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Outline of Research Result

Enhanced central carbon metabolism and its application to metabolite production in *pdhR*-defective *Escherichia coli*

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Research aims

The production of useful chemicals by microbial fermentation has attracted significant attention due to the sustainable nature of bioprocessing, in which biomass is used as the starting material. Thus, improved bioprocessing productivity is an ongoing goal. Our approach to meeting this goal is to enhance cellular central carbon metabolism. In this project, we focused on pyruvate dehydrogenase complex regulator (PdhR), which regulates pyruvate dehydrogenase complex (PDHc) expression (Fig. 1). PdhR is a negative transcription factor that de-represses transcription of the *pdh* operon encoding PDHc in response to an increase in the intracellular concentration of pyruvic acid, resulting in enhanced pyruvic acid metabolism.[1] As PDHc is an enzyme connecting glycolysis and the TCA cycle, deletion of the gene encoding PdhR (*pdhR*) is expected to enhance central carbon metabolism. Moreover, PdhR reportedly not only regulates the transcription of the *pdh* operon but also that of *ndh*, which encodes NADH dehydrogenase-II (NDH-II), and the *cyo* operon, which encodes cytochrome bo_3 oxidase (Cyt bo_3).[2] Therefore, we investigated the possible activation of central carbon metabolism by deletion of *pdhR* and its application to metabolite production. In this study, the metabolism of glucose in wild-type and *pdhR*-deleted (Δ*pdhR*) *Escherichia coli* cells was compared to explore alterations in fermentation characteristics of the mutant.

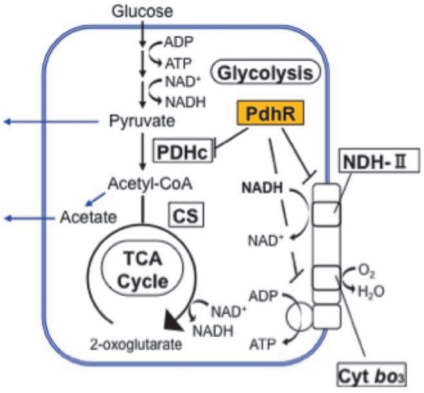

Fig. 1. Enzymes under the control of PdhR in *Escherichia coli*. All enzyme abbreviations are given in the text. ⊣:Repression.

Methods

The Δ*pdhR* mutant was constructed from the wild-type *E. coli* strain W1485 according to the method of Datsenko.[3] The wild-type strain and Δ*pdhR* mutant were cultured in minimal fermentation medium containing 50 g/L glucose in a 2-L jar fermentor under batch fermentation conditions. Cultures were prepared under STAT conditions (the agitation speed was automatically controlled to maintain the dissolved oxygen [DO] concentration above 2 ppm) or fixed agitation conditions (the agitation speed was fixed such that all DO was depleted after the late stationary phase). Growth status, residual glucose level, organic acid formation, and respiration rate were monitored and compared between the two strains. Furthermore, the enzymatic activities of PDHc, citrate synthase (CS), NDH, and Cyt, which play important roles in central carbon metabolism and the respiratory chain, were measured using cells harvested at the late stationary phase. Changes in the levels of transcription of Cyt genes were also assessed by quantitative real-time PCR. In addition, the intracellular NAD^+/NADH ratio, an indicator of intracellular redox balance, was measured using cycling assay methods.

Results

① Construction of the Δ*pdhR* mutant

Deletion of the *pdhR* region (~600 bp) in the Δ*pdhR* mutant was confirmed by PCR analysis. Dramatic increases in the activities of both PDHc and NDH-II in the Δ*pdhR* mutant compared to the wild-type strain were also confirmed (see Fig. 3), as reported previously.[1]

② Fermentation analysis

The wild-type strain and Δ*pdhR* mutant were cultured in minimal salts medium in 2-L jar fermentors. Under fixed agitation at 400, 600, or 800 rpm, DO was depleted after 4, 6, or 8 h of culture, respectively, while under STAT conditions, the DO concentration was maintained above 2 ppm. These results indicate that the oxygen supply was adequately controlled by the agitation speed.

Under STAT conditions, both the wild-type strain and Δ*pdhR* mutant showed good and comparable growth (Fig. 2A). On the other hand, under fixed agitation conditions at

400, 600, and 800 rpm, both the wild-type strain and Δ*pdhR* mutant showed reduced growth; however, the Δ*pdhR* mutant showed greater growth than did the wild-type strain. As shown in Fig. 2A, the difference was remarkable, especially in the culture agitated at 600 rpm (600 rpm conditions); the maximum growth level, indicated by the optical density of the culture broth at 660 nm, of the Δ*pdhR* mutant reached 20, while that of the wild-type strain was 12.

