Respiration and the F$_1$Fo-ATPase Enhance Survival under Acidic Conditions in *Escherichia coli*

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**Abstract**

Besides amino acid decarboxylation, the ADP biosynthetic pathway was reported to enhance survival under extremely acidic conditions in *Escherichia coli* (Sun et al., J. Bacteriol. 193: 3072–3077, 2011). *E. coli* has two pathways for ATP synthesis from ADP: glycolysis and oxidative phosphorylation. We found in this study that the deletion of the F$_1$Fo-ATPase, which catalyzes the synthesis of ATP from ADP and inorganic phosphate using the electro-chemical gradient of protons generated by respiration in *E. coli*, decreased the survival at pH 2.5. A mutant deficient in hemA encoding the glutamyl tRNA reductase, which synthesizes glutamate 1-semialdehyde also showed the decreased survival of *E. coli* at pH 2.5. Glutamate 1-semialdehyde is a precursor of heme synthesis that is an essential component of the respiratory chain. The ATP content decreased rapidly at pH 2.5 in these mutants as compared with that of their parent strain. The internal pH was lowered by the deletion of these genes at pH 2.5. These results suggest that respiration and the F$_1$Fo-ATPase are still working at pH 2.5 to enhance the survival under such extremely acidic conditions.

**Materials and Methods**

**Bacterial Strains and Culture Media**

The bacterial strains used in this study are listed in Table 1. *E. coli* was grown at 37°C in 4 to 10 ml of minimal E medium [17] containing 0.4% glucose (designated EG). The medium pH was adjusted by the addition of NaOH to 7.5 and 7.0 or HCl to 5.5 and 2.5. LB (Luria-Bertani broth) and LB containing 0.4% glucose (designated LBG) media were also used as a rich medium. Antibiotics were used as the following concentrations: tetracyclin, 10 μg/ml; kanamycin, 25 μg/ml. Delta-aminolevulinic acid (ALA) was used at 100 μg/ml.

**Measurement of the AR**

The AR was measured with cells grown in the logarithmic growth phase as described previously [11] with the following modifications. After the cells had been precultured overnight in LB (for the wild type) or LBG (for mutants) with antibiotics in strains resistant to antibiotics, the cells were diluted 500-fold with EG medium at pH 7.5 and cultured at 37°C until the optical density at 600 nm (OD$_{600}$) reached 0.3~0.4. Two ml of the culture medium were centrifuged at 5,000 xg for 5 min, and the cells in the pellet were suspended with 4 ml of EG medium at pH 5.5. The cell suspension was incubated for 4 h without shaking for the acidic oxidative phosphorylation. In addition to ATP synthesis, the respiratory chain has been reported to regulate pHi in *E. coli* [12], and F$_1$Fo-ATPase was shown to regulate pHi in other bacteria [13]. In the present study, we found that both respiration and the F$_1$Fo-ATPase function at pH 2.5 to enhance the AR in *E. coli*.

**Introduction**

*Escherichia coli* has to pass through the extremely acidic stomach before entering the more hospitable gastro-intestinal tract, and hence resistance to extremely acidic environments (AR) is an important mechanism for *E. coli* to survive [1,2]. Multiple metabolic pathways have been reported to function for survival under extremely acidic conditions. Three amino acid-dependent systems have been identified as enhancing the AR in *E. coli* [1]. The most potent system is the glutamate-dependent system (AR2) [3,4]. The other two systems are arginine-dependent (AR3) [5,6] and lysine-dependent (AR4) [7–9] systems. Recently an adenosine-dependent AR system was reported in *E. coli*, and this system was less active than AR2 but more potent than AR4 [10]. These systems were proposed to regulate the intracellular pH (pHi) at a higher level than the pH of the surroundings [1,10].

Why is such pHi regulation required for survival at acidic pH? The most likely explanation may be that some metabolic pathways are required to function for survival under acidic conditions even if cells are unable to grow and that their activity decreases with the decrease in pHi. Our group has reported that the deletion of genes for the metabolic pathway to synthesize ATP was demonstrated to decrease the AR in *E. coli*, suggesting that ATP-dependent metabolic pathways contribute to survive under acidic conditions [11]. Furthermore, it was demonstrated that one such system was the DNA repair system [11].

