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Identification and characterization of a short-chain acyl dehydrogenase from *Klebsiella pneumoniae* and its application for high-level production of L-2,3-butanediol

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Abstract *Klebsiella pneumoniae* synthesize large amounts of L-2,3-butanediol (L-2,3-BD), but the underlying mechanism has been unknown. In this study, we provide the first identification and characterization of an L-2,3-BD dehydrogenase from *K. pneumoniae*, demonstrating its reductive activities toward diacetyl and acetoin, and oxidative activity toward L-2,3-BD. Optimum pH, temperature, and kinetics determined for reductive and oxidative reactions support the preferential production of 2,3-BD during cell growth. Synthesis of L-2,3-BD was remarkably enhanced by increasing gene dosage, reaching levels that, to the best of our knowledge, are the highest achieved to date.

Keywords *Klebsiella pneumonia* · 2,3-Butanediol dehydrogenase/acetoin reductase · L-2,3-butanediol · Stereospecificity

Introduction

2,3-Butanediol (2,3-BD), used extensively as a cryoprotectant and a component of printing inks, is an important platform chemical with a wide range of potential industrial applications. In addition, derivatives of 2,3-BD can be used as a solvent and fuel additive and as a building block for synthetic rubber [1, 7, 8, 21]. Moreover, stereoisomeric forms of 2,3-BD have significant applications in the pharmaceutical, agrochemical, fine chemical, and food industries [20].

A number of microorganisms are capable of producing 2,3-BD in D- (*2R*,*3R*), L- (*2S*,*3S*), and meso-type stereoisomeric forms. The *Klebsiella* species, *K. pneumonia* and *K. oxytoca*, have been found to produce a maximum 2,3-BD concentration of approximately 150 g/l as a 90/10 % mixture of meso and L forms [9]. The 2,3-BD biosynthesis pathway has been extensively studied in *K. pneumoniae* [17], especially for meso-2,3-BD (Fig. 1). In this pathway, pyruvate is first converted to acetolactate by the action of acetolactate synthase (Als) and then decarboxylated to D-acetoin by the action of acetolactate decarboxylase (Adc). D-acetoin is converted to meso-2,3-BD by a reversible reaction mediated by meso-2,3-BD dehydrogenase (Ard), which is crucial for producing this stereospecific isoform. It has been found that genes for these enzymes are clustered within a region of the *K. pneumonia* chromosome.

However, in contrast to the case for meso-2,3-BD, little information is available regarding the synthesis of L-2,3-BD in *K. pneumoniae*. Although L-2,3-BD dehydrogenase genes have been reported for *Brevibacterium saccharolyticum*, *Enterobacter cloacae* and *Rhodococcus erythropolis* [5, 18, 19], BLAST analyses of whole-genome sequences have not yielded an L-2,3-BD dehydrogenase-encoding gene for *K. pneumoniae*. Here, using an *ard*-deficient mutant and complementation experiments, we identified a *K. pneumoniae* short-chain acyl dehydrogenase exhibiting L-2,3-BD dehydrogenase activity. We further characterized the enzymatic properties of the protein and successfully utilized it for enhanced production of L-2,3-BD.

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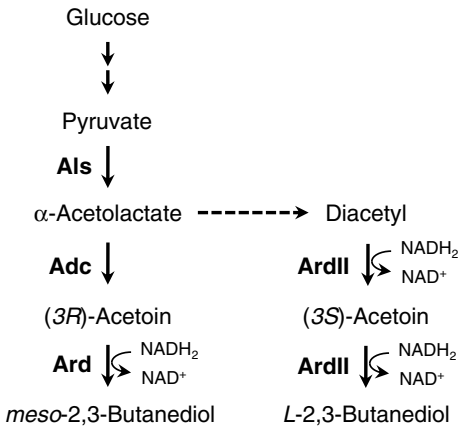

Fig. 1 Proposed metabolic pathway for the synthesis of 2,3-BD in *K. pneumoniae*

Materials and methods

Bacterial strains, plasmids, and media

The *K. pneumoniae* Δ*ldhA* strain, a lactate dehydrogenase-deficient mutant derived from ATCC 200721, was described previously [11]. *Escherichia coli* DH5α was used for DNA manipulations. The λ Red and FLP recombinases were expressed by helper plasmids pKD46 [3] and pCP20 [2], respectively. The replication of these plasmids is temperature-sensitive, allowing them to be easily eliminated. The pIJ773 vector was used as the source of the apramycin-resistance gene. The GEM T Easy vector was used to create the plasmids pGEM-*ard* and pGEM-*ardII*. Microbial cells were grown in LB [0.5 % (w/v) yeast extract (Difco), 1.0 % (w/v) Bactotryptone (Difco), and 1.0 % (w/v)NaCl] or germ medium [10] supplemented with appropriate antibiotics [50 μg/ml ampicillin, 50 μg/ml apramycin, or 10 μg/ml tetracycline].

Deletion of the *ard* gene

For construction of the *ard* deletion mutant, 300-bp DNA sequences located upstream and downstream of *ard* were amplified by polymerase chain reaction (PCR) using the following oligonucleotide pairs: A1 (5′-ATC ACA ATA AGG A AA GGA AA-3′) and A2 (5′-CGG TCA TAT AAT CAG A AT CCG *GTT AAC* CCT TTA ACG TTG ATG TTG-3′) for the upstream region, and A3 (5′-CAA CAT CAA CGT T AA AGG *GTT AAC* CGG ATT CTG ATT ATA TGA CCG-3′) and A4 (5′-ATT TGG TTC CTC AAT TTT ATA G-3′) for the downstream region; italicized bases indicate *Hpa*I sites. The PCR products were annealed via overlapping regions of the A2 and A3 primers, amplified as a single fragment using primers A1/A4, and cloned into the pGEM-T Easy vector. The resulting plasmid was digested with *Hpa*I and ligated to an apramycin-resistance gene [*aac*(*3*)*IV*] obtained from pIJ773 by digestion with *Eco*RI and *Hin*dIII and treatment with the Klenow fragment. The resultant plasmid, designated pT-ard-Apra, was used as a template for PCR amplification of the deletion cassette, which was introduced into *K. pneumoniae* Δ*ldhA* by electroporation [4] to induce homologous recombination. Correct integration of the DNA fragment was confirmed by Southern hybridization using the upstream regions of *ard* and *aac*(*3*)*IV* to probe *Dra*I-digested chromosomal DNA (Fig. S1).

