



Inherent characteristics of gene expression for buffering environmental changes without the corresponding transcriptional regulations

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Gene expression patterning is crucial for environmental nutritional responses such as the nitrogen response in *Escherichia coli*. The nitrogen response is primarily regulated by the expression of glutamine synthetase (GS), which catalyzes the sole reaction of glutamine formation, by cis-logic regulatory circuits. Here, by removing the entire corresponding operator and promoter regions required for the control of GS, we constructed an *E. coli* strain that enables the detection of the basal GS gene expression, which is expressed from a plain promoter unrelated to the nitrogen response, and measured by co-transcribed GFP expression, an indicator of GS expression. Using strain cultures, we found that the GS expression level was able to shift inversely against the change of the environmental glutamine concentration. As a control experiment, we repeated similar experiments with another strain in which the GS regulatory region remained intact and the GFP gene following the plain promoter was introduced into a different chromosomal site. For this strain, we found that the GFP expression level did not shift in accordance with the environmental glutamine concentration. These results showed that GS expression from the plain promoter exhibited a responsive ability to buffer environmental changes, whereas

the GS expression shift did not correlate with the specific characteristics of the plain promoter and GFP expression. This study identifies the inherent characteristics of basal gene expression in response to environmental changes, facilitating a deeper understanding of cellular design principles.

Key words: *Escherichia coli*, nitrogen response, glutamine synthetase, operator-deleted gene expression, robust phenomena

Living organisms adjust their gene expressions to survive and persist against a wide variety of harsh environmental and mutational genetic perturbations. Gene expression patterning can execute various tasks with the functions of decorated molecular systems, such as cis-logic gates¹⁻⁴ and a topological connectivity⁵⁻⁸. Previous studies have elucidated that the highly tuned design of genetic regulatory circuits allows the rational regulation of accurate gene expression timing⁹, the cost minimization of enzyme production¹⁰, etc^{11,12}. In addition, without the quantitative tuning of kinetic parameters, the topological connectivity determines the stable establishment of developmental systems (e.g., the segment polarity in *Drosophila melanogaster*)^{13,14}. These studies focused attention on the characteristics of decorated molecular systems that control the gene expression ensembles. For any particular gene, gene expression can be divided into two components: basal gene expression and

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forced gene expression by the decorated systems; the former component is intrinsically expressed by housekeeping proteins such as polymerase, ribosome etc., while the latter component is extrinsically expressed by other regulatory proteins that comprise decorated systems. Although this concept is effective for measuring cell-cell variation, in which basal gene expression generates intrinsic noise and decorated systems generate extrinsic noise^{15,16}, it remains unclear in relation to other properties of basal gene expression. Knowing these functions is essential for understanding the cellular design principles that control gene expression patterns. Therefore, we investigated the inherent properties of basal gene expression, particularly the response characteristics to environmental nutritional changes.

Environmental nutritional responses have some well-characterized examples such as the nitrogen response in *Escherichia coli*. The nitrogen response is primarily regulated with glutamine synthetase (GS)¹⁷, which catalyzes the sole reaction of glutamine formation, converting glutamate with ammonium to glutamine; its complete loss results in glutamine auxotrophy¹⁸. The response begins with the sensing of nitrogen levels by monitoring the pool of intracellular glutamine¹⁹. The relationships between the glutamine concentration and GS activity are that glutamine limitation increases GS activity, whereas glutamine sufficiency decreases it²⁰. GS activity is controlled in at least three distinct ways, as described below. The structural gene for GS, *glnA*, which is located at the *glnALG* operon, is subject to transcriptional regulation¹⁸. Gene expression of the *glnALG* operon is complicatedly regulated with various signal transmitter proteins such as the uridylyltransferase/uridylyl-removing enzymes, NRII/NRI, and PII^{21,22}. In addition, enzymatic activity is regulated by reversible covalent adenylation on the tyrosine residue of each subunit²³. Finally, the enzymatic activity is regulated by the feedback inhibition of a number of nitrogenous metabolites, which are competitive inhibitors that bind to the glutamate or nucleotide substrate site²⁴. Together, these regulatory mechanisms ensure that GS activity is precisely controlled and can be rapidly adjusted in response to environmental stimuli.

Here, we constructed modified strains (*E. coli* OSU7 and OSU8) to observe how basal GS gene expression responds to changes in the environmental glutamine concentration. These two strains were constructed by removing the entire corresponding operator and promoter regions for the control of GS, and by introducing another plain promoter unrelated to the nitrogen response for the expression of the GS gene. Accordingly, we obtained *E. coli* strains with GS expression that was free from transcriptional regulators. These transcriptional regulators are decorated systems that are required for the control of GS expression levels in the nitrogen response. For both strains, we found that the GS expression level was able to shift in response to the environmental glutamine concentration, so that glutamine limitation increased the GS expression level whereas glutamine suffi-

ciency decreased it. The shifting direction of the GS expression level changed inversely with changing glutamine concentration, suggesting that the basal GS gene expression exhibited a responsive buffering ability against environmental perturbation. This study illustrates the inherent characteristics of basal gene expression in response to environmental nutritional changes and facilitates a deeper understanding of cellular design principles.