*E. coli* has two pathways for ATP synthesis from ADP: glycolysis and oxidative phosphorylation. F$_1$Fo-ATPase catalyzes the synthesis of ATP from ADP and inorganic phosphate using the electro-chemical gradient of protons generated by respiration in oxidative phosphorylation. In addition to ATP synthesis, the respiratory chain has been reported to regulate pHi in *E. coli* [12], and F$_1$Fo-ATPase was shown to regulate pHi in other bacteria [13]. In the present study, we found that both respiration and the F$_1$Fo-ATPase function at pH 2.5 to enhance the AR in *E. coli*. 
Table 1. Bacterial strains and plasmids used in this study.

| Strains          | Genotype/description                  | Reference/source |
|------------------|---------------------------------------|------------------|
| W3110            | λ F- derived from E. coli K-12        | [14]             |
| BW25113          | lac*16 mraT14 AlaCZ10ss hisR234 AaraBAD10ss AtrhaBAD10ss | [15]             |
| JW3710           | BW25113 atpD::km                      | Keio collection(2) |
| JW3715           | BW25113 atpE::km                      | Keio collection   |
| DK8              | HisP01 bglR thi1 relA1 ivc:tn10 ΔatpBFEHAGDC | [16]             |
| ME8366           | HifC glpR glpD hemA30 zch:tn10        | ME collection(2) |
| SE020            | W3110 atpD::km                        | This study: W3110xP1(WJ3710) |
| SE021            | W3110 atpD::km hemA30 zch:tn10        | This study: SE022xP1(WJ3710)(3) |
| SE022            | W3110 hemA30 zch:tn10                 | This study: W3110xP1(ME8366)(3) |
| SE023            | W3110 atpE::km                        | This study: W3110xP1(WJ3715) |

1 km is a gene conferring resistance to kanamycin.
2 Keio and ME collections were obtained from the National BioResource Project (National Institute of Genetics, Mishima, Japan): E. coli.
3 These strains required ALA for growth in LB medium and the growth cessation in E medium containing 0.4% glycerol instead of glucose was complemented by a plasmid having hemA, suggesting the mutation of hemA.

doi:10.1371/journal.pone.0052577.t001

adaptation [18], and the adapted cells were challenged in a 40-fold volume of EG medium at pH 2.5. After incubation at 37°C for 1 or 2 h, the cells were diluted with phosphate-buffered saline [11] and plated on LBG agar plates. Colonies appearing after overnight culture at 37°C were counted, and viability was expressed as described previously [11]. The measurement was repeated three times using separate culture, and the mean value and the standard deviation were calculated.

Measurement of the ATP Content

After the cells had been cultured as described above, the cells were chilled on ice and then centrifuged at 10,000×g for 5 min at 4°C. The pellets were treated with a solution containing 20 mM Tris-HCl, 50 mM MgSO4, 4 mM EDTA, and 50% methanol at pH 7.4 for 30 min at 70°C [19] and then were centrifuged at 10,000×g for 5 min at 4°C. The ATP content of the supernatant was measured using a luminometer (Turner Designs, Inc.) as described previously [20]. Luciferase and standard ATP were purchased from Sigma Chemical Co. The measurement was repeated three times using separate culture, and the mean value and the standard deviation were calculated.

Measurement of Intracellular pH (pHi)

The pHi was determined by the distribution of salicylic acids between outside and inside the cells, as described previously [11,21]. After the cells had been adapted in EG medium at pH 5.5 for 4 h, the harvested cells were suspended in EG medium at pH 5.5 or 2.5 at approximately 1×10^8 cells per ml, and [1^4]C]salicylic acids (10 μM; 0.2 μCi/ml) was added. After incubation at 37°C for 15 min, 1 ml of the medium was centrifuged at 10,000×g for 5 min through an oil mixture (laurylbromide and liquid paraffin). The radioactivity of the supernatant and the pellet were measured to obtain the indicator concentrations outside and inside cells, respectively. The amount of protein in the pellet was measured, and the radioactivity of the pellet was divided by the internal water content calculated from the protein content of the pellet. The pHi was calculated by the following equation:

\[ \text{pHi} = \log \left( \frac{[A]_{\text{in}}}{[A]_{\text{out}}} \right) \left( 10^{\text{pKa}} + 10^{\text{pHout}} \right) - 10^{\text{pKa}} \]

where \([A]_{\text{in}}\) and \([A]_{\text{out}}\) are the concentrations of salicylic acids inside and outside cells, respectively, and \(\text{pHout} \) is the medium pH. The pKa of salicylic acids used was 2.89. The measurement was repeated three times using separate culture, and the mean value and the standard deviation were calculated.

