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ORIGINAL RESEARCH PAPER

Enhanced aldehyde dehydrogenase activity by regenerating NAD^+ in *Klebsiella pneumoniae* and implications for the glycerol dissimilation pathways

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Abstract In *Klebsiella pneumoniae*, 3-hydroxypropaldehyde is converted to 3-hydroxypropionic acid (3-HP) by aldehyde dehydrogenase (ALDH) with NAD^+ as a cofactor. Although ALDH overexpression stimulates the formation of 3-HP, it ceases to accumulate when NAD^+ is exhausted. Here we show that NAD^+ regeneration, together with ALDH overexpression, facilitates 3-HP production and benefits cell growth. Three distinct NAD^+-regenerating enzymes: NADH oxidase and NADH dehydrogenase from *K. pneumoniae*, and glycerol-3-phosphate dehydrogenase (GPD1) from *Saccharomyces cerevisiae*, were individually expressed in *K. pneumoniae*. In vitro assay showed their higher activities than that of the control, indicating their capacities to regenerate NAD^+. When they were respectively co-expressed with ALD4, an ALDH from *S. cerevisiae*, the activities of ALD4 were significantly elevated compared with that expressing ALD4 alone, suggesting that the regenerated NAD^+ enhanced the activity of ALD4. More interestingly, the growth rates of all NAD^+-regenerating strains were prolonged in comparison with the control, indicating that NAD^+ regeneration stimulated cell proliferation. This study not only reveals the reliance of ALD4 activity on NAD^+ availability but also provides a method for regulating the *dha* regulon.

Keywords Aldehyde dehydrogenase · 3-Hydroxypropionic acid · *Klebsiella pneumoniae* · NAD^+ regeneration

Introduction

3-Hydroxypropionic acid (3-HP) is one of the US Department of Energy's top 12 value-added platform compounds among renewable biomass products (Werpy and Petersen 2004). As a versatile precursor, 3-HP can be readily converted into a series of C_3 compounds, including 1,3-propanediol, acrylic acid, 3-hydroxypropaldehyde, and malonic acid. *Klebsiella pneumoniae*, as a natural 3-HP producer, has attracted much attention due to its capacity to metabolize glycerol and having a similar genetic background to *Escherichia coli*, together with rapid cell proliferation (Ashok et al. 2012; Huang et al. 2012). In *K. pneumoniae*, glycerol dissimilation is split into

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oxidation and reduction pathways. In the oxidation pathway, ATP is generated to sustain cell growth. In the reduction pathway, glycerol is converted to 3-hydroxypropaldehyde (3-HPA) by a B_{12}-dependent glycerol dehydratase (GDHt), 3-HPA is subsequently converted to 1,3-propanediol by 1,3-propanediol oxidoreductase or to 3-HP by aldehyde dehydrogenase (AldH) (Forage and Foster 1982; Suthers and Cameron 2001; Zheng et al. 2004). As a promising 3-HP producer, *K. pneumoniae* can synthesize vitamin B_{12} and thus has an advantage over *E. coli* that needs the addition of vitamin B_{12} (Ashok et al. 2011, 2012).

Biological production of 3-HP has mainly focused on the determination of efficient ALDHs and optimization of the fermentation process (Raj et al. 2009; Luo et al. 2011). Since ALDH activity is lower than GDHt, plasmid-dependent overexpression of ALDH is a common strategy for diverting glycerol flux to 3-HP. One obvious drawback of this strategy is the massive NAD^+ consumption by overexpression of ALDH that leads to NAD^+ exhaustion and the cessation of 3-HP formation. Given the topological rigidity of *dha* regulon in *K. pneumoniae*, NAD^+ cannot be recompensed simply by the optimization of fermentation process. Hence, engineering NAD^+ regeneration systems in *K. pneumoniae* seems to be an imperative choice to address this issue.

