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Use of *Escherichia coli add/ade* mutant and *Saccharomyces cerevisiae* WSH2 to construct a highly efficient coupled system for glutathione production

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ABSTRACT

How to supply ATP efficiently and economically is one of the key issues to achieve the commercialization of the enzymatic production of glutathione (GSH). In this work, a highly efficient coupled system for GSH enzymatic production was constructed with *Escherichia coli* Δ*add/ade* and *Saccharomyces cerevisiae* WSH2. In this system, ATP-consuming reactions for GSH synthesis are coupled with ATP-generating reactions. The results indicated that the irreversible transformation from ATP into hypoxanthine (Hx) was completely blocked in *E. coli* Δ*add/ade*, and ATP was mainly transformed into adenosine (Ado) and adenine (Ade). Due to the facts that Ado and Ade could be both used to generate ATP by *S. cerevisiae* WSH2, ATP-regenerating reaction was established in this coupled system. As a result, GSH production in this system reached 10.88 mM within 6 h, which was 4.03-fold of the coupled system of *E. coli* BW25113 and *S. cerevisiae* WSH2. The results are helpful for investigating the enzymatic production of GSH and other valuable ATP-dependent products.

1. Introduction

Glutathione (γ-glutamyl-L-cysteinylglycine, GSH) is the most abundant free thiol compound in cells [1]. GSH acts as the principal redox buffer, plays an important role in oxidative stress response, and influences several essential processes such as gene expression, cell proliferation, and apoptosis [2–5]. Therefore, nowadays GSH finds wide applications in pharmaceutical, food and cosmetic industries [6–8], and the commercial demand for GSH is expanding.

GSH is synthesized by the consecutive action of γ-glutamylcysteine synthetase (GSH I, EC 6.3.2.2) and GSH synthetase (GSH II, EC 6.3.2.3) with the consumption of ATP. Compared with the fermentative method commercialized in the early 1980s, the enzymatic production of GSH can achieve a higher concentration and be more beneficial to its final purification. However, to date, the enzymatic production of GSH has not been commercialized. On one hand, the requirement for ATP (2 M ATP for 1 M GSH) in GSH biosynthesis makes this process difficult to scale-up, for it is impractical from an economic point of view to add ATP directly on an industrial scale. On the other hand, a great deal of GSH I and GSH II with high catalytic activity are also essential to the enzymatic synthesis of GSH.

In our previous study, to achieve high catalytic activity of GSH I and GSH II, a recombinant plasmid (pBV03) containing two copies of *gsh I* gene and one copy of *gsh II* gene was constructed in *Escherichia coli* [9]. Moreover, GSH was produced by *E. coli* BW25113 (pBV03) or *E. coli* JW1615 (pBV03) coupled with an ATP regeneration system, in which ATP was regenerated from adenosine (Ado) and glucose by the glycolytic pathway of *Saccharomyces cerevisiae* WSH2 [10]. The results indicated that ATP consumed for GSH production was transformed quickly and irreversibly into hypoxanthine (Hx) by *E. coli* (Fig. 1), moreover, Hx could not be utilized for ATP regeneration by *S. cerevisiae*, resulting in the low ATP-regenerating efficiency of the coupled system [11]. Although the irreversible transformation from ATP into Hx could be reduced greatly by the knockout of adenosine deaminase (*add*, EC 3.5.4.4) gene, however, it is not blocked completely. Therefore in this work, to completely block the irreversible transformation from ATP into Hx (Fig. 1), *E. coli* Δ*add/ade* was constructed by the disruption of adenosine deaminase and adenine deaminase (*ade*, EC 3.5.4.2). The resultant recombinant strain of *E. coli* was then used to construct a new coupled system for GSH enzymatic synthesis. As a result, GSH production of this coupled system increased greatly due to the enhancement of ATP-regenerating efficiency. To the best of our knowledge, this is the first report regarding the construction of *E. coli* host for ATP-regenerating system by knocking out the genes of both *add* and *ade*. The results obtained in this work are expected

