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ORIGINAL PAPER

Identification and characterization of *Klebsiella pneumoniae* aldehyde dehydrogenases increasing production of 3-hydroxypropionic acid from glycerol

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Abstract *Klebsiella pneumoniae* produces 3-hydroxypropionic acid (3-HP) from glycerol with oxidation of 3-hydroxypropionaldehyde (3-HPA) to 3-HP in a reaction catalyzed by aldehyde dehydrogenase (ALDH). In the present study, two putative ALDHs of *K. pneumoniae*, YneI and YdcW were identified and characterized. Recombinant YneI was specifically active on 3-HPA and preferred NAD^+ as a cofactor, whereas YdcW exhibited broad substrate specificity and preferred $NADP^+$ as a cofactor. Overexpression of ALDHs in the glycerol oxidative pathway-deficient mutant *K. pneumoniae* AK resulted in a significant increase in 3-HP production upon shake-flask culture. The final titers of 3-HP were 2.4 and 1.8 g L^{-1} by recombinants overexpressing YneI and YdcW, respectively. Deletion of the ALDH gene from *K. pneumoniae* did not affect the extent of 3-HP synthesis, implying non-specific activity of ALDHs on 3-HPA. The ALDHs might play major roles in detoxifying the aldehyde generated in glycerol metabolism.

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Introduction

3-Hydroxypropionic acid (3-HP) is a valuable C3 building block, and is used as an intermediate in the synthesis of many commercially valuable chemicals employed in the production of adhesives, fibers, and resins. Despite the industrial importance of 3-HP, it is available commercially as an aqueous solution from only a few suppliers because of the high cost of production and the low yields obtained [1, 2]. Glycerol is an attractive substrate for development of a low-cost fermentation route to 3-HP because glycerol is a major by-product of biodiesel manufacturing and is, therefore, available in large quantities [3].

Two models of 3-HP biosynthesis from glycerol have been suggested in *Klebsiella pneumoniae*. In the coenzyme A (CoA)-dependent pathway, 3-hydroxypropionaldehyde (3-HPA) is converted to 3-HP by a CoA-dependent aldehyde dehydrogenase (PduP) via a series reactions. In the CoA-independent pathway, 3-HPA is directly converted to 3-HP by the action of aldehyde dehydrogenase (ALDH). The CoA-dependent pathway involving PduP has been described in previous study [4, 5], but ALDH involved in CoA-independent pathway has never been characterized.

To explore ALDH involved in the CoA-independent pathway, putative 176 dehydrogenase genes identified from genome sequences of *K. pneumoniae* were overexpressed and, as the result, three ALDHs, termed AldHk, YdcW and YneI, were revealed to increase production of 3HP. We have previously reported on the role of AldHk enhancing 3-HP production [6]. In here, two other ALDHs (YdcW

and YneI) were characterized and gene effects on 3-HP biosynthesis were also investigated.

Materials and methods

Bacterial strains, plasmids and media

Klebsiella pneumoniae Cu, a derivative of strain ATCC 700721, was obtained by curing of cryptic plasmids by ethidium bromide. The *K. pneumoniae* AK strain (Cu Δ[*orfY-dhaT-orfW-orfX-dhaR-dhaD*::Apr^R]) has been previously described [7]. *E. coli* DH5α was used for DNA manipulation. The plasmid pGEM-T Easy (Promega, Madison, WI) was employed for cloning, whereas pET28a (Novagen, Darmstadt, Germany) and pBR322 were used for expression of genes in *E. coli* and *K. pneumoniae*, respectively. Microbial cells were grown in LB medium (yeast extract [Difco, Sparks, MD, USA], 0.5 %; Bacto-tryptone [Difco], 1.0 %; and NaCl, 1.0 % [all w/v]) supplemented with appropriate antibiotics (ampicillin [50 μg mL^{-1}] and/or tetracycline [10 μg mL^{-1} for *E. coli* and 50 μg mL^{-1} for *K. pneumoniae*]). The pIJ773 vector was the source of the apramycin-resistance gene, and the temperature-sensitive plasmid pKD46 was employed when homologous recombination was required [8].

Chemical and reagents

T4 DNA ligase and restriction enzymes were obtained from Takara Bio. Inc. (Otsu, Shiga, Japan). Coomassie Brilliant blue R-250 staining solution was purchased from Noble Biosciences Inc. (Suweon, Kyounggi, Korea). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and 30 % (w/v) acrylamide/bis-acrylamide were the products of Biosesang Inc. (Seongnam, Kyounggi, Korea). Ammonium persulfate was obtained from Bio-Rad Laboratories, Inc. (Richmond, CA, USA). Ethidium bromide was the product of Promega. The 3-HP standard was purchased from Tokyo Chemical Industry Co. (Chuo, Tokyo, Japan). Other chemicals were the products of the Sigma Chemical Company (St. Louis, MO, USA).