In this study, three distinct NAD^+ regeneration enzymes: NADH oxidase and NADH dehydrogenase from *K. pneumoniae*, and glycerol-3-phosphate dehydrogenase (GPD1) from *Saccharomyces cerevisiae*, were individually employed for regenerating NAD^+ in *K. pneumoniae*. Detailed analysis of cell concentration, glycerol consumption, enzymatic activity and formation of 3-HP and byproducts could comprehensively assess the efficiency of engineered NAD^+ regeneration systems. More importantly, this study aimed to unravel the potential limitations on 3-HP production and provide a better understanding of the *dha* regulon, a typical pathway fork mediating carbon flux allocation, cofactor balance and cell growth.

Materials and methods

Plasmids, strains, medium and reagents

The vector pET-28a (Novagen) was used in this study with minor modifications. The original T7 promoter was replaced by a native promoter *pk* of *dhaB1* gene, the first subunit of *dhaB* gene cluster (GenBank U30903) in *K. pneumoniae* DSM 2026. The resulting vector was designated as pET-pk. *Escherichia coli* DH5α, *S. cerevisiae* and *K. pneumoniae* DSM 2026 strains were from DSMZ GmbH, Germany. *E. coli* DH5α was grown in LB medium. *S. cerevisiae* was grown in yeast extract/peptone/dextrose medium (g/L): yeast extract, 10; peptone, 20; glucose, 20. The medium for generating 3-HP by recombinant *K. pneumoniae* contained (per liter): $K_2HPO_4{\cdot}3H_2O$, 3.4 g; KH_2PO_4, 1.3 g; $(NH_4)_2SO_4$, 4 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; $CaCO_3$, 0.1 g; yeast extract, 3 g; glycerol, 20 g; and 1.25 mL trace element solution. The trace element solution contained (per liter): $ZnCl_2{\cdot}6H_2O$, 2.72 g; $FeSO_4$, 32 g; $MnCl_2{\cdot}4H_2O$, 0.68 g; $CoCl_2{\cdot}6H_2O$, 1.88 g; H_3BO_3, 0.24 g; Na_2MoO_4, 0.02 g; $CuCl_2{\cdot}2H_2O$, 1.88 g; and 40 mL conc. HCl. The recombinant was microaerobically grown in 50 mL Erlenmeyer flask containing 25 mL medium with 50 μg kanamycin/mL at 37 °C and 120 rpm continuous shaking. The microaerobic condition was achieved using a foam stopper. Taq DNA polymerase and restriction enzymes were purchased from TaKaRa (Dalian, China). 3-HP was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Other standard chemicals were products of Sigma. DNA synthesis and sequencing were performed by Beijing Sunbiotech Co. Ltd., China.

Construction of the recombinants

The genes encoding NADH dehydrogenase and NADH oxidase were cloned by PCR from the genomic DNA of *K. pneumoniae*. The GPD1 coding gene, *gpd1*, (Hubmann et al. 2011) and aldehyde dehydrogenase coding gene, *ald4,* were cloned by PCR from the genomic DNA of *S. cerevisiae*. The native *pk* promoter of the gene *dhaB* was used to drive gene expression. The gene *ald4* and NAD^+-regenerating enzyme genes share the T7 terminator.

Primers and restriction enzymes are shown in Supplementary Table 1. Schematic diagram of vector construction is shown in Fig. 1. PCR used: 94 °C for 4 min; 94 °C for 1 min, 55 °C for 45 s, 72 °C for X min, 30 cycles; 72 °C for 10 min, 16 °C hold where X depends on the length of amplified gene. All other molecular manipulations followed standard protocols. The recombinant plasmids were transformed into

