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Identification of acetoin reductases involved in 2,3-butanediol pathway in *Klebsiella oxytoca*

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ABSTRACT

The acetoin reductase (AR) of *Klebsiella oxytoca* is responsible for converting acetoin into 2,3-butanediol (2,3-BDO) during sugar fermentation. Deleting the AR encoding gene (*budC*) in the 2,3-BDO operon does not block production of 2,3-BDO, as another similar gene exists in addition to *budC* called diacetyl/acetoin reductase (*dar*) which shares 53% identity with *budC*. In the present study, both *budC* and *dar* of *K. oxytoca* were independently cloned and expressed in *Escherichia coli* along with *budA* (acetolactate decarboxylase) and *budB* (acetolactate synthase), which are responsible for converting pyruvate into acetoin. The recombinant *E. coli* expressing *budABC* and *budAB-dar* produced 2,3-BDO from glucose but *E. coli* expressing only *budAB* did not and produced acetoin alone. This demonstrates that Dar functions similar to BudC. Mutants of *budC*, *dar*, and both genes together were developed in *K. oxytoca* Δ*ldhA* (lactate dehydrogenase). *K. oxytoca* Δ*ldhA* Δ*budC* Δ*dar*, deficient in both AR genes, showed reduced 2,3-BDO concentration when compared to *K. oxytoca* Δ*ldhA* and *K. oxytoca* Δ*ldhA* Δ*budC* by 84% and 69%, respectively. Interestingly, *K. oxytoca* Δ*ldhA* Δ*dar* resulted in a significant reduction in the reversible conversion of 2,3-BDO into acetoin than that of *K. oxytoca* Δ*ldhA*, which was observed in a glucose depleted fermentation culture. In addition, we observed that Dar played a key role in dissimilation of 2,3-BDO in media containing 2,3-BDO alone.

1. Introduction

Production of fuels and chemicals from renewable resources has attracted much attention due to increasing concerns about the environment and the limited availability of fossil fuel resources. Several chemicals have been suggested, and they can be supplied to chemical industries by fermentative microbial production using renewable biomass. The production of 2,3-butanediol (2,3-BDO), with a history of more than 100 years, is one such example (Palsson et al., 1981). 2,3-BDO has extensive industrial applications as a synthetic rubber and has roles in the solvent industry (Ji et al., 2011).

Many bacterial species can produce 2,3-BDO using a variety of carbohydrates via mixed acid fermentation, and *Enterobacter*, *Klebsiella*, *Bacillus*, and *Serratia* species have been considered industrially important genera due to their efficient production of 2,3-BDO (Celinska and Grajek, 2009; Ji et al., 2011). In these 2,3-BDO natural producers, pyruvate, which is converted from monosaccharides, is channeled into 2,3-BDO by three key enzymes, including catabolic α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC), and acetoin (diacetyl) reductase (AR, also called 2,3-BDO dehydrogenase). First, ALS is involved in the formation of α-acetolactate from pyruvate under limited O_2 conditions. Then, α-acetolactate is decarboxylated into acetoin by ALDC. Finally, AR catalyzes the reversible reduction of acetoin to 2,3-BDO (Syu, 2001). When O_2 is present, α-acetolactate undergoes spontaneous decarboxylation to produce diacetyl, which is irreversibly converted to acetoin by AR (Johansen et al., 1973). AR involves both irreversible reduction of diacetyl to acetoin and reversible reduction of acetoin to 2,3-BDO (Bryn et al., 1971).