Preparation of the Membrane Fraction

The membrane fraction was prepared as described previously [22,23] with the following modifications. The cells were cultured in 100 ml of EG medium at pH 7.5 until OD$_{600}$ reached approximately 0.3. The cells were harvested by centrifugation at 5,000×g for 5 min, washed with 0.1 M potassium phosphate buffer pH 6.6, and resuspended in 5 ml buffer A containing 10 mM Tris-HCl, 140 mM KC1, 2.0 mM β-mercaptoethanol, and 10% glycerol, at pH 8.0. The cells were disrupted through a French pressure cell (Aminco) at 10,000 pounds per inch$^2$, and unbroken cells were removed by centrifugation at 10,000×g for 10 min. The membrane fraction was obtained by centrifugation at 100,000×g for 1 h. The obtained membranes were washed once with buffer A and then resuspended with buffer A containing 10% glycerol at 2 to 4 mg protein per ml. All steps were performed at 4°C. The membranes were stored at −20°C until use.

Measurement of ATPase Activity

The ATP hydrolysis activity in the membranes was determined by the amount of inorganic phosphate (Pi) released from ATP, as previously described [24,25]. After 5 μg of the membranes had been added to the reaction buffer containing 300 μl of buffer solution (200 mM Tris-maleate and 5 mM MgCl$_2$ at pH 9.0) and 270 μl of water, the mixture was incubated at 37°C for 5 min, and then 30 μl of 100 mM ATP was added. After incubation at 37°C for 20 min, the reaction was stopped by the addition 300 μl of cold 15% trichloroacetic acid and immediately chilled on an ice bath. The resulting mixture was centrifuged at 3,000×g for 10 min at 4°C, and 800 μl of the supernatant was mixed with 1.87 ml of the reagent (10 ml of 5 N H$_2$SO$_4$, 10 ml of 2.5% ammonium molybdate, 10 ml of the solution containing 3% NaHSO$_3$ and 1% β-methylamino-phenol sulfate, and 40 ml of H$_2$O$_2$). The mixture was incubated at 18°C for 10 min, and the absorbance at 660 nm was measured. K$_2$HPO$_4$ (10 mM) was used as a
standard phosphate. One unit of ATPase activity was defined as the activity to release 1 μmol of Pi from ATP for 1 min. The measurement was repeated three times using separate culture, and the mean value and the standard deviation were calculated.

Measurement of Proton Pumping Activity
The proton pumping activity of the membranes was determined using the quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) as described previously [26,27]. The membranes were suspended with the buffer containing 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) and 10 mM MgCl₂ (pH 7.5) at 60 μg/ml of membrane protein. After 1 μl of 0.1% ACMA was added to the reaction mixture (1 ml), 5 μl of 200 mM ATP was added. The fluorescence intensity from ACMA was measured with excitation and emission wavelengths of 410 nm and 490 nm, respectively.

Measurement of the Membrane Permeability to Protons
The membrane permeability to protons was measured as described previously [28] with the following modifications. The E. coli cells cultured overnight in LB medium were diluted 1000-fold with EG medium of pH 7.5 and then were grown at 37°C until OD₆₀₀ reached 0.3~0.4. The cells were resuspended in the same volume of EG medium at pH 5.5, and cultured for 4 h at 37°C for the acidic adaptation. The adapted cells were collected by centrifugation at 10,000 × g for 5 min at room temperature, washed with H₂O and resuspended in 3 ml H₂O containing 20 mM MgCl₂ at 5 × 10⁷ cells/ml. The measurement was carried out at 25°C with stirring. After 20 μl of 0.2 M HCl had been added, the change of pH was measured with a pH meter connected to a computer. The membrane permeability to protons was represented as one pH unit change per min per mg protein [25]. The measurement was repeated three times using separate culture, and the mean value and the standard deviation were calculated.

