S7-3

Global Gene Expression Profiling in L-Lysine Producing *Escherichia coli*

Seong-Jun Kim[1,3], Mun Su Rhee[1], Young-Hoon Park[1], Jae-Gu Pan[2], and Yu-Sam Kim[3]

[1]*R&D Center for Bioproducts, CJ Corp., Ichon, Korea;* [2]*Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea;* [3]*Department of Biochemistry, Yonsei University, Seoul, Korea*

In order to isolate a lysine overproducing mutant strain, *Escherichia coli* W3110 was subjected to a mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine, NTG, and applied to a series of selection procedure to obtain resistancy to a threonine analog, α-amino-β-hydroxyvaleric acid, AHV, and a lysine analog, S-(2-aminoethyl) L-cysteine, AEC. AEC-susceptibility revealed that the selected mutant strain, K225, exhibited a high level of resistance (20mg/ml). The K225 strain produced 12g/l of lysine. DNA array analysis of metabolic changes between wild type, W3110 and mutant strain, K225 identified several differentially expressed genes in the lysine biosynthetic pathway and the central carbon metabolism. The expression levels of lysine biosynthetic pathway genes, *thrA* (aspartokinase), *dapD* (tetrahydropyridine-2-carboxylate N-succinyltransferase), *dapE* (succinyl-diaminopimelate desuccinylase), *dapA* (dihydrodipicolinate synthase), *lysA* (diaminopomelate decarboxylase), were increased. The lysine importer genes, *cadB* and *lysP,* were repressed, and exporter gene, *lysE* (*yggA*) was increased in K225. In central metabolic pathway, the expression levels of the genes related to tricarboxylic acid cycle were diminished particularly. These results indicated that because of extremely low TCA cycle fluxes, and the increased levels of phosphoenolpyruvate and oxaloacetate, K225 exhibited extensive overflow into lysine biosynthetic pathway in order to overproduce lysine.

Introduction

Amino acids are the basic building blocks for proteins and nutritionally important key compounds. They have many potential uses, e.g., food additives, pharmaceuticals, feed supplements, polymer materials, and agricultural chemicals, and demand for amino accompanied by development of mass production technology for each type of amino acid acids has grown rapidly (4). L-Lysine is an essential amino acid that has to be available in sufficient amounts in feed-stuffs to meet the nutritional requirements of animals. Because corn, wheat, and barley have a few lysine, therefore, supplement of a lysine-rich source is necessary (6). The dosed addition of L-lysine saves raw materials and reduces nitrogen excretion. Addition of 0.5% L-lysine increases feed quality as much as adding approximately 20% soybean meal. Because most of the other amino components of soybean meal are not used by the animals, the amount of nitrogen excretion is

increasing significantly. Using low protein diets supplemented with crystalline amino acids can help to solve this problem (2). Owing to the ability of *Corynebacterium glutamicum* to secrete amino acid, large-scale production of lysine was started by using mutants of coryneform bacteria. L-Lysine fermentation has the long history for more than 40 years using traditional *C. glutamicum* mutants. Because the fermentation processes are usually carried out at temperatures below 35°C, with the aim of reducing cooling cost, thermo-tolerant bacteria, such as *Bacillus licheniformis, Bacillus methanolicus, and Corynebacterium thermoaminogenes*, have been studied widely (5,6). Another approach to solve this problem is a development of *E. coli* strain. Threonine and Tryptophan have been manufactured using *E. coli* already. The application of *E. coli* to L-lysine fermentation has another merit that lots of information and knowledge about *E. coli* have been accumulated.

In this study, a lysine overproducing mutant was isolated by combing a mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine, NTG, and a series of selection procedure to obtain resistance to a threonine analog, α-amino-β-hydroxyvaleric acid, AHV, and a lysine analog, S- (2-aminoethyl) L-cysteine, AEC. Furthermore, we tried to understand the mechanism how and why the mutant stain, K225 strain, could overproduce L-lysine using DNA array technique.

Materials and Methods

Bacterial strains. The strains used throughout this study were *E. coli* wild type strain W3110 [F- λ- IN(*rrnD-rrnE*)*1 rph-1*], threonine producing strain, TH2387, and lysine producing strain, K225. Mutant strains, TH2356 and K225 were constructed by a mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG).

NTG mutagenesis. W3110 was grown in LB broth at 37°C overnight, washed 0.1M citrate buffer three times, and then treated with NTG (500ug/ml for final concentration) for 20 min with shaking. After NTG treatment, cells were washed twice in chilled phosphate buffer, resuspended in potassium phosphate buffer and spreaded on the selective media.

