Analysis of Fluctuation in Gene Expression Based on Continuous Culture System

Tadashi Yamada, Makoto Sadamitsu, Keisuke Nagahisa, Akiko Kashiwagi, Chikara Furusawa, Tetsuya Yomo, and Hiroshi Shimizu

Department of Bioinformatic Engineering, Graduate School of Information Science and Technology, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
{nukumori, sadamitsu, nagahisa, akikok, furusawa, yomo, shimizu}@ist.osaka-u.ac.jp

Abstract. The emergence of heterogeneous cellular state in uniform environment was studied. Using a continuous culture system which provides homogeneous culture environment, we investigated the fluctuation in expression level of *glnA* gene in a cell population. As results, we found that the expression level of *glnA* gene in the cells exhibit a large fluctuation (with two orders of magnitude of protein number), even though expression of the gene is essential for cellular growth and the environment is homogeneous. Furthermore, among several steady states, the transient processes of such heterogeneous cell population were investigated, by changing environmental conditions. The results showed that cells can respond to environmental changes even when their intra-cellular state is accompanied by fluctuations. These results may provide a clue to understand why biological systems can maintain and reproduce themselves robustly.

1 Introduction

In studies of biological systems, it is generally assumed that intra-cellular state of isogenic cells in a fixed environment are homogeneous, and analysis of the systems are based on this assumption. For example, cell concentration is usually measured at the macroscopic level, such as measurement of turbidity of culture medium, which relies on the homogeneity of cells. However, recent developments of experimental technique enable us to measure the intra-cellular state at the single-cell level, and such measurements suggest that heterogeneity of cellular state is ubiquitous in cellular system [1][2]. Thus, to clarify the nature of such cellular heterogeneity is important for further development of biological studies. For this purpose, in this study we investigate the heterogeneity of cellular state in a uniform environment, and adaptation process of such heterogeneous cellular state to environmental changes, using bacteria cells in continuous culture system.

In this study, to characterize intra-cellular state of cells, we adopt expression level of *glnA* gene, since the expression of this gene is essential for nitrogen assimilation in our culture medium, and then it can represent cell viability under

A.J. Ijspeert et al. (Eds.): BioADIT 2006, LNCS 3853, pp. 113–127, 2006.

nitrogen-limited growth condition. In our system, a glutamine synthetase gene *glnA* deficient mutant of *Escherichia coli* YMC21 [3] was used as a host bacterium. The plasmid pKGN-EGFP, carrying the *egfp-glnA* [4] fusion gene was introduced into the *glnA*-deficient mutant. This cells were cultured in a minimal medium, containing glucose and glutamate as sole carbon and nitrogen sources, respectively. Glutamine synthetase only catalyze the conversion reaction from glutamate to glutamine. This pathway is essential for cell growth under the medium condition using in this study, so that cells must express the *glnA* gene to grow in the system. Using this recombinant strain, we can monitor the expression level of *glnA* gene at a single cell level, by measuring fluorescence of EGFP-GlnA fusion protein. This *egfp-glnA* gene is not regulated by wild-type promoter of *glnA* gene[5][6], instead the expression of this gene is controlled by *tac* promoter[7], which is artificially constructed and is not regulated directly by other factors in the *E. coli* YMC21 cells. And this strain and plasmid used in this system do not have repressor gene (*lacI*) of *tac* promoter. Use of this promoter allows us to the nature of heterogeneity in gene expression levels regardless of regulations in the wild-type strain.

To maintain the environmental conditions constant, we used a continuous culture system. In this culture system, growth rate, cell concentration, and substrate concentration are kept constant at a steady state by a continuous input flow of medium and output flow of culture (medium and cells), where the growth rate of cells is identical to the dilution rate of the medium. At such steady state of continuous culture, intra-cellular state is also expected to be constant, and the heterogeneity of cellular state was studied at such steady state conditions.

Furthermore, after reaching such steady state of cell population, we changed the environmental conditions to study the transitions of intra-cellular state between these steady states. Here, we added two types of environmental changes. In one experiment, we changed glutamate concentration in the feeding medium, to change the importance of *glnA* gene under the condition. For example, when the concentration of glutamate in the medium decrease, it is expected that more expression of *glnA* is required for cell growth. On the other hand, when the concentration of glutamate in the medium becomes enough high, the importance of *glnA* expression decreases. In another experiment, we used the glutamate medium as basis and switched to the mixed nitrogen medium containing glutamate and glutamine. Here, by this environmental change, the importance of *glnA* is expected to decrease, since glutamine is the product of the enzymatic reaction which is catalyzed by *glnA* protein.

Using this system, we investigated the heterogeneity of cellular state at a steady state of the continuous culture, and the transient process of cellular state between such steady states. As a result, first we found that even at the steady state of continuous culture, the expression level of *glnA* gene in the cells showed a broad distribution (with two orders of magnitude of protein number), even though the reaction catalyzed by *glnA* protein is essential for cell viability under our medium condition. Second, it was shown that such heterogeneous cellular state responded to environmental changes. Since the heterogeneity of cellular

state was maintained in uniform environments, these observations indicate that the fluctuations in cellular states inevitably emerges, and biological systems can be sustained even under such fluctuations in intra-cellular dynamics. To clarify the mechanism that biological systems can work under such fluctuations will allow us to construct the robust artificial systems, such as IT networking.