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

To construct a plasmid permitting expression of YneI and YdcW in *E. coli*, the relevant genes were amplified from chromosomal DNA of *K. pneumoniae* using appropriate primers (YneI-F: 5′-**ccatgg**atgatgaatttatctgcaacg-3′; YneI-R: 5′-**ctcgag**gcgacggtctttccaga-3′; YdcW-F: 5′-**ccatgg**atga aacaggataatgctatg-3′; YdcW-R: 5′-**ctcgag**gctatgcttaaccatc acat-3′; the bases in bold indicate *Nco*I and *Xho*I sites, respectively). The PCR conditions featured initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, with a final hold at 72 °C for 7 min. The PCR products were cloned into pGEM-T-Easy, and clones were subjected to nucleotide sequencing to confirm the absence of any error. After digestion with *Nco*I and *Xho*I, DNA fragments were ligated into the corresponding restriction sites of pET28a, yielding pET-*yneI* and pET-*ydcW*, respectively.

Construction of plasmids for expression of ALDHs in *K. pneumoniae*

The *yneI* (GenBank accession no. ABR77066) and *ydcW* (GenBank accession no. ABR77355) genes were amplified from chromosomal DNA of *K. pneumoniae* using appropriate primers (YneI-F: 5′-tctagaatgatgaatttatctgcaacgca-3′; YneI-R: 5′-ctcgaggcgacggtctttccaga-3′; YdcW-F: 5′-tctagaatgaaacaggataatgctatgca-3′; YdcW-R: 5′-actagtctcgagttagcgacggtctttccaga-3′). To introduce the PlacZ promoter upstream of these genes, promoter of PlacZ was amplified from pBluescript by PCR. DNA fragments were cloned into the pGEM-T-Easy vector, and clones were subjected to sequencing to confirm the absence of any error. The gene fragments, including the ALDH genes, were next inserted between the corresponding restriction sites downstream of the *lacZ* promoter sequence. Subsequently, pVOT (pBR- PlacZ -*dhaT*- PlacZ-*orfWX*), pGEM-PlacZ-*yneI,* and pGEM-PlacZ-*ydcW* were cut with *Xho*I, and ligated, yielding plasmids pBR-PlacZ-*dhaT*-PlacZ-*yneI*-PlacZ-*orfWX* (pVOTYI) and pBR-PlacZ-*dhaT*-PlacZ-*ydcW*-PlacZ-*orfWX* (pVOTYW). Plasmid pVOT, described in a previous report [7], includes the *orfW* gene encoding a factor reactivating DhaB. Final plasmids were transformed into the *K. pneumoniae* AK strain by electroporation.

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

Recombinants of *E. coli* BL21 (DE3) pLysS clone harboring pET-*yneI* and pET-*ydcW* were grown to mid-exponential phase at 37 °C, with aeration, in 250 mL shake flasks, until the A_{600} value attained 0.4–0.6. Expression of YneI and YdcW was induced by addition of IPTG to 0.5 mM, followed by incubation for 4 h at 37 °C. Cells were harvested by centrifugation at 4,278*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 18,510*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 (using a

buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole), ALDHs linked to the His_6 tag were eluted in elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole. Protein quantification was achieved using the Bradford protein assay reagent, employing BSA as a standard. After boiling for 5 min, SDS-PAGE was performed on 12 % (w/v) polyacrylamide gel. Proteins were stained with Coomassie Brilliant Blue R-250.

Enzyme activity assay

ALDH activity was measured by the method of Leal et al. [9], with slight modifications. The reaction mixture contained 50 mM potassium phosphate buffer (pH 8.0), 4 mM NAD^+ (or $NADP^+$), 2 mM of an aldehyde substrate, and a protein source (37.0 μg mL^{-1} of soluble cell lysate or 43.5 μg mL^{-1} of a purified ALDH); incubation proceeded at 37 °C for 10 min. Enzyme activity was determined by measuring the amount of NADH (or NADPH) produced from NAD^+ (or $NADP^+$), by absorbance at 340 nm. One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 μmol NADH per min. All activity measurements were performed in triplicate.

Substrate specificity was examined at 37 °C and pH 8.0 using various aldehydes including 3-hydroxypropionaldehyde (3-HPA), propionaldehyde (PA), butyraldehyde (BA), valeraldehyde (VA), isovaleraldehyde (IVA) and furaldehyde (FA), in the presence of NAD^+ or $NADP^+$ as cofactor.

Cultivation of *K. pneumoniae* strains in glycerol-containing medium

Klebsiella pneumoniae strains were cultivated in medium containing 20 g L^{-1} glycerol, 30 mM potassium phosphate buffer (pH 7.0), 1 g L^{-1} yeast extract, 2 g L^{-1} $(NH_4)_2SO_4$, 0.2 g L^{-1} $MgSO_4$, 0.002 g L^{-1} $CaCl_2{\cdot}2H_2O$, 1 mL L^{-1} Fe solution (5 g L^{-1} $FeSO_4{\cdot}7H_2O$ and 4 mL HCl [37 %, w/v]), and 1 mL L^{-1} of trace element solution (70 mg L^{-1} $ZnCl_2$, 100 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 60 mg L^{-1} H_3BO_3, 200 mg L^{-1} $CoCl_2{\cdot}4H_2O$, 20 mg L^{-1} $CuCl_2$ $2H_2O$, 25 mg L^{-1} $NiCl_2{\cdot}6H_2O$, 35 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$, and 4 mL HCl [37 %, w/v]) [10].