Media and culture conditions. A mutant K225 strain was cultured in the yield medium (60g glucose, 2g yeast extract, 17g $(NH_4)_2SO_4$, 1g NaCl, 1g $MgSO_4 \cdot 7H_2O$, 0.3g KH_2PO_4, 0.6 K_2HPO_4 per liter) in order to estimate lysine productivity. For the preparation of DNA array samples, W3110, TH2387, K225 cells were cultured in the seed medium (10g Yeast extract, 10g Tryptone, 10g NaCl, 5g Glucose per liter) till late log phase, and then inoculated to 5L jar fermenter (Ko Biotech, Korea) containing the fermenter medium (30g glucose, 1.5g K_2HPO_4, 5g $(NH_4)_2SO_4$, 1g $MgSO_4 \cdot 7H_2O$, 5g yeast extract, 0.2g $CaCl_2$, Trace element per liter). Using this culture medium as seed, strains were inoculated to 30L jar fermenter containing fermenter medium. Samples were harvested at inoculation time point, mid log, early stationary, and late stationary phase, respectively.

RNA preparation. Total RNA isolation of *E. coli* was carried out by using the modified hot-phenol method (1,7).

DNA array analysis. *E. coli* oligo chips were purchased from GenomicTree, Inc. (Daejeon, Korea). The

mixture of W3110 #1,2,3,4 total RNA samples was labeled with Cy3-dUTP as control and the RNA samples of each growth stage of each strain, labeled with Cy5-dUTP, respectively. Differently labeled probes from different samples were mixed and applied to a microarray slide following incubation at 65℃ overnight under humidified conditions. The fluorescent image of the hybridized microarray slide was scanned with an Axon scanner GenePix 4000B and analyzed using the GenePix Pro 3.0 (Axon Instruments, Inc. CA, USA).

Results and Discussion

Properties of the *E. coli* mutant, K225. *E. coli* mutant was isolated by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as described in materials and methods. The analysis of the regulatory properties of dihydrodipicolinate synthase (DDPS) of this mutant was performed by investigation of growth resistancy to AEC sensitivity (Fig.1). The growth of *E. coli* Wild type, W3110, was decreased about 30% in the present of 1mg/ml AEC. In contrast to wild type strain, K225 strain was able to grow in the present of 15mg/ml AEC. This result indicated that DDPS of K225 was desensitized to feedback inhibition by L-lysine. The growth pattern and a profile of glucose consumption were described in Fig.2. The productivity of L-lysine was 12.9g/L and the yield was 21.5% (Fig.2).

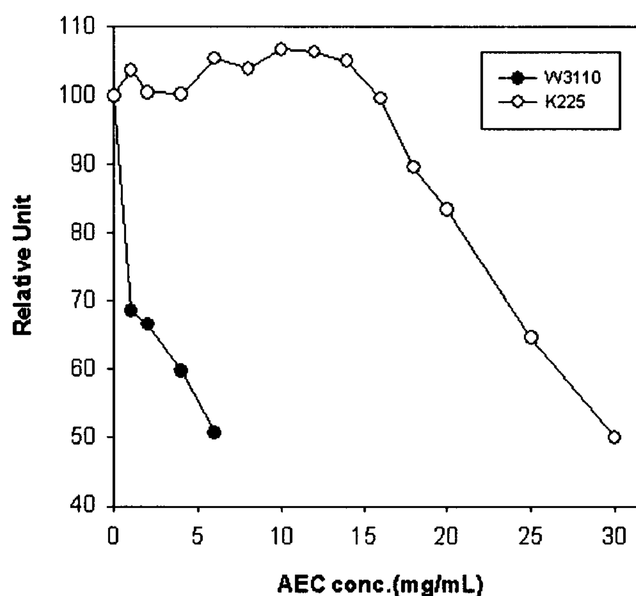

Fig. 1. AEC-susceptibility test of W3110 and K225. Wild type, W3110 (●) and mutant, K225 (○) grown overnight in LB broth were inoculated in M9 broth with supplement of various concentration of AEC (S- (2-aminoethyl) L-cysteine), and incubated at 33°C. Shaking incubation and measurement for growth at A562 were performed using Bioscreen C®.