2 Materials and Methods

2.1 Strain and Plasmid

A glutamine synthetase gene (*glnA*) deficient mutant of *Escherichia coli* YMC21 [3] (genotype is shown in Table 1) was used as a host. The plasmid pKGN-EGFP (shown in Figure 1), carrying the *egfp-glnA* fusion gene downstream of *tac* promoter was introduced into the determined *glnA*-deficient mutant. The plasmid has β-lactamase gene.

2.2 Cultivation Methods

-80°C stock of experimental strain is streaked on M9 like glutamate medium agar (KH_2PO_4 4.5g/L, K_2HPO_4 10.5g/L, Glutamate 1mM, Glucose 0.4%,

Table 1. The strains used in this study

strain	genotype
Escherichia coli YMC21	*Δ(glnA-glnG), ΔlacU169, endA1, hsdR17, thi-1, supE44*

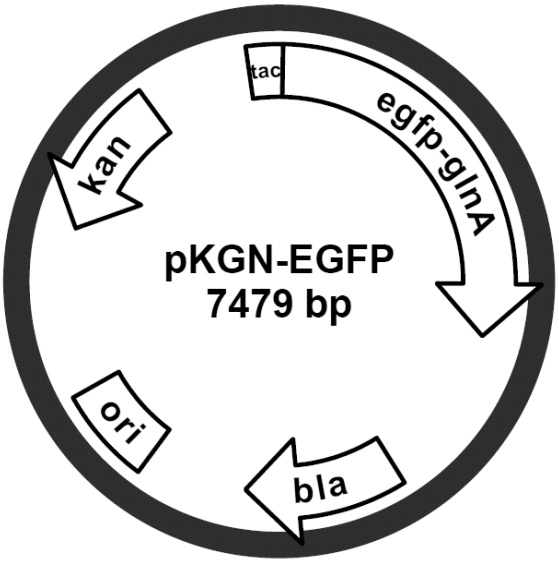

Fig. 1. Vector map of pKGN-EGFP

Thiamine·HCl 5mg/L, $MgSO_4 \cdot 7H_2O$ 50mg/L, Ampicilin 50mg/L, agar 1.5%) plate and stored at 37°C for 2 days. Prewormed fresh M9 like glutamate medium (KH_2PO_4 4.5g/L, K_2HPO_4 10.5g/L, Glutamate 1mM, Glucose 0.4%, Thiamine·HCl 5mg/L, $MgSO_4 \cdot 7H_2O$ 50mg/L, Ampicilin 50mg/L) is added to the plate. The colonies are suspended by glass stick. In preculture, the suspended cells are inoculated into 100mL M9 like glutamate medium in Sakaguchi flask and cultured at 37°C, 120rpm for a day(MM-10, Titec Japan). The precultured cells are collected into two 50ml tubes and centrifuged at 6000rpm for 10min twice. The pellet is resuspended by the fresh 10mL fresh M9 like glutamate medium. The 10mL suspended cells are added into the 125mL M9 like glutamate medium in the 200mL fermenter (Titec Japan) which aeration and temperature are controlled by fermentation controller (BMJ-01, ABLE Japan). In main culture system, cultivation is performed at the scale of 125mL. Cultivation conditions of aeration, temperature and dilution rate are 1vvm, 37°C and 0.075(1/h), respectively. The fermenter is prewormed at 37°C before inoculation and stored for 30 minutes after inoculation. After storing for 30 minutes, feeding pumps of input and output flows of medium start to operate and this time is set as a start time (0h) of the culture. The sample from output medium is taken for analysis the cell concentration, glucose concentration, glutamate concentration and population dynamics by flow cytometer (EPICS ELITE, Beckman Coulter), periodically. Cell concentration is analyzed by the particle characterization analyzer (SD-2000, Sysmex) and spectrophotometer (UV mini 1240, Shimazu). The glucose concentration is measured by glucose analyzer (MODEL2700, Y.S.I Co. Ltd.). The glutamate concentration is measured by the Glutamate F Kit (Roche Diagnostics Swiss).

3 Continuous Culture System

In this study, we adopt the continuous culture system to maintain a uniform environment in space and time. Figure 2 illustrates a reactor tank. The configurations of this cultivation method are also called chemostat. The principle of continuous culture is described below in detail[8][9].

At a steady state, concentrations of components in the reactor are kept constant, so we can apply the following material balance equations to any components of the system:

$$\frac{dXV}{dt} = \mu XV - F_{out}X = 0 \tag{1}$$

$$\frac{dSV}{dt} = F_{in}S_f - F_{out}S - \nu XV = 0 \tag{2}$$

where X = cell concentration in the reactor and in the effluent stream
F_{in} = volumetric flow rate of feed liquid stream
F_{out} = volumetric flow rate of effluent liquid stream
V = total volume of culture
μ = specific growth rate of the cells
S_f = substrate concentration in the feed stream

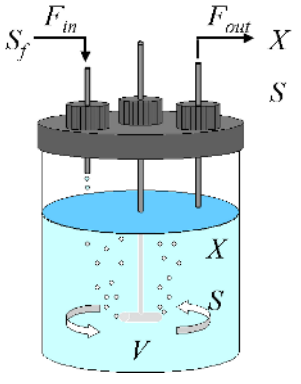

Fig. 2. Schematic diagram of reactor tank

S = substrate concentration in the reactor and in the effluent stream
ν = specific substrate consumption rate.