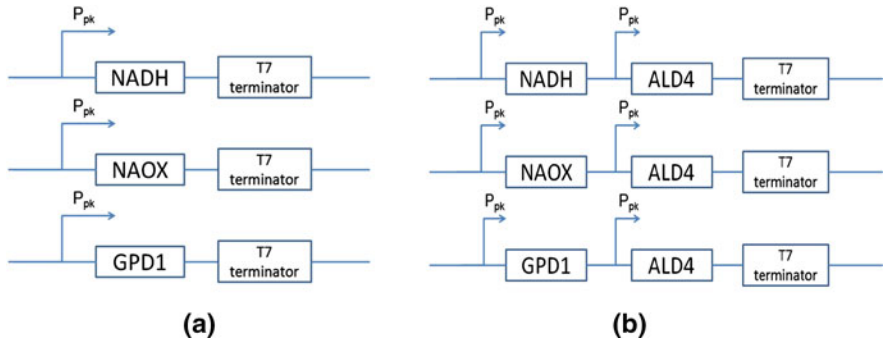

Fig. 1 Construction of recombinant vectors expressing either NAD^+ regeneration enzyme alone or in combination with aldehyde dehydrogenase (ALD4) using pET-pk vector. **a** Vectors expressing NAD^+-regenerating enzymes (NADH, NAOX, and GPD1) alone were to generate NAD^+ and test their activities and serve as controls. **b** Co-expression of two enzyme genes was to regenerate NAD^+ and produce 3-hydroxypropionic acid. NADH and NAOX respectively, refer to NADH dehydrogenase and NADH oxidase from *K. pneumoniae*; GPD1 stands for glycerol-3-phosphate dehydrogenase from *S. cerevisiae*; ALD4 indicates aldehyde dehydrogenase from *S. cerevisiae*. pk is the native promoter of *dhaB* gene in *K. pneumoniae*

K. pneumoniae, and the positive recombinants were screened by LB kanamycin plate and further identified by sequencing.

Enzymatic activity assay

Recombinant strains were grown in fermentation medium with kanamycin added as appropriate. After 9 h cultures were centrifuged at 10,000×*g* for 10 min and cells washed with 5 mL PBS (pH 7.0). 50 μL PMSF (10 mg/mL) was added to inhibit protease activity. Cells were disrupted ultrasonically (800 W, working 3 s, pause 2 s, 50 times). Total protein was measured using the Bradford method. For NADH oxidation, 100 μL cell-free extract was added to 0.2 mM NADH and incubated at 37 °C for 5 min. The amount of consumed NADH was determined by measuring the decrease of absorbance at 340 nm. The unit of enzyme activity was defined as the amount of protein in total cell-free extract to produce 1 μmol NAD^+ per min. The experiment was performed in triplicate. ALD4 activity was measured similarly except 2 mM NAD and 2 mM propionaldehyde were added into the reaction mixture. The unit of ALD4 activity was defined as the amount of protein to produce 1 μmol NADH per min.

Flask cultivation

The recombinants were grown in LB medium containing (per liter): yeast extract 5 g, NaCl 10 g, peptone 10 g, and kanamycin 50 mg. 1 % of an overnight culture was inoculated in to the medium containing the same concentration of antibiotic. Microaerobic condition was achieved by using a 250 mL Erlenmeyer flask containing 100 mL medium and shaking at 150 rpm at 37 °C.

Analytical methods

Cell concentrations were measured by using microplate reader at 600 nm with 200 μL fermentation medium added to the well. The metabolites, 3-HP, lactic acid and acetic acid, were determined by HPLC equipped with a C18 column and a UV detector. The mobile phase was 95 % H_2O/5 % methanol/0.05 % phosphoric acid at 0.8 ml/min. All samples were filtered through 0.22 μm membrane filter. The residual glycerol concentration was monitored every 3 h by a titration method with $NaIO_4$ (for control of glycerol).

Results

Characterization of the recombinant strains

All recombinants were characterized by restriction digestion and further confirmed by sequencing. All cloned genes showed >98 % homology with the reported sequences in the GeneBank, suggesting that the desired genes had been correctly obtained. As shown in Fig. 1, the NAD^+-regenerating enzyme gene

was inserted upstream of *ald4* gene and both were under control of *pk* promoter. For the expression of the NAD^+-regenerating enzyme in *K. pneumoniae*, considering that NADH dehydrogenase (KPN_01106) and NADH oxidase (KPN_01757) are native enzymes from the host *K. pneumoniae*, they would be prone to matching the transcriptional machinery. In contrast, GPD1 may conflict with the transcriptional machinery because it is a heterologous enzyme from *S. cerevisiae* (Hubmann et al. 2011).