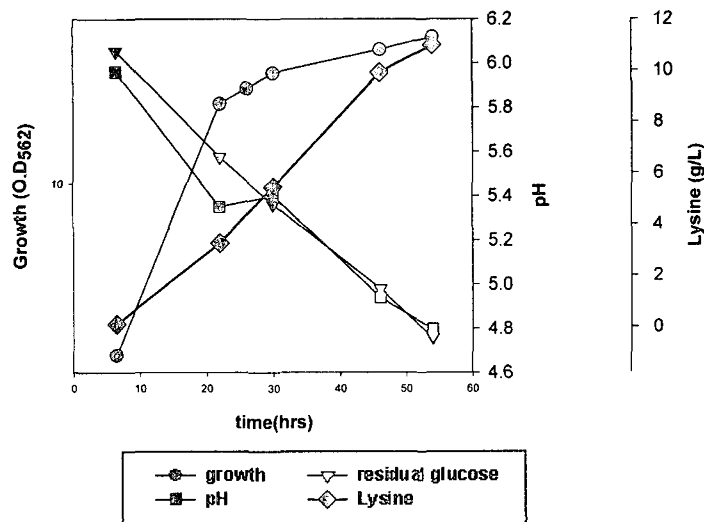

Fig. 2. Growth and productivity of the *E. coli* mutant, K225. K225 was cultured in 250ml cornered baffled flask and samples were taken periodically and measured for growth (●), pH (■), concentration of residual glucose (▽) and productivities of L-lysine (◆).

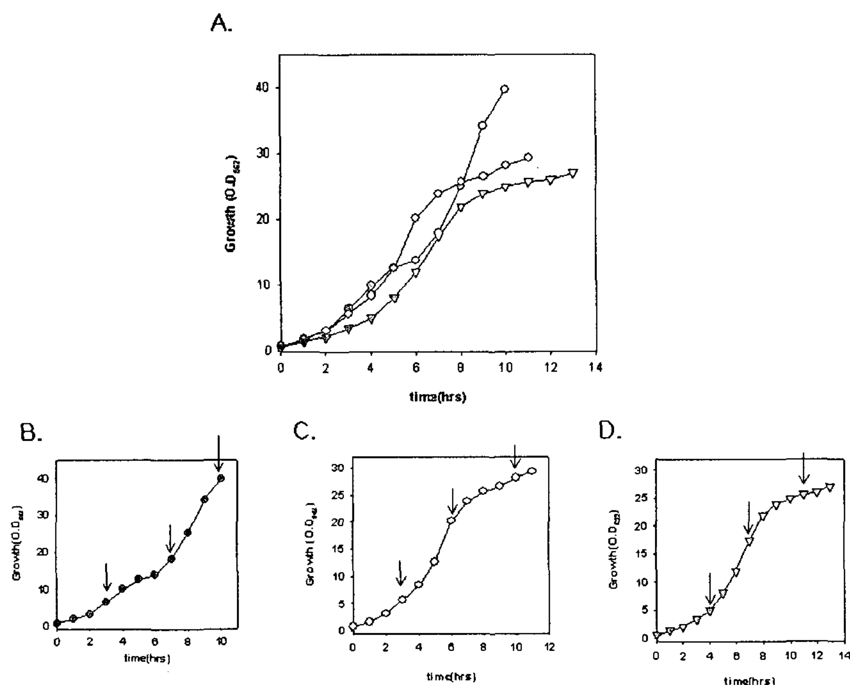

Fig. 3. Growth curve of W3110, TH2356, and K225 for sample preparation of DNA array analysis. W3110 (●, B), TH2356 (▼, C), and K225 (○, D) grown in 5L jar fermenter following flask culture were inoculated in 30L jar fermenter (Materials and methods). Samples were taken at appropriate time points and arrows indicate the sampling times.

DNA array analysis. In order to identify the differentially expressed genes between wild type and mutant, we performed a DNA array analysis. Samples were prepared (Fig.3) and DNA array analysis was performed as described in materials and methods. A number of genes involved in amino acids biosynthesis showed changes in expression profile in K225 strain (Fig.4). Amino acid biosynthetic pathway genes,