In the continuous culture system, culture volume V is a constant.

$$\frac{dV}{dt} = 0 \tag{3}$$

$$F_{in} = F_{out} \tag{4}$$

By applying Eq. 3 and Eq. 4 to Eq. 1 and Eq. 2, we obtain the following equations:

$$\frac{dX}{dt} = \mu X - DX = 0 \tag{5}$$

$$\frac{dS}{dt} = D(S_f - S) - \nu X = 0. \tag{6}$$

Here, as noted in Eq. 5 and Eq. 6, the parameter D, called the dilution rate and defined by

$$D = \frac{F_{in}}{V} = \frac{F_{out}}{V} \tag{7}$$

characterizes the holding time or processing rate of the continuous culture system. In Eq. 5, We have equality if

$$X = 0 \tag{8}$$

or

$$\mu = D \tag{9}$$

is approved. In the case of Eq. 8, no cell exists in the reactor. This case is called "wash-out", where all cells are flowed out because their maximum specific growth rate is lower than the dilution rate. In this case, the substrate concentration S becomes S_f. In the case of Eq. 9, the cells can survive in the reactor. The specific

growth rate of the cells becomes identical to the dilution rate. This means that the number of the increased cells by growth is equivalent to the number of cells flowed-out. At the steady-state, by applying the Eq. 8 to Eq. 9 the equation of substrate concentration is written as

$$\frac{S_f - S}{X} = \frac{\nu}{\mu}. \tag{10}$$

In Eq. 10, the righthand side is constant value. In this case, the cell concentration is proportional to the substrate concentration of feeding medium if the cells consume the substrate completely (S is almost zero). Then, we can control the cell concentration at the steady-state by changing the substrate concentration. In this study, we use M9 like glutamate medium including glutamate as sole nitrogen source. We kept the glutamate concentration in the feeding medium lower than 1mM, since in this range the culture environment is maintained as the glutamate limited condition. We can cultivate cells under uniform environment and change the environment in the reactor by changing the feeding medium.

4 Results

We performed two kinds of experiments with environmental changes. First, we performed the continuous culture with change in glutamate concentration. After the environmental conditions reached at the steady state, the condition was kept constant until 20 or more generations of cells passed, to obtain the steady state of intra-cellular reaction dynamics. Then, the concentration of glutamate in the feeding medium was changed, in the order of 1mM → 0.1mM → 1mM. Figure 3 shows time courses of cell concentration, glutamate concentration and glucose concentration in the reactor, respectively. Cell concentration was estimated by optical density at 600nm (OD_{600}) and particle number measured by the particle characterization analyzer. At each steady state, we confirmed that glutamate concentration was almost zero and cell concentration was proportional to the glutamate concentration in the feeding medium. Soon after switching high glutamate concentration to lower one (at 270h in Fig. 3), cell concentration started to decrease, and after about 48 hours it reached to the new steady state. In a similar way, soon after switching low concentration to higher one (at 510h in Fig. 3), cell concentration increased and after about 48 hours it reached to the original steady state.

Figures 4 and 5 show the distributions of cellular states at each sampling time, measured by flow cytometer. The axis from front to right back indicates Forward Scattering intensity (FS) of each cell, and the axis from front to left back indicates GFP fluorescent intensity of each cell. The vertical axis indicates the fruquency (%) of each FS-GFP region. Upper layer of each figure shows the 3D histogram of FS-GFP and lower layer shows the density plot. Here, GFP fluorescence intensity indicates the expression level of *glnA* gene, and FS represents the cell size, respectively. In Fig. 4, distributions at the same steady state are compared with each other. The shapes of distributions at the same steady state

