



Title	Alterations of cellular physiology in Escherichia coli in response to oxidative phosphorylation impaired by defective F1-ATPase
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1        **Alterations of cellular physiology in *Escherichia coli* in response to**  
2                    **oxidative phosphorylation impaired by defective F<sub>1</sub>-ATPase**

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19

20       Running Title: Physiology of F<sub>1</sub>-ATPase mutant of *E. coli*

21

## 1 **Abstract**

2 The physiological changes in an F<sub>1</sub>-ATPase-defective mutant of *E. coli* W1485 growing  
3 in a glucose-limited chemostat included a decreased growth yield (60%) and increased  
4 specific rates of both glucose consumption (168%) and respiration (171%). Flux analysis  
5 revealed that the mutant showed approximately twice as much flow in glycolysis but only  
6 an 18% increase in the tricarboxylic acid (TCA) cycle, owing to the excretion of acetate,  
7 where most of the increased glycolytic flux was directed. Genetic and biochemical  
8 analyses of the mutant revealed the downregulation of many TCA cycle enzymes,  
9 including citrate synthase, and the upregulation of the pyruvate dehydrogenase complex  
10 in both transcription and enzyme activities. These changes seemed to contribute to acetate  
11 excretion in the mutant. No transcriptional changes were observed in the glycolytic  
12 enzymes, despite the enhanced glycolysis. The most significant alterations were found in  
13 the respiratory chain components. The total activity of NADH dehydrogenases (NDHs)  
14 and terminal oxidases increased about twofold in the mutant, which accounted for its  
15 higher respiration rate. These changes primarily arose from the increased (3.7-fold)  
16 enzyme activity of NDH-2 and an increased amount of cytochrome *bd* in the mutant.  
17 Transcriptional upregulation appeared to be involved in these phenomena. As NDH-2  
18 cannot generate an electrochemical gradient of protons and as cytochrome *bd* is inferior  
19 to cytochrome *bo*<sub>3</sub> in this ability, the mutant was able to recycle NADH at a higher rate  
20 than the parent and avoid generating an excess proton-motive force. We discuss the  
21 physiological benefits of the alterations in the mutant.

# 1 INTRODUCTION

2

3           The elucidation of the regulatory mechanism of glycolytic flux is critical for  
4 developing effective fermentation processes for the production of useful metabolites by  
5 microorganisms. Glycolytic flux in *Escherichia coli* is controlled primarily by the ATP  
6 demand of the cells, rather than by glycolytic enzymes (22). For example, defects in the  
7 activity of F<sub>1</sub>F<sub>o</sub>-ATP synthase that impair oxidative phosphorylation (21, 38, 40) or  
8 increased ATPase activity in hydrolyzing ATP (22), which both lead to a reduced  
9 [ATP]/[ADP] ratio (21, 22), result in enhanced rates of glucose consumption. The  
10 enhancement of glucose consumption by defective F<sub>1</sub>F<sub>o</sub>-ATP synthase activity has also  
11 been reported in the Gram-positive bacteria *Bacillus subtilis* (32) and *Corynebacterium*  
12 *glutamicum* (34), which are industrially important. Several attempts have been made to  
13 apply these findings to the production of useful metabolites from glucose by fermentation.  
14 Our group reported the first successful application of pyruvate production, using an *E.*  
15 *coli* mutant with a defective F<sub>1</sub>-ATPase (40). In this case, enhanced pyruvate production  
16 was achieved with an increased rate of glucose consumption. The effectiveness of  
17 F<sub>1</sub>F<sub>o</sub>-ATP synthase defects for the production of acetate (9), as well as pyruvate (8), has  
18 also been reported in different *E. coli* mutants. Recently we demonstrated that the  
19 mutation also works for the improvement of glutamate production in *C. glutamicum* (1).

20           Although the [ATP]/[ADP] ratio is well accepted as a controlling factor of  
21 glycolysis, the underlying mechanisms by which enhanced glucose metabolism is  
22 established in response to an energy shortage are still not well understood. The allosteric  
23 activation of the key enzymes in the glycolytic pathway, *i.e.*, phosphofructokinase I (2)

1 and pyruvate kinase II (23), under a reduced [ATP]/[ADP] ratio is thought to contribute to  
2 this phenomenon. However, previous works (21, 38) have suggested the possibility that  
3 qualitative changes in certain cell components, such as an increase in *b*-type cytochrome  
4 contents, as well as allosteric control, are involved in the mechanism of enhanced glucose  
5 metabolism. To address this important question, we investigated the alterations in cellular  
6 physiology that occur in *E. coli* in response to impaired oxidative phosphorylation due to  
7 a defective F<sub>1</sub>-ATPase. To avoid any metabolic distortion from unnecessary genetic  
8 background, we constructed a simple F<sub>1</sub>-ATPase-defective mutant from the wild-type *E.*  
9 *coli* W1485. Glucose-limited chemostat culture was employed to ensure that cell samples  
10 grew at the same rate in the exponential phase. We conducted detailed analyses of  
11 metabolic flux, gene expression profiles, and central carbon metabolic and respiratory  
12 chain enzyme activities to elucidate the mechanism(s) of enhanced glucose metabolism.

13

## 14 **MATERIALS AND METHODS**

15

16 **Bacterial strains and culture conditions.** The wild-type strain *E. coli* W1485  
17 (ATCC12435) was used. An F<sub>1</sub>-ATPase-defective mutant, HBA-1 (*atpA401*, *bgl*<sup>+</sup>), was  
18 constructed by the P1kc transduction of *atpA401* (4), a defective gene for the  $\alpha$  subunit of  
19 F<sub>1</sub>-ATPase, into strain W1485. This mutant allele was first isolated in 1971 by Butlin et  
20 al., (4) as a gene (*uncA401*) that causes uncoupling of phosphorylation associated with  
21 electron transport. The *E. coli* K12 strain carrying *uncA401* showed negligible activity of  
22 Ca<sup>2+</sup>, Mg<sup>2+</sup>-activated ATPase (4). A series of intensive investigations of this mutant allele  
23 has located this mutation in the  $\alpha$  subunit of F<sub>1</sub>-ATPase (13) and the sequence analysis

1 has revealed a single base change that resulted in the replacement of Ser 373 into Phe (29).  
2 This mutant F<sub>1</sub>-ATPase has been shown to have virtually no ATPase activity yet retain the  
3 same subunits ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$ ) organization in terms of molecular weight, stoichiometry  
4 ( $\alpha_3\beta_3\gamma\delta\epsilon$ ) and arrangement (6). This mutant F<sub>1</sub>-ATPase has been suggested to bind to  
5 ATPase-depleted membranes and keep proton impermeability of the membrane (6). This  
6 was further confirmed in our preliminary experiment in which similar levels of  
7 valinomycin-induced artificial membrane potential were monitored using inside-out  
8 membrane vesicles prepared from strains W1485 and HBA-1 as monitored by  
9 fluorescence quenching method (probe: bis-(1,3-dibutylbarbituric acid)pentamethine  
10 oxonol (DiBAC<sub>4</sub>(5)) (data not shown). Therefore, the membrane of the strain HBA-1 has  
11 also been confirmed to be sealed and maintain normal level of proton impermeability. To  
12 obtain transductants effectively, *atpA401* was co-transduced with *bgl*<sup>+</sup>, as described  
13 previously, using AN718bgl-7 as the donor strain (40). Almost no ATPase activity was  
14 detected in strain HBA-1, when enzyme activity was measured as described previously  
15 (40). Both strains were cultured in a glucose-limited chemostat in modified M9 minimal  
16 medium containing trace elements to stabilize the continuous culture. The medium  
17 contained 14.7 g/l Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 3.0 g/l KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/l NaCl, 1.0 g/l NH<sub>4</sub>Cl, 1.0  
18 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 1.0  $\mu$ M FeCl<sub>3</sub>, 0.03  $\mu$ M (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 4  $\mu$ M H<sub>3</sub>BO<sub>3</sub>, 0.3  
19  $\mu$ M CoCl<sub>2</sub>, 0.1  $\mu$ M CuSO<sub>4</sub>, 0.8  $\mu$ M MnCl<sub>2</sub>, 0.1  $\mu$ M ZnSO<sub>4</sub>, and 2 g/l glucose as a carbon  
20 source. The continuous chemostat culture was conducted at a dilution rate of 0.2 h<sup>-1</sup>, with  
21 a working volume of 750 ml, in a 2-L jar fermentor. The cultures were aerated at 1.5 l/min  
22 (2 vvm), with stirring at 700 rpm. Dissolved oxygen (DO) in the culture broth of both  
23 parent and mutant was monitored by a DO electrode and was maintained at about 90%.

