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Pyruvate dehydrogenase complex regulator (PdhR) gene deletion boosts glucose metabolism in *Escherichia coli* under oxygen-limited culture conditions

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Pyruvate dehydrogenase complex regulator (PdhR) is a transcriptional regulator that negatively regulates formation of pyruvate dehydrogenase complex (PDHc), NADH dehydrogenase (NDH)-2, and cytochrome bo_3 oxidase in *Escherichia coli*. To investigate the effects of a PdhR defect on glucose metabolism, a *pdhR* deletion mutant was derived from the wild-type *E. coli* W1485 strain by λ Red-mediated recombination. While no difference in the fermentation profiles was observed between the two strains under oxygen-sufficient conditions, under oxygen-limited conditions, the growth level of the wild-type strain was significantly decreased with retarded glucose consumption accompanied by by-production of substantial amounts of pyruvic acid and acetic acid. In contrast, the mutant grew and consumed glucose more efficiently than did the wild-type strain with enhanced respiration, little by-production of pyruvic acid, less production yield and rates of acetic acid, thus displaying robust metabolic activity. As expected, increased activities of PDHc and NDH-2 were observed in the mutant. The increased activity of PDHc may explain the loss of pyruvic acid by-production, probably leading to decreased acetic acid formation, and the increased activity of NDH-2 may explain the enhanced respiration. Measurement of the intracellular NAD^+/NADH ratio in the mutant revealed more oxidative or more reductive intracellular environments than those in the wild-type strain under oxygen-sufficient and -limited conditions, respectively, suggesting another role of PdhR: maintaining redox balance in *E. coli*. The overall results demonstrate the biotechnological advantages of *pdhR* deletion in boosting glucose metabolism and also improve our understanding of the role of PdhR in bacterial physiology.

[**Key words:** PdhR; Pyruvate dehydrogenase complex; NADH dehydrogenase-2; NAD^+/NADH ratio; Acetic acid; Redox balance]

Escherichia coli is one of the most important microbes for industrial production of useful materials through bioprocessing, such as biofuels and bioplastics. The importance of *E. coli* in bioprocessing depends on its rapid growth, ability to grow under both aerobic and anaerobic conditions, established gene engineering systems, abundant biochemical, genetic information, and so on. Therefore, new methods to enhance the metabolic activity of *E. coli* are always in demand.

Our research group is focused on the enhancement of central metabolism in industrially important bacteria as a universal method to improve bioprocessing for rapid consumption of fermentation substrates for metabolite production. In general, our studies have revealed that impaired energy generation leads to enhanced glucose metabolism to compensate for the reduced energy level in cells. For example, we discovered that a defective mutation in H^+-ATPase in both *E. coli* and *Corynebacterium glutamicum* dramatically activated glycolysis with increased respiration (1,2). In the case of *E. coli*, this mutation led to improved production of pyruvic acid (3,4) and L-alanine (5). Furthermore, we have shown that simultaneous elimination of the activities of two respiratory-chain enzymes in *E. coli*, NADH dehydrogenase (NDH)-1 and cytochrome bo_3 oxidase (Cyt bo_3) (both having a higher efficiency of proton-motive force generation compared with their counterpart enzymes), leads to enhanced glucose metabolism with a concomitant increase in respiration (6).

In this study we focused on pyruvate dehydrogenase complex regulator (PdhR), a pyruvate-sensing transcriptional regulator that represses the *pdh* operon (*pdhR-aceE-aceF-lpdA*) encoding pyruvate dehydrogenase complex (PDHc) (7) (Fig. 1). PdhR was later found to also regulate two respiratory chain enzyme genes, *ndh* encoding NDH-2 and the *cyo* operon (*cyoABCDE*) encoding Cyt bo_3 (8) (Fig. 1). This is not surprising considering that the PDHc reaction yields NADH, which must be re-oxidized by respiration. PdhR is now recognized as a global regulator controlling more than 20 genes largely involved in aerobic glucose metabolism in *E. coli* (9). Thus, it was speculated that a *pdhR* deletion mutation (Δ*pdhR*) may enhance central metabolism by increasing the activities of PDHc, NDH-2, and Cyt bo_3 during aerobic growth of *E. coli* on glucose (Fig. 1). At the same time, ^{13}C-flux analysis during glucose

