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Process Biochemistry 41 (2006) 2160–2169

Process Biochemistry

www.elsevier.com/locate/procbio

Overexpression of genes of the *dha* regulon and its effects on cell growth, glycerol fermentation to 1,3-propanediol and plasmid stability in *Klebsiella pneumoniae*

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Received 15 March 2006; received in revised form 22 May 2006; accepted 15 June 2006

Abstract

Glycerol dehydratase that catalyses the conversion of glycerol to 3-hydroxypropionaldehyde was previously supposed to be a limiting step in the 1,3-propanediol production from glycerol. In this study, glycerol dehydratase was over-expressed separately or coordinately with 1,3-propanediol oxidoreductase in *Klebsiella pneumoniae* DSM2026 to investigate its effects on the glycerol fermentation. The overexpression of glycerol dehydratase surprisingly led to a serious impairment of growth of *K. pneumoniae* in both continuous and batch cultures. Instability of the plasmids bearing the genes encoding glycerol dehydratase and/or 1,3-propanediol oxidoreductase was observed in recombinant cells, especially in anaerobic cultures using glycerol as sole carbon source. It is postulated that an imbalanced conversion of glycerol to the intermediate 3-hydroxypropionaldehyde and its further conversion to 1,3-propanediol and the associated accumulation and toxicity of 3-hydroxypropionaldehyde are responsible for the phenomena observed. Furthermore, the putative regulatory gene *dhaR* of the *dha* regulon was also overexpressed in *K. pneumoniae*. The increased expression of 1,3-propanediol oxidoreductase confirmed the role of DhaR as a positive regulator of the *dhaT* gene.

Keywords: Glycerol metabolism; Glycerol dehydratase; 1,3-Propanediol oxidoreductase; 3-Hydroxypropionaldehyde; *DhaR*; *Klebsiella pneumoniae*

1. Introduction

Anaerobic glycerol metabolism is of fundamental importance for the microbial production of 1,3-propanediol (1,3-PD). Genes corresponding to anaerobic glycerol metabolism are typically organized as a gene cluster called *dha* regulon. It has been identified in natural 1,3-PD producers such as *Klebsiella pneumoniae* [1,2], *Citrobacter freundii* [3], C*lostridium pasteurianum* [4,5] and *Clostridium butyricum* [6]. Due to the industrial interest on using 1,3-PD as a monomer for a novel polymer polytrimethylene terephthalate (PTT), a glucose-utilizing strategy by metabolically engineered *E. coli* strain expressing a glycerol 3-phosphate dehydrogenase (DAR1) and glycerol 3-phosphate phosphatase (GPP2) genes from *Saccharomyces cerevisiae* and the *dhaB* genes of *K. pneumoniae* was developed by Genencor and Dupont [7]. In Europe, the glycerol-utilizing process is of particular interest due to the abundance of glycerol as a by-product in the biodiesel production from rapeseed oil.

In the model organism *K. pneumoniae* for glycerol metabolism it primarily involves two branch pathways: the reductive branch and the oxidative branch. 1,3-PD is produced by the reductive branch in two successive enzymatic reactions. Glycerol dehydratase (GDHt), encoded by three genes *dhaB*-alpha, *dhaB*-beta and *dhaB*-gamma, catalyzes the first reaction from glycerol to 3-hydroxypropionaldehyde (3-HPA) [8,9]. The latter is then reduced to 1,3-PD by the enzyme 1,3-propanediol oxidoreductase (PDOR) encoded by the gene *dhaT* under the consumption of reducing power $NADH_2$ [1,10]. The initial steps of the oxidative pathways are catalyzed by the enzymes glycerol dehydrogenase and dihydroxyacetone kinase. The enzymes GDHt and PDOR obtained from *K. pneumoniae* have been overexpressed in *E. coli* [2,11] and 6.3 g/L of 1,3-PD was produced in a fed-batch fermentation

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1359-5113/$ – see front matter
doi:10.1016/j.procbio.2006.06.012

using glucose and glycerol as co-substrates. Kinetic and pathway analysis suggested that GDHt is a major rate-limiting enzyme for the consumption of glycerol and for the formation of 1,3-PD in *K. pneumoniae*, especially at high glycerol concentrations [12]. It has also been shown that GDHt activity is a limiting step for 1,3-PD production in *C. butyricum* [13]. Therefore, increasing the dehydratase activity in the native producer *K. pneumoniae* could probably increase the productivity of 1,3-PD fermentation.

DhaR, encoded by the gene *dhaR* in the *dha* operon, shows a high similarity to the AAA^+ (ATPases associated with various cellular activities) superfamily of enhancer binding proteins, consisting of a N-terminal sensing domain, a central structurally conserved ATPase domain and a C-terminal helix-turn-helix motif. It was suggested to be a transcriptional activator due to its role on the up-regulation of glycerol dehydrogenase in *C. freundii* [3]. Its close homolog in *E. coli* has been recently shown to stimulate the transcription of the *dhaKLM* operon (corresponding to the *dhaK* operon in *K. pneumoniae* [1]) from a sigma 70 promoter and its transcription is negatively auto-regulated [14]. Genetic and biochemical studies indicate that the enzyme subunits DhaL and DhaK act antagonistically as coactivator and corepressor of the transcription activator by mutually exclusive binding to the sensing domain of DhaR [14]. It is interesting to investigate its putative role on the expression of enzymes of the reductive pathway of 1,3-PD production.