Identification and expression of *ardII* in *K. pneumoniae*

In order to identify putative L-2,3-BD dehydrogenase of *K. pneumonia*, 19 dehydrogenase genes including the gene for meso-2,3-BD dehydrogenase (Ard) were introduced into the *ard*-deficient mutant. The genes were amplified from *K. pneumoniae* chromosomal DNA using the primer pairs (Table S1). The *lacZ* promoter was introduced upstream of dehydrogenase genes by amplifying the corresponding DNA from pBluescript by PCR using the primers P*lacZ*-F (5′-*TCT A GA* GCG CAA CGC AAT TAA TG-3′; italicized bases indicate an *Xba*I site) and P*lacZ*-R (5′-*GGA TCC* AGC T GT TTC CTG TGT-3′; italicized bases indicate a *Bam*HI site). DNA fragments were cloned into the pGEM-T-Easy vector, and the fidelity of cloning steps were confirmed by direct sequencing of clones. Dehydrogenase genes were next inserted into the corresponding restriction sites downstream of the *lacZ* promoter sequence. Subsequently, the tetracycline resistance gene was inserted, and the resultant plasmids were transformed into *K. pneumoniae* strains.

Construction of plasmids for expression of ArdII in *E. coli*

A plasmid permitting expression of ArdII in *E. coli* was constructed by amplifying the 744-bp ArdII gene from chromosomal DNA of *K. pneumoniae* using the primers ArdII-F (5′-GCT AGC ATG CAG ATC GAT TTA ACA G GT A-3′) and ArdII-R (5′-CTC GAG TCA GGC GTT C AA TCC GCC-3′). The PCR conditions featured an initial denaturation at 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 53 °C for 1 min, and 72 °C for 1 min, with a final hold at 72 °C for 7 min. The PCR product was cloned into pGEM-T Easy, and the fidelity of cloning steps were confirmed by direct sequencing of clones [6]. After digestion with *Nhe*I and *Xho*I, the ArdII DNA fragment was ligated into the corresponding restriction sites of pET28a. The resultant plasmid was introduced into *E. coli* BL21(DE3) by heat-shock transformation.

Expression and purification of ArdII in/from *E. coli*

Recombinants of *E. coli* BL21 (DE3) pLysS clone harboring pET-*ardII* were grown to mid-exponential phase

Table 1 Metabolites of flask-cultivated *K. pneumoniae* mutant strains

Metabolites (g/l)	Δ*ldhA*	Δ(*ldhA ard*)	Δ(*ldhA ard*)/pGEM-*ard*	Δ(*ldhA ard*)/pGEM-*ardII*
Acetoin	0.8	10.3	10.7	8.3
Meso-2,3-BD	13.9	0.6	2.9	1.6
L-2,3-BD	1.2	0.9	1.0	6.8

at 37 °C with aeration in 250-ml shaking flasks until the optical density value at 600 nm (OD_{600}) reached 0.4–0.6. Expression of ArdII was induced by addition of IPTG (isopropyl β-D-1-thiogalactopyranoside) to 0.5 mM, followed by incubation for 4 h at 37 °C. Cells were harvested by centrifugation at 4,000×*g* for 10 min at 4 °C. Each cell pellet was washed twice in 50 mM potassium phosphate buffer (pH 7.0) and suspended in 40 ml lysis buffer (pH 8.0) containing 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole. Next, the cells were sonicated and the resulting solutions centrifuged at 18,000×*g* for 20 min. Each supernatant was loaded onto a Ni^{2+}-nitrilotriacetic acid (NTA) chromatography column equilibrated with 5 ml lysis buffer. After washing with a buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole, His_6-tagged ArdII was eluted in elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole. Protein was quantified using the Bradford protein assay reagent, employing bovine serum albumen (BSA) as a standard. After boiling for 5 min, proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % (w/v) gels. Proteins in gels were stained with Coomassie Brilliant Blue R-250.

Enzyme activity assay

Enzyme activity was determined spectrophotometrically by measuring the changes in absorbance at 340 nm corresponding to the oxidation of NADH or the reduction of NAD^+ [21]. The reaction mixtures, containing 50 mM potassium phosphate buffer (pH 7.0) and 4 mM NAD^+ for oxidation reactions or 50 mM sodium acetate buffer (pH 5.0) and 0.2 mM NADH for reduction reactions, were incubated at 37 °C for 10 min. One unit (U) of enzyme activity was defined as the amount of enzyme required to reduce 1 μmol of NAD(H) in 1 min. All enzyme activities were determined in triplicate. Protein concentrations were determined with a protein assay kit (Bio-Rad) using BSA as the standard (Takara Bio. Inc.). All activity measurements were performed in triplicate.

Cultivation of *K. pneumoniae* strains

Klebsiella pneumoniae strains were cultivated in medium containing 20 g/l glucose, 20 mM potassium phosphate buffer (pH 7.0), 1 g/l yeast extract, 2 g/l $(NH_4)_2SO_4$, 0.2 g/l $MgSO_4$, 0.002 g/l $CaCl_2{\cdot}2H_2O$, 1 ml/l Fe solution [5 g/l $FeSO_4{\cdot}7H_2O$ and 4 ml HCl (37 %, w/v)], and 1 ml/l of trace element solution [70 mg/l $ZnCl_2$, 100 mg/l $MnCl_2{\cdot}4H_2O$, 60 mg/l H_3BO_3, 200 mg/l $CoCl_2{\cdot}4H_2O$, 20 mg/l $CuCl_2$ $2H_2O$, 25 mg/l $NiCl_2{\cdot}6H_2O$, 35 mg/l $Na_2MoO_4{\cdot}2H_2O$, and 4 ml HCl (37 %, w/v)] supplemented with appropriate antibiotics.