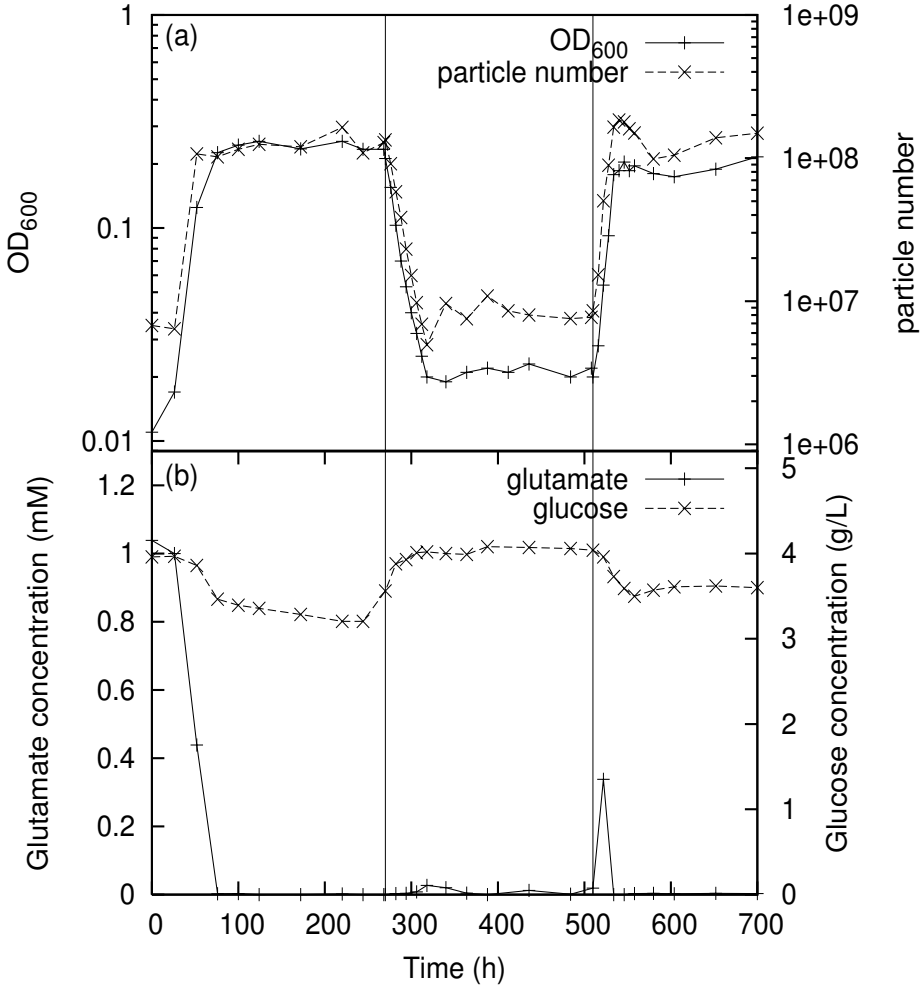

Fig. 3. Time courses of glutamate concentration change experiment. (a) the time courses of cell concentration, and (b) the time courses of substrate concentration. In the graph (a), (+) mark indicates the cell concentration measured by turbidity method (corresponding left vertical axis) and (×) mark indicates cell concentration measured by particle characterization analyzer (corresponding right vertical axis), respectively. In the graph (b), (+) mark indicates the glutamate concentration (corresponding left vertical axis) and (×) mark indicates the glucose concentration (corresponding right vertical axis), respectively. Two vertical lines at 270h and 510h indicate the time of changing the glutamate concentration in the feeding medium.

are similar. An important point here is that the distribution of *glnA* expression level exhibits a large standard deviation (with two orders of magnitude of protein number), even though the environment is homogeneous and the reaction catalyzed by *glnA* protein is essential for cell viability under the medium condi-

tion in this study. We confirmed that these diversities of gene expression levels are not due to experimental errors of flow cytometory, by measuring standard beads (BD Living Colors^{TM}EGFP Calibration Beads, BD Biosciences) which have known sizes and fluorescences, and comparing these standard data to the data of the cells.

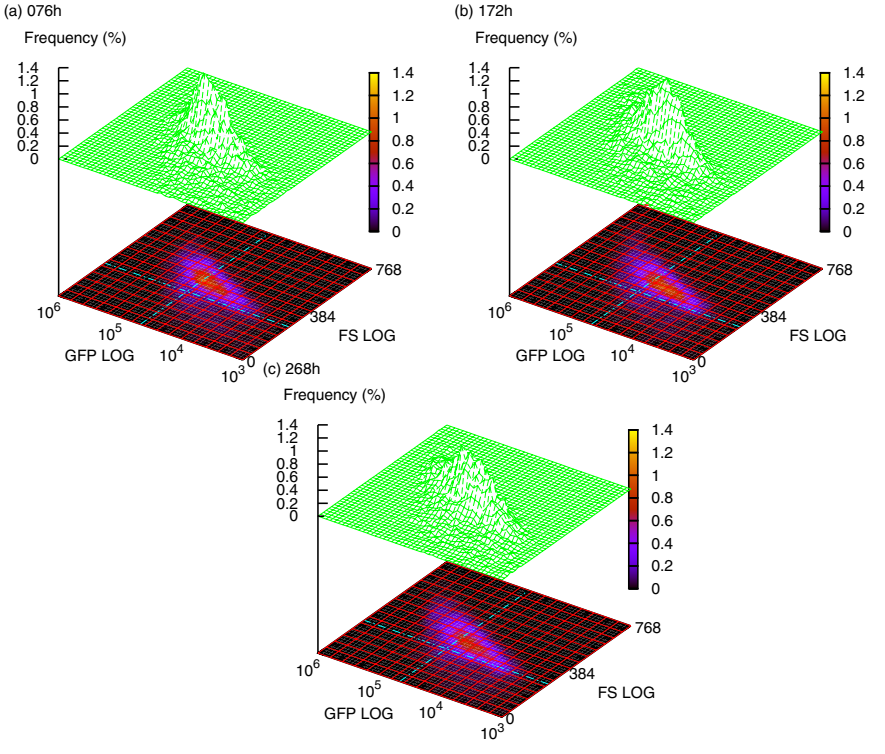

Fig. 4. The distribution of GFP fluorescence and FS signal at the initial steady-state of glutamate concentration 1mM. The figures show the distribution at (a) 76h, (b) 172h, and (c) 268h, respectively. The shapes of distribution at the same steady state are similar each other.

When the glutamate concentration in the feeding medium was changed, dynamic changes of cellular states were observed. When the glutamate concentration in the feeding medium decreased, the cell population with a high GFP fluorescence intensity (*glnA* expression level) became the major group in the system, and the number of cells with a low GFP fluorescence intensity (*glnA* expression level) decreased (Figure 5 (a-b)). On the other hand, when the glutamate concentration in the feeding medium increased, the cell population which had a low GFP fluorescence intensity (*glnA* expression level) became the major group in the system, and the number of cells which had a high GFP fluorescence intensity (*glnA* expression level) decreased (Figure 5 (c-d)). Interestingly, the