1 The culture temperature was controlled at 37°C, and the pH was adjusted to 7.0 with  
2 NaOH.

3 **Fermentation analysis.** Growth was measured by the spectrophotometric  
4 absorbance of the culture broth at 660 nm. The concentration of glucose remaining in the  
5 culture broth was determined by the glucose oxidase method, using Glucose C2 (Wako  
6 Pure Chemical Industries, Ltd., Osaka, Japan). Organic acids in the culture broth were  
7 determined by HPLC (column: AMINEX HPX-87H, Bio-Rad Laboratories, Hercules,  
8 CA, USA; mobile phase: 0.01 N H<sub>2</sub>SO<sub>4</sub>; flow rate: 0.6 ml/min; detection: absorbance at  
9 210 nm). The respiration rate of the bacterial cells during chemostatic culture was  
10 measured using a dissolved oxygen analyzer (Model MD-1000, Iijima Electronics  
11 Corporation, Gamagori, Aichi, Japan) equipped with a Clark-type oxygen electrode.  
12 Measurements were conducted at 37°C in the air-tight chamber within the range yielding  
13 a linear relationship between the cell concentration and the oxygen-consumption rate.  
14 Our calculation assumed the oxygen solubility in the 37°C medium to be 0.214 mM. The  
15 results were expressed as mmol O<sub>2</sub> h<sup>-1</sup> (g dry cell weight)<sup>-1</sup>. The dry cell weight of strains  
16 W1485 and HBA-1 was determined from the correspondence of one optical density unit  
17 at 660 nm to 0.414 mg and 0.411 mg dry cell weight per ml, respectively.

18 **Flux analysis.** The metabolic fluxes of the wild type strain and the mutant were  
19 estimated using the stoichiometric approach described by Holms (18). This method  
20 provides the way to calculate metabolic fluxes within the central metabolic pathways in *E.*  
21 *coli* growing on various single carbon sources at a constant growth rate. The idea is to  
22 balance the metabolic events in the conversion of feedstock (glucose) to biomass and  
23 by-products using the defined metabolic pathways and the experimental data of growth

1 rate, glucose consumption, by-product formation and biomass production. The kinetic  
2 parameters (specific rates of glucose consumption and metabolites production) in  
3 chemostat culture and the amounts of precursor metabolites required for the biosynthesis  
4 of building blocks (27) were used to calculate the fluxes in the central metabolic  
5 pathways.

6 **Extraction of total RNA.** Cells in the chemostat culture were withdrawn and  
7 immediately mixed with crushed ice prepared at  $-80^{\circ}\text{C}$ . The mixtures were centrifuged at  
8  $8,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min, and the supernatants were discarded. The RNA was isolated  
9 from the cell pellet with ISOGEN (Nippon Gene Co., Ltd., Toyama, Tomaya, Japan),  
10 according to the manufacturer's instructions. The RNA was treated with RQ1 RNase-Free  
11 DNase (Promega Corporation, Madison, WI, USA) and extracted again with ISOGEN.  
12 The concentration and quality of the total RNA yield were determined  
13 spectrophotometrically and by agarose gel electrophoresis. The extracted RNA was kept  
14 at  $-80^{\circ}\text{C}$  until used.

15 **DNA array analysis.** For *E. coli*-specific primed cDNA synthesis, 2  $\mu\text{g}$  total  
16 RNA and 4  $\mu\text{l}$  *E. coli* cDNA labeling primers (Sigma-Aldrich Corporation, St. Louis, MO,  
17 USA) were added to the transcription mixture (6  $\mu\text{l}$  5x first-strand buffer and 1  $\mu\text{l}$  each of  
18 10 mM dATP, 10 mM dGTP, and 10 mM dTTP), and the total volume was adjusted to  
19 26.5  $\mu\text{l}$  with RNase-free water. The samples were incubated at  $90^{\circ}\text{C}$  for 2 min and were  
20 kept at  $42^{\circ}\text{C}$  for 20 min. Then, 0.5  $\mu\text{l}$  RNase inhibitor (20 U RNase OUT, Invitrogen  
21 Corporation, Carlsbad, CA, USA), 1  $\mu\text{l}$  reverse transcriptase (200 U, SuperScript II,  
22 Invitrogen) and 2  $\mu\text{l}$  [ $\alpha$ - $^{33}\text{P}$ ]dCTP (20  $\mu\text{Ci}$ ; GE Healthcare Bio-Sciences Corp.,  
23 Piscataway, NJ, USA) were added to the reaction mixture. After incubation at  $42^{\circ}\text{C}$  for

1 2.5 h, the labeled cDNA was purified on a Sephadex G-25 spin column (GE Healthcare  
2 Bio-Sciences). The purified cDNA was denatured at 94°C for 10 min and immediately  
3 chilled on ice. The cDNA probe thus prepared was used to perform the hybridization  
4 experiment using Panorama *E. coli* Gene Arrays (Sigma-Aldrich) as described in the  
5 manufacturer's instructions. After hybridization, the arrays were exposed to Imaging  
6 Plates (Fuji Photo Film Co., Ltd., Minami-Ashigara, Kanagawa, Japan) for 48 h. The  
7 exposed Imaging Plates were scanned with BAS-5000 (Fuji Photo Film). Data analysis  
8 was performed with Array Gauge software (v 1.2; Fuji Photo Film). The data were  
9 calculated as the average and standard deviation of eight independent experiments and  
10 expressed as a fraction of the total hybridization signal on each DNA array filter. A  
11 two-tailed Student's *t*-test *p* value < 0.05 was considered statistically significant.

12 **Northern blot analysis.** The extracted total RNA was separated by  
13 formaldehyde-agarose gel electrophoresis (9% formaldehyde, 1x MOPS buffer, pH 5.0, 5  
14 mM sodium acetate, 1 mM EDTA, 1% agarose). The separated RNA was transferred onto  
15 a Hybond-N<sup>+</sup> membrane (GE Healthcare Bio-Sciences) by the capillary method. To  
16 detect *hns* gene expression with the hybridization probe, a 0.41-kb DNA fragment was  
17 amplified by PCR using the following primer set: 5'-CGAAGCACTTAAAATTCTGA-3'  
18 and 5'-TTATTGCTTGATCAGGAAAT-3'. Northern hybridization was carried out using  
19 AlkPhos Direct and ECF substrate (GE Healthcare Bio-Sciences). The signals were  
20 quantified by Typhoon 8600 (GE Healthcare Bio-Sciences) and ImageQuant software (v  
21 5.2; Molecular Dynamics, Sunnyvale, CA, USA).