The total time to consume all glucose in the medium was approximately 14 h in both strains under STAT conditions. In contrast, under 600 rpm conditions, more than 30 h were required by the wild-type strain, while the Δ*pdhR* mutant consumed all available glucose in about 24 h (Fig. 2B). The specific respiration rate of the Δ*pdhR* mutant was increased 1.2-fold compared to that of the wild-type strain. Therefore, the Δ*pdhR* mutant showed greater metabolic activity than the wild-type strain under oxygen-depleted conditions.

The concentrations of 2-oxoglutarate, pyruvate, and acetate in the culture medium were measured. Under STAT conditions, both strains produced around 2 g/L of 2-oxoglutarate, and little pyruvate and acetate. Under 600 rpm conditions, however, the Δ*pdhR* mutant produced more 2-oxoglutarate (1.71 g/L) than did the wild-type strain (0.85 g/L). Pyruvate production was observed in the wild-type strain (maximum level, ~4 g/L), while the Δ*pdhR* mutant produced little pyruvate. Acetate formation was observed in both strains; however, the maximum yield in the Δ*pdhR* mutant (~5 g/L) was lower than that in the wild-type strain (~10 g/L). These results suggest that, in the Δ*pdhR* mutant, the carbon flux was maintained beyond pyruvate and a greater amount of carbon flowed into the TCA cycle.

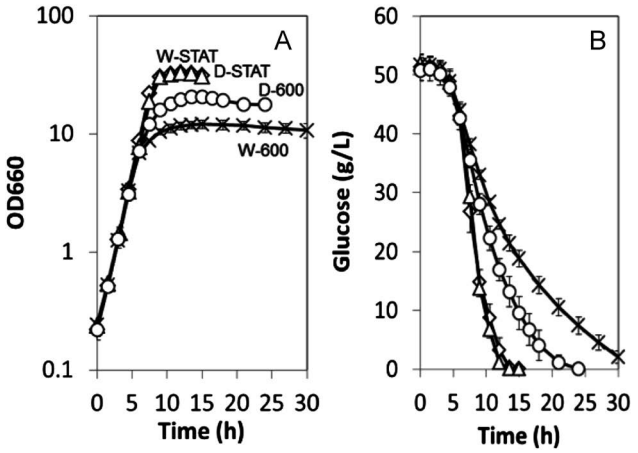

Fig. 2. Growth and glucose consumption under the two culture conditions (STAT and 600 rpm). W, wild-type strain; D, Δ*pdhR* mutant; STAT, STAT conditions; 600, 600 rpm conditions.

③ Enzymatic activities of the central carbon metabolic pathway and the respiratory chain

To investigate the mechanisms underlying the metabolic changes observed in the Δ*pdhR* mutant, the activities of PDHc, CS, NDH, and Cyt were measured in cell extracts prepared from log phase cells (Fig. 3). PDHc activity was significantly increased in the Δ*pdhR* mutant up to 6.1- and 3.0-fold the levels in the wild-type strain under both STAT and 600 rpm conditions, respectively. CS activity was relatively constant (~300 nmol/min/mg) in both strains irrespective of culture conditions (data not shown). NDH activity was measured separately in terms of NDH-I and -II activity. The level of NDH-I activity was similar and constant in both strains under both culture conditions. In contrast, the levels of NDH-II activity in the Δ*pdhR* mutant under both the STAT and 600 rpm conditions were increased 5.1- and 4.2-fold compared to those in the wild-type strain, respectively. Cyt activity was measured as the total activity of three enzymes, Cyt bo_3, Cyt *bd*-I, and Cyt *bd*-II. The total Cyt activity level did not differ significantly between the two strains under either culture condition. Compared to the STAT conditions, however, both strains showed a higher activity level with agitation at 600 rpm. This may have been caused not by the deletion of *pdhR*, but by some global regulators that respond to anaerobic conditions.

④ Analysis of terminal oxidase transcription

In *E. coli*, Cyt consists of three isozymes, Cyt bo_3 (encoded by *cyo*), Cyt *bd*-I (encoded by *cyd*), and Cyt *bd*-II (encoded by *app*). These three enzymes could not be evaluated separately by enzymatic assays. Therefore, transcriptional levels of the three genes (*cyo*, *cyd*, and *app*) were independently quantified and compared by real-time PCR analysis. Our results revealed no significant difference between the wild-type strain and Δ*pdhR* mutant under either culture condition. These results indicate that the effect of the deletion of *pdhR* on the expression of Cyt bo_3 was limited.