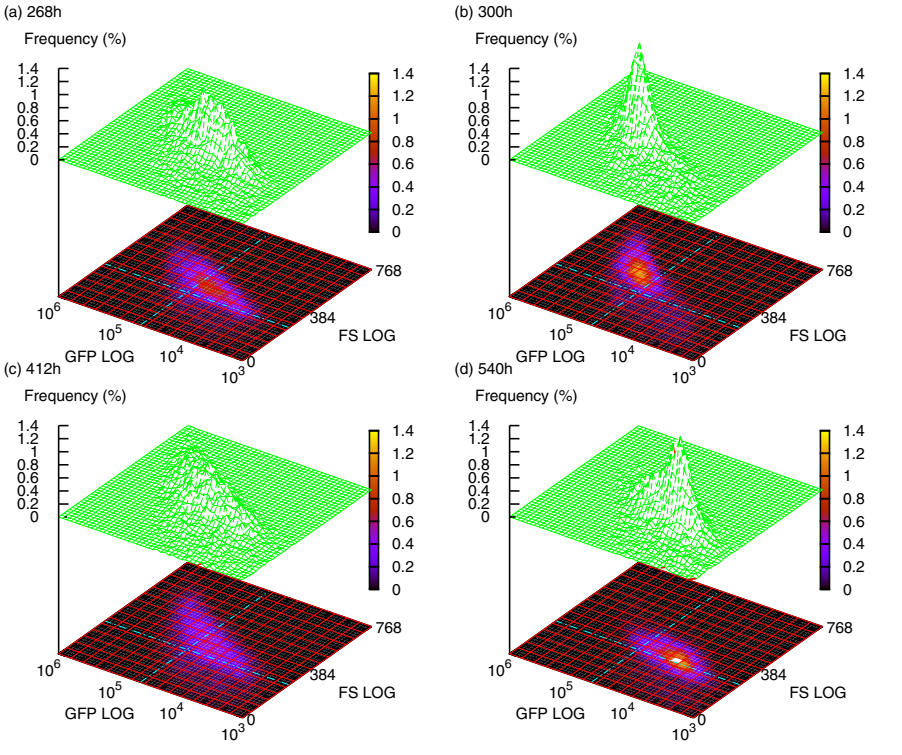

Fig. 5. The distributions of GFP and FS signal at the transient state of glutamate concentration change. Figures (a) and (b) show the distributions at the transient when the glutamate concentration in feeding medium was changed from 1mM to 0.1mM. Figure (a) shows the distribution at the initial steady state (268h) at 1mM glutamate, and figure (b) shows the distributions at 30h after change of the glutamate concentration (300h) during this transient process, subpopulation with low GFP intensity (low expression level of *glnA*) disappeared. This change was not maintained and the distribution was recovered to the original one around 48h after change of the glutamate concentration. Figures (c) and (d) show the distributions at the transient when the glutamate concentration in feeding medium was changed from 0.1mM to 1mM. Figure (c) shows the distribution at the initial steady state (508h) at 0.1mM, and figure (d) shows the distributions at 30h after change of the glutamate concentration (540h). In this case, subpopulation with high GFP intensity (high expression level of *glnA*) disappeared. Also, this change was not maintained and the distribution was recovered to the original one around 48h after change of the glutamate concentration.

cell population with the high or low *glnA* expression level was not maintained, and the original distribution with a large standard deviation reappeared, after reaching the steady state.

Next, we performed the continuous culture in which the feeding medium was changed from the medium containing glutamate as a sole nitrogen source to one containing both glutamate and glutamine. In a similar way to the previous ex-

