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Alterations of glucose metabolism in *Escherichia coli* mutants defective in respiratory-chain enzymes

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ABSTRACT

The effects of reduced efficiency of proton-motive force (pmf) generation on glucose metabolism were investigated in *Escherichia coli* respiratory-chain mutants. The respiratory chain of *E. coli* consists of two NADH dehydrogenases and three terminal oxidases, all with different abilities to generate a pmf. The genes for isozymes with the highest pmf-generating capacity (NADH dehydrogenase-1 and cytochrome bo_3 oxidase) were knocked out singly or in combination, using a wild-type strain as the parent. Analyses of glucose metabolism by jar-fermentation revealed that the glucose consumption rate per cell increased with decreasing efficiency of pmf generation, as determined from the growth parameters of the mutants. The highest rate of glucose metabolism was observed in the double mutant, and the lowest was observed in the wild-type strain. The respiration rates of the single-knockout mutants were comparable to that of the wild-type strain, and that of the double mutant was higher, apparently as a result of the upregulation of the remaining respiratory chain enzymes. All of the strains excreted 2-oxoglutaric acid as a product of glucose metabolism. Additionally, all of the mutants excreted pyruvic acid and/or acetic acid. Interestingly, the double mutant excreted L-glutamic acid. Alterations of the fermentation profiles provide clues regarding the metabolic regulation in each mutant.

1. Introduction

The development of effective fermentation processes for the production of useful compounds by microorganisms is a major goal of biotechnology, and our laboratory has focused on the enhancement of the central metabolism of microorganisms. Central metabolism, which consists of the glycolytic pathway and tricarboxylic acid (TCA) cycle, is primarily regulated by the energy status of the cells and is stimulated by low ATP levels (Atkinson, 1970). In *Escherichia coli*, activities of key enzymes of the glycolytic pathway and TCA cycle are controlled by adenine nucleotides and/or NADH as part of the regulation of central metabolism, according to the energy status of the cell. For example, the increased ADP and AMP concentrations that occur under high energy demands allosterically activate phosphofructokinase I (Babul, 1978) and pyruvate kinase II (Kotlarz et al., 1975), respectively, thereby increasing the rate of catabolism. On the other hand, when the ATP levels are sufficient, these enzymes are no longer activated, and NADH, which generates ATP during oxidative phosphorylation, inhibits the activities of both citrate synthase (CS) (Weitzman, 1981) and the pyruvate dehydrogenase complex (PDHc) (Kim et al., 2008), thereby downregulating the glycolytic pathway and TCA cycle. In previous studies (Yokota et al., 1994a, 1994b), we derived a mutant with a defect in the F_1F_o-ATP synthase, a key enzyme of oxidative phosphorylation that generates the bulk of cellular ATP, to produce cells with lower energy status. Although the mutant showed reductions in both growth rate and growth yield, it exhibited an increased rate of glucose consumption. Thus, we succeeded in the efficient production of pyruvate, an end product of glycolysis. Detailed analyses of the simple F_1F_o-ATP synthase-defective mutant revealed an increased respiration rate and altered expression of many genes, including those for respiratory enzymes, which together increased the central metabolism (Noda et al., 2006; Yokota and Wada, 2009).

During oxidative phosphorylation, the proton-motive force (pmf) generated by the respiratory chain drives the F_1F_o-ATP synthase to produce ATP. We predicted that the energy status of cells could be reduced and catabolism could be enhanced through muta-

Abbreviations: pmf, proton-motive force; TCA, tricarboxylic acid; CS, citrate synthase; PDHc, pyruvate dehydrogenase complex; NDH, NADH dehydrogenase; Cyt bo_3, cytochrome bo_3 oxidase; Cyt *bd*-I, cytochrome *bd*-I oxidase; Cyt *bd*-II, cytochrome *bd*-II oxidase; MS, minimal salts; OD_{660}, optical density at 660 nm; TLC, thin-layer chromatography; ODHc, 2-oxoglutarate dehydrogenase complex; ICDH, isocitrate dehydrogenase; Q_1H_2, ubiquinol-1; PdhR, pyruvate dehydrogenase complex regulator.