When *K. pneumoniae* strains were grown in a 5-L bioreactor (Kobiotech Co. Ltd, Incheon, Korea), seed cells were cultivated in a 250-mL flask containing 50 mL of the preculture medium described above for 12 h at 120 rpm, and were next inoculated into the bioreactor, containing the same medium, at 2 % (v/v) with IPTG induction (0.5 mM). Batch cultivation was conducted at 37 °C, with shaking at 200 rpm, at pH 7.0. Air (0.5 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

The concentrations of 3-HP, glycerol, and other metabolites in culture broth were determined by high-performance liquid chromatography (Agilent System 1200; Santa Clara, CA); the system was equipped with a refractive index detector and an ion-exchange column (300 × 78 mm; Rezex ROA-organic acid; Phenomenex, Torrance, CA). The mobile phase was 0.0025 mol L^{-1} H_2SO_4 and the flow rate was 0.5 mL min^{-1}. The column and cell temperatures were 65 and 45 °C, respectively [4].

Results and discussion

Sequence analysis of ALDHs

YneI contained 462 amino acids and was 76, 74, 75, and 87 % homologous to YneI of *E. coli* (EHX11321), YneI of *Salmonella enterica* (NP_460484), YneI of *Citrobacter youngae* (ZP_06352898), and Ald of *Enterobacter aerogenes* (YP_004593910), respectively (Fig. 1). Residues putatively forming the active site of an ALDH, Asn-137, Glu-234, Gly-265, and Cys-268, and $NAD(P)^+$ binding sites were evident in YneI.

YdcW contained 481 amino acids and 85, 84, 92, and 97 % homologous to YdcW of *E. coli* (YP_001462716), YdcW of *S. enterica* (YP_002114622), Ald of *E. aerogenes* (YP_004594212), and Ald of *K. variicola* (YP_003439305), respectively (Fig. 2). The figure shows the catalytic residues (triangles) of Asn-155, Glu-252, Gly-283, and Cys-286; the substrate-binding sites (circles) of Asp-285 and Leu-444, and the $NAD(P)^+$ binding sites (underline) (151, 152, 154, 178, 180, 181, 210, 215, 229–231, 234, 237, 252, 254, 286, 333, 384).

Expression and purification of *K. pneumoniae* ALDHs in/from *E. coli*

To explore ALDH activities, recombinant proteins were purified from *E. coli* BL21 harboring pET28a-based clones. SDS-PAGE analysis of cytoplasmic fractions of such strains confirmed that recombinant proteins approximately 55 kDa in size were prominently expressed. No such band was observed in the cytoplasmic fraction of control cells harboring vector pET28a. His_6-tagged recombinant ALDHs in cytoplasmic fractions of *E. coli* were purified by Ni–NTA affinity column chromatography (Fig. 3).

Fig. 1 Alignment, emphasizing homology, of YneI with other aldehyde dehydrogenases: YneI of *Escherichia coli* (EHX11321), YneI of *Salmonella enterica* (NP_460484), YneI of *Citrobacter youngae* (ZP_06352898), and Ald of *Enterobacter aerogenes* (YP_004593910.1). The putative active sites (*triangles*) of the aldehyde dehydrogenases featuring Asn-137, Glu-234, Gly-265, and Cys-268; and the NAD(P)$^+$ binding sites (*underlined*) (amino acid residues 133–137, 145, 160, 162, 163, 210–213, 216, 219–220, 234–236, 268, 365, 367, 393, 431) are shown

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Kp_YneI   1:MMNLSATHAVSVNPTTGEVVSSLPWASEREVDAAITLAAAGYRQWRQTPLADRADALRRI 60
Ec_YneI   1:MTITPATHAISINPATGEQLSVLPWAGANDIENALQLAAAGFRDWRETNIDYRAEKLRGI 60
Se_YneI   1:MTMMTATQALSVNPATGQTLAAMPWANAQEIEHALSLAASGFKKWKMTSVAQRAQTLRDI 60
Cy_YneI   1:MTITAATHAISVDPTSGERLTAMPWASAEEIAQALSLAASGYSEWKQTTVAHRALTLRHI 60
Ea_Ald    1:MN-LSATHAVSVNPTTGEVVSSLPWSTEQQVDSAIALAQQGFRQWRQVTVADRAAALRKV 59
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Kp_YneI  61:GAALRARGEEVAQMITLEMGKPIAQARGEVAKSANLCDWYAEHGPAMLATEATLVENNQA 120
Ec_YneI  61:GKALRARSEEMAQMITREMGKPINQARAEVAKSANLCDWYAEHGPAMLKAEPTLVENQQA 120
Se_YneI  61:GQALRAHAEEMAQCITREMGKPIKQARAEVTKSAALCDWYAEHGPAMLNPEPTLVENQQA 120
Cy_YneI  61:GQALRNRAEEMAQCISREMGKPIKQARGEVAKSAALCDWYAEHGPGMLNPEPTLVENNQA 120
Ea_Ald   60:GSAMRARGEELAQMISLEMGKPIAQARGEVSKSANLCDWYAEHGPAMLATEATQVEDNKA 119
             * *.*.. **.**.*..****** ***.**.***.**********.**  *.*.**...*