Assay of enzymatic activities

To screen enzymes for regenerating NAD^+, three candidate enzymes, NADH oxidase, NADH dehydrogenase and GPD1, were individually overexpressed in *K. pneumoniae* and their activities were analyzed. In vitro assay of crude extract showed that these three enzymatic activities were 58, 45 and 36 U/mg, respectively, which were all higher than that of the control (32 U/mg), indicating that the enzyme have the capability to regenerate NAD^+ in *K. pneumoniae*.

To investigate the effect of NAD^+ on ALD4 activity the three NAD^+-regenerating enzymes were each co-expressed with ALD4 and the resulting ALD4 activity was analyzed. ALD4 activities were 34, 55.5, and 33.5 U/mg when ALD4 was co-expressed with NADH oxidase, NADH dehydrogenase and GPD1, respectively. In contrast, the ALD4 activity in the control strain (Ald4 expressed alone) was 25 U/mg, suggesting that NAD^+ regeneration enhanced the activity of ALD4.

Effect of NAD^+ regeneration on glycerol consumption and cell growth

As shown in Fig. 2, the engineered NAD^+ regeneration systems affected glycerol metabolism and cell proliferation. For glycerol consumption, the recombinant strains that co-expressed *ald4* and one of three NAD^+-regenerating enzyme genes consumed less glycerol during 24 h fermentation than the control which only expressed *ald4* gene (50-2-1), especially for the strains OXLD (co-expressed NADH oxidase and ALD4) and GLD (co-expressed GPD1 and ALD4), whose growth rates were significantly prolonged and the stationary phases were delayed. However, the recombinant strain NALD4 that co-expressed NADH dehydrogenase and ALD4s consumed more glycerol than OXLD and GLD, and its growth rate was even shorter than that of the control strain 50-2-1(9 h). The possible explanation for this phenomenon may be from the reaction catalyzed by NADH dehydrogenase, which is closely related to respiration pathways (Gyan et al. 2006), and thus more glycerol could be consumed to sustain cell oxidation.

For cell growth, the control strain 50-2-1 gave the highest biomass over 18 h cultivation. However, cells departed from the main growth phase much earlier than the NAD^+-regenerating strains. This may be attributed to NAD^+ exhaustion caused by overexpression of *ald4*. Although strain NALD4 also displayed a short growth phase, the fundamental reason may be different from that of strain 50-2-1. Instead of NAD^+ exhaustion, it is ascribed to active oxidation caused by overexpression of NADH dehydrogenase. Unlike the above two strains, strains OXLD and GLD grew poorly in the first 0–18 h, however, they showed nearly equal cell concentrations to 50-2-1 after 18 h growth, implying that NAD^+ regeneration facilitated cell growth. In fact, many enzymes in glycolytic pathway utilize NAD^+ as cofactor. Hence, the regenerated NAD^+ not only enhances the activity of Ald4 but also accelerates glycolysis and thus stimulates cell proliferation.

3-HP and byproducts in NAD^+-regenerating recombinants

To evaluate the effectiveness of different NAD^+-regenerating enzymes, the strain expressing only ALD4 was chosen as the control (strain 50-2-1). As shown in Fig. 3, the control strain presented a fluctuant concentration of 3-HP during 24 h fermentation. Two 3-HP peaks occurred at 9 and 18 h. In the first 9 h, cells grew actively and 3-HP accumulated rapidly, indicating a close relationship between 3-HP formation and cell growth. From 9 to 15 h, however, 3-HP decreased rapidly due to the depletion of NAD^+ caused by overexpression of Ald4. Interestingly, from 15 to 18 h, 3-HP concentration increased which is attributed to NAD^+ compensation from glycerol oxidation pathway.