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tions that disrupt the respiratory chain. From a biotechnological point of view, the mutation of respiratory chain genes has been used for the production of anaerobic fermentation products through the engineering of the metabolic redox balance. For example, the complete shutdown of the respiratory chain has been reported to increase lactic acid production in *E. coli* under aerobic culture conditions, owing to the impairment of NAD^+/NADH recycling (Becker et al., 1997; Portnoy et al., 2008). However, modulation of the cellular energy status by respiratory chain mutation has never been used as a strategy to enhance overall central carbon metabolism. The respiratory chain of *E. coli* includes NADH dehydrogenase (NDH) and terminal oxidase. Each of these respiratory components consists of a few isozymes with different efficiencies for coupling electron transfer to the generation of an electrochemical proton gradient: NDH-1 ($2\ H^+/e^-$) and NDH-2 ($0\ H^+/e^-$), and cytochrome bo_3 oxidase (Cyt bo_3) ($2\ H^+/e^-$), cytochrome *bd*-I oxidase (Cyt *bd*-I) ($1\ H^+/e^-$) and cytochrome *bd*-II oxidase (Cyt *bd*-II) ($0\ H^+/e^-$) (Bekker et al., 2009; Bogachev et al., 1996; Gennis and Stewart, 1996; Matsushita et al., 1987). Furthermore, three additional putative NADH dehydrogenases [NAD(P)H:quinone oxidoreductase], namely, WrbA, QOR and YhdH, have been reported (Patridge and Ferry, 2006; Sulzenbacher et al., 2004; Thorn et al., 1995). However, they are not integral membrane proteins and assumed to be not electrogenic. Accordingly, we constructed mutants with defects in the most efficient isozymes in pmf generation, namely NDH-1 and Cyt bo_3, either singly or in combination, to achieve different levels of impaired efficiency of pmf generation. An analysis of the batch fermentation of glucose by these mutants was conducted to evaluate the effect of reduced efficiency of pmf generation on the central metabolism.

2. Materials and methods

2.1. Bacterial strains, plasmids and bacteriophage

The *E. coli* strains, plasmids, and bacteriophage used in this work are listed in Table 1.

2.2. Construction of respiratory-chain mutants

2.2.1. NDH-1-defective mutant

The *nuo* operon encoding NDH-1 consists of 14 genes, making it too large to be deleted. Thus, we constructed an NDH-1-defective mutant by deleting the promoter region of the *nuo* operon through double crossover replacement (Fig. 1A). An approximately 0.7-kbp region downstream of the *nuo* promoter was amplified by PCR using two synthetic primers, YHPn11 (5′-GTATGTCAACACCCGGGGAAGTCATCGCTCATCACTG-3′) and YHPn12 (5′-CCATCAGGTCAGAATTCCGCGGCGAAGCACGCAATAC-3′). The underlined bases denote *Sma*I and *Eco*RI restriction sites, respectively. The PCR-generated DNA fragment was digested with *Sma*I and *Eco*RI and then ligated into pMAN997 (Ap^r, Matsui et al., 2001) with a temperature-sensitive replication origin, yielding pMnuo1. Similarly, an approximately 0.7-kbp region upstream of the *nuo* promoter was PCR-amplified using the primers YHPn13 (5′-GCGTTTAAGGCTCTAGACCTGCGTACCTCGCCAACAC-3′) and YHPn14 (5′-GAGTATTTTCTCCCGGGGTGACACGCTTTTGTCATTCC-3′). The underlined bases denote *Xba*I and *Sma*I restriction sites, respectively. The PCR product and pMnuo1 were digested with *Xba*I and *Sma*I and then ligated to obtain pMnuo2. The chloramphenicol resistance marker gene (*cat*) was obtained by digestion of pHSG396 (Takara Bio Inc., Otsu, Japan) with *Acc*II and was inserted into the *Sma*I site of pMnuo2 to generate pMnuo3 for homologous recombination. Sequence analysis confirmed that the orientation of the *cat* gene was the same as that of the *nuo* operon. Wild-type strain W1485 was transformed with pMnuo3, and the transformants showing resistance to both ampicillin (50 μg/mL) and chloramphenicol (25 μg/mL) on LB agar plates (see Section 2.3) were selected as the single-crossover strains. After the single-crossover strains were cultured in LB medium containing these same concentrations of both antibiotics at 42 °C, the colonies that showed resistance to chloramphenicol, but not ampicillin, on LB agar plates were selected as the double-crossover strains. The deletion of the promoter region of the *nuo* operon on the chromosome was confirmed by sequencing, and the strain YN28 was obtained. The promoter region in strain YN28 was transferred into strain W1485 by P1kc transduction. To exclude unwanted secondary mutations, three transductants were randomly selected and checked for swarming ability (flagellation) on LB agar plate but containing 2.5 g/L agar at 30 °C for 6 h. After that, they were cultured in MS2 medium (see Section 2.3) for checking their growth in minimal medium (glucose consumption, growth rate and growth level). The same criteria for checking were applied in all the following strain constructions. The promoter region of an appropriate strain was confirmed again by sequencing, and the NDH-1-defective mutant, designated ΔNDH-1, was finally constructed.