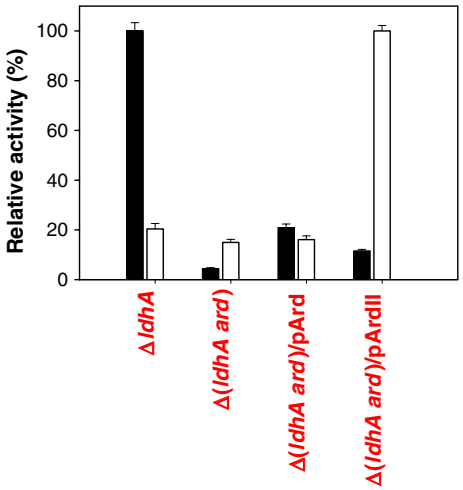

Fig. 2 Activities of meso-2,3-BD dehydrogenase (*closed bars*) and L-2,3-BD dehydrogenase (*open bars*) in *K. pneumoniae* mutant strains

For culture of *K. pneumoniae* strains in a 5-l bioreactor (Kobiotech Co. Ltd, Incheon, Korea), seed cells were first grown in a 1-l flask containing 200 ml of the preculture medium described above for 12 h with shaking (200 rpm) and then inoculated into the bioreactor containing the same medium at 10 % (v/v) with IPTG induction (0.5 mM). Fed-batch cultivation was conducted at 37 °C with shaking at 200 rpm (initial pH 7.0). pH was continuously monitored and maintained at 6.0 by automatic addition of NH_4OH. Air (2.0 vvm) was supplied to the stirred reactor containing 2 l of fermentation medium. All results reflect data obtained from three independent experiments. Average values are shown.

Analytical methods

Metabolites and sugars in the culture broth were determined using a high-performance liquid chromatography system (Agilent 1200) equipped with a refractive index

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Ba   D23BD      1:MKAARWHNQKDIRIENIDEPKAEPGKVK-IKVKWCGICGSDLHEYLGGPIFIPVGKPHPL 59
Bs   L23BD      1:MS---------K-VAMVTGGAQGIGRGISEKLAADGF---DIAVADLPQ---Q-EEQAAE 43
Kp   ArdII      1:MQI----DLTGK-KALVTGASRGLGRAIALSLARAGA---DVVITYEKS---A-DKAQAV 48
Kp   Ard        1:MK---------K-VALVTGAGQGIGKAIALRLVKDGF---AVAIADYND---A-TAKAVA 43
                   *          .  . ...   . *  .   .   *    .

BaA  D23BD     60:TNEMAPVT-MGHEFSGEVVEVGEGVKNYSVGDRVVVEPIFATHGHQGAYNLDEQMGFLGL 118
BrA  L23BD     44:T--IKLIEAADQKAVF--VGLDVTDK-----AN-F-DSA--IDEAAE------K---LG- 80
KpN  Dha19     49:ADE---IKALGRHGEA--VQADSASA-----QAIQ-EAV--THAARS----------LG- 84
KpN  M23BD     44:S-EIN--QAGG-RAMA--VKVDVSDR-----DQVF-AA---VEQARK------T---LG- 78
                     .    . .       *  .                    .           **

BaA  D23BD    119:AGGGGFSEYVSVDEELLFKLPEELSYEQGALVEPSAVALYAVRQSKLKAGD-KAAVFGC 177
BrA  L23BD     81:-GF-DVLVNNAGIA-----Q--------IKPLL-E--VTEEDLKQ--IYSVNVFSVFFGI 120
KpN  Dha19     85:-GL-DILVNNAGIA-----R--------GGPLE-S--MTLADIDA--LINVNIRGVVIAT 124
KpN  M23BD     79:-GF-DVIVNNAGVA-----P--------STPIE-S--ITPEIVDK--VYNINVKGVIWGI 118
                   *  .  ..... .                ..      .           .   .  .

BaA  D23BD    178:GPIGLLVIEALKAAGATDIYAVELSPERQEKAKELGAIIIDPSKTDDVVEEIAKRTNGGV 237
BrA  L23BD    121:QAA-SRKFD--ELGVKGKI--INAASIAAIQGF-PILSAYSTTKFAVR--GLTQ---AAA 169
KpN  Dha19    125:QEA-LVHMA--DG---GRI--INIGSCLANRVAMPGIAVYAMTKSALN--ALTR---GLA 171
KpN  M23BD    119:QAA-VEAFK--KEGHGGKI--INACSQAGHVGN-PELAVYSSSKFAVR--GLTQ---TAA 167
                   . .            . *  ..  .       .    .  * .     ..      .

BaA  D23BD    238:-DVSYEVTGVPVVLRQAIQSTN-IAGETVIVSIWEKGAEIHPNDIVIKERTVKGIIGYR- 294
BrA  L23BD    170:QELAPK--GHTVNAYAPGIVGTGMWEQ-IDAELSKINGKPIGENFKEYSSSIA-LGRPSV 225
KpN  Dha19    172:RDLGPR--GITVNLVHPGPTNSDM-----N---------PEDGEQAEAQRQMIAVGHYGQ 215
KpN  M23BD    168:RDLAPL--GITVNGYCPGIVKTPMWAE-IDRQVSEAAGKPLGYGTAEFAKRIT-LGRLSE 223
                    .. .   * .*.   ..     .               .      .        .