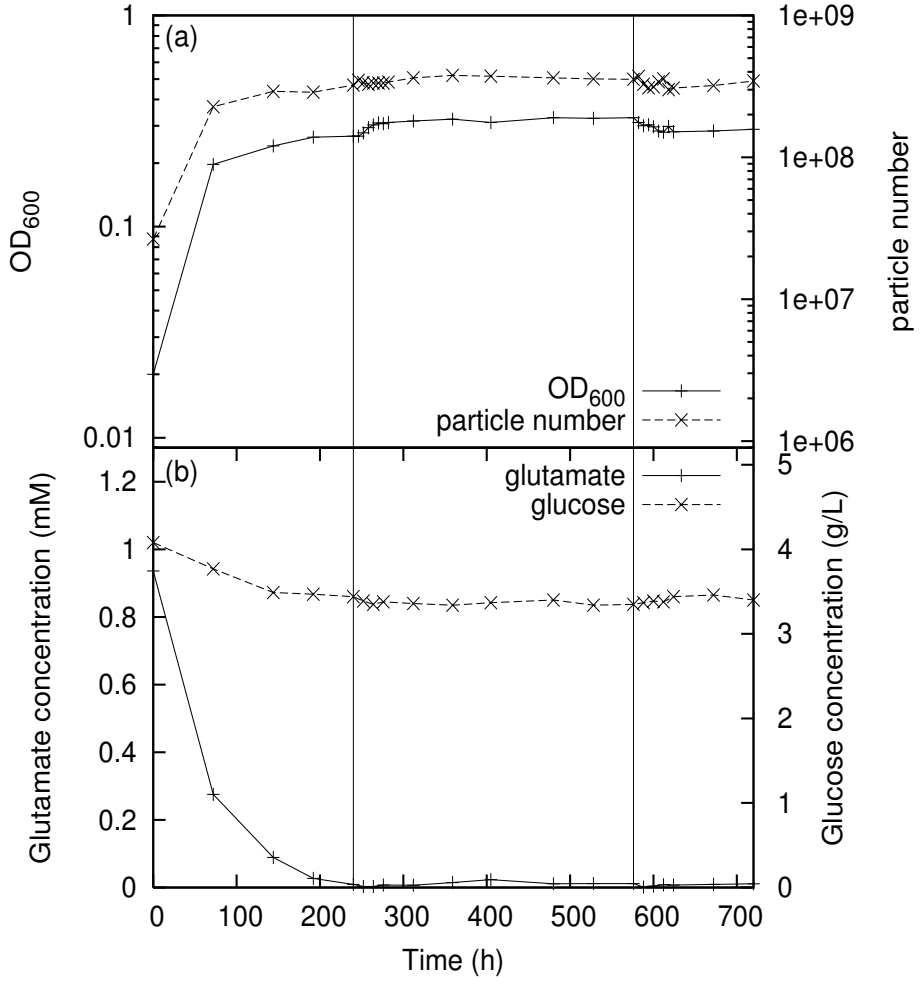

Fig. 6. Time courses of glutamate concentration change experiment. Figure (a) shows the time course of cell concentration, and figure (b) shows the time courses of substrate concentration, respectively. In the figure (a), (+) mark indicates the cell concentration measured by turbidity method (corresponding left vertical axis) and (×) mark indicates cell concentration measured by particle characterization analyzer (corresponding right vertical axis), respectively. In the figure (b), (+) mark indicates the glutamate concentration (corresponding left vertical axis) and (×) mark indicates the glucose concentration (corresponding right vertical axis), respectively. Two vertical lines at 240h and 576h indicate the time of changing the glutamine concentration in the feeding medium.

periment, to obtain the steady intra-cellular state, we kept the system for 20 or more generations passed after the environmental condition settled down. After that, the feeding medium was changed in the order of (1mM glutamate) → (1mM glutamate and 0.1mM glutamine) → (1mM glutamate). Figure 6 shows

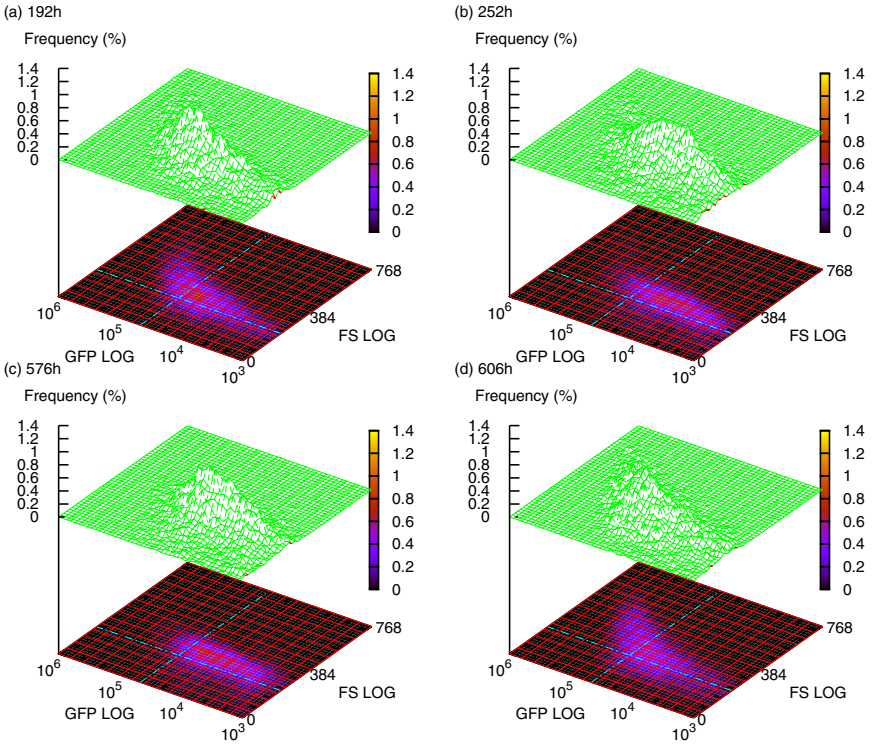

Fig. 7. The distributions of GFP and FS signal at the transient when glutamine is added to or removed from the feeding medium. Figures (a) and (b) show the distributions at the transient when glutamine concentration in feeding medium was changed from 0mM to 0.1mM. Figure (a) shows the distribution at the initial steady state (192h) at 1mM glutamate only medium, and figure (b) shows the distributions at 30h after change to the glutamine added medium (252h). After addition of glutamine, subpopulation with high GFP intensity (high expression level of *glnA*) disappears. Figures (c) and (d) show the distributions at the transient when glutamine concentration in feeding medium was changed from 0.1mM to 0mM. Figure (c) shows the distribution at the initial steady state (576h) at 0.1mM glutamine, and figure (d) shows the distributions at 30h after removal of glutamine (606h). In this case, Also, this change was not maintained and the distribution was recovered to the original one around 48h after change of the glutamate concentration. In this case, subpopulation with low GFP intensity (low expression level of *glnA*) disappears. These distributions were maintained as long as the culture conditions were kept constant.

the time courses of the glutamine addition culture. At all the steady states, that cell concentration and glucose concentration were constant, and glutamate concentration was almost zero. The addition of glutamine to the feeding medium made the cell concentration increasing about 20%. In the microscopic viewpoint, when the feeding medium was switched from glutamate only medium to glutamine added glutamate medium, dynamic change of intra-cellular states was