                             ▼
Kp_YneI 121:VIEYRPLGAILAVMPWNFPVWQVMRGAVPILLAGNSYLLKHAPNVMGSARLLGEIFAAAG 180
Ec_YneI 121:VIEYRPLGTILAIMPWNFPLWQVMRGAVPIILAGNGYLLKHAPNVMGCAQLIAQVFKDAG 180
Se_YneI 121:VIEYRPLGVILAIMPWNFPLWQVLRGAVPILLAGNSYLLKHAPNVTGCAQMIARILAEAG 180
Cy_YneI 121:VIEYRPVGTILAIMPWNFPLWQVMRGAIPILLAGNGYLLKHAPNVTGCAAIIAQVFADAD 180
Ea_Ald  120:VIEYRPLGPILAVMPWNFPVWQVLRGAVPILLAGNSYLLKHAPNVMGSAQLLGELFAKAG 179
             ******.* ***.******.***.***.**.****.*********.*.*....  .. *.

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Kp_YneI 181:LPDGVFGWVNATNDGVSQIINDDRIAAVTVTGSVRAGKAIGAQAGAALKKCVLELGGSDP 240
Ec_YneI 181:IPQGVYGWLNADNDGVSQMIKDSRIAAVTVTGSVRAGAAIGVQAGAALKKCVLELGGSDP 240
Se_YneI 181:TPAGVYGWVNANNEGVSQMINDPRIAAVTVTGSVRAGAAIGAQAGAALKKCVLELGGSDP 240
Cy_YneI 181:VPAGVYGWVNADNAGVSQMINDPRIAAVTVTGSVRAGAAIGAQAGAALKKCVLELGGSDP 240
Ea_Ald  180:LPAGVFGWINATNEGVSQIINDDRIAAVTVTGSVRAGRAIGAQAGAALKKCVLELGGSDP 239
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                                     ▼  ▼
Kp_YneI 241:FIVLNDADLDEAVKAAVTGRYQNSGQVCAASKRFILEAGIAEAFTRKFVDAVAALKMGDP 300
Ec_YneI 241:FIVLNDADLELAVKAAVAGRYQNTGQVCAAAKRFIIEEGIASAFTERFVAAAAALKMGDP 300
Se_YneI 241:FIVLNDADLELAVKAAVAGRYQNTGQVCAAAKRFIVEEGIAQAFTDRFVAAAAALKMGDP 300
Cy_YneI 241:FIVLNDADLDLAVKAAVAGRYQNTGQVCAAAKRFIIEEGIAAEFTARFVAAASALKMGDP 300
Ea_Ald  240:FIVLNDADLDEAVKAAAVGRYQNSGQVCAASKRFIVEAGIADAFTKKFVAAVAALKMGDP 299
             *********..*****..*****.******.**** *.***  .** .**.*..*******

Kp_YneI 301:RDEQNYVGPMARFDLRDELHQQVTATLDEGATLLLGAEKIEGAGNYYAPTVLGNVTAGMT 360
Ec_YneI 301:RDEENALGPMARFDLRDELHHQVEKTLAQGARLLLGGEKMAGAGNYYPPTVLANVTPEMT 360
Se_YneI 301:LVEENDLGPMARFDLRDELHQQVQASVAEGARLLLGGEKIAGEGNYYAATVLADVTPDMT 360
Cy_YneI 301:LDEENDLGPMARFDLRDELHQQVEASLAEGARLLLGGEKISGPGNYYAVTVLGGVTPEMT 360
Ea_Ald  300:RDEQNYIGPMARFDLRDELHQQVEATLKEGATLLLGGEKIAGAGNYYAPTVLANVTPEMT 359
             ..*.* .*************.**......**.****.**..*.****..***..**..**

Kp_YneI 361:GFRQELFGPVATLTTARDADHALALANDSEFGLSATVYTTDEAQAQRFARELECGGVFLN 420
Ec_YneI 361:AFREEMFGPVAAITVAKDAEHALELANDSEFGLSATIFTTDETQARQMAARLECGGVFIN 420
Se_YneI 361:AFRQELFGPVAAITVAKDAAHALALANDSEFGLSATIFTADDTLAAEMAARLECGGVFIN 420
Cy_YneI 361:AFRQELFGPVAAITVAKNAEHALALANDSDFGLSATVFTSDETLAQKMASRLECGGVFIN 420
Ea_Ald  360:GFRQELFGPVATISEARDAAHALELANDSEFGLSATVFTSSSAEAERFARGLECGGVFIN 419
             .**.*.*****....*..* ***.*****.******..* ... *   .* .*******.*