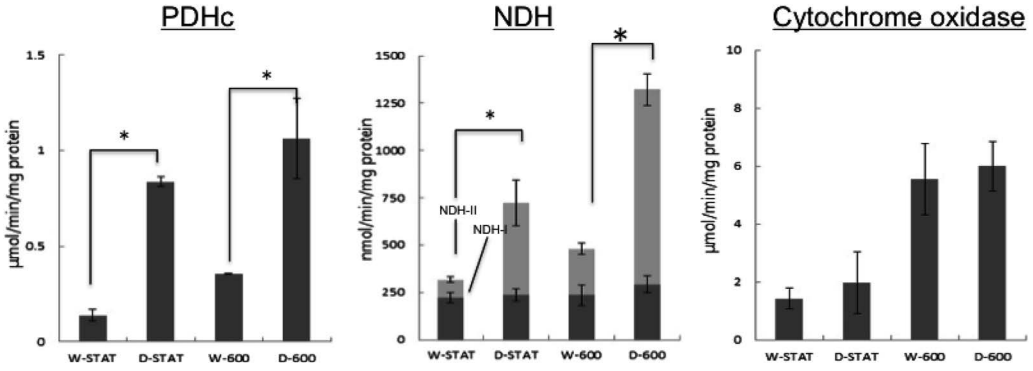

Fig. 3. Activity levels of PDHc and respiratory enzymes in the wild-type strain and Δ*pdhR* mutant under both culture conditions (STAT and 600 rpm). Error bars represent the standard deviation (n=3). *, significant *t*-test differences ($p<0.05$). For NDH activity, the difference in total activity levels of NDH-I and -II was evaluated. The abbreviations are the same as those in Fig. 2.

⑤ Intracellular NAD^+/NADH ratio

Based on the measured enzyme activity levels, PDHc and NDH-II activities were significantly increased in the Δ*pdhR* mutant compared to that in the wild-type strain. As these enzymes are related to NADH production and consumption in cells, respectively, the intracellular NAD^+/NADH ratio was determined and compared in both strains. Under STAT conditions, the NAD^+/NADH ratio of the Δ*pdhR* mutant was similar to or slightly higher than (~1.2-fold increase) that in the wild-type strain, suggesting that a slightly oxidative environment existed in the Δ*pdhR* mutant cells. On the other hand, the NAD^+/NADH ratio in the Δ*pdhR* mutant was significantly decreased 0.6-fold that in the wild-type strain under the 600 rpm conditions. These results suggest that the intracellular environment of the Δ*pdhR* mutant was more reductive than that of the wild-type strain under 600 rpm conditions.

Conclusions

The Δ*pdhR* mutant showed both a higher glucose consumption rate and increased growth under 600 rpm conditions. This may be due to the increased availability of precursor metabolites for growth, which originated from the higher carbon flux into the TCA cycle in the Δ*pdhR* mutant. Therefore, we conclude that the Δ*pdhR* mutation enabled energy-saving fermentation processes in *E. coli*, in which agitation or aeration costs were reduced.

In the Δ*pdhR* mutant, the formation of pyruvate and acetate was remarkably relieved under 600 rpm conditions, although the by-production of these organic acids is generally observed in *E. coli* cultures along with insufficient aeration; this interferes with normal growth and metabolite production. The disappearance of pyruvate formation in the Δ*pdhR* mutant is believed to be due to increased PDHc activity. This must have increased the intracellular concentration of acetyl-CoA. However, in the Δ*pdhR* mutant, the excretion of acetate, a typical anaerobic metabolite of acetyl-CoA, was also decreased, while 2-oxoglutarate formation was increased. One possible explanation for the decrease in acetate formation is the activation of CS activity by acetyl-CoA, a known activator of *E. coli* CS, leading to the increased flow of acetyl-CoA into the TCA cycle.

Our enzyme activity measurements revealed dramatic increases in the activities of both PDHc and NDH-II, which are under the control of PdhR, in the Δ*pdhR* mutant under both STAT and 600 rpm conditions. However, the total Cyt activity level and transcriptional level of *cyo*, which are also controlled by PdhR, did not differ between the two strains, while an increase in respiration rate was observed in the Δ*pdhR* mutant. These results indicate that the NDH-II reaction is the rate-limiting step in the respiratory chain. Indeed, we observed that the total NDH activity level was only one-tenth that of Cyt in the wild-type strain under 600 rpm conditions, suggesting that NDH-II controls the rate of respiration in *E. coli*.

The much lower NAD^+/NADH ratio observed in the Δ*pdhR* mutant compared to the wild-type strain under 600 rpm conditions indicates a reduced intracellular environment in the mutant compared to the parent. However, this result is unexpected as the Δ*pdhR* mutant exhibited a higher respiration rate, which enabled enhanced NAD^+/NADH recycling. In the Δ*pdhR* mutant, enhanced formation of NADH caused by the increase in PDHc activity was not balanced even by the increased re-oxidation of NADH driven by the increased activity of NDH, resulting in NADH accumulation in the mutant.

References

(1) Ogasawara, H., Ishida, Y., Yamada, K., Yamamoto, K., and Ishihama, A. (2007) PdhR (pyruvate dehydrogenase complex regulator) controls the respiratory electron transport system in *Escherichia coli*. *J. Bacteriol.* **189**: 5534–5541.

(2) Göhler A. K. *et al*. (2011) More than just a metabolic regulator-elucidation and validation of new targets of PdhR in *Escherichia coli*. *BMC Syst. Biol.* **5**: 197. doi: 10.1186/1752-0509-5-197.

(3) Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA*. **97**: 6640–6645.