BaA  D23BD    295:-DIFPSVLALMKEGYFSADKLVTKKIVLDDLIEEGFGALIKEKNQVKILVKPN         346
BrA  L23BD    226:PEDVAGLVSFLAS--ENSNYVTGQVM-L---VDGGML----------YN---         258
KpN  Dha19    216:PEDIAAAVTFLAS--PAAGQISGTG--LD--VDGGLN----------A----         247
KpN  M23BD    224:PEDVAACVSYLAS--PDSDYMTGQSL-L---IDGGMV----------FN---         256
                   ... .  .  ...         .    *    ..*
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Fig. 3 Alignment of amino acid sequences homologous to ArdII. N-terminal coenzyme binding motif (GxxxGxG) is indicated in *box*

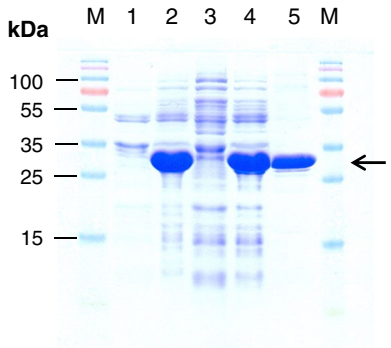

Fig. 4 Expression and purification of ArdII in/from *E. coli*. *Lane 1* total cell lysates of *E. coli* BL21 harboring pET28a (control); *lane 2* total cell lysates of *E. coli* BL21 harboring pET-*ardII*; *lane 3* insoluble fraction; *lane 4* soluble fraction; *lane 5* purified ArdII (*arrow*)

detector and an Aminex HPX-87H column (300 × 78 mm; Bio-Rad, Hercules, CA, USA). The mobile phase was 2.5 mM H_2SO_4 and the flow rate was 0.5 ml/min. The column and cell temperatures were maintained at 65 and 45 °C, respectively [13]. Cell growth was determined from measurements of OD_{600} using a UV–Vis spectrophotometer (Ultrospec 3100 Pro; Amersham Biosciences, Piscataway, NJ, USA).

Nucleotide sequence accession number

The nucleotide sequences of the *ard* and *ardII* gene from *K. pneumoinae* were presented in the GenBank database under accession no. ABR77489 and ABR76070, respectively.

Results

Identification of a putative *K. pneumoniae* 2,3-BD dehydrogenase

We found that the level of 2,3-BD, especially meso-2,3-BD, was severely decreased in an *ard*-deficient mutant of *K. pneumoniae,* which accumulated acetoin (Table 1). Reintroduction of the *ard* gene restored the level of meso-2,3-BD, although acetoin still accumulated. In agreement with these results, meso-2,3-BD dehydrogenase activity in the *ard*-deficient mutant was remarkably decreased and was complemented by reintroduction of the *ard* gene (Fig. 2). These results are consistent with previous reports that Ard is crucial for this synthesis pathway, especially for meso-2,3-BD (Fig. 1). However, the level of L-2,3-BD was not affected by manipulation of the *ard* gene.

Table 2 Activities of ArdII toward various substrates

Substrate	Activity (%)
Oxidation	
L-2,3-BD	100 (ND)
Meso-2,3-BD	14.3 ± 1.1
D-2,3-BD	ND
1,4-BD	20.4 ± 1.3
1,3-BD	10.2 ± 0.7
Glycerol	2.1 ± 0.1
Reduction	
Acetoin	100 (20.3 ± 1.3)
Diacetyl	48.6 ± 1.4
3-Hydroxy-3-methly-2-butanone	16.7 ± 0.3

Parentheses indicated activity measured using $NADP^+$ (L-2,3-BD) and NADPH (acetoin) as a cofactor

ND not detected

No previous information was available regarding the synthesis of L-2,3-BD in *K. pneumoniae*. In order to identify the pathway, we carried out a set of complementation experiments with the *ard*-deficient mutant using various dehydrogenase genes from whole genome sequences. Over 175 genes annotated as dehydrogenase were found in genome sequences of *K. pneumonia*. Among them, first, we selected a set of genes encoding a protein of similar size to Ard (771 amino acids) (Table S1), then the genes were introduced into the the *ard*-deficient mutant to identify the mutant producing increase amount of L-2,3-BD. As a result, a dehydrogenase gene, named *ardII*, was identified (Table 1), and the resulting complemented strain exhibited high levels of L-2,3-BD dehydrogenase activity (Fig. 2). These results identify ArdII as the putative *K. pneumoniae* 2,3-BD dehydrogenase involved in L-2,3-BD synthesis.

Sequence analysis of ArdII

The putative 2,3-BD dehydrogenase gene (*ardII*) from *K. pneumoniae* encoded a polypeptide of 247 amino acids with a predicted molecular weight of about 27 kDa.