Western Blot Analysis of ATPase Subunits in the Membranes
Western blot analysis of the membrane fraction was carried out as described previously [29,30] using rabbit antiserum against F₁ part of E. coli F₁F₀-ATPase which was donated by M. Futai (School of Pharmacy, Iwate medical University, Iwate, Japan). The protein content in the membrane fraction was quantified as described below. Two μg of membrane proteins were mixed with 4 × SDS-PAGE sample buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.05% bromophenol blue), boiled for 90 seconds, and then applied to a 10% polyacrylamide gel containing 0.1% SDS. Proteins separated by the gel electrophoresis were transferred to a PVDF membrane at 50 volt/cm for 60~70 min. After the PVDF membrane had been incubated with PBS (157 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄, pH 7.4) containing 3% BSA for blocking, the membrane was overlaid with 1 ml of antibody diluted solution (3 mM Tris-HCl buffer containing 45 mM NaCl, 3% BSA, and 10% FBS, pH 7.6) containing 1 μl of antisera against F₁ part of E. coli F₁F₀-ATPase. The membrane was washed 2 times with TBS-Tween (10 mM Tris-HCl buffer containing 150 mM NaCl and 0.1% Tween 20, pH 7.6) and overlaid with 1 ml of antibody diluted containing 5 μl of anti-rabbit antibodies conjugated with alkaline phosphatase (Biosource, USA). After the membrane was washed 2 times by TBS-Tween, staining was carried out as described previously [29,30].

Other Methods
Transduction with P1φ5 was performed as described previously [31]. Protein was measured as described previously [32], and bovine serum albumin was used as a standard.

Results
Enzyme Activities in the F₁F₀-ATPase Mutants
Oxidative phosphorylation is mediated by the respiratory chain and the F₁F₀-ATPase in E. coli [33]. E. coli F₁F₀-ATPase consists of two parts, F₁ and Fo, which contain five subunits (α, β, γ, δ, and ε) and three subunits (a, b, and c), respectively [34,35]. We used mutants deficient in atpD (SE020) and atpE (SE023) in this study. atpD and atpE encode the β and c subunits, respectively [36]. We also used DK8 [16] in which all genes for the F₁F₀-ATPase were deleted.

Since the optimum pH of the ATP hydrolysis activity was 9.0 [24], the ATP hydrolysis activity was measured at pH 9.0. The activity was 0.52 ± 0.17 μmol Pi/min/mg protein in the wild type, while the activity was not detected (less than 0.01 μmol Pi/min/mg protein) in any of the F₁F₀-ATPase mutants at pH 9.0. The proton pumping activity was impaired in these mutants (Fig. 1). The wild type strain grew in the E medium containing lactic acid instead of glucose at pH 5.5, but none of the F₁F₀-ATPase mutants grew under this condition, indicating that the oxidative phosphorylation was still active at pH 5.5 in the wild type strain but not in the F₁F₀-ATPase mutants. These results suggested that the F₁F₀-ATPase activity was negligible in these mutants.

The AR of Mutants Deficient in a Gene for the F₁F₀-ATPase or Heme Synthesis
The stationary-phase cells may be resistant to various stresses [4]. To minimize the responses to stresses other than acidic stress, cells growing logarithmically were used in the present study. F₁F₀-ATPase mutants SE020, SE023 and DK8 showed AR of E. coli decreased about 20-fold compared with that of the wild type W3110 after the cells were challenged at pH 2.5 for 1 h (Fig. 2), and none of these mutants survived after 2 h challenge at pH 2.5 (data not shown). The mutant deficient in hemA encoding glutamyl tRNA reductase (SE022) had a low ability to survive at pH 2.5. Glutamyl tRNA reductase is the enzyme that synthesizes glutamate 1-semialdehyde in E. coli [37,38]. Delta-aminolevulinic acid (ALA) is a precursor of heme biosynthesis and is synthesized from glutamate 1-semialdehyde. Therefore, ALA was added to produce heme in the hemA mutant as indicated.