Kp_YneI 421:GYCASDARVAFGGVKKSGFGRELSHFGLHEFCNAQTVWKDRR                   462
Ec_YneI 421:GYCASDARVAFGGVKKSGFGRELSHFGLHEFCNIQTVWKDRI                   462
Se_YneI 421:GYSASDARVAFGGVKKSGFGRELSHFGLHEFCNVQTVWKNRV                   462
Cy_YneI 421:GYSASDARVAFGGVKKSGFGRELSHFGLHEFCNVQTVWKNRL                   462
Ea_Ald  420:GYSASDARVAFGGVKKSGFGRELSHFGLHEFCNIQTVWKDRR                   461
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Functional analysis of ALDHs produced in *E. coli*

The specific activities of purified YneI and YdcW were 962 and 644 U mg^{-1} proteins, respectively, when 3-HPA was the substrate and NAD$^+$ the cofactor (Table 1). Substrate specificity was examined using several aldehydes including 3-HPA, PA, BA, VA, IVA, and FA, in the presence of NAD$^+$ and NADP$^+$ as cofactors. YneI was active on 3-HPA specifically and utilized both NAD$^+$ and NADP$^+$ as cofactor, but with a preference for NAD$^+$ (Fig. 4a). YdcW was active on all aldehydes tested with exception of FA. The highest activity was evident when PA served as substrate. YdcW utilized both NAD$^+$ and NADP$^+$ as cofactors but preferred NADP$^+$ (Fig. 4b).

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Kp_YdcW     1:MKQD-NAMQHNLLINGKLVAGEGEKVPVYNPATGEVILEIAEATAAQVDAAVEAADRAFD  59
Ec_YdcW     1:M------Q-HKLLINGELVSGEGEKQPVYNPATGDVLLEIAEASAEQVDAAVRAADAAFA  53
Se_YdcW     1:MTIWENAMQYQLLINGVLVDGEGERQSVYNPATGEVILEIAEASPAQVDAAVLAADSAFA  60
kv_Ald      1:MQ-------HNLLINGKLVAGEGEKVPVYNPATGDVILEIAEATAAQVDAAVEAADRAFD  53
Ea_Ald      1:MQ-------HNLLINGKLVAGEGEVLPVFNPANGEAIIEIAEATEAQVDAAVNAADRAFV  53
              *        ..*****.**.****. .*.***.*....*****...****** ***.**

Kp_YdcW    60:AWSQTTPKTRAECLLKLADAISAQAETLAQLESLNCGKPLHCVINDEMPAIVDVFRFFAG 119
Ec_YdcW    54:EWGQTTPKVRAECLLKLADVIEENGQVFAELESRNCGKPLHSAFNDEIPAIVDVFRFFAG 113
Se_YdcW    61:EWGQTTPKARAECLLKLADSIEQNALEFARLESQNCGKPLHCVINDEIPAIVDVFRFFAG 120
kv_Ald     54:AWSQTTPKTRSECLLKLADAIAAQAETLAQLESLNCGKPLHCVINDEMPAIADVFRFFAG 113
Ea_Ald     54:SWSQTTPKTRAECLLKMADVITEHAETLAKLESLNCGKPLHCVINDEMPAIADVFRFFAG 113
               *.*****.*.*****.** *    ....* ***.*******...***.***.********

                                            ▼
Kp_YdcW   120:AARCLPGMAAGEYLEGHTSMIRRDPVGVVASIAPWNYPLMMAAWKLAPALAAGNCVVIKP 179
Ec_YdcW   114:AARCLNGLAAGEYLEGHTSMIRRDPLGVVASIAPWNYPLMMAAWKLAPALAAGNCVVLKP 173
Se_YdcW   121:AARCLSGLAAGEYLEGHTSMIRRDPIGVVASIAPWNYPLMMAAWKLAPALAAGNCVVIKP 180
kv_Ald    114:AARCLPGIAAGEYLEGHTSMIRRDPVGVVASIAPWNYPLMMAAWKLAPALAAGNCVVIKP 173
Ea_Ald    114:AARCLPGMAAGEYLEGHTSMIRRDPVGVVASIAPWNYPLMMAAWKLAPALAAGNCVVIKP 173
              *****.* ****************.*******************************.**