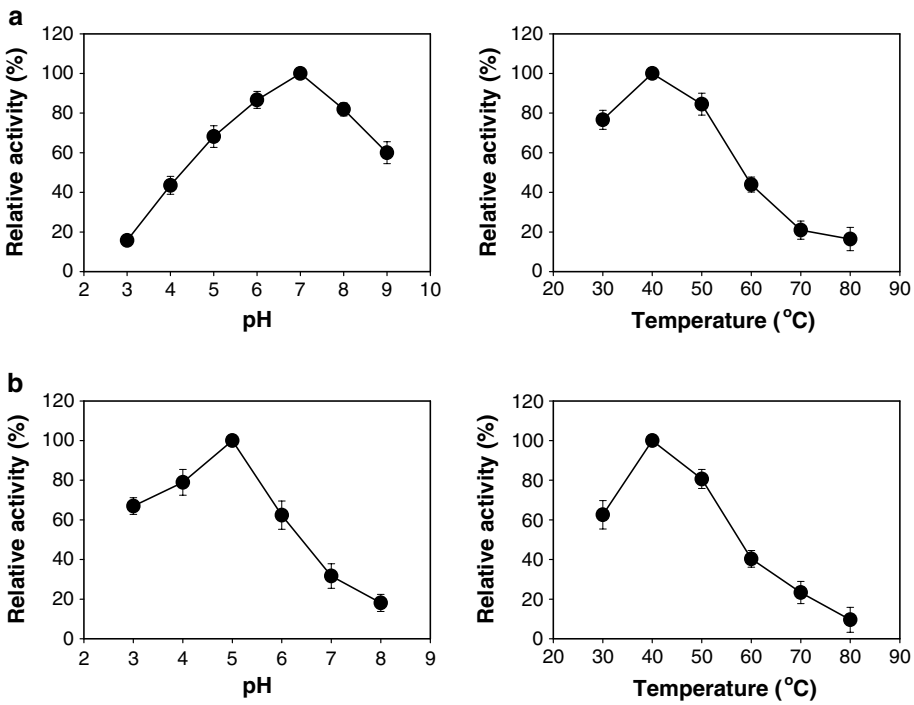

Fig. 5 Effects of pH and temperature on ArdII activity toward the substrates, L-2,3-BD (**a**) and acetoin (**b**)

A BLAST analysis showed that its deduced amino acid sequence was 33.0 % homologous to the L-2,3-BD dehydrogenase from *Brevibacterium saccharolyticum* (GenBank accession no. AB009078), 39.5 % homologous to the meso-2,3-BD dehydrogenase from *K. pneumoniae* (GenBank accession no. ABR77489), and 17.5 % homologous to the D-2,3-BD dehydrogenase from *Bacillus amyloliquefaciens* (GenBank accession no. KF358987) (Fig. 3).

Analysis of *K. pneumoniae* ArdII revealed an N-terminal coenzyme binding motif (GxxxGxG) (Fig. 3), which is a characteristic glycine-rich consensus sequence found in all short-chain alcohol dehydrogenases/reductases.

Characterization of the enzymatic properties of ArdII

An *E. coli* strain harboring pET-*ardII* was constructed to produce high levels of recombinant ArdII. An SDS-PAGE analysis of the cytoplasmic fraction from this strain confirmed prominent expression of the recombinant protein (Fig. 4). No such band was observed in the cytoplasmic fraction of control cells harboring the empty pET28a vector. His_6-tagged recombinant ArdII in the soluble fraction of crude cell lysates of pET-*ardII*-expressing *E. coli* was purified by Ni–NTA affinity column chromatography.

The substrate specificity of purified ArdII was examined using several substrates for oxidative and reductive reactions. The highest oxidative enzyme activity was observed with L-2,3-BD as substrate in the presence of NAD^+ (Table 2), whereas lower or no activity was observed with meso- and D-2,3-BD, indicating that ArdII is an L-2,3-BD dehydrogenase. Additionally, ArdII showed reductive activity toward diacetyl as well as acetoin (Table 2), like other L-2,3-BD dehydrogenases. As shown in Fig. 1, the sequential reductive activities toward diacetyl and acetoin led to the synthesis of L-2,3-BD in *K. pneumoniae*. The preferred cofactor for oxidative and reductive activities of ArdII was NAD(H) not NADP(H) (Table 2).

As shown in Fig. 5, the optimal activity was observed at pH 7 and 40 °C for L-2,3-BD (oxidative reaction) and at pH 5 and 40 °C for acetoin (reductive reaction). To estimate kinetic parameters, we measured the Michaelis constant (K_m) and the maximum velocity (V_{max}) for oxidative and reductive reactions. The K_m of ArdII was 5.51 ± 0.31 (mM), 0.58 ± 0.11, and 3.80 ± 0.23 for L-2,3-BD, acetoin, and diacetyl, respectively; the corresponding V_{max} values were 6.73 ± 0.55 (μm/min), 36.36 ± 0.34, and 9.50 ± 0.61, respectively (Fig. 6). Similarly, K_m values of ArdII for cofactors NAD of the oxidative reaction and NADH of reductive reaction were 4.86 ± 0.61 (mM) and 0.53 ± 0.12, respectively. These enzymatic properties are consistent with the synthesis of 2,3-BD from acetoin during cell growth.

