is assimilated via both reductive and oxidative pathways. In the reductive pathway, glycerol is converted to 1,3-PD by the coenzyme B12-dependent glycerol dehydratase (DhaB) and the 1,3-propanediol oxidoreductase (DhaT) in successive steps. In the oxidative pathway, glycerol is oxidized to dihydroxyacetone then pyruvate via the Embden-Meyerhof-Parnas (EMP) pathway before conversion to byproducts such as acetic acid, lactic acid, ethanol and 2,3-butanediol (BDO). This pathway competes for NADH and a carbon source with the 1,3-PD pathway.

Of all byproducts, BDO is the most abundant and shares a similar boiling point with 1,3-PD, which impedes the downstream purification of 1,3-PD.[3] In *Klebsiella*, the bud operon includes the transcriptional activator-like protein (*bud*R)[4] and 3 enzymes; α-acetolactate synthase (*bub*B), α-acetolactate decarboxylase (*bub*A), and 2,3-butanediol dehydrogenase (*bub*C). Together, these proteins are responsible for the synthesis of BDO[5,6] (Fig. 1). Based on this knowledge, genetic manipulation has been performed to reduce the accumulation of BDO.[7,8] However, detailed knowledge of the role of these genes in BDO synthesis remains elusive.

Previous studies indicated that *bud*R positively regulates *bud*ABC and BDO biosynthesis.[4,8,9] Generally, gene knockout is the method of choice for studying gene function, but manipulation of essential genes is not possible. The bud operon could also be responsible for the synthesis of branched chain amino acids such as valine and leucine, which are important to cell growth.[10] Unlike gene knockout, RNA-based

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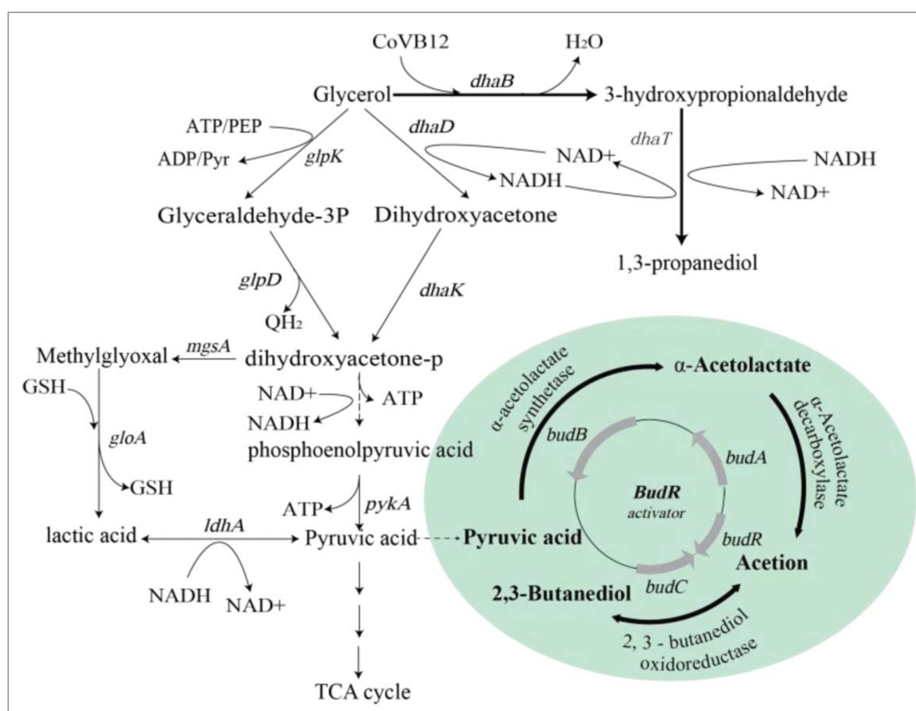

Figure 1. The glycerol metabolic pathway in *Klebsiella*.

approaches can be applied to repress the expression of essential genes in a more controlled manner.[11] In this study, expression of *bud*A-C in *K. pneumoniae* CICIM B0057 was repressed using antisense RNAs to investigate the effect on 1,3-PD and BDO biosynthesis.