Compared to the control, NAD^+-regenerating strains, OXLD and NALD4, showed continuous accumulation of 3-HP instead of a fluctuation. We speculate that NAD^+ regeneration together with ALD4 overexpression mitigated the shortage of

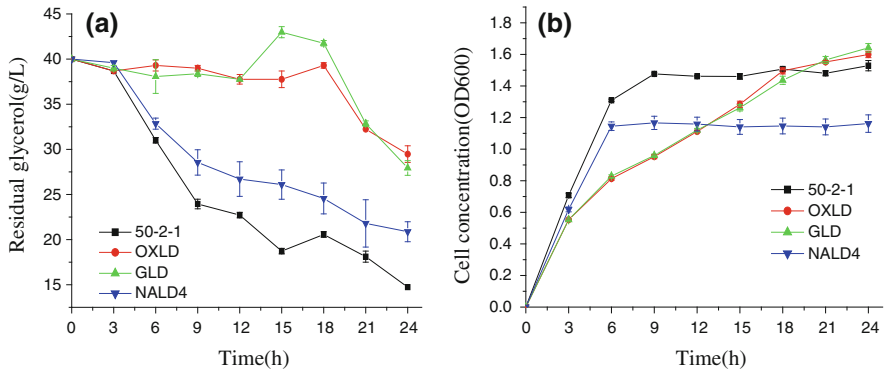

Fig. 2 Glycerol consumption and cell growth of recombinant *Klebsiella pneumoniae*. **a** Residual glycerol levels in different recombinants detected at several time points; 50-2-1, the recombinant *K. pneumoniae* harboring *ald4* in vector pET-pk; OXLD, the recombinant *K. pneumoniae* harboring NADH oxidase gene and *ald4*; GLD, the recombinant *K. pneumoniae* harboring *gpd1* and *ald4* gene; NALD4, the recombinant *K. pneumoniae* harboring NADH dehydrogenase gene and *ald4*. **b** Time *curve* of cell density in the same recombinants as described in A at corresponding time *points*. *Error bars* represent standard deviation

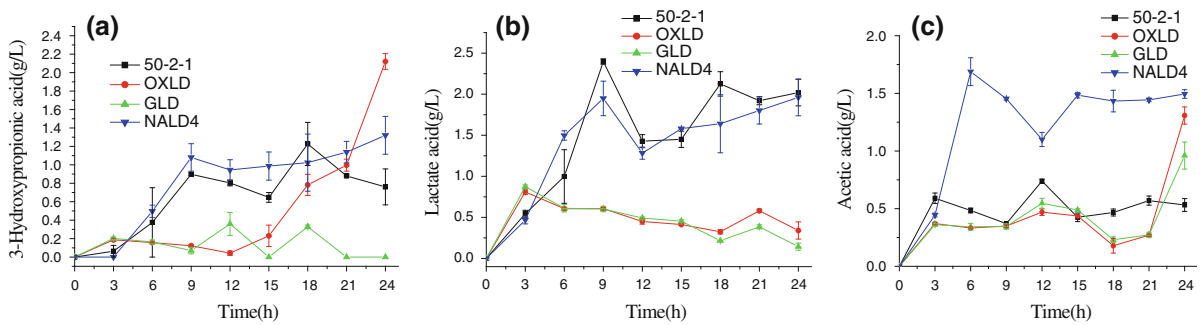

Fig. 3 Metabolites produced by recombinant *Klebsiella pneumoniae* expressing vectors as described in Fig. 1 **a** 3-hydroxypropionic acid; **b** lactic acid; **c** acetic acid. 50-2-1, the recombinant *K. pneumoniae* harboring *ald4* in vector pET-pk; OXLD, the recombinant *K. pneumoniae* harboring NADH oxidase gene and *ald4*; GLD, the recombinant *K. pneumoniae* harboring glycerol 3-phosphate dehydrogenase gene and *ald4*; NALD4, the recombinant *K. pneumoniae* harboring NADH dehydrogenase gene and *ald4*. The *error bars* represent standard deviation from three independent experiments

NAD^+ and thus facilitated 3-HP formation. For strain OXLD, 3-HP accumulation was very low in the first 12 h but after 15 h, it increased and reached to 2.12 g/L at 24 h. Surprisingly, 3-HP accumulation in strain GLD, that co-expressed GPD1 and ALD4, was even lower than that of the control. One possible reason for this may be the heterologous expression of *gpd1* gene from *S. cerevisiae*. The underlying mechanism needs to be further studied.