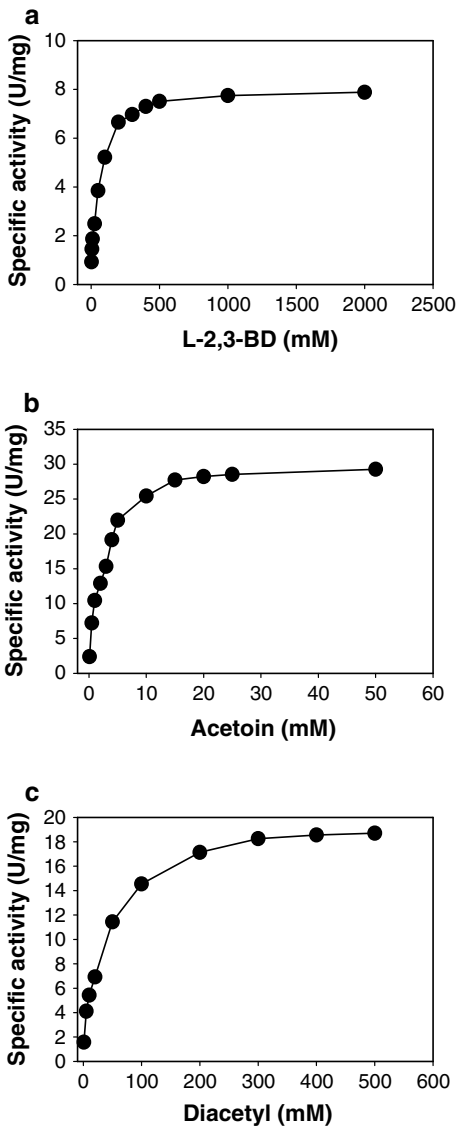

Fig. 6 Enzyme kinetics of ArdII. **a** L-2,3-BD; **b** acetoin; **c** diacetyl

High level production of L-2,3-BD by expression of *ardII*

Finally, we evaluated the effect of *ardII* gene copy number on 2,3-BD production in *K. pneumoniae*. Efficient fed-batch fermentation could enhance the concentrations of target products [12, 15]. To achieve a higher product concentration, a fed-batch fermentation was carried out with glucose as the carbon and energy source (Figs. 7, 8). Multiple copies of *ardII* dramatically increased production level of L-2,3-BD in the *ard*-deficient mutant strain with decrease of acetoin with complementation of defects in glucose consumption and cell growth (Table 3). In the *ard*-deficient mutant, the levels of acetate, ethanol, and succinate were similar or slightly higher compared to the parent ldhA strain although the glucose consumption

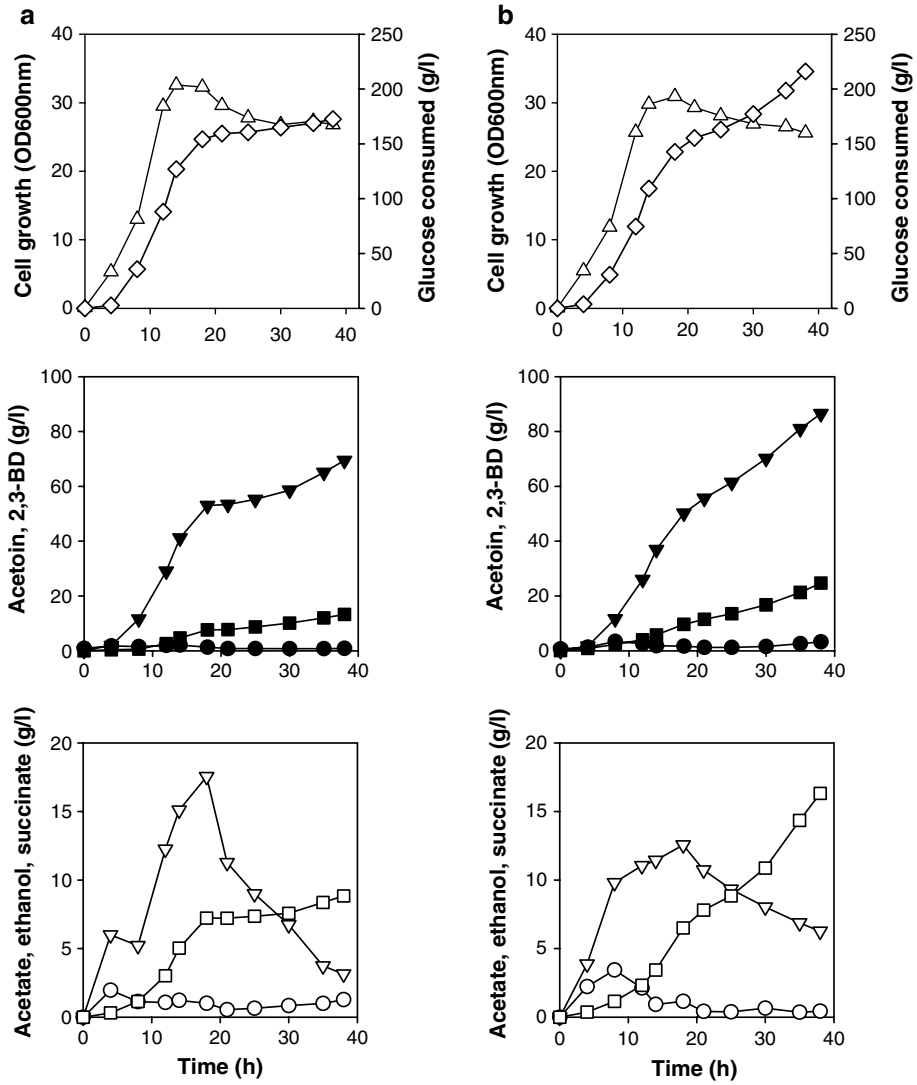

Fig. 7 Fed-batch fermentation of a *K. pneumoniae* Δ*ldhA* harboring pGEM (**a**) and pGEM-*ardII* (**b**). *Open triangles up* cell growth, *open diamonds* glucose consumed, *closed circles* acetoin, *closed triangles down* meso-2,3-BD, *closed squares* L-2,3-BD, *open circles* acetate, *open triangles down* ethanol, *open squares* succinate

was significantly decreased, which might be due to increased level of NADH by elimination of synthesis of meso-2,3-BD.

Discussion

2,3-BD is a crucial chiral compound with a 3 isomeric forms: D- (*2R,3R*), L- (*2S,3S*) and meso [1]. Especially, L-2,3-BD is an important precursor of asymmetric synthesis and potentially valuable liquid fuel [14, 16].

In this study, we isolated a short-chain acyl dehydrogenase/reductase from *K. pneumoniae*, called ArdII, and demonstrated that it possesses L-2,3-BD dehydrogenase activity. This is the first report to describe the identification and characterization of L-2,3-BD dehydrogenase activity in *K. pneumoniae*, in which high levels of L-2,3-BD—up to approximately 25 %, as meso-2,3-BD are produced (Table 3).

ArdII showed enzymatic activity toward diacetyl as well as acetoin, in agreement with the predicted metabolic pathway for L-2,3-BD in *K. pneumoniae* (Fig. 1). α-Acetolactate, produced from pyruvate by the enzymatic activity of Als, was converted through a non-enzymatic reaction to diacetyl, from which L-2,3-BD was produced via acetoin by subsequent catalysis by ArdII. This was confirmed by the increase in L-2,3-BD production in *K. pneumoniae* induced by expression of *ardII*. The effect of gene expression was more marked in the *ard*-deficient mutant strain, indicating that the two 2,3-BD dehydrogenase activities—Ard for D-2,3-BD and ArdII for L-2,3-BD—compete for the common intermediate α-acetolactate. We recently constructed an *ardII*-deficient mutant strain to further examine the role of ArdII.