Material and methods

Strains, plasmid and reagents

K. pneumoniae CICIM B0057 was stored in our lab and used as the parent strain. *Escherichia coli* JM109 (Invitrogen) and pEtac[12] were utilized for plasmid construction. 1,3-propandiol and 2,3-butanediol were purchased from Sigma-Aldrich (Steinheim, Germany). DNA polymerase, restriction endonuclease, ligase (solution I), and Genomic DNA and Gel DNA Purification Kits were purchased from Takara (Dalian, China). Tryptone and yeast extract were bought from Oxoid (Basingstoke, UK). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and kanamycin were bought from Sangon (Shanghai, China). All other compounds were of reagent grade or higher quality.

Plasmid construction

Primers P1 and P2 (Table 1) were used for the construction of pETR by PCR using pEtac as a template. A hairpin structure was incorporated to improve RNA stability. Antisense DNA fragments of *bud*A, *bud*B, and *bud*C were cloned using PCR and genomic DNA from *K. pneumoniae* CICIM B0057 based on genomic information from *K. pneumoniae* 342 (GenBank Accession Number: CP000964.1). The resultant DNA fragments were inserted into the *Bam*HI and *Hin*dIII sites of pETR to generate pETR/anti-*bud*A, pETR/anti-*bud*B, and pETR/anti-*bud*C. PCR amplification conditions were as follows: initial denaturation at 94°C for 10 min followed by 30 cycles of 98°C for 15 s, 60°C for 15 s, and 72°C for 2 min. Plasmids were transformed into *K. pneumoniae* CICIM B0057 to generate *K. pneumoniae* CICIM B0057 (pETR/anti-*bud*A), *K. pneumoniae* CICIM B0057 (pETR/anti-*bud*B), and *K. pneumoniae* CICIM B0057 (pETR/anti-*bud*C).

Media and cultivation

Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% sodium chloride) was used for the cultivation of *K. pneumoniae* CICIM B0057 and *E. coli*. The fermentation medium for *K. pneumoniae* CICIM B0057 contained the following (g/l): glycerol, 40; glucose, 5; K_2HPO_4, 7.5; $(NH_4)_2SO_4$, 2; $MgSO_4·7H_2O$, 2; $FeSO_4·7H_2O$, 0.005; yeast extract, 7; vitamin B_{12}, 0.015, and 1 ml trace element solution. The

Table 1. Primers used in this study.

Name	Sequence	Remarks
P1	TCCTCCTTAATTGGTACGTCACCCACCACCACCACCACGGATCC	The forward primer for pETR
P2	AGGAGGAATTAACCATGCAGTGGGTGGTGGTGGTGGTGAAGCTT	The reverse primer for pETR
P3	CCCAAGCTTGAACGAGGAAGTGGTATATG	The forward primer for antisense DNA of budA
P4	CGCGGATCCTAGAGCACGCTCTCGGGATG	The reverse primer for antisense DNA of budA
P5	CCCAAGCTTCGTAGAAAGTTAAGGGGTTC	The forward primer for antisense DNA of budB
P6	CGCGGATCCCAGTGAGTCGAACACCTTGTC	The reverse primer for antisense DNA of budB
P7	CCCAAGCTTCACAATAAGGAAAGGAAAATG	The forward primer for antisense DNA of budC
P8	CGCGGATCCGTGGCGTCGTTATAATCGGC	The reverse primer for antisense DNA of budC
P9	CGCGGATCCATCGAAAACGTCTCAAACCAG	The forward primer for antisense DNA of budC
P10	CGCAAGCTTATACCACTTC CTCGTTCAAC	The reverse primer for antisense DNA of budC

composition of the trace element solution was as follows (g/l): $ZnCl_2$, 0.7; $MnCl_2 \cdot 4H_2O$, 1; H_3BO_3, 0.6; $CoCl_2 \cdot 6H_2O$, 2; $CuCl_2$, 0.2; $NiCl_2 \cdot 6H_2O$, 0.25; $Na_2MoO_4 \cdot 2H_2O$, 0.35. Kanamycin was added at a final concentration of 100 μg/ml where needed. Fermentation of *K. pneumoniae* CICIM B0057 was performed in 250 ml gauze and paper-covered conical flasks containing 50 ml medium at 37°C in a rotary shaker incubator at 100 rpm.