especially lysine biosynthetic pathway genes were up regulated by 2- to 4-fold in K225. In *E. coli*, there are three lysine transporter genes such as lysine importer genes, *cadB* and *lysP*, and exporter gene, *lysE* (*yggA*). It shown that both *cadB* and *lysP* were down regulated by 2.7- and 2-fold and *yggA* was up regulated by 8.8-fold. This data indicated that not only lysine biosynthesis-related genes but also others, e.g. central metabolism pathway genes and two component regulator genes unexpectedly, caused the increase of L-lysine productivity. Especially, expression levels of genes involved in central metabolism pathway genes changed tremendously. TCA cycle genes, *sdhCDAB*, *sucABCD*, *fumCA*, and *acnA* were down regulated by 2- to 10-fold; therefore, the activity of TCA cycle was mostly inactivated in a K225 strain. These results may suggest that high level of the important intermediate for lysine biosynthesis, oxaloacetate, owing to inactivation of TCA cycle and up-regulation of lysine biosynthetic pathway genes caused K225 to overproduce L-lysine. Phosphoglucose isomerase and G6P dehydrogenase are located at the first juncture of the two important routes of central metabolism, the Embden-Meyerhof-Parnas (EMP) and pentose phosphate (PP) pathways (3). The *zwf* gene was up regulated by 3-fold in mutant strain, so K225 might produce more NADPH to supply reducing power than W3110, which is used essentially in lysine biosynthesis. These results may indicate that levels of phosphoenolpyruvate, oxaloacetate, and NADPH are increased in K225 strain and K225 exhibits extensive overflow into lysine biosynthetic pathway in order to overproduce lysine. In this study, we could speculate that the genes of K225 were mutated appropriately in order to overproduce L-lysine. However, some other genes were regulated in the opposite direction to lysine overproduction unexpectedly. If these genes were modified by genetic engineering technique, the strain for large-scale production of lysine would be developed.

[This work was supported by a grant from Microbial Genomics & Application Center in 21C Frontier R&D Program by Korean Ministry of Science and Technology]

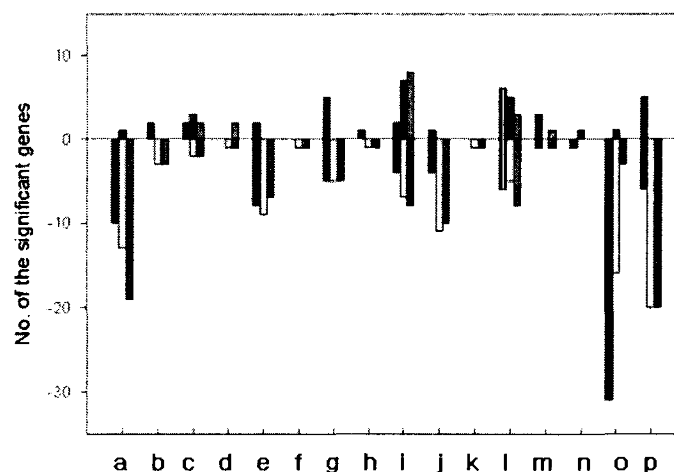

Fig. 4. A global view of gene expression changes in W3110, TH2356, and K225 using DNA microarray analysis. The significant genes were grouped by physiological functions, amino acid biosynthesis (a), carbon catabolism (b), cell process (c), structure (d), central intermediate metabolism (e), DNA replication (f), energy metabolism (g),fatty acid metabolism (h), hypothetical genes (i), nucleotide (j), phage (k), putative protein (l), regulatory protein (m), transcription (n), translation (o), and transporter (p). Each bar represents strains, Black and left bar is W3110, light gray and middle bar is TH2356, and dark gray and right bar is K225.

References

1. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl. 1999. Short protocols in Molecular Biology. John Wiley and Sons. Co.
2. Herman, T. 2003. Industrial production of amino acids by coryneform bacteria. *J. Biotechnol.* **104**: 155-72.
3. Hua, Q., C. Yang, T. Baba, H. Mori, and K. Shimizu. 2003. Responses of the central metabolism in Escherichia coli to phosphoglucose isomerase and glucose-6-phosphate dehydrogenase knockout. *J. Bacteriol.* **185**: 7053-7067.
4. Ikeda, M. 2003. Amino acid production processes. *Adv Biochem Eng Biotechnol.* **79**: 1-35.
5. Ohnishi, J., M. Hayashi, S. Mitsuhashi, and M. Ikeda. 2003. Efficient 40°C fermentation of L-lysine by a new *Corynebacterium glutamicum* mutant developed by genome breeding. *Appl Microbiol Biotechnol.* **62**: 69-75
6. Pfefferle, W., B. Mőckel, B. Bathe, and A. Marx. 2003. Biotechnological manufacture of Lysine. *Adv Biochem Eng Biotechnol.* **79**: 59-112
7. Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular cloning. A laboratory manual. Cold spring Harbor Lab. Press. Cold Spring Harbor. New York.