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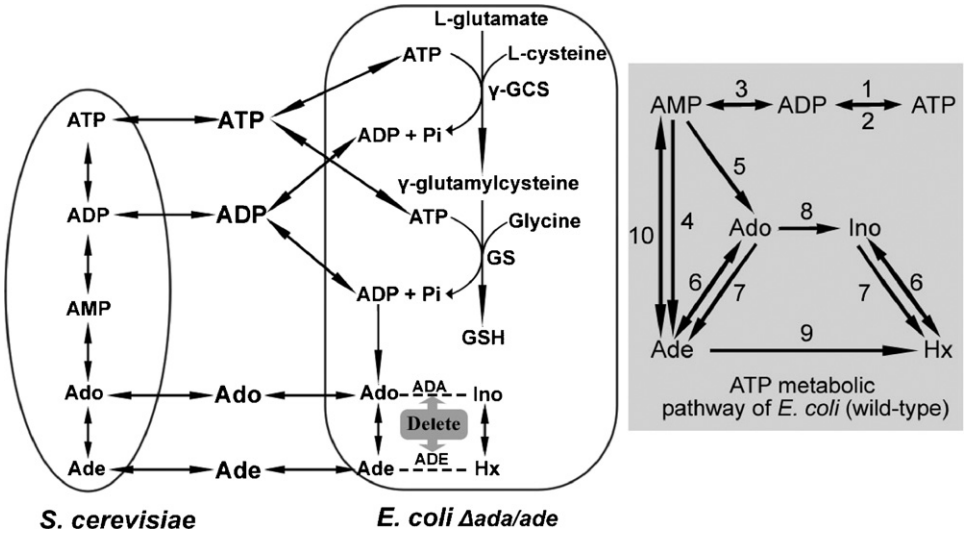

Fig. 1. Schematic diagram of GSH production and ATP regeneration by the coupled system constructed with *E. coli* and *S. cerevisiae* (pathways of purine metabolism in *E. coli* referring to KEGG 2009). ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; Ado, adenosine; Ade, adenine; Ino, inosine; Hx, hypoxanthine; γ-GCS, γ-glutamylcysteine synthetase; GS, GSH synthetase; ADA, adenosine deaminase; ADE, adenine deaminase. 1, Nucleoside diphosphate kinase; 2, pyruvate kinase; 3, adenylate kinase; 4, AMP nucleosidase; 5, adenosine 5′-monophosphate phosphohydrolase; 6, purine-nucleoside phosphorylase; 7, ribonucleoside hydrolase; 8, adenosine deaminase; 9, adenine deaminase; 10, adenine phosphoribosyltransferase.

to be helpful to achieve the commercialization of the enzymatic production of GSH and construct the coupling ATP-consuming and ATP-generating systems.

2. Materials and methods

2.1. Chemicals

ATP, ADP, AMP, Hx, Ado, Ade, inosine (Ino), GSH reductase, nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) and 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma–Aldrich (Shanghai, China). Taq DNA polymerase, ampicillin, kanamycin, L-arabinose, and primers were purchased from Sangon (Shanghai, China). All other chemicals were reagent grade, and those used for high performance liquid chromatography (HPLC) were redistilled and filtered prior to use.

2.2. Bacterial strains and plasmids

The bacterial strains used in this study are *E. coli* JW1615 (*add* mutant), *E. coli* JW3640 (*ade* mutant) and their parent strain *E. coli* BW25113. The mutants were constructed at Keio University, Japan using the method of Datsenko and Wanner [12] by Baba et al. [13]. *E. coli Δadd/ade* was constructed by disrupting *ade* gene of *add* mutant (*E. coli* JW1615) as described below. The Red helper plasmid pKD46 and the FLP helper plasmid pCP20 were generous gifts from Dr. B.L. Wanner.