Enzyme activity assay

Recombinant *K. pneumoniae* CICIM B0057 cells were collected by centrifugation at 10,000×g for 10 min, washed twice, and suspended in 100 mM potassium phosphate buffer (pH 7.0). Cells were disrupted by sonication and centrifuged at 10,000×g for 10 min. The supernatant was collected as the crude protein extract. All procedures were performed at 4°C. Protein concentration was measured by the Bradford method[13] using bovine serum albumin as the standard.

BudA activity was analyzed in 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.0)], 0.5 M NaCl, 0.2% Triton X-100, and 5.3 mM α-acetolactate at 30°C for 20 min as previously reported, and the substrate α-acetolactate was freshly prepared by saponifying ethyl-2-acetoxy-2-methylacetate at pH 11.5.[14] The acetoin content was analyzed as previously reported.[15] One unit of activity was defined as the formation of 1 μmol of acetoin in 1 min.

BudB activity was analyzed in 70 mM sodium acetate buffer (pH 5.3), 0.17 mM thiamine pyrophosphate and 80 mM pyruvate as previously reported.[16] The reaction was started by addition of cell-free extract at 37°C. The resultant α-acetolactate was converted to acetoin at 45°C for 30 min in the presence of 65 mM HCl, and acetoin was measured as previously reported.[15] One unit of activity was defined as the formation of 1 μmol of acetoin in 1 min.

BudC activity was analyzed in 120 mM BDO and 4 mM NAD^+ in 33 mM sodium pyrophosphate (pH 8) as previously described.[17] The reaction was started by addition of cell-free extract at 37°C. One unit of activity was defined as the formation of 1 μmol of NADH in 1 min. The change in the concentration of NADH (ε_{340} = 6220 $M^{-1} \cdot cm^{-1}$) was measured at 340 nm using a spectrophotometer (UV-2450; Shimadzu Co., Kyoto, Japan).

Analytical methods

Glycerol, 1,3-PD, 3-HP and other metabolites were analyzed by High Performance Liquid Chromatography fitted with a refractive index detector and a Bio-Rad Aminex organic acids HPX-87H column at 60°C with 5 mM H_2SO_4 as the mobile phase and an elution rate of 0.6 ml/min.[18]

Results and discussion

Construction of an antisense RNA expression system

To improve the stability of antisense RNA (asRNA) against degradation by RNAase, a hairpin structure[19] was inserted into the multiple cloning site of the plasmid pEtac, resulting in plasmid pETR (Fig. 2). A previous study suggested that an antisense DNA sequence complementary to a nucleotide sequence close to the ribosome-binding site (RBS) is better able to inhibit the expression of target genes.[20] Thus, we designed asRNAs based on the DNA sequence between -50 and 50 nucleotides from the RBS for all 3 genes (Fig. 2). The obtained antisense fragments were cloned into pETR, resulting in recombinant plasmids pETR/anti-*budA*, pETR/anti-*budB*, and pETR/anti-*budC*. These plasmids were transformed into *K.*

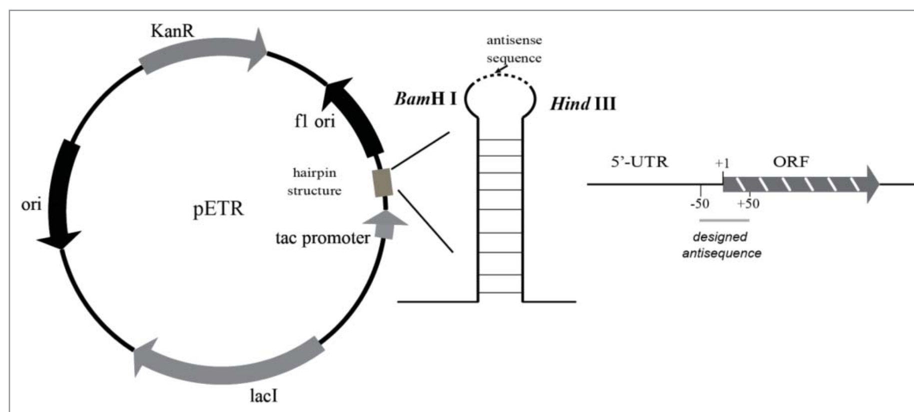

Figure 2. The antisense RNA tools constructed in this paper.