Depending on 2,3-BDO producing bacteria, the gene encoding AR is either clustered in an operon called 2,3-BDO operon or located separately in the genome (Fig. 1). In *Klebsiella terrigena*, *Klebsiella pneumoniae*, and *Enterobacter aerogens*, these three key enzymes are encoded by the genes *budA* (ALDC), *budB* (ALS), and *budC* (AR), which are a single operon together (Blomqvist et al., 1993; Ui et al., 1998). The 2,3-BDO operon, *budABC*, is regulated at the transcriptional level by a transcriptional activator (TA), which belongs to the LysR family of TAs, and is encoded by the *budR* gene (Mayer et al., 1995). The divergently transcribed *budR* is located adjacent to the *budABC* operon constituting *budRABC*. In other representative 2,3-BDO producers, such as *Bacillus* and *Serratia* species, the acetoin operon comprises ALDC and ALS. However, the AR encoding gene is located far from those genes (Moons et al., 2011; Nicholson, 2008; Rao et al., 2012). This acetoin operon, *budAB*, is also under the control of the TA, a neighboring and divergently transcribed LysR

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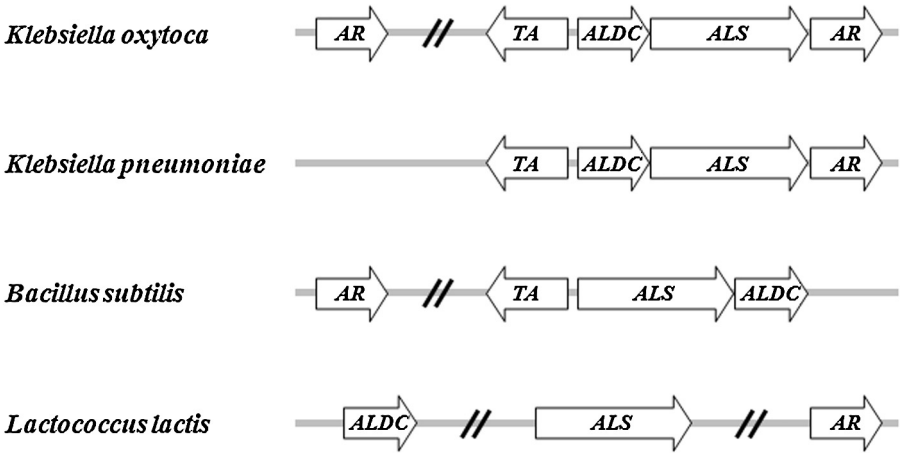

Fig. 1. Genetic organization of the genes involved in 2,3-BDO production from representative natural producers. *TA*, gene encoding the transcriptional activator; *ALDC*, gene encoding α-acetolactate decarboxylase; *ALS*, gene encoding α-acetolactate synthase; *AR*, gene encoding acetoin (diacetyl) reductase/2,3-BDO dehydrogenase.

homologue, as in the 2,3-BDO operon, but transcription of the AR encoding gene is not regulated by the TA.

Among several 2,3-BDO producers described above, *Klebsiella oxytoca* and *K. pneumoniae* have shown unbeatable efficiency in 2,3-BDO production for their broad substrate spectrum and cultural adaptability (Garg and Jain, 1995). Furthermore, the increasing number of full genome sequences and genetic tools available for strain development make *Klebsiella* species industrially important 2,3-BDO producers. In contrast to *K. pneumoniae*, the full genome sequences of *K. oxytoca* strains were revealed recently (Liao et al., 2012; Shin et al., 2012), and a search of the KEGG database (http://www.genome.jp/kegg/) indicated that the genes involving 2,3-BDO synthesis in *K. oxytoca* KCTC 1686 are clustered in an operon under the control of a divergently transcribed TA, as in other *Klebsiella* species (*K. pneumoniae* and *K. terrigena*) and *Enterobacter aerogenes*. We found a gene in *K. oxytoca*, other than *budC* and annotated as AR, which was designated as diacetyl/acetoin reductase (*dar*), and located separately far from the *budABC* operon. Dar belongs to the short chain dehydrogenase/reductase (SDR) family and also to the same ortholog group (K03366 in KEGG database) as BudC. The existence of two ARs involving 2,3-BDO metabolism is a unique feature of *K. oxytoca* because, until now, most 2,3-BDO producers in *Enterobacteriaceae* are known to possess only one AR (part of a *budABC* operon or monocistronic) as described above.