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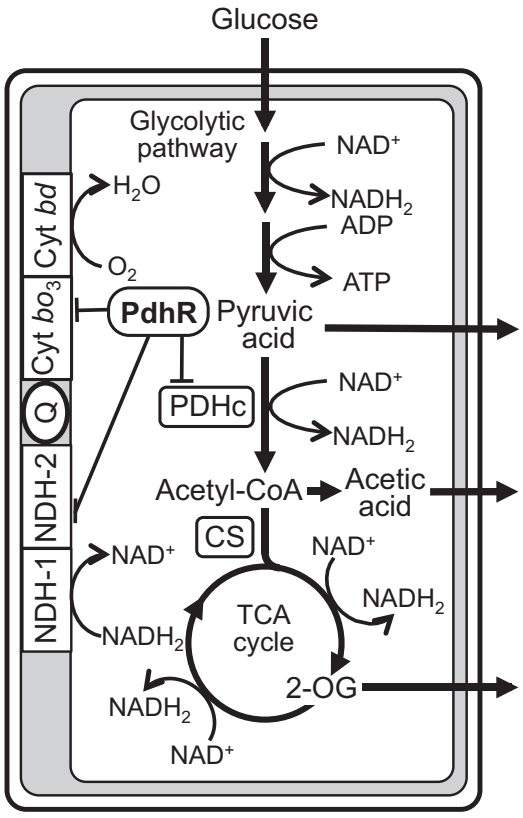

FIG. 1. Enzymes under the control of PdhR and the central metabolic pathway in *Escherichia coli*. PdhR, pyruvate dehydrogenase complex regulator; ⊦, negative control of enzyme formation; PDHc, pyruvate dehydrogenase complex; CS, citrate synthase; NDH-1, NADH dehydrogenase-1; NDH-2, NADH dehydrogenase-2; Cyt bo_3, cytochrome bo_3 oxidase; Cyt *bd*, cytochrome *bd* oxidase; 2-OG, 2-oxoglutaric acid; Q, quinones.

metabolism in a Δ*pdhR* mutant revealed a reduced acetate secretion rate (78%) and increased TCA cycle flux (134%) compared with the wild-type strain (10). Thus, the Δ*pdhR* mutation also has the potential to reduce acetate accumulation, which is an undesirable metabolite in bioprocessing due to its toxicity as a weak acid and resulting loss of carbon, both of which decrease the yield of the desired metabolites (11). Thus, *pdhR* seems to be an attractive target for improving the efficiency of fermentative production systems using *E. coli*.

In this study, the detailed fermentation profile and physiology of a simple Δ*pdhR* mutant strain were investigated. In the experiments, the wild-type *E. coli* W1485 strain and a Δ*pdhR* mutant derived from the wild-type strain were cultured in a glucose minimal medium using a 2-L jar fermentor under various oxygen supply conditions to explore the effects of the Δ*pdhR* mutation on glucose metabolism. Activities of the relevant enzymes and the intracellular NAD^+/NADH ratio were measured to determine the effect of Δ*pdhR* on the physiology of *E. coli* cells.

Our study provides, for the first time, detailed characterization of a Δ*pdhR* mutant during aerobic metabolism of glucose, which will be beneficial not only for the fermentation industry, but also for better understanding bacterial physiology.

MATERIALS AND METHODS

Bacterial strains and plasmids The bacterial strains and plasmids used in this study are listed in Table 1.

Construction of the ΔpdhR mutant The *pdhR* gene was deleted from the wild-type *E. coli* W1485 strain (ATCC 12435) using λ Red-mediated recombination, as described previously (12). A DNA fragment containing the chloramphenicol acetyltransferase gene (*cat*) with 50-nucleotide and 49-nucleotide extensions homologous to the upstream and downstream sequences of *pdhR*, respectively, was amplified by TaKaRa *Ex Taq* DNA polymerase (Takara Bio Inc., Kusatsu, Japan) using pKD3 as a template and the primer set pdhR-Pup and pdhR-Pdown (Table 2). The PCR product was then introduced into *E. coli* W1485/pKD46 competent cells, in which expression of λ Red recombinase had been induced in the presence of 10 mM L-arabinose, and the transformants were cultured on LB agar containing 25 μg/mL chloramphenicol. The chloramphenicol-resistant colonies that appeared on the plates were picked, and introduction of the desired insertion was then confirmed by PCR using the primer set pdhR-Vup and pdhR-Vdown (Table 2), which yielded a 1600-bp PCR product. Elimination of the *cat* gene was conducted using the pCP20 (conferring ampicillin resistance) helper plasmid, as described previously (13); this was then confirmed by PCR using the primer set pdhR-Vup and pdhR-Vdown. A candidate strain yielding an 800-bp PCR product was selected and then induced to lose pCP20 by culturing in LB medium without antibiotics at 42°C for 12 h. The resultant ampicillin-sensitive strain was further verified for increased PDHc activity as a result of the *pdhR* deletion (see Fig. 3A). Based on these criteria, successful derivation of the Δ*pdhR* mutant was confirmed, and the strain was designated W1485Δ*pdhR* (hereafter Δ*pdhR* mutant).