When glutamate or arginine was added, the survival of the ATPase mutants was increased, but the survival rate was still lower than that of the wild type strain (Fig. 2). The addition of glutamate increased the survival of the hemA mutant, but surprisingly the addition of arginine decreased survival in the hemA mutant (Fig. 2). The reason for this decrease is still unknown. We next constructed a double mutant deficient in both atpD and hemA. After the double mutant had been cultured overnight in LB with 100 μg/ml ALA, the cells were transferred to the EG medium at pH 7.5 and then to pH 5.5 medium without the addition of ALA. Although the double mutant could grow in the medium at both 7.5 and 5.5 at a slower rate than that of the single mutant, the double mutant could not survive after 1 h challenge at pH 2.5. Even if glutamate or arginine was added, the survival of the double mutant was very low (less than 0.0001%, Fig. 2). These results suggest that either respiration, or the F₁F₀-ATPase, is essential for survival at pH 2.5 in E. coli since both could not be eliminated simultaneously.
ATP Content of the Mutants Deficient in the F1Fo-ATPase and Heme Protein

In order to examine whether the ATPase mutants and the respiratory chain mutant affect the ATP content, we investigated the ATP content in the mutants. The ATP content was decreased at pH 7.5 in the F1Fo-ATPase mutants, but not at pH 5.5 (Fig. 3). In contrast, the ATP content of the hemA mutant was lower than that of its parent strain at pH 5.5 (Fig. 3). These data indicated that the ATP synthetic activity of glycolysis is enough to compensate the ATP consumption at pH 5.5 but the activity of oxidative phosphorylation is not. The ATP content of these mutants decreased more rapidly at pH 2.5 than that of the wild type strain, and the decrease was more rapid in the hemA mutant than that in the F1Fo-ATPase mutants (Fig. 3). The ATP content in the hemA mutant was lower at pH 5.5 and decreased more rapidly at pH 2.5 as compared with that of the purA and purB mutants reported previously [11], although the survival was almost the same between the hemA and purB mutants after the acidic challenge at pH 2.5 for 1 h (data not shown). The survival of the hemA mutant was significantly lower than that of the purB mutant after 2 h challenge at pH 2.5 (data not shown). The ATP content of the double mutant deficient in atpD and hemA at pH 5.5 was less than 0.01 nmol per mg protein. These data support the previous result that ATP content is an important factor for survival of E. coli in acidic conditions [11].

Effect of Acidic pH on the Expression of the F1Fo-ATPase

The F1 portion of the ATPase is not composed of integral membrane proteins and is associated with the membrane-embedded Fo subunits. The expression of the F1 part of the F1Fo-ATPase in the membranes was investigated with Western blot analysis. The results implied that the expression of the F1 subunits was not affected significantly by the pH change (Fig. 4), ruling out the possibility that the elevated ATP content at pH 5.5 was due to the increase in the amount of the ATPase. The amount of the F1 α subunit was decreased in the atpE mutant that is deficient in the Fo c subunit (Fig. 4), indicating that proper assembly of the holoenzyme was impaired in this strain.

Intracellular pH (pHi) in the Mutants Deficient in the F1Fo-ATPase and Heme Protein

The pH values of all of the F1Fo-ATPase mutants used in this study were lower than that of the wild type strain (Table 2). The pHi of the hemA mutant was also low, but higher than that of the F1Fo-ATPase mutants (Table 2). These data indicated that the F1Fo-ATPase and the respiratory chain were important for pH regulation.
We measured the membrane permeability to protons as described previously [2,28]. The initial velocities of pH change after acid pulse were $0.022 \pm 0.009$ and $0.021 \pm 0.007$ pH (n = 3) per min per mg protein in the wild type W3110 and DK8, respectively, in the pH range from 4.1 to 4.7. Similar results were obtained with the $atpD$ and $atpE$ mutants (data not shown). The $hemA$ mutant showed similar permeability ($0.022 \pm 0.006$ pH per min per mg protein, n = 3). These data indicated that the decreased pHi in the mutants was not due to an increase in the membrane permeability to protons.