22 **Real-time PCR analysis.** The reaction mixture containing 5 µg total RNA, 1  
23 µl random primers (300 ng, Invitrogen), and 1 µl 10 mM dNTP mixture in a total volume

1 of 12  $\mu$ l was incubated at 65°C for 5 min and immediately chilled on ice. Then, 4  $\mu$ l of 5x  
2 first-strand buffer, 2  $\mu$ l 0.1 M dithiothreitol (DTT) and 1  $\mu$ l RNase inhibitor (40 U RNase  
3 OUT, Invitrogen) were added, and the mixture was incubated at 25°C for 10 min and then  
4 at 42°C for 2 min. After that, 1  $\mu$ l reverse transcriptase (200 U; SuperScript II, Invitrogen)  
5 was added, and the mixture was incubated at 42°C for 90 min, and then 70°C for 15 min.  
6 The real-time PCR reaction was carried out in a 50- $\mu$ l (total volume) mixture containing  
7 25  $\mu$ l 2x TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA,  
8 USA), 900 nM each of forward and reverse primers, 200 nM TaqMan probe specific for  
9 the target gene, and 5  $\mu$ l of the cDNA sample. The amplification and detection of specific  
10 products were performed with the ABI PRISM 7000 sequence detection system (Applied  
11 Biosystems) using the following profile: incubation at 50°C for 2 min, 95°C for 10 min,  
12 and 40 cycles at 95°C for 15 s and at 60°C for 1 min. Data analysis was performed using  
13 the ABI PRISM 7000 sequence detection system software (v 1.0; Applied Biosystems).  
14 Each sample was analyzed in duplicate. The sequences of the TaqMan probes and primers  
15 for the target genes were as follows: *ndh* (Probe:  
16 5'-FAM-CTGCTGCGGCCCAACGAG-TAMRA-3'; Forward primer:  
17 5'-TGCGTTACCGCCACGTATC-3'; Reverse primer:  
18 5'-ACGCGAACGCCAAGTTTC-3'); *cyoA* (Probe:  
19 5'-FAM-TTCCCGCAATCTTGATGGCT-TAMRA-3'; Forward primer:  
20 5'-GGCCTGATGTTGATTGTCGTT-3'; Reverse primer:  
21 5'-GGTACTTCCAGGCGAAACCA-3'); *cydA* (Probe:  
22 5'-FAM-TTGCCTTGACCGCGATGTACCACTTC-TAMRA-3'; Forward primer:  
23 5'-TCGAACTGTCGCGCTTACAG-3'; Reverse primer:

1 5'-CGAGCGTCAGTGGCACAA-3'). For the endogenous control, the 16S rRNA gene  
2 *rrsA* was used (Probe: 5'-FAM-CCGGGCCTTGTACACACCGCC-TAMRA-3'; Forward  
3 primer: 5'-GAATGCCACGGTGAATACGTT-3'; Reverse primer:  
4 5'-ACCCACTCCCATGGTGTGA-3'). A relative standard curve method was used to  
5 calculate the relative expression level of the target gene. The expression ratio was  
6 obtained by dividing the relative expression level of the mutant by that of the parent.

7 **Measurement of enzymes in central carbon metabolism.** Cells were  
8 harvested by centrifugation, washed with an appropriate buffer, and kept at  $-20^{\circ}\text{C}$  until  
9 use. The cells were disrupted by sonication in the same buffer, and the cell debris was  
10 removed by centrifugation at  $39,000 \times g$  at  $4^{\circ}\text{C}$  for 40 min. The supernatant was  
11 gel-filtered using a PD-10 column (GE Healthcare Bio-Sciences) with the same buffer to  
12 remove low-molecular-weight materials. The eluate was used as the crude enzyme for the  
13 assay. The composition of the buffer system is described in the assay conditions of the  
14 respective enzymes. Enzyme activity was monitored spectrophotometrically using a  
15 Beckman DU 7400 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) at  
16  $25^{\circ}\text{C}$ . The protein concentration of the crude enzyme was determined using the Bio-Rad  
17 Protein Assay (Bio-Rad Laboratories), with bovine serum albumin as the standard. The  
18 specific activity of each enzyme under the assay conditions was expressed as  $\text{nmole min}^{-1}$   
19  $(\text{mg protein})^{-1}$ . For pyruvate dehydrogenase (PDH), 50 mM potassium phosphate buffer  
20 (pH 8.1) was used as the washing and extraction buffer. The reaction mixture consisted of  
21 50 mM potassium phosphate buffer (pH 8.1), 0.05 mM CoASH, 3 mM L-cysteine, 2.33  
22 mM  $\text{NAD}^{+}$ , 0.2 mM thiamine pyrophosphate, 1 mM  $\text{MgSO}_4$ , 2 mM sodium pyruvate, and  
23 the crude enzyme. The reaction was initiated by the addition of sodium pyruvate, and the

1 NADH concentration increase was monitored at 340 nm (39). For acetate kinase (ACK),  
2 50 mM of imidazole-HCl buffer (pH 7.3) containing 10 mM MgCl<sub>2</sub> was used as the  
3 washing and extraction buffer. The reaction mixture consisted of 50 mM imidazole-HCl  
4 buffer (pH 7.3), 10 mM MgCl<sub>2</sub>, 12 mM acetyl phosphate, 5 mM ADP, 10 mM glucose,  
5 1.6 mM NADP, hexokinase (56 U/ml), and glucose 6-phosphate-dehydrogenase (1.5  
6 U/ml). The reaction was initiated by the addition of ADP, and ATP formation was  
7 monitored by the increase of the NADPH concentration at 340 nm (31). For citrate  
8 synthase (CS), 20 mM Tris-HCl (pH 8.0) containing 10 mM MgCl<sub>2</sub> and 1 mM EDTA was  
9 used as the washing and extraction buffer. Activity was measured in the reaction mixture,  
10 which contained 100 mM Tris-HCl buffer (pH 8.0), 0.16 mM acetyl-CoA, 0.2 mM  
11 oxaloacetic acid, and 0.1 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The reaction  
12 was initiated by the addition of oxaloacetic acid. The CoA yield was monitored by the  
13 absorbance increase at 412 nm. The molecular extinction coefficient of 13,600 M<sup>-1</sup> cm<sup>-1</sup>  
14 for 5-mercapto-2-nitrobenzoic acid was used to calculate the enzyme activity (36). For  
15 succinyl-CoA synthetase (SCS) (7), 20 mM potassium phosphate buffer (pH 7.2)  
16 containing 20 mM MgCl<sub>2</sub> was used as the washing and extraction buffer. The reaction  
17 mixture contained 50 mM Tris-HCl buffer (pH 7.2), 10 mM MgCl<sub>2</sub>, 100 mM KCl, 10 mM  
18 sodium succinate, 0.1 mM CoASH, and 0.4 mM ATP. The reaction was initiated by the  
19 addition of ATP, and the formation of succinyl-CoA was monitored by the absorbance  
20 increase at 230 nm. For succinyl-CoA, we used the molar extinction coefficient of 4900  
21 M<sup>-1</sup>cm<sup>-1</sup> at 230 nm to calculate enzyme activity. For malate dehydrogenase (MDH), 7 mM  
22 potassium phosphate buffer (pH 7.0) containing 30% glycerol and 3.5 mM DTT was used  
23 as the washing and extraction buffer. The reaction mixture consisted of 100 mM

1 potassium phosphate buffer (pH 7.2), 0.13 mM NADH, and 0.33 mM oxaloacetic acid.  
2 The reaction was initiated by the addition of oxaloacetic acid. The decrease of NADH,  
3 coupled with the formation of malate, was monitored at 340 nm (26).