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant genotypes or characteristics[a]	Reference or source[b]
Escherichia coli		
W1485 (ATCC 12435)	F^+, λ^-, *rpoS396*(Am), *rph-1*	ATCC
W1485Δ*pdhR*	Δ*pdhR*, Cm^S	This study
Plasmid		
pKD3	*oriR6Kγ*, *rgnB*, *FRT*, *cat*, *bla*	12
pKD46	*oriR101*, *RepA101*^{ts}, *araC*, P_{araB}-*gam-bet-exo*, *tL3*, *bla*	12
pCP20	*FLP*$^+$, λcI857$^+$, λp_R, Rep^{ts}, *cat*, *bla*	13

[a] Cm^S, Chloramphenicol-sensitive.
[b] ATCC, American Type Culture Collection.

Media and culture conditions The LB agar contained (per L): 10 g Polypepton (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 5 g yeast extract (Nacalai Tesque, Inc., Kyoto, Japan), 10 g NaCl, 15 g agar, and NaOH to adjust the pH to 7.0. A minimal salt (MS) medium, MS3, was used for batch fermentation culture. MS3 medium differed from MS1 medium (6) in that the iron concentration was reduced by half, and glucose was added separately. MS3 medium contained (per L): 50 g glucose, 10 g $(NH_4)_2SO_4$, 2 g K_2HPO_4, 2 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$ (added separately), 14.7 mg $CaCl_2 \cdot 2H_2O$ (added separately), 81.1 mg $FeCl_3 \cdot 6H_2O$, and 2.5 mL micronutrient solution (in 0.05 N·HCl, added separately), which contained (per L) 396 mg $MnCl_2 \cdot 4H_2O$, 703.2 mg $CuSO_4 \cdot 5H_2O$, 720 mg $ZnSO_4 \cdot 7H_2O$, 720 mg $CoCl_2 \cdot 6H_2O$, 30 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 98.96 mg H_3BO_3. The pH of the medium was adjusted to 7.0 using 1 N HCl. The seed medium, MS4, had the same composition as that of MS3, except that the concentrations of glucose and K_2HPO_4 were reduced to 20 g/L and 1 g/L, respectively, and $CaCl_2 \cdot 2H_2O$ was replaced with 20 g/L $CaCO_3$.

Cultures were conducted in the same manner as described previously (6). Briefly, bacterial strains were refreshed twice on LB agar plates, and then precultures were inoculated into 50 mL MS4 medium in 500-mL Sakaguchi flasks and incubated with shaking for 12 h until the early stationary phase. After $CaCO_3$ was removed from the broth by centrifugation at 20 ×g at room temperature, the supernatants were inoculated in MS3 medium to give an initial optical density at 660 nm (OD_{660}) of 0.2. Batch fermentation was conducted in a 2-L jar fermentor (BMJ-02PI; ABLE Corp., Tokyo, Japan) with a working volume of 1 L. In this study, two conditions were used to operate the jar fermentor, namely, DO-STAT conditions or fixed-agitation conditions. DO-STAT in this study is defined as automatic stirring rate control, which enable the dissolved oxygen (DO) concentration in the medium to be kept above the setting value (2 ppm) by increasing the stirring rate up to 980 rpm. Under DO-STAT conditions, the initial culture conditions were aeration at 2 volumes air per volume culture per min (2 vvm) and stirring at 600 rpm. When the DO value dropped below 2 ppm, the stirring rate was automatically increased to maintain the DO value above 2 ppm. Under the fixed-agitation conditions, a fixed rate of stirring at either 400, 600, or 800 rpm was used throughout the culture. All of the cultures were conducted at 37°C, and the pH was maintained at 6.0 using 2 N NaOH.

Fermentation analysis Growth, residual glucose, organic acids, the specific rate of glucose consumption, and respiration were analyzed as described previously (6). The specific rate of glucose consumption during the log phase of culture was expressed as the decrease in glucose (mM) per increase in cells (OD_{660}) per h. The same calculation method was applied for organic acid production rate analyses except that the increase in organic acid (mM) was used. The specific rate of

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