In the present study, we investigated the function of Dar and BudC from *K. oxytoca* KCTC 12133BP on the production of 2,3-BDO in *Escherichia coli* and *K. oxytoca*. The synthetic operons *budRAB*, *budRABC* and *budRAB-dar* were constructed for *E. coli* expression. *K. oxytoca* mutants deficient in Dar, BudC, or both were developed, and their fermentation profiles were examined to assess the role of each AR on 2,3-BDO production.

2. Materials and methods

2.1. Bacterial strains and plasmids

All strains, plasmids, and primers used in this study are listed in Table 1. The *K. oxytoca* strain was isolated from a cattle farm and deposited at the Korean Collection for Type Cultures under accession number KCTC 12133BP (Kim et al., 2013). *E. coli* JM109 was used in all standard cloning procedures as the host strain to express the 2,3-BDO operon from *K. oxytoca*. Detailed procedures for constructing the strains and plasmids are described below. All primers for PCR amplification were designed based on the sequence information available from NCBI database (accession no. CP003218). Primer synthesis and DNA sequencing were carried out by Genotech (Daejeon, Korea). Enzymes and related reagents for DNA manipulation were purchased from New England Biolabs (Beverly, MA, USA), TaKaRa Shuzo (Shiga, Japan), Solgent (Daejeon, Korea), and Sigma–Aldrich (St. Louis, MO, USA). The plasmids and DNA fragments were prepared with Qiagen kits (Qiagen, Chatsworth, CA, USA). All other chemicals used were of analytical grade and purchased from Sigma–Aldrich.

2.2. Cloning of 2,3-butanediol operon and dar gene from K. oxytoca

The genes involved in 2,3-BDO synthesis (*budR*, *budA*, *budB*, *budC*, and *dar*) were amplified using the primers listed in Table 1, and cloned as illustrated in Fig. 2. First, the *budRABC* operon, including the genes encoding TA (*budR*, locus tag KOX_22360), ALDC (*budA*, locus tag KOX_22365), ALS (*budB*, locus tag KOX_22370), and AR (*budC*, locus tag KOX_22375) was amplified by polymerase chain reaction (PCR) using the genomic DNA of *K. oxytoca* KCTC 12133BP as a template and the primers budRABCF and budRABCR flanked with the *Apa*I and *Xba*I sites, respectively. The *budRAB* gene fragment and the downstream region of the *budC* gene were combined by overlap extension PCR using the primers budRABCF, budRABR, budRABF, and budRABCR. This PCR fragment was cloned into the pBBR1MCS vector between the *Apa*I and *Xba*I sites resulting in pBRbudRAB. To facilitate replacement of the *budC* gene in the *budRABC* operon with other AR gene, such as *dar*, the *Bsr*GI site was inserted between the *budB* and *budC* genes by overlap extension PCR using the primers budRABCF, BsrGIR, BsrGIF, and budRABCR. Then, this modified *budRABC* operon was cloned into the pBBR1MCS vector between the *Apa*I and *Xba*I sites resulting in pBRbudRABC. Finally, the *budC* gene in pBRbudRABC was replaced by the *dar* gene (locus tag KOX_01940) at the *Bsr*GI and *Xba*I sites. The *dar* gene and the downstream region of the *budC* gene were combined by overlap extension PCR using the primers darF1, darR, darF2, and budRABCR. The resulting plasmid, designated pBRbudRABD, contained the *budR*, *budA*, *budB*, and *dar* genes.

2.3. Development of K. oxytoca mutant strains

In-frame deletions of the *budC* and *dar* genes in the genome of *ldhA* (lactate dehydrogenase) deficient *K. oxytoca* KCTC 12133BP (KoΔL) were performed using a method described previously (Kim et al., 2013). Briefly, two DNA fragments of each gene with overlapping ends were amplified from *K. oxytoca* genomic DNA using