Kp_YdcW   180:SEITPLTALKLAELAKDIFPEGVINVLFGRGKTVGDPLTAHVKVRMVSLTGSIATGAHII 239
Ec_YdcW   174:SEITPLTALKLAELAKDIFPAGVINVLFGRGKTVGDPLTGHPKVRMVSLTGSIATGEHII 233
Se_YdcW   181:SEITPLTALKLAALAKDIFPPGVLNVLFGRGQTVGDVLTGHEKVRMVSLTGSIATGEHIL 240
kv_Ald    174:SEITPLTALKLAELAKDIFPEGVINVLFGRGKTVGDPLTSHAKVRMVSLTGSIATGAHII 233
Ea_Ald    174:SEITPLTALKLAELAKDIFPAGVINVLFGRGPTVGDPLTGHSKVRMVSLTGSIATGAHII 233
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Kp_YdcW   240:GHTASSIKRTHMELGGKAPVIVFDDADIDAVVDGVRTFGFYNAGQDCTAACRIYAQQGIY 299
Ec_YdcW   234:SHTASSIKRTHMELGGKAPVIVFDDADIEAVVEGVRTFGYYNAGQDCTAACRIYAQKGIY 293
Se_YdcW   241:RHTAPAIKRTHMELGGKAPVIVFDDADLDAVAQGVRTFGFYNAGQDCTAACRIYAQRGIY 300
kv_Ald    234:SHTASSIKRTHMELGGKAPVIVFDDADIDAVVDGVRTFGFYNAGQDCTAACRIYAQQGIY 293
Ea_Ald    234:GHTASSIKRTHMELGGKAPVIVFDDADIDAVVEGIRTFGFYNAGQDCTAACRIYAQQGVY 293
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Kp_YdcW   300:DQLVEKLGAAVASLKMGAPEDAATELGPLSSLAHLERVSAAVEAARALPHIKVVTGGSRA 359
Ec_YdcW   294:DTLVEKLGAAVATLKSGAPDDESTELGPLSSLAHLERVSKAVEEAKATGHIKVITGGEKR 353
Se_YdcW   301:DALVEKLGNAVSSLKMGAPEDKSTELGPLSSLAHLKRVTAAVEEAKALSHIRVITGGSQT 360
kv_Ald    294:DQLVEKLGAAVASLKMGAPEDASTELGPLSSLAHLERVSAAVEAAKVLPHIKVVTGGRRA 353
Ea_Ald    294:DRLVEKLGAAVASLKMGQPDDPSTELGPVSSLAHLERVTAAVNAARALPHIKVVTGGSRA 353
              * ******.**..**.*.*.* .*****.******.**..**..*....**.*.***...

Kp_YdcW   360:DGAGYYFQPTLLAGARQEDAIVQREVFGPVVSVTPFSDEAQALSWANDSQYGLASSVWTK 419
Ec_YdcW   354:KGNGYYYAPTLLAGALQDDAIVQKEVFGPVVSVTPFDNEEQVVNWANDSQYGLASSVWTK 413
Se_YdcW   361:EGKGYYFAPTLLADAKQEDAIVQREVFGPVVSITVFDDEDQVLRWANDSRYGLASSVWTQ 420
kv_Ald    354:DGAGYYFQPTLLAGARQEDAIVQREVFGPVVSVTPFSDEAQALSWANDSQYGLASSVWTK 413
Ea_Ald    354:AGNGYYFQPTLLAGARQQDAIVQREVFGPVVSVTSFSDEEQVLTWANDSQYGLASSVWTQ 413
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Kp_YdcW   420:DVGRAHRLSARLQYGCTWVNTHFMLVSEMPHGGQKLSGYGKDMSMYGLEDYTVVRHVMVK 479
Ec_YdcW   414:DVGRAHRVSARLQYGCTWVNTHFMLVSEMPHGGQKLSGYGKDMSLYGLEDYTVVRHVMVK 473
Se_YdcW   421:DVGRAHRLSARLQYGCTWINTHFMLVSEMPHGGQKQSGYGKDMSLYGLEDYTLVRHIMVK 480
kv_Ald    414:DVGRAHRLSARLQYGCTWVNTHFMLVSEMPHGGQKLSGYGKDMSMYGLEDYTVVRHVMVK 473
Ea_Ald    414:DVGRAHRLSARLQYGCTWVNTHFMLVSEMPHGGQKLSGYGKDMSMYGLEDYTVVRHVMVK 473
              *******.**********.***************.********.*******.***.***