Lactic acid and acetic acid are two major byproducts in 3-HP biosynthesis. They are the products of lactate dehydrogenase and phosphotransacetylase, respectively. Since both enzymes use NADH as cofactor, engineering a NAD^+-regenerating system in *K. pneumoniae* would lead to a competition for NADH between the heterologous NAD^+-regenerating enzymes and lactate dehydrogenase and phosphotransacetylase. Therefore, the amounts of lactic acid and acetic acid in the NAD^+ regeneration strains would be decreased compared to the control. As expected, NAD^+-regenerating strains produced less byproduct than the control strain 50-2-1 (see Fig. 3). This is because overexpression of Ald4 consumed NAD^+, which was generated via the formation of lactic acid and acetic acid. Compared with the control strain 50-2-1, NAD^+-regenerating strains, OXLD and GLD, produced less lactic acid and acetic acid. Overexpression of NADH oxidase or GPD1 mitigated the requirement for lactic acid and acetic acid. Lactic acid decreased more than acetic acid, mainly because

there is only one-step from lactic acid to pyruvate. In contrast, metabolic flux from pyruvate to acetic acid involves three reactions. One exceptional strain is NALD4, as discussed above, plasmid-dependent overexpression of NADH dehydrogenase accelerated the oxidation reaction and caused byproducts to accumulate under microanaerobic conditions. Given the rigidity of *dha* regulon, 3-HP biosynthesis is intrinsically dependent on several variants including enzyme expression, NADH/NAD^+ ratio and cell proliferation. Only intensification of glycerol reductive pathway cannot significantly divert carbon flux to 3-HP.

Discussion

To our knowledge, this is the first systematic exploration of NAD^+ regeneration together with ALDH overexpression for producing 3-HP in *K. pneumoniae*. This study provides an insight into glycerol metabolism in *K. pneumoniae*. Collectively, NAD^+ regeneration enhanced 3-HP production by increasing ALD4 activity and reallocating carbon flux towards oxidation and reduction pathways, and thus provided a method for regulating glycerol dissimilation pathways, a typical metabolic fork mediated by *dha* regulon (Forage and Foster 1982). Under anaerobic or microaerobic conditions, *K. pneumoniae* can survive using glycerol as sole carbon and energy source (Forage and Lin 1982). Indeed, glycerol metabolism in *K. pneumoniae* is mediated by a powerful *dha* regulon that governs parallel oxidation and reduction pathways. This strict metabolic fork tightly controls gene expression, cofactor balance, glycerol allocation and even cell proliferation. This fine regulation is evidenced by the previous metabolic engineering of *K. pneumoniae*. For example, both overexpressing the genes in glycerol reduction pathway and deleting the genes in oxidation pathway failed to significantly increase the carbon flux towards reduction pathway, clearly indicating the structural rigidity of *dha* regulon (Zhao et al. 2009; Seo et al. 2009). In contrast, cofactor regeneration powerfully impinges upon this regulon because the NAD^+/NADP ratio dynamically regulates the expression of enzymes related to glycerol oxidation and reduction pathways. Any oscillations of the NAD^+/NADP ratio may elicit a global response. As reported here, the regeneration of NAD^+ interrupted the original balance and reprogrammed cell metabolism including enzyme activity, glycerol consumption and cell growth. Hence, management of cofactor as a promising means can be applied to future metabolic engineering.

Overall, this study not only reveals the reliance of ALDH activity on NAD^+ availability but also provides new insight into the rigidity of *dha* regulon. More importantly, this study suggests a novel strategy for accumulating 3-HP: measurement of cofactor for coordinated regulation of glycerol dissimilation pathways rather than only intensification of reduction pathway or attenuation of oxidation pathway. Conceivably, cofactor regeneration could be a general strategy to allocate metabolic flux towards adjacent pathways.

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