4 **Measurement of enzymes in the respiratory chain.** Cells were washed with  
5 50 mM potassium phosphate buffer (pH 7.5) containing 5 mM MgSO<sub>4</sub>, 1 mM DTT, and  
6 10% glycerol, resuspended in the same buffer, and then disrupted twice using a French  
7 pressure cell (Ohtake Works, Tokyo, Japan) at 16,000 psi. The mixtures were centrifuged  
8 at 8,000 x g at 4°C for 10 min, and the supernatant was ultracentrifuged at 120,000 x g, at  
9 0°C for 2 h. The membrane fraction was suspended by homogenization with a  
10 teflon-coated homogenizer in the same buffer and used as the crude enzyme for the  
11 NADH dehydrogenase (NDH) assay. The activities of the NDHs were measured by  
12 monitoring the decrease of the NADH or deamino-NADH concentration at 340 nm. The  
13 reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.5), 5 mM MgSO<sub>4</sub>,  
14 and 0.125 mM of either NADH or deamino-NADH substrate. The reaction was initiated  
15 by the addition of the crude enzyme. As deamino-NADH is the substrate for NDH-1 but  
16 not for NDH-2 (25), the NDH-2 activity was calculated by subtracting the  
17 deamino-NADH oxidase activity (NDH-1 activity) from the NADH oxidase activity  
18 (total NDH activity). The protein concentration of the crude enzyme was determined  
19 using the Bio-Rad Protein Assay (Bio-Rad Laboratories), with bovine serum albumin as  
20 the standard. The molar extinction coefficient of 6220 M<sup>-1</sup>cm<sup>-1</sup> at 340 nm for both  
21 substrates was used to calculate the specific activity, which was expressed as nmole min<sup>-1</sup>  
22 (mg protein)<sup>-1</sup>. The aerobic respiratory chain of *E. coli* contains two types of terminal  
23 oxidases, cytochrome *bo*<sub>3</sub> oxidase and cytochrome *bd* oxidase. The *bo*<sub>3</sub>-type oxidase is

1 more efficient ( $2\text{H}^+/\text{e}^-$ ) than the *bd*-type oxidase ( $1\text{H}^+/\text{e}^-$ ) in creating the electrochemical  
2 gradient of protons. The activities of these oxidases cannot be measured separately, so the  
3 total activity was measured as ubiquinol-2 ( $\text{Q}_2\text{H}_2$ ) oxidase activity. The crude enzyme for  
4 the assay of  $\text{Q}_2\text{H}_2$  oxidase activity was prepared in the same manner as described for the  
5 NDHs, except that DTT and glycerol were omitted from the buffer used for cell washing  
6 and disruption. The measurement was conducted with  $30\ \mu\text{M}$   $\text{Q}_2\text{H}_2$  in  $50\ \text{mM}$  potassium  
7 phosphate buffer (pH 7.5) containing 0.1% Tween 20. The absorbance increase at 275 nm  
8 was monitored after the enzyme was added to start the reaction. The protein concentration  
9 of the crude enzyme was measured by the modified Lowry method with bovine serum  
10 albumin as the standard (12). The molar extinction coefficient of  $12,250\ \text{M}^{-1}\text{cm}^{-1}$  at 275  
11 nm was used to calculate the specific activity, which was expressed in  $\text{nmole min}^{-1}$  ( $\text{mg}$   
12  $\text{protein}^{-1}$ ).

13           **Immunoblot analysis of the terminal oxidases.** Immunoblot analysis was  
14 conducted to investigate the abundance of each of the terminal oxidases. The membrane  
15 preparations used for the  $\text{Q}_2\text{H}_2$  oxidase assay were subjected to SDS-polyacrylamide gel  
16 electrophoresis (SDS-PAGE;  $23\ \mu\text{g}$  protein per lane). After electrophoresis, the protein  
17 bands in the gel were transferred electrophoretically onto a polyvinylidene fluoride  
18 (PVDF) membrane (Millipore Corporation, Billerica, MA, USA) at 100 mA for 4 h. After  
19 blocking with 3% gelatin and washing, the membrane was incubated for 2 h with  
20 anti-cytochrome *bo*<sub>3</sub> or anti-cytochrome *bd* antibody. After incubated for 2 h with Protein  
21 A-peroxidase, the protein bands were visualized by the addition of color reagents and  
22  $\text{H}_2\text{O}_2$ . Prestained marker proteins (Bio-Rad Laboratories) were used to estimate the  
23 relative molecular weights. Anti-cytochrome *bo*<sub>3</sub> serum was obtained against the

1 cytochrome *bo*<sub>3</sub> purified from *E. coli* (Matsushita, K., unpublished) and was used at a  
2 50-fold dilution. Dr. Tatsushi Mogi (ATP System Project, ERATO, JST) kindly supplied  
3 the anti-cytochrome *bd* serum. The following pretreatment was carried out before use: the  
4 anti-cytochrome *bd* serum (0.1 ml) was mixed with 1 ml (~10 mg/ml) of the membranes  
5 suspension prepared from *E. coli* GO103 ( $\Delta$ *cydAB*) (30) and then incubated at 30°C for 2  
6 h. The mixture was centrifuged at 10,000 x *g* for 10 min to obtain the supernatant, which  
7 was used as the antibody after a 20-fold dilution.

8

## 9 **RESULTS**

10

11 **Enhanced glucose metabolism in an F<sub>1</sub>-ATPase-defective mutant HBA-1,**  
12 **as revealed by chemostat cultures.** To evaluate the effects of an F<sub>1</sub>-ATPase defect on  
13 glucose metabolism, the mutant HBA-1 and its parent W1485 were cultured in M9  
14 minimal medium in a glucose-limited chemostat at the same growth rate ( $D = 0.2 \text{ h}^{-1}$ ), and  
15 the fermentation parameters of both strains were calculated. As shown in Table 1, the  
16 mutant exhibited reduced biomass production (60%), with increased specific rates of both  
17 glucose consumption (168%) and respiration (171%), compared with the parent. Analysis  
18 of the fermentation products revealed a substantial excretion of acetate by the mutant,  
19 whereas no acetate excretion was observed for the parent. These results clearly  
20 demonstrated that glucose metabolism by the F<sub>1</sub>-ATPase-defective mutant was enhanced,  
21 in agreement with previous observations in different *atp* mutants (21, 40).

22 **Flux analysis.** Based on the observed fermentation parameters (Table 1), the  
23 metabolic fluxes within the central metabolic pathways were estimated by a

1 stoichiometric approach. As shown in Fig. 1, the glycolytic flux distribution in HBA-1  
2 appeared to be approximately twice that in W1485. However, its distribution in the TCA  
3 cycle was only 18% higher in the mutant than in the parent. This difference was due to the  
4 acetate excretion observed for HBA-1, whereby the increased flux within the glycolytic  
5 pathway overflows, thereby reducing the flow of acetyl-CoA entering the TCA cycle.  
6 Increased flow of carbon through glycolytic pathway and in TCA cycle and increased  
7 excretion of acetate were also observed in a different *atp* mutant growing in glucose  
8 minimal medium under batch culture conditions (21).