Kp_YdcW 480:HS                                                           481
Ec_YdcW 474:H-                                                           474
Se_YdcW 481:H-                                                           481
kv_Ald  474:HG                                                           475
Ea_Ald  474:H-                                                           474
            *
```

Fig. 2 Alignment, emphasizing homology, of YdcW with aldehyde dehydrogenases YdcW of *Escherichia coli* (YP_001462716), YdcW of *Salmonella enterica* (YP_002114622), Ald of *Enterobacter aerogenes* (YP_004594212), and Ald of *Klebsiella variicola* (YP_003439305). The putative catalytic residues (*triangles*) of Asn-155, Glu-252, Gly-283, and Cys-286; the substrate-binding sites (*circles*) of Asp-285 and Leu-444; and the NAD(P)$^+$ binding sites (*underlined*) (amino acid residues 151, 152, 154, 178, 180, 181, 210, 215, 229–231, 234, 237, 252, 254, 286, 333, and 384) are shown

Increased 3-HP production upon overexpression of ALDHs in *K. pneumoniae* AK

When ALDHs were overexpressed in *K. pneumoniae*, 3-HP production remarkably enhanced, compared to that of the control strain (the basal level was 0.6 g L^{-1}) upon shake-flask culture over 48 h. The concentrations of 3-HP yielded by the recombinant strains were 2.4 g L^{-1} when YneI was expressed and 1.8 g L^{-1} when YdcW was synthesized (Table 2). Batch fermentation was conducted in a 5-L

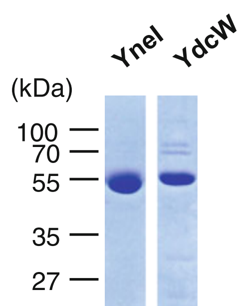

Fig. 3 SDS-PAGE analysis of purified YneI and YdcW from cell lysates of *E. coli* harboring pET-*yneI* and *E. coli* harboring pET-*ydcW*, respectively

bioreactor. The recombinant strain harboring pVOTYI consumed all added glycerol (20 g L^{-1}) by 20 h of culture and the maximal 3-HP production was 3.9 g L^{-1} at 30 h (Fig. 5b). The recombinant strain harboring pVOTYW consumed glycerol at a slower rate than did the control (measured at 24 h; Fig. 5a) and the maximal 3-HP production was 2.2 g L^{-1} at 30 h (Fig. 5c). *K. pneumoniae* first dehydrates glycerol via the action of a coenzyme B_{12}-dependent glycerol dehydratase (DhaB) to yield 3-HPA, which is next oxidized to 3-HP by NAD^{+}-dependent ALDH. As YdcW prefers NADP+ as cofactor, the enzyme may not efficiently catalyze the synthesis of 3-HP from 3-HPA.

Probable roles of ALDHs on glycerol metabolism in *K. pneumoniae*

It has been suggested that 3-HP is synthesized from glycerol, in *K. pneumoniae,* via two distinct routes. In the CoA-dependent pathway, 3-HPA is converted to 3-HP in a series of reactions involving catalysis by PduP and in the CoA-independent pathway, 3-HPA is directly converted into 3-HP by an ALDH. Various ALDHs have been screened with a view toward improving 3-HP production. However, this CoA-independent pathway featuring direct oxidation of 3-HPA to 3-HP has not yet been well-characterized in native organisms. Deletion of *aldHk* from *K. pneumoniae* did not affect the extent of 3-HP synthesis from glycerol [6].

To examine the role played by YneI and YdcW in 3-HP production, *yneI* and *ydcW* were precisely deleted from *K. pneumoniae* using a homologous recombination method

Table 1 Enzyme activities of YneI and YdcW prepared from *E. coli*

Strain	Total activity (μmol NADH mL^{-1} min^{-1})	Protein (mg mL^{-1})	Activity (U mg^{-1} protein)
E. coli/ pET28a	0.00	0.04	ND
E. coli/ pET-*yneI*	0.72	0.04	19.54
E. coli/ pET-*ydcW*	1.53	0.04	41.46
Purified YneI	41.87	0.04	962.41
Purified YdcW	28.02	0.04	644.05

ND not detected

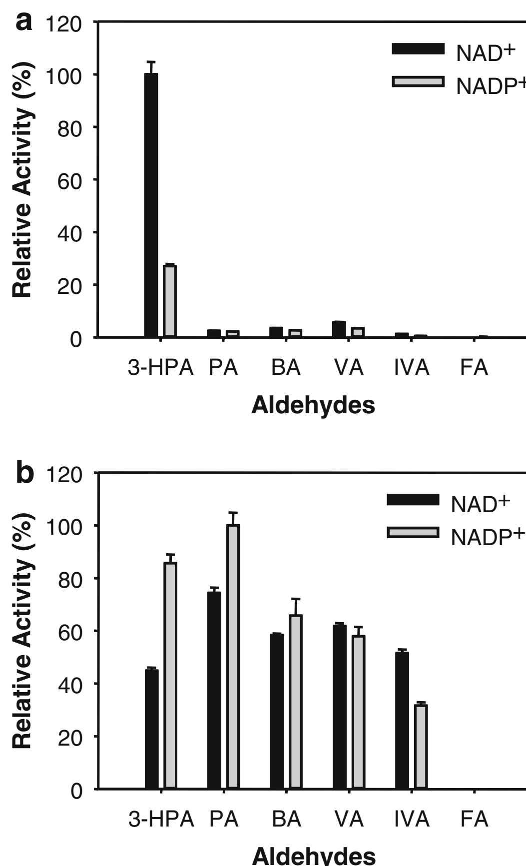