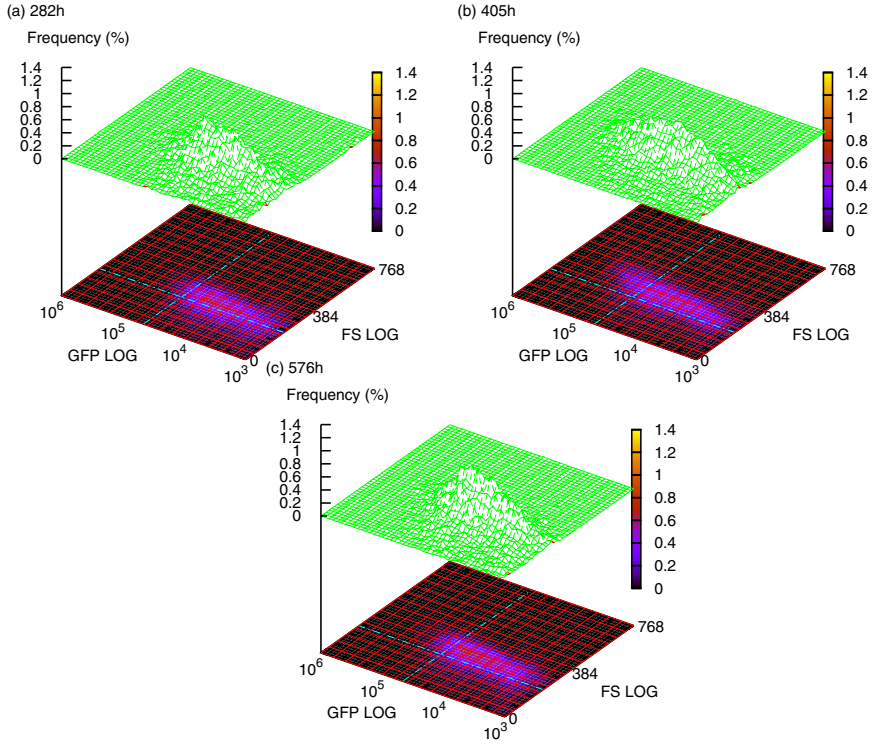

Fig. 8. The distributions at the steady state of glutamine added medium. The figures show the distribution at (a) 252h, (b) 405h, and (c) 576h, respectively. The shapes of distribution with low GFP intensity are maintained at the same steady state.

observed as shown in Figure 7 (a-b). In the steady state of glutamate medium, the distributions of *glnA* expression exhibited a large standard deviation as the previous experiments. On the other hand, when feeding medium was changed to the glutamine added medium, the number of the cells which had a high expression level of *glnA* decreased. This population change continued to the next steady state and this distribution was maintained during the steady state in this condition(Figure 8). When the addition of glutamine was cut, the original distribution was recovered as shown in Fig. 7 (c-d).

5 Discussions

Continuous cultures of the isogenic cells in an uniform environment were carried out, to investigate the nature of heterogeneity in intra-cellular state which is characterized by the analysis using the flow cytometer. As results, first we found that even in uniform environments in space and time, the cells generally show heterogeneity of intra-cellular states. Even though the expression level of *glnA* gene is essential for cell viability under the medium condition in this study,

the distribution of the expression level exhibited a broad distribution (with two orders of magnitude of protein number) in the uniform environment. This result clearly showed that such heterogeneity is inevitable in cell population, presumably due to the stochastic nature of intra-cellular dynamics[10].

Second, we found that the distribution of *glnA* gene expression dynamically changed when the substrate concentrations in the feeding medium were changed. When the glutamate concentration in the feeding medium decreased, cells with low *glnA* expression disappeared, and only cells having high *glnA* expression levels remained. Conversely, when glutamate concentration increased, cells with high *glnA* expression disappeared, and only cells with low *glnA* expression remained. Furthermore, we found that at the steady state of cells in the medium containing both glutamate and glutamine, the subpopulation with a high *glnA* expression level decreased. This change of distribution was maintained as long as glutamine was added to the feeding medium.

One may cast a question whether this dynamical change of cell population is given by flow out of subpopulation with lower growth speed, or it is due to the change of intra-cellular state in response to the environmental change. To answer it, we analyzed the change in the distribution of *glnA* expression in detail. In the continuous culture with the addition of glutamine to the feeding medium (Figure 7 (a-b)), the histograms of *glnA* expression level at the steady state in glutamate only medium and at the transient state (12h after switching to glutamine added medium) were plotted in Fig. 9. As shown in the figure, the