9       **Transcriptome analysis.** To determine whether these alterations in glucose  
10 metabolism were accompanied by alterations in gene expression, a DNA array analysis  
11 was conducted to compare the genomic expression profiles of the parent and mutant. In  
12 general, the differences in the expression levels of most genes appeared to be relatively  
13 small (less than two-fold) and not statistically significant. Moreover, when differences  
14 were detected, most of the genes showed decreased expression levels in the mutant. The  
15 similar tendency was observed in the DNA array analysis using cells cultured at a 50%  
16 higher dilution rate ( $D = 0.3 \text{ h}^{-1}$ ; data not shown). The results are summarized in Table 2,  
17 which lists only the genes of known function and relevance and shows the different  
18 expression levels in the two strains. Most striking is the absence of the genes involved in  
19 glycolysis, despite the enhanced glucose metabolism in the mutant. In contrast, the  
20 expression levels of several genes (*gltA*, *icdA*, *sucA*, *sucB*, *sucD*, and *mdh*) coding for  
21 enzymes in the TCA cycle were significantly decreased, to about 50% of the parental  
22 levels. Of these, the decreased expression of *gltA* (44% of the parental level), which codes  
23 for citrate synthase, appears to be important in the downregulation of the flux entering

1 into the TCA cycle. Also *aceE* and *aceF* (126% of the parental level), which code for  
2 enzymes of the PDH complex, showed a tendency to upregulate. We consider these  
3 changes, in combination with reduced expression of *gltA*, to favor the redirection of the  
4 glycolytic flux into acetate formation in the mutant (Table 1, Fig. 1). Moreover, the  
5 expression of the genes coding for the glyoxylate-shunt enzymes *aceA* and *aceB* were  
6 significantly decreased in the mutant (about 60% of the parental level). However, the  
7 most interesting finding involves the elevated expression of some genes coding for  
8 respiratory chain enzymes. Although the differences were not as prominent, the  
9 expression of *ndh*, coding for NDH-2, and *cydA*, coding for cytochrome *bd* oxidase  
10 subunit I, were elevated to about 130% of the parental levels. The *cyoA* expression also  
11 showed a tendency toward upregulation, but this was not statistically significant. These  
12 data suggest that increased respiration in the mutant may be accompanied by alterations  
13 in the composition of the respiratory enzymes, which are regulated at the transcription  
14 level. In addition to the genes involved in central metabolism, several genes coding for  
15 flagella formation and cellular structures (*ompF*) appeared to be significantly repressed.  
16 The expression levels of *flhC* and *flhD*, which code for a flagella transcriptional activator,  
17 were about 45% below those of the parent, and other flagellar genes (*fliC*, *fliD*, *flgB*, *flgC*,  
18 and *flgL*) were also repressed. Among the regulatory function genes, only *hns*, coding for  
19 the histone-like protein H-NS, was repressed in the mutant (~50%). Given that H-NS is  
20 an abundant DNA-binding protein involved in numerous cellular processes, including the  
21 replication, recombination, and transcriptional regulation of a large number of genes, a  
22 decrease in H-NS protein may have profound effects on *E. coli* cell physiology (33).  
23 Besides genes listed in Table 2, the following genes were worth to be referred to as

1 significantly downregulated ones in the mutant (~50%) with known function but with  
2 apparently less physiological relevance in response to bioenergetic stress: *hupA* and *hupB*  
3 (DNA-binding protein HU), *topA* (DNA topoisomerase I, omega protein I), *grpE* (heat  
4 shock protein GrpE), *htpG* (heat shock protein HtpG), *mopB* (GroES protein), *cspD* (cold  
5 shock-like protein CspD).

6           To verify the results of the DNA array analysis (Table 2), the expression of four  
7 selected genes was monitored by either real-time PCR assay (*ndh*, *cyoA*, and *cydA*) or  
8 Northern blot analysis (*hns*). The results, summarized in Table 3, showed comparable  
9 tendencies between the DNA array experiments and the real-time PCR assay or Northern  
10 blot analysis. Although we did not monitor all the genes listed in Table 2, these results  
11 illustrate the reliability of the DNA array experiments for screening and locating  
12 important changes in the mutant at the level of transcription.

13           **Enzyme activity in central carbon metabolism.** Based on the results of the  
14 fermentation analysis (Table 1, Fig. 1) and the determination of the genomic expression  
15 profile (Table 2), several enzymes important to central carbon metabolism were measured  
16 to substantiate the observed metabolic changes, especially acetate production, in the  
17 mutant (Table 4). In the mutant, the activity of the PDH complex, a key enzyme complex  
18 in supplying acetyl-CoA, was twice that in the parent, whereas the activities of three TCA  
19 cycle enzymes (citrate synthase, succinyl-CoA synthetase, and malate dehydrogenase) in  
20 the mutant appeared to be about half those in the parent. These results agree well with the  
21 results obtained in the transcriptome analysis (Table 2), thereby providing validation at  
22 the level of enzyme activity. For further insight into the mechanism of acetate production  
23 in the mutant, we measured acetate kinase activity, although the transcriptome analysis

1 (Table 2) detected no difference in this enzyme. However, as shown in Table 4, the acetate  
2 kinase activity in the mutant was 155% that in the parent. Thus, acetate production in the  
3 mutant appears to be triggered by increased acetyl-CoA accumulation attributable to the  
4 elevated activity of the PDH complex and the reduced activity of TCA cycle enzymes,  
5 especially citrate synthase. The accumulated acetyl-CoA is then readily metabolized into  
6 acetate through the acetate kinase-phosphotransacetylase pathway, in which acetate  
7 kinase activity is elevated.

8         **Total activities of NDHs and terminal oxidases.** The enhanced rate of  
9 respiration (Table 1) as well as the transcriptional upregulation of *ndh* and *cydA* (Tables 2  
10 and 3) observed in the mutant led us to measure the total activity of NDHs and terminal  
11 oxidases. Two types of NDHs are known in the *E. coli* respiratory chain, NDH-1 and  
12 NDH-2 (25). NDH-1 couples the oxidation of NADH to the creation of the  
13 electrochemical gradient of protons, whereas NDH-2 does not. Thus, NDH-1 is primarily  
14 important for energy recovery from NADH oxidation, and NDH-2 is thought to work as a  
15 bypass to modulate electron flow in response to the growth environment. As shown in Fig.  
16 2A, the total activity of the NDHs (NDH-1 + NDH-2) was increased 2.3-fold in the  
17 mutant compared with the parent. The mutant also showed a 1.8-fold increase in the total  
18 activity of the terminal oxidases (cytochrome *bo*<sub>3</sub> oxidase + cytochrome *bd* oxidase; Fig.  
19 2B). These changes in respiratory enzyme activity correspond to the increased respiration  
20 rate in the mutant (1.7-fold; Table 1).

21         **Analysis of the proportions of the NDHs.** The proportion of each NDH  
22 isozyme in the mutant (Fig. 2A) was particularly striking. In the parent, total NDH  
23 activity was composed of about 60% NDH-1 and 40% NDH-2. However, the NDH-2

1 activity in the mutant was dramatically elevated to 3.7-fold that in the parent, whereas  
2 only a slight increase (1.3-fold) was detected in the NDH-1 activity. Therefore, we  
3 concluded that the increased total activity of the NDHs was attained primarily through the  
4 preferential increase of NDH-2 activity in the mutant, which concurs with the observed  
5 upregulation of *ndh* transcription in the mutant (Tables 2 and 3). Consequently, NDH-2  
6 activity is predominant in the mutant, comprising up to about 70% of total NDH activity,  
7 in contrast to about 40% in the parent.