Fig. 4 Substrate and cofactor specificity of YneI (**a**) and YdcW (**b**). 3HPA, 3-hydroxypropionaldehyde; PA, propionaldehyde; BA, butyraldehyde; VA, valeraldehyde; IVA, isovaleraldehyde; FA, furaldehyde. Data are expressed as mean ± SD. Three independent experiments were made for each end-point. Statistical differences were determined by the student's *t* test. There were no statistically significant differences

Table 2 Metabolites of recombinant *K. pneumoniae* strains grown under shake-flask conditions

Metabolite (g L^{-1})	Recombinant *K. pneumoniae* strain		
	Expressing pVOT	Expressing pVOTYI	Expressing pVOTYW
Residual glycerol	0	0	1.7
1,3-PD	9.3	8.8	6.5
3-HP	0.6	2.4	1.8

Initial glycerol level: 20 g L^{-1}

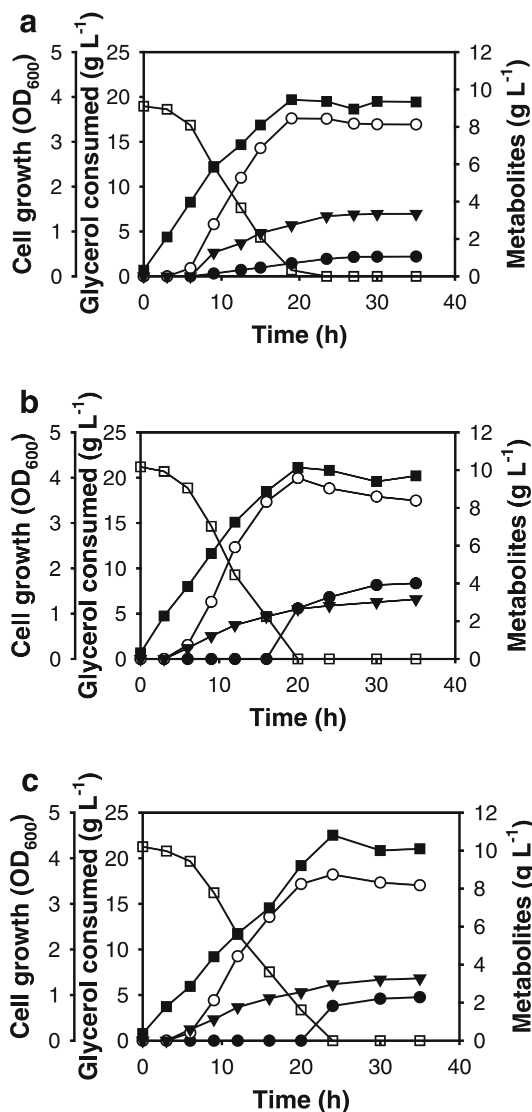

Fig. 5 Metabolite analysis of recombinant *K. pneumoniae* harboring pVOT (**a**), pVOTYI (**b**) or pVOTYW (**c**). Glycerol consumed, *open squares*; 3-HP, *closed circles*; 1,3-PD, *open circles*; acetic acid, *closed triangles*; OD_{600}, *closed squares*

[11]. Southern blotting of the wild-type and mutant strain probes specific for the genes of interest confirm that deletions had occurred and the mutants were stable. The wild-type and deletion mutant strains were subjected to batch fermentation in a 5-L bioreactor supplemented with glycerol as carbon source. All strains attained maximum optical density levels (at A_{600} nm) above 5.4 and consumed almost all added glycerol (20 g L^{-1}) by 9 h of culture. The maximal 3-HP productions noted were approximately 2 g L^{-1}. No significant difference was evident among wild-type and mutant strains (data not shown).

In previous reports, we investigated a CoA-dependent pathway of *K. pneumoniae* in which 3-HPA is metabolized via the action of PduP (a CoA-dependent ALDH) to yield 3-HP. As 3-HP was still produced by a Δ*pduP* mutant, we speculated that a CoA-independent pathway featuring an additional ALDH might participate in 3-HP synthesis from glycerol [4]. Aldehydes vary in length and in characteristics of the alkyl chains but all are toxic because of high chemical reactivity [12]. ALDHs play major roles in detoxifying aldehydes that are generated both endogenously and exogenously. Some ALDHs oxidize only a few aldehydes; others exhibit broad substrate specificities [13]. Therefore, *K. pneumoniae* may produce 3-HP from glycerol via a pathway featuring the activity of a non-specific ALDH (or several such enzymes).

Some reports on the non-specific ALDHs increasing 3-HP production by overexpression have appeared. Available ALDHs include ALD4 of *Saccharomyces cerevisiae*; ALDH2 of *Homo sapiens*; AldA, AldB, AldH, and YdcW of *Escherichia coli*; KGSADH of *Azospirillum brasiliense*; AldA, AldB, PuuC, AldH, YdcW, EtuE, FeaB, GabD, BadH, and PduP of *K. pneumoniae*; AdhB of *Zymomonas mobilis*; and AldH and PduQ of *Lactobacillus collinoides* [14–18].

Conclusion

In the present study, we identified and characterized two putative ALDHs involved in 3HP production from glycerol in *K. pneumoniae*. Overexpression of the ALDHs obviously increased 3-HP production in *K. pneumoniae*. However, deletion of the gene from *K. pneumoniae* did not affect the extent of 3-HP synthesis from glycerol, indicating non-specific activity of ALDHs on 3-HPA. The ALDHs probably play major roles in detoxifying the aldehyde generated in glycerol metabolism. In addition, overexpression of genes would contribute to efficient production of 3-HP from glycerol.

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