2.2.2. Cyt bo_3-defective mutant

A strategy similar to that used for the construction of strain ΔNDH-1 above was applied to generate a Cyt bo_3-defective mutant. However, in this case, the entire region of the *cyo* operon (*cyoABCDE*) encoding Cyt bo_3 was deleted by double crossover replacement (Fig. 1B). An approximately 1.2-kbp region upstream of the *cyo* operon and containing *Sal*I site was amplified by PCR using two synthetic primers, AZPcyo1 (5′-TTCTTCTCTCAAGCTTGCCAGGCTTACGTT-3′) and AZPcyo2 (5′-CAGATGCGTTTTTCGTAGCGCCAGATAATC-3′). The underlined bases denote a *Hind*III site. The PCR-generated DNA fragment was digested with *Hind*III and *Sal*I and then ligated into pMAN997 to yield pMcyo1. Similarly, an approximately 1.3-kbp region downstream of the *cyo* operon and containing *Sal*I site was PCR-amplified with the primers AZPcyo3 (5′-GCTTTCCGACTCCATCTGGGGAATTATTCT-3′) and AZPcyo4 (5′-AGGCGCAAATTCGAATTCATGAAATCCGCG-3′). The underlined bases denote an *Eco*RI site. The PCR-generated fragment and pMcyo1 were digested with *Sal*I and *Eco*RI and then ligated to obtain pMcyo2. The kanamycin resistance marker gene (*kan*) was amplified from pHSG299 (Takara Bio) by PCR using two synthetic primers, AZPkan4 (5′-TCGTGAAGAAGGTGTCGACGACTCATACCA-3′) and AZPkan6 (5′-AGGAAGCGGAAGAAGCTCGCACATTCAGC-3′). The underlined bases denote a *Sal*I site. The *kan* gene and pMcyo2 were digested with *Sal*I and ligated to generate pMcyo3 for homologous recombination. Sequence analysis showed the orientation of the *kan* gene to be opposite that of the original *cyo* operon. Strain W1485 was transformed with pMcyo3, and the transformants showing resistance to both ampicillin (100 μg/mL) and kanamycin (50 μg/mL) on LB agar plates were selected as the single-crossover strains. Double-crossover strains, in which the *cyo* operon was deleted, were obtained in a manner similar to that of the NDH-1 defective mutant, except that kanamycin resistance was used as the selection marker. Sequence analysis identified strain AZ28 as a *cyo* operon-deleted mutant. Then, the disrupted region in strain AZ28 was transferred into strain W1485 by P1kc transduction, using kanamycin resistance as the selection marker. After the deletion was confirmed by sequence analysis, the Cyt bo_3-defective mutant, designated ΔCytbo, was finally obtained.

2.2.3. Double mutant having both NDH-1- and Cyt bo_3-defects

To obtain the double mutant, the Cyt bo_3-disrupted region (Km^r) from strain AZ28 was transduced into strain ΔNDH-1 (Cm^r) by P1kc, and the candidates were screened for resistance to both