8           **Analysis of the proportions of the terminal oxidases.** The increased total  
9 activity of the terminal oxidases in the mutant (Fig. 2B) prompted us to analyze in detail a  
10 possible alteration in the proportion of the terminal oxidases (cytochrome *bo*<sub>3</sub> oxidase +  
11 cytochrome *bd* oxidase). Thus, we have conducted an immunoblot analysis for the  
12 terminal oxidases to gain insight into the cytochrome components of the membrane (Fig.  
13 3). The band corresponding to subunit I of the *bd*-type oxidase was more abundant in the  
14 membrane of the mutant (lane 1) than of the parent (lane 2), whereas the bands for  
15 subunits I and II of the *bo*-type oxidase in the membranes did not differ much between the  
16 mutant (lane 3) and parent (lane 4). These results correspond to the transcriptional  
17 upregulation observed for *cydA* in the mutant (Tables 2 and 3) and indicate an increase in  
18 the concentration of the *bd*-type oxidase relative to the *bo*-type oxidase in the mutant.

19

## 20 **DISCUSSION**

21

22           This study illustrated the overall alterations in an *E. coli* K-12 cell that are  
23 associated with defective oxidative phosphorylation due to a mutation in the F<sub>1</sub>-ATPase.

1 The results appear to explain how metabolic changes leading to enhanced glucose  
2 consumption are possible in response to energy shortages caused by the F<sub>1</sub>-ATPase  
3 defect.

4 The use of a glucose-limited chemostat for culturing the F<sub>1</sub>-ATPase-defective  
5 mutant and its parent enabled a precise characterization of both strains growing  
6 exponentially at the same rate ( $D = 0.2 \text{ h}^{-1}$ ). The enhanced glucose metabolism in the  
7 mutant, as revealed by the fermentation parameters (Table 1), was further substantiated  
8 by flux analysis (Fig. 1). We calculated twice as much flux through the glycolytic  
9 pathway in the mutant. However, we obtained no evidence from the DNA array analysis  
10 to suggest the transcriptional upregulation of genes involved in glycolysis (Table 2). Thus,  
11 we suggest that the enhanced glycolytic flux was brought about by the allosteric  
12 activation of the key enzymes of this pathway, phosphofructokinase I (activation by  
13 ADP) (2) and pyruvate kinase II (activation by AMP) (21), under the reduced  
14 [ATP]/[ADP] ratio. Interestingly, in the mutant, the flux through the TCA cycle was only  
15 18% higher than that in the parent, owing to a redirection of the flux into acetate (Fig. 1),  
16 which suggests a stringent metabolic regulation to prevent the flow of the glycolytic  
17 pathway from entering the TCA cycle. Results from the enzymatic activities (Table 4)  
18 revealed increased activities of the PDH complex and acetate kinase and decreased  
19 activities of several TCA cycle enzymes, including citrate synthase. The flux of the TCA  
20 cycle in *E. coli* has been shown to be controlled by citrate synthase through feedback  
21 inhibition by NADH as a negative effector (37). As the rate of NADH formation in the  
22 mutant with enhanced glucose metabolism would be higher than that in the parent, this  
23 inhibition, together with the observed alterations in the enzyme activities of the PDH

1 complex, citrate synthase, and acetate kinase, might direct the glycolytic flux into acetate.  
2 From an energetic point of view, the number of ATPs generated by substrate-level  
3 phosphorylation from either the metabolism of acetyl-CoA through the TCA cycle (ATP  
4 generation at the succinyl-CoA synthetase reaction) or the acetate pathway (generation at  
5 the acetate kinase reaction) is the same (Fig. 1). Therefore, the physiological importance  
6 of the redirection of the glycolytic flux into acetate is not thought to be related to  
7 substrate-level phosphorylation in the acetate pathway, but rather to result from a  
8 different aspect. The best explanation is the suppression of additional NADH formation  
9 through the TCA cycle, because the F<sub>1</sub>-ATPase-defective mutant had already generated  
10 excess NADH by enhanced glycolysis. As shown in Table 2, the downregulated genes of  
11 the TCA cycle enzymes include three (*icdA*, *sucA*, and *mdh*) coding for dehydrogenases  
12 generating NADH. These changes, together with reduced citrate synthase activity, appear  
13 to reduce NADH formation by the reduced metabolism of acetyl-CoA through the TCA  
14 cycle. Furthermore, the downregulation of genes (*aceA*, *aceB*) coding for enzymes in the  
15 glyoxylate shunt was also demonstrated (Table 2), which could also reduce NADH  
16 formation through malate dehydrogenase, while saving acetyl-CoA for substrate-level  
17 phosphorylation coupled with acetate formation (acetate kinase) or the TCA cycle  
18 (succinyl-CoA synthetase).

19 In this study, we showed that both the reduction of flow in the TCA cycle and  
20 the alteration of respiratory chain components to increase the respiration rate are  
21 necessary for the F<sub>1</sub>-ATPase-defective mutant to achieve enhanced glucose metabolism.  
22 As shown in Figs. 2A and 3, preferential increases in NDH-2 activity and cytochrome *bd*  
23 oxidase content were discovered in the respiratory chain of the F<sub>1</sub>-ATPase-defective

1 mutant. As components of the respiratory chain of *E. coli*, each NDH and terminal  
2 oxidase isozyme exhibits a different efficiency in generating the electrochemical gradient  
3 of protons coupled with electron transfer (5, 14): NDH-1 ( $2\text{H}^+/\text{e}^-$ ) and NDH-2 ( $0\text{H}^+/\text{e}^-$ );  
4 *bo*-type oxidase ( $2\text{H}^+/\text{e}^-$ ), and *bd*-type oxidase ( $1\text{H}^+/\text{e}^-$ ). The increased components of the  
5 respiratory chain are bioenergetically less effective, and the net result is that the mutant  
6 can recycle the excess NADH formed in its enhanced central metabolism, thus avoiding  
7 the generation of excess proton-motive force. In fact, a 20% higher membrane potential  
8 has been measured in the *atp*-deletion mutant (21). Therefore, this alteration in the  
9 respiratory components seems beneficial from a bioenergetics point of view for enabling  
10 the mutant to maintain homeostasis. The observed alterations in the respiratory chain  
11 components are a novel finding of an adaptive response in the  $\text{F}_1$ -ATPase-defective  
12 mutant (probably common to all *atp* mutants), and this is in accord with the  
13 aforementioned metabolic redirection strategy that limits NADH formation in the TCA  
14 cycle. Another interesting aspect was the mechanism for the transcriptional upregulation  
15 of NDH-2 and *bd*-type oxidase in response to the *atp* mutation (Tables 2 and 3). Under  
16 anaerobic conditions, the expression of *ndh*, which codes for NDH-2, is subject to  
17 repression by Fnr, the *fnr* (fumarate nitrate reduction) gene product (17). Under aerobic  
18 conditions, Fis (the *fis* gene product; a factor for inversion stimulation) exhibits a growth  
19 phase-dependent modulation of transcription from the *ndh* promoter. In the early  
20 logarithmic growth phase, when Fis expression is maximal, *ndh* expression is repressed  
21 by Fis, thus ensuring that energetically efficient NDH-1 is used. This repression is  
22 relieved at the stationary phase, when Fis expression decreases. Thus, NDH-2 seems to be  
23 fully expressed when cellular energy is sufficient (16). In this context, the mechanism of