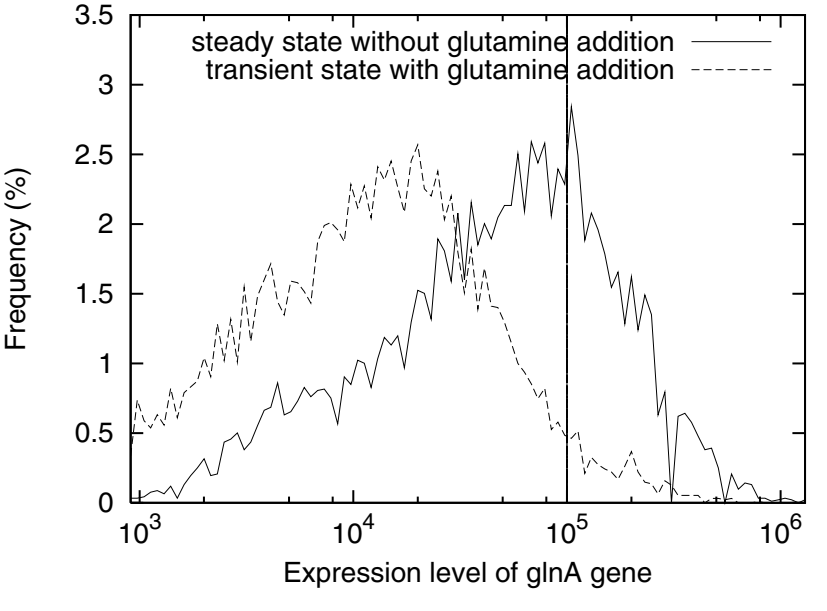

Fig. 9. The histogram of GFP intensities (expression level of *glnA* gene) at the steady state (dotted line) and transient state (solid line) of glutamine addition. The distribution of GFP intensity (expression level of *glnA* gene) moved to lower side for 12h.

expression level decrease in the transient state, and the question here is whether this change of the distribution can be explained by the difference in the growth speeds of cell populations or not. Since the flow out of the medium and cells is kept constant in the continuous culture system, we can determine the maximal change of cell population in a certain period, when the intra-cellular state of cells do not change and there are only differences in the growth speed of cells. Assume that there is a subpopulation of cells which cannot grow in the new environmental condition, their specific growth rate is zero ($\mu = 0$). The change in the population of such cells is represented as follows.

$$\frac{dX_{sub}}{dt} = -DX \tag{11}$$

Here, a survival ratio of subpopulation is defined as

$$\frac{X_{sub}(t)}{X_{sub}(0)} = \exp(-D \cdot t). \tag{12}$$

Since the dilution rate D was set at 0.075(1/h) in our experiments, when a subpopulation of cells stop to grow due to the environmental change, we can estimate that about 40% of these cells remains in the reactor 12h after the environmental change. This indicates the maximal change of cell population in the reactor without changing their intra-cellular state. In the transient process shown in Fig. 9, the fraction of cells which have more than 10^5 GFP intensity (a.u.) remains only 15% at 12h after the environmental change. This means that, the decrease of such subpopulation cannot explained by the difference of growth speed among cells, instead, the *glnA* expression level of cells changes in response to the environmental change.

It should be noted that in our experiments, the change of *glnA* expression level seems to be regulated in accordance with its requirement. For example, when the concentration of glutamate in the feeding medium is decreased, more *glnA* expression may be required to cell growth, and then the expression of *glnA* actually increases. On the other hand, when glutamine, which is product of reaction catalyzed by GlnA protein, is added to the feeding medium, the importance of *glnA* expression may be decrease, and in fact the expression of the gene decreases. The fact that such regulation is possible even though the promoter of this gene is not directly controlled by other factors suggests that the existence of another mechanism for the adaptation of cellular state. Such mechanism may provide a clue to understand why biological systems can maintain and reproduce themselves robustly, even though their intra-cellular reaction network are inevitably accompanied by large fluctuations. Also, we believe that to understand such nature of this mechanism will be available to make a robust artificial systems, such as IT networking.

Acknowledgements

This work was partially supported by the grant of the 21st century center of excellent (COE) program from the Japan Society for Promotion of Science (JSPS).

References

1. Natarajan A and Srienc F: Glucose uptake rates of single *E. coli* cells grown in glucose-limited chemostat cultures. J Microbiol Methods, 42(1). Sep, 2000, 87-96
2. Natarajan A and Srienc F: Dynamics of glucose uptake by single *Escherichia coli* cells. Metab Eng, 1(4). Oct, 1999, 320-333
3. Xu,W-Z and A.Kashiwagi and T. Yomo and I. Urabe: Fate of a mutant emerging at the initial stage of evolution. Researches on Population Ecology, Vol.38. (1996) 231-237
4. Zhang G and Gurtu V and Kain SR: An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. Biochem Biophys Res Commun, 227(3). Oct, 1996 707-711
5. Mariette R. Atkinson and Timothy A. Blauwkamp and Vladamir Bondarenko and Vasily Studitsky and Alexander J. Ninfa: Activation of *glnA*, *glnK*, and *nac* Promoters as *Escherihia coli* Nitrogen Starvation. Journal of Bacteriology, 184(19). Oct, 2002, 5358-5363
6. Shizue Ueno-Nishio and Keith C. Backman and Boris Magasanik: Regulation at the *glnL*-Operator-Promoter of the Complex *glnALG* Operon of *Escherichi coli*. Journal of Bacterioloty, Mar, 1983 1247-1251
7. Herman A. De Boar and Lisa L. Comstock and Mark Vaser: The *tac* promoter: A functional hybrid derived from the *trp* and *lac* promoters. PNAS, Vol.80. Jan, 1983 (1)21-25
8. Butler, G.J. and G.S.K. Wolkowicz: A mathematical model of the chemostat with a general class of functions describing nutrient uptake. SIAM Journal of Applied Mathematics, Vol.45. (1985) 138-151
9. James Edwin Bailey and David F. Ollis: Biochemical Engineering Fundamentals. McGrow-Hill, 1986
10. Nitzan Rosenfeld and Jonathan W. Young and Uri Alon and Reter S. Swain and Michael B. Elowitz: Gene Regulation at the Single-Cell Level. SCIENCE, Vol. 307. Mar, 2005 1962-1965