pneumoniae CICIM B0057 to generate *K. pneumoniae* CICIM B0057 (pETR/anti-*budA*), *K. pneumoniae* CICIM B0057 (pETR/anti-*budB*), and *K. pneumoniae* CICIM B0057 (pETR/anti-*budC*).

Effect of antisense RNAs on enzyme activity and cell growth

In *K. pneumoniae*, pyruvate is decarboxylated by BduB to form α-acetolactate, which is converted to acetoin and 2,3-butanediol by BudA and BudC, respectively. To repress the expression of these enzymes, antisense RNAs were transformed into the host to generate *K. pneumoniae* CICIM B0057 (pETR/anti-*bud*A), *K. pneumoniae* CICIM B0057 (pETR/anti-*bud*B), and *K. pneumoniae* CICIM B0057 (pETR/anti-*bud*C). The BudB activity of the anti-*bud*B strain was reduced by 60.5% (from 11.90 to 4.69 U/mg), the BudC activity of the anti-*bud*C strain was decreased by 70.5%, and the BudA activity of the anti-*bud*A strain was reduced by 68% (0.015 to 0.0048 U/mg). The antisense RNAs therefore successfully repressed the expression of their corresponding enzymes, leading to dramatically decreased enzyme activity.

Antisense RNAs for *bud*A and *bud*C also repressed cell growth at the log phase (Fig. 3). However, the final biomass of the anti-*bud*C strain was comparable to the parent *K. pneumoniae* CICIM B0057 (pETR) strain. The final biomass of *K. pneumoniae* CICIM B0057 (pETR/anti-*bud*A) and *K. pneumoniae* CICIM B0057 (pETR/anti-*bud*B) was reduced by 14.8% and 13.2%, respectively, suggesting that anti-*bud*A and anti-*bud*B led to a decreased biomass.

Effect of antisense RNAs on 1,3-PD and BDO production

As shown in Fig. 4, the BDO concentration in all strains was similar before 12 h. However, after this time, BDO accumulation in the anti-*bud*A and anti-*bud*B strains was reduced from ~5 g/l to 2 g/l. In contrast, BDO production in the parent and anti-*bud*C strains peaked at ~7 g/l after 20 h, then decreased to ~3 g/l at the end of the fermentation. These results indicated that BDO synthesis was impeded by the introduction of antisense RNAs for *bud*A and *bud*B. The 1,3-PD yield of the anti-*bud*A and anti-*bud*B strains increased by less than 10% compared to the parent strain, indicating a positive effect of the 2 antisense RNAs on 1,3-PD biosynthesis. No significant differences in by-products were detected between the strains tested; ethanol, lactic acid and succinic acid all accumulated to ~0.6 g/l, whereas acetic acid reached 2.5 g/l.

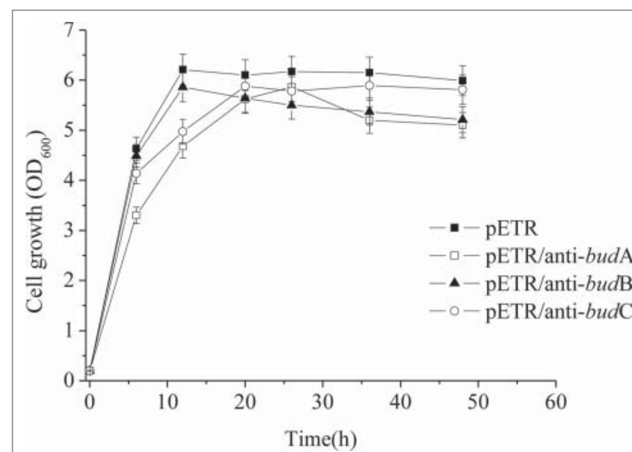

Figure 3. The cellgrowth of the recombinants.