Ui et al. [18] analyzed the structural basis for the stereospecific features of the two 2,3-BD dehydrogenases using the meso-type from *K. pneumoniae* and the L-type from

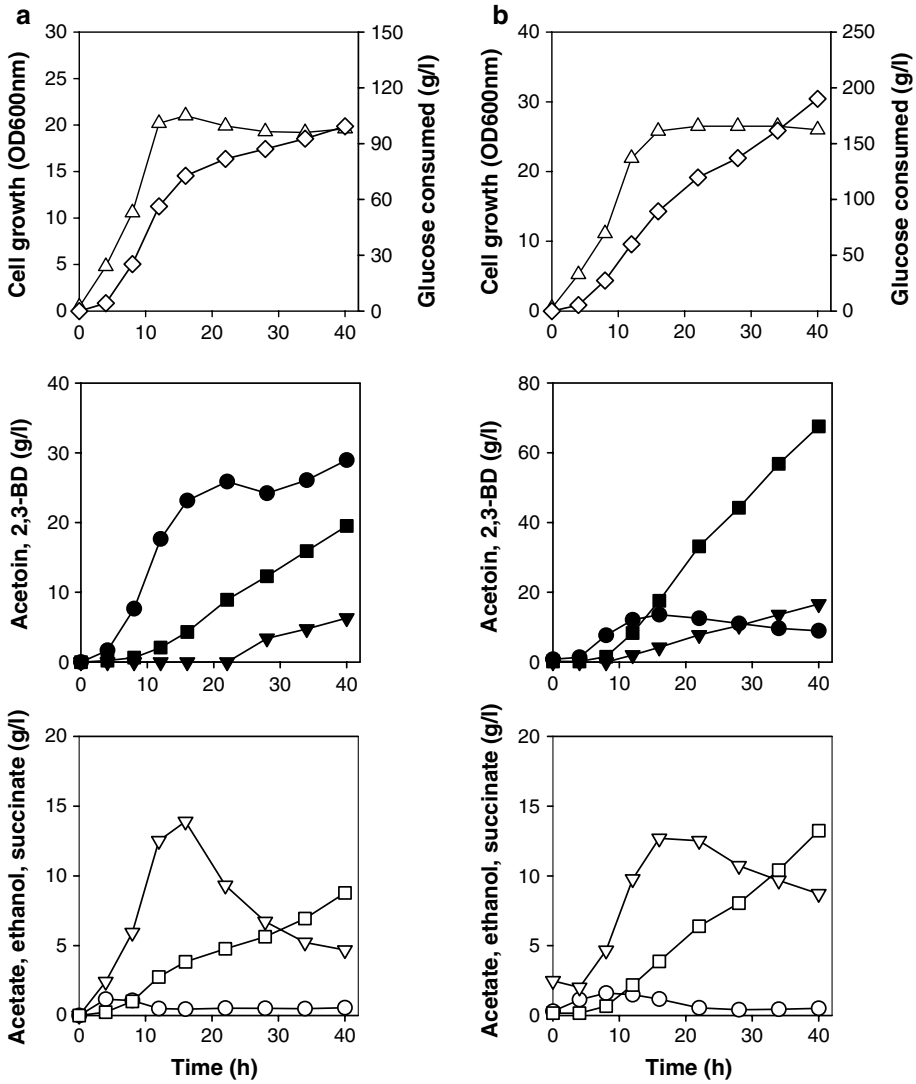

Fig. 8 Fed-batch fermentation of a *K. pneumoniae* Δ(*ldhA ard*) harboring pGEM (**a**) and pGEM-*ardII* (**b**). *Open triangles up* cell growth, *open diamonds* glucose consumed, *closed circles* acetoin, *closed triangles down* meso-2,3-BD, *closed squares* L-2,3-BD, *open circles* acetate, *open triangles down* ethanol, *open squares* succinate

Table 3 Analysis of metabolites of fed-batch–fermented *K. pneumoniae* mutant strains

	Δ*ldhA*		Δ(*ldhA ard*)	
	pGEM	pGEM-*ardII*	pGEM	pGEM-*ardII*
Glucose consumed (g/l)	172.6	216.0	99.3	190.1
Cell growth (OD600 nm)	26.8	25.6	19.6	26.0
Acetoin (g/l)	0.9	3.3	29.0	9.0
Meso-2,3-BD (g/l)	69.5	86.5	6.3	16.6
L-2,3-BD (g/l)	13.3	24.7	19.5	67.5
Acetate (g/l)	1.3	0.4	0.6	0.5
Ethanol (g/l)	3.1	6.3	4.7	8.7
Succinate (g/l)	8.8	16.3	8.8	13.2
Meso-/L-2,3-BD conversion yield (g/g)	0.4/0.08	0.4/0.11	0.06/0.2	0.09/0.46

Brev. Saccharolyticum. Here, we identified the two types of 2,3-BD dehydrogenases in a single microorganism for the first time. We expect that further comparative analyses of the structural and catalytic properties of these enzymes will provide additional insights that should prove helpful in understanding and applying the stereospecific properties of enzymes.

In practice, expression of *ardII* markedly stimulated L-2,3-BD production, especially in the *ard*-deficient mutant strain. The maximal level of L-2,3-BD obtained in this

study was ~77.6 g/l, which, to the best of our knowledge, is the highest level shown by a natural or recombinant microbial strain. We anticipate that production could be further increased through strain engineering and optimization of fermentation conditions.