In this study, the effects of overexpression of *dhaB* (solely or together with *dhaT*) and *dhaR* on the cell growth and glycerol fermentation in *K. pneumoniae* DSM2026 were studied. The stability of plasmids in the different recombinants and under different fermentation conditions was also examined. The results lead to a better understanding of many experimentally observed phenomena of the glycerol fermentation and have important implications for developing better strategies for the metabolic engineering of 1,3-propanediol production.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

Bacterial strains and plasmids used in this study are given in Table 1. LB medium was used as a rich medium for the routine growth of *K. pneumoniae* strains. The composition of the continuous culture and batch culture media for *K. pneumoniae* was described previously [15,16]. The initial glycerol concentration for the continuous culture was 30 g/L and the concentration in the feed medium was 50–175 g/L. Around 100 g/L glycerol was used in the batch cultivation. For the cultivation of *K. pneumoniae* 150 μg/mL carbenicillin (Cb) plus 100 μg/mL ampicillin (Ap) were used in flask culture while 200 μg/mL Cb plus 133 μg/mL Ap were used in the continuous or batch cultures.

Shake flask cultivation were used for seed culture, initial investigation of the overexpression of GDHt and PDOR by different plasmids and the study of plasmid stability in variant conditions. If not specified, anaerobic flask cultivation was performed in septum bottles at 37 °C using medium containing 20 g/L glycerol described elsewhere [17].

For continuous culture and batch culture a 4 L Setric Bioreactor (Set 4V, Setric Genie Industrial, Toulouse, France) with a working volume of 2 and 3 L was used, respectively. The reactor was connected to a real-time computer control system (UBICON, ESD, Hannover, Germany) for on-line data acquisition. All cultivations were carried out at 37 °C, pH 7.0 and 300 rpm. To ensure anaerobic condition, the bioreactor was sparged with nitrogen at a flow rate of 0.4 vv m. The cultivation strategy was described previously by Menzel et al. [16].

2.2. DNA manipulations

General DNA manipulations were performed as described by Sambrook and Russel [18]. Plasmids were isolated with the plasmid purification kit Jetstar (Genomed, Bad Oeynhausen, Germany). Genomic DNA from *K. pneumoniae* was extracted with a DNA Kit (Qiagen, Hilden, Germany). DNA fragments were recovered from gels with the Jetsorb kit (Genomed, Bad Oeynhausen, Germany). Electroporation of *K. pneumoniae* was done with the gene pulser II (BioRad, Germany) at 2.5 kV, 50 μF, and 150 Ω by using a 2 mm cuvette. T4 DNA-polymerase and restriction enzymes (Promega, Madison, USA) were used as recommended by the suppliers. DNA sequencing was performed by using the ABI PRISM Dye Terminator (Perkin-Elmer, Weiterstadt, Germany).

2.3. Construction of plasmids pKM13, pKM15 and pKM16

To construct the plasmid pKM13, the plasmid pTC3 [19] bearing the *dha*-regulon (*dhaB*, *dhaT*, *dhaD*, *dhaK* and *dhaR* gene) from *Klebsiella* was digested with *Kpn*I and the resultant sticky end was treated with T4-DNA polymerase to create a blunt end. A second digestion was performed with *Mlu*I and a 3.343 kb fragment containing the intact *dhaR* and partial *dhaT* (3′-end) genes was isolated for further construction. The plasmid pTC18 [20], a derivative of pTC3, was digested with *Sal*I, and the end was filled with Klenow polymerase. The created fragment was then digested with *Mlu*I. A 10.664 kb big fragment containing the intact *dhaB* and partial *dhaT* (5′-end) genes was recovered from gel and ligated with the 3.343 kb fragment, generating a plasmid named as pKM13 (14.007 kb) (Fig. 1). It contains the *dhaB*, *dhaT* and *dhaR* genes from *K. pneumoniae* ATCC 25955.

Plasmids pKM15 and pKM16 are derivatives of pKM13. For this purpose, the plasmid pKM13 was double digested with *Kpn*I and *Mlu*I and a 1.224 kb small fragment containing the partial *dhaT* gene was removed. The rest big fragment was treated with T4-DNA polymerase and was then self-ligated. The created plasmid was designated as pKM15 (12.783 kb) (Fig. 1), containing the

Table 1
Bacterial strains and plasmids used in this study

Strains or plasmids	Genotype/phenotype	Reference
Strains		
K. pneumoniae DSM2026	Wild type	DSMZ, Germany
Plasmids		
pBR322	Cloning vector, Ap^r, Tc^r	[42]
pTC3	Derivative of plasmid pBR322, bearing *K. pneumoniae dhaB*, *dhaT*, *dhaD*, *dhaK* gene	[19]
pTC18	pTC3 without *Sal*I–*Sac*I fragment	[20]
pKM13	pTC3 without *Sal*I–*Kpn*I (10.06 kb) fragment, bearing *K. pneumoniae dhaB*, *dhaT*, *dhaR* gene	This study
pKM15	pKM13 without *Kpn*I–*Mlu*I fragment, bearing *K. pneumoniae dhaB*, *dhaR* gene	This study
pKM16	pKM13 without *Aat*II–*Mlu*I (6.794 kb) fragment, bearing *K. pneumoniae dhaR* gene	This study