1 the transcriptional upregulation of *ndh* in the F<sub>1</sub>-ATPase-defective mutant is difficult to  
2 interpret and needs to be clarified in future work. On the other hand, the *cydAB* operon,  
3 coding for the *bd*-type oxidase, has been shown to be regulated by the interplay of three  
4 global regulatory proteins, Fnr, ArcA (aerobic respiration control; *arcA* gene product),  
5 and H-NS, in such a way that its expression is maximal under microaerobic conditions  
6 (11, 15, 19, 20). This is physiologically important because the *bd*-type oxidase has a high  
7 affinity for oxygen, thereby working effectively under microaerobic conditions. Under  
8 aerobic conditions, however, the expression of the *cydAB* operon is normally regulated at  
9 a low level because of repression by H-NS (15). In the F<sub>1</sub>-ATPase-defective mutant,  
10 *bd*-type oxidase content increased even under aerobic conditions (Fig. 3). Interestingly, in  
11 the mutant, the expression of H-NS appeared to be repressed to half that of the parent  
12 (Tables 2 and 3). Thus, it seems reasonable to attribute the increase of *bd*-type oxidase  
13 content to the decrease of H-NS protein.

14           The transcription of seven genes involved in flagellar biogenesis and *ompF*  
15 coding for porin was found to be downregulated in the mutant (Table 2). The genes *flhC*  
16 and *flhD* constitute the master operon the expression of which switches on the expression  
17 of all the other genes involved in flagellar biogenesis (10). As *flhC* and *flhD* are  
18 downregulated to less than half in the mutant (Table 2), the expression of the other genes  
19 (*flgB*, *flgC*, *flgL*, *fliC* and *fliD*) seems to be affected accordingly. Once again, a decreased  
20 expression of *hns* is implicated in these phenomena, because H-NS has been  
21 demonstrated to be the positive transcriptional regulator of the *flhDC* operon in vivo (35),  
22 and the *hns* mutation has been shown to cause a loss of motility due to the lack of flagella  
23 (3). The advantages of these responses are not clear. However, the reduced synthesis of

1 such a large multi-component apparatus (flagellum) and one of the most abundant  
2 proteins in *E. coli* in terms of mass (porin) may contribute to cost-savings in biosynthesis  
3 (24, 28), especially in an *atp* mutant, in which the ATP supply is limited.

4           In this study, we clarified a series of physiological changes associated with an  
5 F<sub>1</sub>-ATPase-defective mutation in *E. coli*. The mutation produced not only alterations in  
6 central carbon metabolism but also changes in respiratory chain and cellular structure  
7 components. The overall results illustrate a novel, yet reasonable, strategy enabling *E.*  
8 *coli* to survive energetically difficult conditions brought about by impaired oxidative  
9 phosphorylation. Although experimental evidence is lacking, the observed qualitative  
10 changes in the mutant, especially the downregulation of TCA cycle enzymes and the  
11 upregulation of cytochrome *bd* oxidase, are associated with the operation of the global  
12 control network(s), such as the Arc two-component system. The possibility of the  
13 involvement of some global control network in the adaptive response of the *atp* mutant  
14 and the identification of the signal that is sensed by the network(s) remain to be  
15 elucidated.

16

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18

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3

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1 Table 1. Parameters in the chemostat cultures.

2

3 Strain	Y <sub>CELL</sub> <sup>a</sup>	4 Fluxes <sup>b</sup>			Respiration <sup>c</sup>
		Glucose	Acetate	2-OGA	
5 W1485	0.056 (1) <sup>d</sup>	3.57 (1)	0.00	0.20	14.4 (1)
6 HBA-1	0.033 (0.6)	6.00 (1.68)	4.30	0.10	24.6 (1.71)

7

8 An F<sub>1</sub>-ATPase-defective mutant, HBA-1, and its parent strain, W1485, were cultured  
9 under glucose-limited chemostat conditions in M9 minimal medium at a dilution rate of  
10 0.2 h<sup>-1</sup>, as described in Materials and Methods.

11 <sup>a</sup> Y<sub>CELL</sub>, cell yield in g dry cell/mmol glucose.

12 <sup>b</sup> Fluxes, consumption of glucose or excretion of acetate or 2-oxo-glutarate (2-OGA) in  
13 mmol (g dry cell)<sup>-1</sup> h<sup>-1</sup>.

14 <sup>c</sup> mmol O<sub>2</sub> h<sup>-1</sup> (g dry cell)<sup>-1</sup>.

15 <sup>d</sup> Fold difference between strains W1485 and HBA-1.

TABLE 2. Summary of genes showing different expression levels between strains W1485 and HBA-1 grown in glucose-limited chemostat culture, as revealed by DNA array analysis<sup>a</sup>.

Gene	Gene product	Averaged spot intensities <sup>b</sup>		Standard deviation		Ratio <sup>c</sup>
		W1485	HBA-1	W1485	HBA-1	(HBA-1/ W1485)
<i>aceE</i>	pyruvate dehydrogenase E1 component	1.18E -04	1.48E -04	2.09E -05	3.27E -05	1.26
<i>aceF</i>	E2 of pyruvate dehydrogenase	1.52E -04	1.92E -04	2.79E -05	3.52E -05	1.26*
<i>gltA</i>	citrate synthase	4.05E -04	1.80E -04	7.80E -05	6.03E -05	0.45*
<i>icdA</i>	isocitrate dehydrogenase	2.95E -04	1.80E -04	4.53E -05	2.27E -05	0.61*
<i>sucA</i>	2-oxoglutarate dehydrogenase E1 component	1.34E -04	7.63E -05	3.45E -05	2.25E -05	0.57*
<i>sucB</i>	dihydroliipoamide succinyltransferase component (E2)	1.29E -04	6.19E -05	2.94E -05	1.52E -05	0.48*
<i>sucD</i>	succinyl-CoA synthetase $\alpha$ chain	4.86E -04	1.72E -04	1.87E -04	8.94E -05	0.35*
<i>mdh</i>	malate dehydrogenase	3.18E -04	1.34E -04	1.14E -04	3.66E -05	0.42*

<i>aceA</i>	isocitrate lyase	2.37E -04	1.52E -04	5.90E -05	3.01E -04	0.64*
<i>aceB</i>	malate synthase A	3.82E -04	2.22E -04	7.72E -05	9.11E -05	0.58*
<i>ndh</i>	NADH dehydrogenase II	8.25E -05	1.09E -04	1.18E -05	1.94E -05	1.32*
<i>nuoA</i>	NADH dehydrogenase I chain A	5.78E -05	4.96E -05	1.27E -05	8.07E -06	0.86
<i>cydA</i>	cytochrome <i>d</i> oxidase subunit I	3.00E -04	4.02E -04	1.61E -04	2.43E -04	1.34*
<i>cyoA</i>	cytochrome <i>o</i> oxidase subunit II	3.24E -04	4.19E -04	1.51E -04	2.49E -04	1.30
<i>flhC</i>	flagellar transcriptional activator	1.99E -04	8.96E -05	4.14 E-07	8.26E -06	0.45 *
<i>flhD</i>	flagellar transcriptional activator	2.57E -04	1.12E -04	2.68E -07	2.28E -05	0.44*
<i>fliC</i>	flagellin	8.68E -04	3.64E -04	4.55E -05	1.00E -04	0.42*
<i>fliD</i>	flagellar hook-associated protein 2	1.43E -04	8.07E -05	1.68E -05	2.70E -05	0.56*
<i>flgB</i>	putative flagellar basal-body rod protein FlgB	3.19E -04	1.85E -04	1.85E -05	6.18E -05	0.58*
<i>flgC</i>	putative flagellar basal-body rod protein FlgC	1.52E -04	8.88E -05	6.24E -06	2.77E -05	0.58*
<i>flgL</i>	flagellar hook-associated protein 3	2.18E -04	1.20E -04	9.01E -06	1.79E -05	0.55*
<i>ompF</i>	outer membrane protein F precursor	2.58E -03	1.21E -03	5.83E -04	3.00E -04	0.47*

<i>nmpC</i>	outer membrane porin protein NmpC precursor	1.60E -04	1.10E -04	5.19E -05	1.84E -05	0.69*
<i>hns</i>	histone-like protein H-NS	1.20E -04	6.06E -04	3.18E -04	3.57E -04	0.50*

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<sup>a</sup> The results are from eight independent DNA array analyses.