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References

1. Celińska E, Grajek W (2009) Biotechnological production of 2,3-butanediol-current state and prospects. Biotechnol Adv 27:715–725. doi:10.1016/j.biotechadv.2009.05.002
2. Cherepanov PP, Wackernagel W (1995) Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158:9–14. doi:10.1016/0378-1119(95)00193-A
3. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97:6640–6645. doi:10.1073/pnas.120163297
4. Fournet-Fayard S, Joly B, Forestier C (1995) Transformation of wild type *Klebsiella pneumoniae* with plasmid DNA by electroporation. J Microbiol Methods 24:49–54. doi:10.1016/0167-7012(95)00053-4
5. Li L, Wang Y, Zhang L, Ma C, Wang A, Tao F, Xu P (2011) Biocatalytic production of (2S,3S)-2,3-butanediol from diacetyl using whole cells of engineered *Escherichia coli*. Bioresour Technol 115:111–116. doi:10.1016/j.biortech.2011.08.097
6. Luo LH, Seo JW, Baek JO, Oh BR, Heo SY, Hong WK, Kim DH, Kin CH (2011) Identificaiton and characterization of the propanediol utilization protein PduP of *Lactobacillus reuteri* for 3-hydroxypropionic acid production from glycerol. Appl Microbiol Biotechnol 89:697–703. doi:10.1007/s00253-010-2887-6
7. Ji XJ, Huang H, Ouyang PK (2011) Microbial 2,3-butanediol production: a state-of-the-art review. Biotechnol Adv 29:351–364. doi:10.1016/j.biotechadv.2011.01.007
8. Jurchescu IM, Hamann J, Zhou X, Ortmann T, Kuenz A, Prußе U, Lang S (2013) Enhanced 2,3-butanediol production in fed-batch cultures of free and immobilized *Bacillus licheniformis* DSM 8785. Appl Microbiol Biotechnol 97:6715–6723. doi:10.1007/s00253-013-4981-z
9. Ma C, Wang A, Qin J, Li L, Ai X, Jiang T, Tang H, Xu P (2009) Enhanced 2,3-butanediol production by *Klebsiella pneumoniae* SDM. Appl Microbiol Biotechnol 82:49–57. doi:10.1007/s00253-008-1732-7
10. Oh BR, Seo JW, Heo SY, Hong WK, Luo LH, Joe MH, Park DH, Kim CH (2011) Efficient production of ethanol from crude glycerol by a *Klebsiella pneumoniae* mutant strain. Bioresour Technol 102:3918–3922. doi:10.1016/j.biortech.2010.12.007
11. Oh BR, Seo JW, Heo SY, Hong WK, Luo LH, Kim SH, Park DH, Kim CH (2012) Optimization of culture conditions for 1,3-propanediol production from glycerol using a mutant strain of *Klebsiella pneumoniae*. Appl Biochem Biotechnol 166:127–137. doi:10.1007/s12010-011-9409-6
12. Oh BR, Hong WK, Heo SY, Joe MH, Seo JW, Kim CH (2013) The role of aldehyde/alcohol dehydrogenase (AdhE) in ethanol production from glycerol by *Klebsiella pneumoniae*. J Ind Microbiol Biotechnol 40:227–233. doi:10.1007/s10295-012-1224-8
13. Oh BR, Heo SY, Lee SM, Hong WK, Park JM, Jung YR, Kim DH, Sohn JH, Seo JW, Kim CH (2014) Production of isobutanol from crude glycerol by a genetically-engineered *Klebsiella pneumoniae* strain. Biotechnol Lett 36:397–402. doi:10.1007/s10529-013-1383-3
14. Oliver JW, Machado IM, Yoneda H, Atsumi S (2013) Cyanobacterial conversion of carbon dioxide to 2,3-butanediol. Proc Natl Acad Sci 110:1249–1254. doi:10.1073/pnas.1213024110
15. Park JM, Oh BR, Seo JW, Hong WK, Yu A, Sohn JH, Kim CH (2013) Efficient production of ethanol from empty palm fruit bunch fibers by fed-batch simultaneous saccharification and fermentation using *Saccharomyces cerevisiae*. Appl Biochem Biotechnol 170:1807–1814. doi:10.1007/s12010-013-0314-z
16. Syu MJ (2001) Biological production of 2,3-butanediol. Appl Microbiol Biotechnol 55:10–18. doi:10.1007/s002530000486
17. Ui S, Matsuyama N, Masuda H, Muraki H (1984) Mechanism for the formation of 2,3-butanediol stereoisomers in *Klebsiella pneumoniae*. J Ferment Technol 62:551–559. doi:10.1007/s00253-014-5526-9
18. Ui S, Takusagawa Y, Ohtsuki T, Mimura A, Ohkuma M, Kudo T (2001) Stereochemical applications of the expression of the L-2,3-butanediol dehydrogenase gene in *Escherichia coli*. Lett Appl Microbiol 32:93–98. doi:10.1046/j.1472-765x.2001.00869
19. Wang Z, Song Q, Yu M, Wang Y, Xiong B, Zhang Y, Zheng J, Ying X (2014) Characterization of a stereospecific acetoin (diacetyl) reductase from *Rhodococcus erythropolis* WZ010 and its application for the synthesis of (2S,3S)-2,3-butanediol. Appl Microbiol Biotechnol 98:641–650. doi:10.1007/s00253-013-4870-5
20. Yan YJ, Lee CC, Liao JC (2009) Enantioselective synthesis of pure (R, R)-2,3-butanediol in *Escherchia coli* with stereospecific secondary alcohol dehydrogenase. Org Biomol Chem 7:3914–3917. doi:10.1039/b913501d
21. Zhang GL, Wang CW, Li C (2012) Cloning, expression and characterization of meso-2,3-butanediol dehydrogenase from *Klebsiella pneumoniae*. Biotechnol Lett 34:1519–1523. doi:10.1007/s10529-012-0933-4