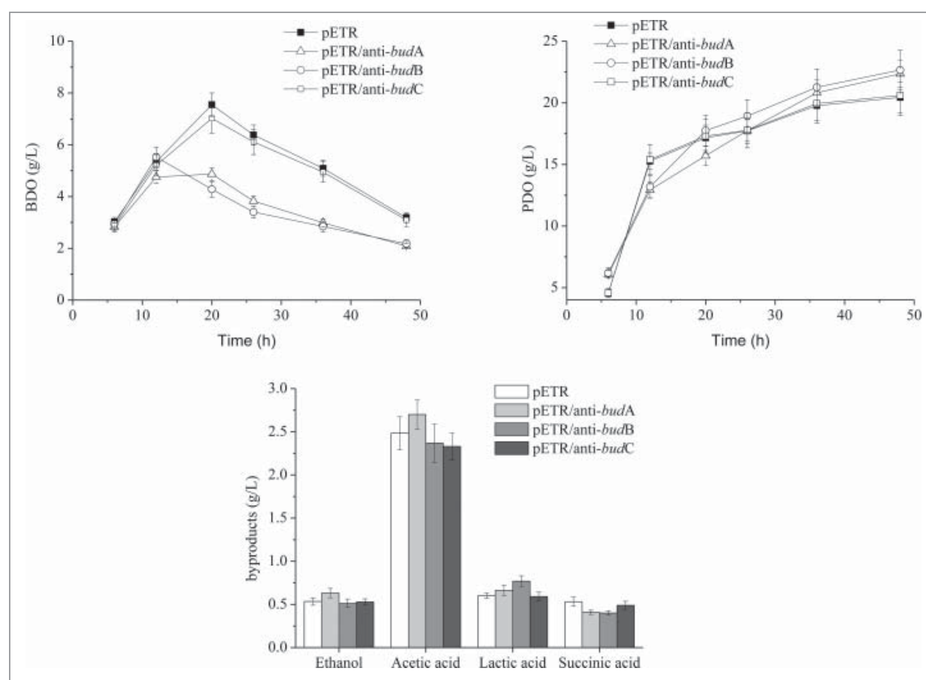

Figure 4. Time course of the fermentation of recombinants.

Although the designed antisense RNAs exhibited similar target enzyme inhibition efficiencies, cell growth and BDO and 1,3-PD production differed significantly. Unlike BudC, a reduction in BudB and BudA activity decreased BDO production, suggesting BudA and BudB might be rate-limiting factors in BDO biosynthesis. Actually, BDO synthesis in the *bud*C deficient *K. pneumoniae* CICIM B0057 was still observed in our previous report.[7] It has been reported that glycerol dehydrogenase might also contribute to the bioconversion of acetoin to BDO,[21] suggesting isoenzymes of BudC may be present in *Klebsiella*. This may explain why a reduction in BudC activity did not appreciably decrease the BDO concentration. Previous studies suggested that BDO protects against acidification.[10,22] The reduced BDO synthesis capacity of the anti-*bud*B and anti-*bud*A strains may also decrease their tolerance to acetic acid, which could explain the decreased biomass. Interestingly, BDO production decreased during the late stages of fermentation in all tested strains. This phenomenon can be explained by the reversibility of the BDO synthesis pathway.[23] The introduced *bud*B and *bud*A antisense RNAs lowered the production of BDO, which decreased NADH consumption by the BDO pathway. This resulted in increased NADH availability for the synthesis of 1,3-PD, explaining the increased abundance of this compound in the anti-*bud*B and anti-*bud*A strains.

In summary, the activities of BudA and BudB proved to be the limiting factors affecting BDO biosynthesis in *K. pneumoniae* CICIM B0057. This knowledge on the role of *bud*ABC in glycerol metabolism could prove useful for reducing BDO and other by-products, and improving the downstream purification of 1,3-PD from glycerol in future commercial bioproduction.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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