**Discussion**

Multiple metabolic pathways may be required for survival of *E. coli* under extremely acidic conditions [1,39]. Our group reported that adenosine deamination increased survival under extremely acidic conditions [1,39]. The results obtained using separate cultures are represented. One asterisk, $p < 0.01$ compared with the wild type; two asterisks, $p < 0.005$ compared with the wild type.
acidic conditions, in addition to amino acid decarboxylation [10]. Furthermore, our group implied that ATP is required for survival under acidic conditions and that one of the ATP-dependent systems is a DNA repair system in *E. coli* [11]. It was found in the previous study that the deletion of *purA* and *purB* genes for purine biosynthesis, and the gene for ADP synthesis from AMP decreased the ATP content and the AR in *E. coli* [11]. In the present study, we investigated the effect of the deletion of genes required for ATP synthesis from ADP on the AR. Both mutants deficient in the genes for the F1Fo-ATPase and the biosynthesis of heme showed rapid decreases in ATP content and low survival at pH 2.5. The F1Fo-ATPase consists of two parts, F1 and Fo, which contain five subunits, respectively [34]. Mutants deficient in *atpD* encode the subunit of F1 and the c subunit of Fo, respectively [36]. The present data suggested that the membrane permeability to protons was not impaired by the deletion of these enzymes. It has been argued that respiration has an essential role in pH regulation in *E. coli* [27]. Consistent with this hypothesis, the pH regulation was impaired in the *hemA* mutant (SE022). The pH regulation was also impaired in the F1Fo-ATPase mutants even if the respiration was working suggesting an additional level of control. Two possibilities can be argued. The first one is that the F1Fo-ATPase extrudes protons to regulate pH instead of the ATP synthesis at acidic pH. Such a function of the F1Fo-ATPase was first demonstrated in *E. hinae* (formerly *S. faecalis*) [13], and was also argued for in *E. coli* [1], although there has been no direct evidence to suggest it is in *E. coli*. The second one is that *E. coli* has an unidentified system for pH regulation whose operation is driven by ATP. The activity of this putative system is diminished by a decrease in the ATP level. The pH was still regulated at a higher level in the medium even though no ATP was detected in the *hemA* mutant after the acidic challenge for 1 h (Table 2, and Fig. 3). This supports that ATP-independent systems such as amino acid decarboxylation operate to regulate pH. The addition of glutamate and arginine could increase the viability of the F1Fo-ATPase mutants, but the survival was still lower than that of the wild type (Fig. 2), indicating that the amino acid systems alone are not sufficient for AR.

Conclusions

Intracellular pH affects the enzyme activity, protein stability, structure of nucleic acids, and functions of many other biological molecules. We found in the present study that respiration and the F1Fo-ATPase participate in pH regulation and maintenance of the ATP content at a high level to enhance the AR of *E. coli*. Since pH regulation is important for survival at acidic pH, *E. coli* is likely to have multiple systems for pH regulation. In any case, it was strongly suggested that the ATP-dependent metabolic processes enhance the survival at acidic pH even if growth stops and that pH regulation is indispensable to keep such metabolic processes active.

Table 2. Intracellular pH in various mutants.

| Strains | pH 5.5 | pH 2.5 |
|---------|--------|--------|
|         | 15 min | 30 min | 60 min |
| W3110   | 7.16±0.09 | 4.08±0.03 | 3.94±0.04 | 3.82±0.04 |
| DK8     | 6.98±0.20** | 3.69±0.04** | 3.54±0.07** | 3.50±0.10** |
| SE020 (atpD) | 7.04±0.14* | 3.61±0.04** | 3.57±0.13** | 3.54±0.12** |
| SE023 (atpE) | 7.13±0.15* | 3.56±0.21** | 3.58±0.18** | 3.54±0.20** |
| SE022 (hemA) | 7.15±0.06 | 3.79±0.03** | 3.71±0.07** | 3.64±0.02** |

pH was measured as described in Materials and Methods. pHo is the pH values of the medium. The p-values compared with the wild type were calculated.

* p<0.05 (n=6); ** p<0.005 (n=6).

doi:10.1371/journal.pone.0052577.t002

Figure 4. Expression of the F1Fo-ATPase. DK8, W3110 (wild type), and SE023 (atpE) were grown at the pH indicated, and the amounts of F1 subunits were measured with Western blot analysis as described in Materials and Methods. M, molecular weight marker.

doi:10.1371/journal.pone.0052577.g004
Acknowledgments

We would like to express our thanks to T. Horie and M. Futai for use of the luminometer and supply of antiserum against the F₁ part of the FₐF₀-ATPase, respectively.

Author Contributions

Conceived and designed the experiments: YS TF HS HK. Performed the experiments: YS. Analyzed the data: YS TF HS HK. Contributed reagents/materials/analysis tools: YS TF HS HK. Wrote the paper: YS TF HS HK.

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