<sup>b</sup> Expressed as the percentage of the total intensity of all the spots on the DNA array.

<sup>c</sup> Expression ratio of HBA-1 to W1485. The asterisk indicates significant *t*-test differences ( $p < 0.05$ ).

Table 3. Comparison of the expression ratios of several genes, as determined by DNA array and Northern blot analysis or real-time PCR analysis.

Gene	Expression ratio (HBA-1/W1485)		
	DNA array <sup>a</sup>	Northern blot <sup>c</sup>	Real-time PCR <sup>c</sup>
<i>ndh</i>	1.32*	ND <sup>b</sup>	2.40
<i>cyoA</i>	1.30	ND	1.67
<i>cydA</i>	1.34*	ND	1.38
<i>hns</i>	0.50*	0.67	ND

<sup>a</sup> Adapted from Table 1. The asterisk indicates significant *t*-test differences ( $p < 0.05$ ).

<sup>b</sup> Not determined.

<sup>c</sup> The assays were repeated two to three times.

Table 4. Activity of several central carbon metabolism enzymes.

Enzyme	Specific activity <sup>a</sup>		Ratio (HBA-1/ W1485)
	(nmol/min/mg)		
	W1485	HBA-1	
PDH complex	74	152	2.05
Acetate kinase	2532	3933	1.55
Citrate synthase	597	375	0.63
Succinyl-CoA synthetase	384	217	0.57
Malate dehydrogenase	13064	6039	0.46

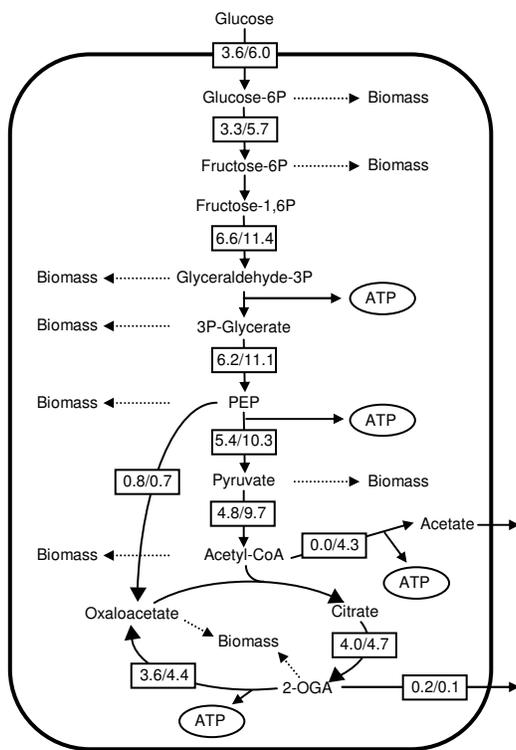
<sup>a</sup> Average values from at least two independent experiments.

(Figure legends)

Fig. 1. Flux analysis in the central metabolic pathways of the parent and mutant. The values in the boxes show the flux of the parent, W1485 (left), and the mutant, HBA-1 (right), in  $\text{mmol (g dry cell)}^{-1} \text{ h}^{-1}$ .

Fig. 2. Analysis of respiratory chain components based on enzyme activity measurements, expressed as  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ . (A) NDH-1 activity: W1485,  $267 \pm 125$  (n = 10); HBA-1,  $338 \pm 110$  (n = 8). NDH-2 activity: W1485,  $197 \pm 87$  (n = 10); HBA-1,  $723 \pm 112$  (n = 8). (B)  $\text{Q}_2\text{H}_2$  oxidase activity: W1485,  $1400 \pm 14$  (n = 2); HBA-1,  $2460 \pm 594$  (n = 2).

Fig. 3. Immunoblot analysis of terminal oxidases. After SDS polyacrylamide gel electrophoresis of the membrane preparations of the parent and mutant, the protein bands were transferred to PVDF membranes, and then probed with anti-cytochrome  $bo_3$  serum (*bo*) or anti-cytochrome *bd* serum (*bd*). Lanes 1 and 3, HBA-1; Lanes 2 and 4, W1485; M, marker proteins. The data shown are representative of at least three independent experiments that gave similar results.



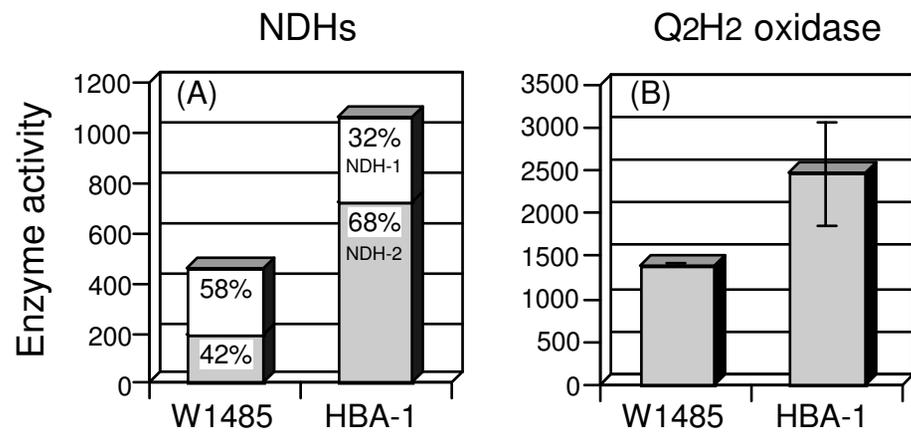


FIG. 2. Noda et al.

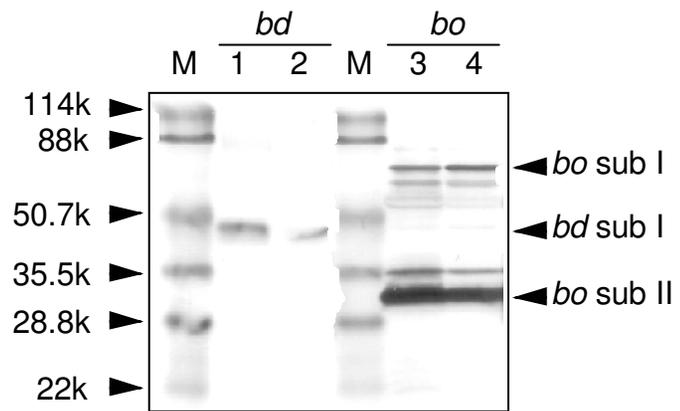


FIG. 3. Noda et al.