2.3. Construction of E. coli add/ade double mutant

E. coli Δadd/ade was constructed by disrupting *ade* gene of *add* mutant (*E. coli* JW1615) by the method of Datsenko and Wanner [12] with some modifications. *E. coli* JW1615 (*add* mutant) was transformed with plasmid pKD46 encoding the L-arabinose inducible Red recombination system. This strain was further transformed with an inactivation cassette which was constructed as follows: the kanamycin resistance gene flanked by two FRT sequences (FLP Recognition Target) was amplified by PCR, using the chromosomal gene of *E. coli* JW3640 (*ade* mutant) as a template. Primers used in the PCR amplification of inactivation cassette were A1 (5′-GAGGATTTGCGGGTTCACA-3′) and A2 (5′-TGGCGATTCAGGGCTTTAC-3′). PCR reactions were carried out in 50 μl reaction solution containing 2.5 U of Taq DNA polymerase, 10 ng chromosomal gene, 1.0 μM of each primer, and 200 μM dNTPs. Reactions were performed for 30 cycles: 94 °C for 30 s, 59 °C for 30 s, 72 °C for 2 min, plus an additional 2 min at 72 °C. PCR products were ethanol-precipitated and then were suspended in 6 μl ddH_2O, and then, 1 μl of PCR product was analyzed by 1% agarose gel electrophoresis using 0.5 × Tris-Boric acid (TBE) buffer. PCR products were electroporated into *E. coli* JW1615 (*add* mutant with plasmid pKD46) and transformants were selected at 37 °C on LB plates (with 30 μg/ml kanamycin). Several kanamycin-resistant clones were picked. The correct structure of each mutant allele was firstly confirmed by PCR using the primers of A1 and A2, and then the knockout of *add* and *ade* gene was verified by DNA sequencing of PCR products. For the production of GSH, the plasmid pBV03 was also introduced into *E. coli Δadd/ade* by electroporation.

2.4. Ado (and Ade) metabolism and GSH production experiments

E. coli BW25113 (pBV03) and *E. coli Δadd/ade* (pBV03) were grown on LB (Luria–Bertani) media (1% tryptone, 0.5% yeast extract, and 1% NaCl) with 100 mg/l ampicillin at 30 °C to an optical density (OD_{600}) of 3.0. When OD_{600} reached 3.0, temperature was adjusted to 42 °C, and the culture was grown for another 4 h. Medium was sterilized at 121 °C for 20 min.

The cells were harvested by centrifugation at 8000 × *g* and 4 °C for 5 min, and then washed three times with 20 mM potassium phosphate buffer (pH 7.0). The washed cells were recollected by centrifugation at 8000 × *g* and 4 °C for 5 min, and stored at −80 °C until further use. GSH production and Ado (and Ade) metabolism experiments are listed in Table 1. For the construction of coupled system, cells of *E. coli* and *S. cerevisiae* grown separately were combined in common reaction vessel (25 ml) and the reaction volume was 2 ml. The cell concentrations of *E. coli* and *S. cerevisiae* were both 200 g wet weight cells per liter. To permeabilize cells, cells were treated with 0.5% (v/v) toluene. The reaction mixture also contained 400 mM glu-

Table 1
GSH enzymatic production experiment.

No.	Description	Cells[a]	Substrate added (mM)[b]
1	Ado or Ade metabolism by *E. coli Δadd/ade*	*E. coli Δadd/ade*	Ado (5) or Ade (5)
2	GSH production with the addition of ATP	*E. coli Δadd/ade* (pBV03)	L-Glu (60), L-Cys (25), Gly (25), ATP (30)
3	GSH production by coupled system	*S. cerevisiae* WSH2 and *E. coli* BW25113 (pBV03) or *E. coli Δadd/ade* (pBV03)	L-Glu (60), L-Cys (25), Gly (25), glucose (400), AMP (1), Ado (10), NAD (0.1)

[a] To permeabilize cells, cells were treated with 0.5% (v/v) toluene. The cells concentration was 200 g wet weight cells per liter.
[b] The reaction mixture also contained 30 mM $MgCl_2$ and 150 mM potassium phosphate buffer (pH 7.0). The mixture was incubated at 37 °C with gentle shaking. Samples were taken and immediately heated in boiling water for 10 min, the cell debris was removed by centrifugation at 8000 × *g* for 5 min, and the supernatants were used for the analysis.