Results

To assess the performance of basal gene expression, which is expressed without cis-logic gates and topological connectivity, in response to environmental nutritional changes, we constructed two *E. coli* strains, OSU8 and OSU7. OSU8 was constructed so that the partial region of the *glnALG* operon, including the entire operator and promoter regions and the *glnA* gene, was replaced by the reporter cassette sequentially containing the P_{tetA} promoter, the *gfpuv5* gene for a GFP variant, and the *glnA101* gene for a GS variant (Fig. 1). OSU7 was constructed in the same way as strain OSU8 except for the presence of the introduced gene for GS, *glnA100*, instead of *glnA101* (Fig. 1). Gene expression from the P_{tetA} promoter is constitutive, as its repressor, TetR, is not carried in this strain. As the genes for GFP and GS are co-transcribed from the P_{tetA} promoter, the fluorescence intensity of a cell is expected to indicate the GS expression level. The *glnA101* gene has mutations of Asn-219 to Asp and Tyr-397 to His and was obtained from a library generated by the random mutagenesis of a progenitor mutant gene for GS, *glnA100*, which has a mutation of Tyr-397 to His. Tyr-397 is known to be a target site for the regulation of activity by covalent adenylation; therefore, GS-Y397H and GS-N219D/Y397H are free from regulation by reversible adenylation^{21,22}. The enzymatic activity of GS-N219D/Y397H seems to be lower than that of GS-Y397H as the *E. coli* cells expressing GS-N219D/Y397H grow much more slowly than those expressing GS-Y397H (data not shown). For a control experiment, we constructed an *E. coli* strain, OSU9, by replacing the *intA* gene (an unrelated prophage gene with metabolic functions) in a wild-type cell with the cassette sequentially containing the P_{tetA} promoter and the GFP gene (Fig. 1C). In this strain, the *glnALG* operon is wild-type and, as the genes for GFP and GS are not co-transcribed from the P_{tetA} promoter, the fluorescence intensity of a cell is expected to be independent of the GS expression level.

To acclimate cells to culture conditions, the *E. coli* strain OSU8 was cultured with serial-transfer (see **Materials and Methods**) under the culture conditions used to start the environmental glutamine transition experiments. Assuming logarithmic cell growth, the specific growth rate was calculated for each subculture (see **Materials and Methods**). As the acclimation proceeded, the specific growth rate increased and finally reached a plateau level characteristic of the

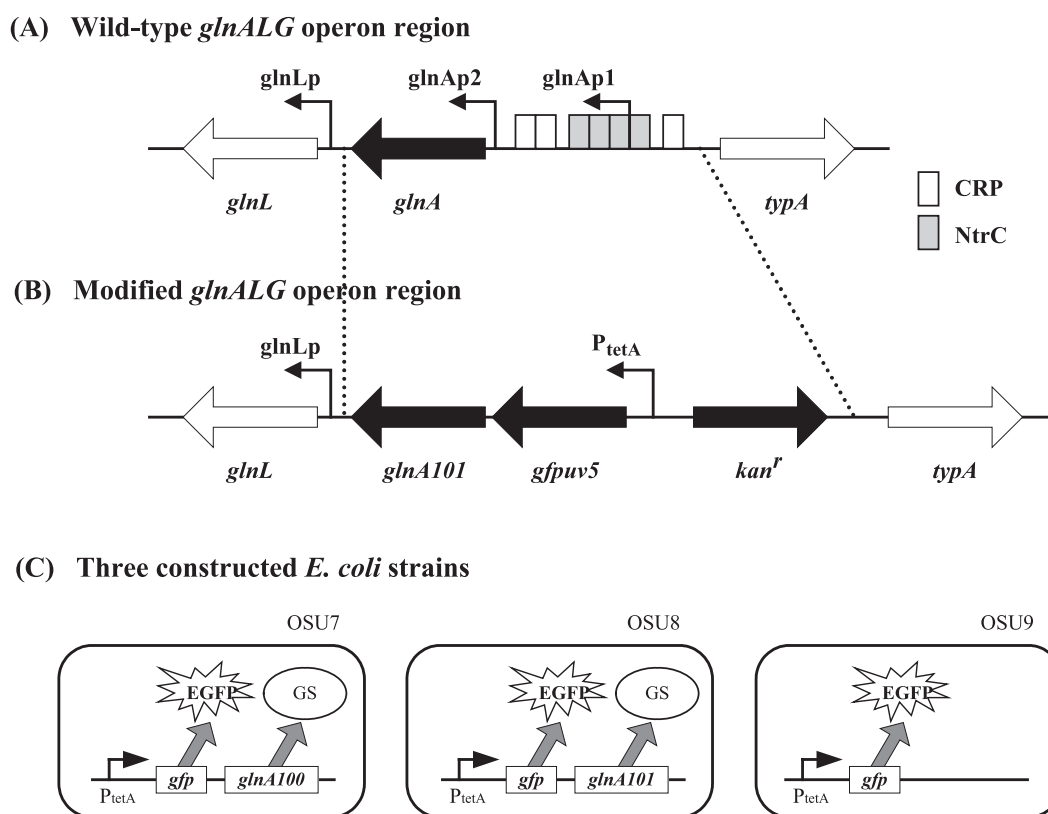


Figure 1 Structure of *glnALG* operon mutations. (A) The region near the wild-type *glnALG* operon. (B) The region after modification. (C) The design of three *E. coli* strains, OSU7, OSU8, and OSU9.

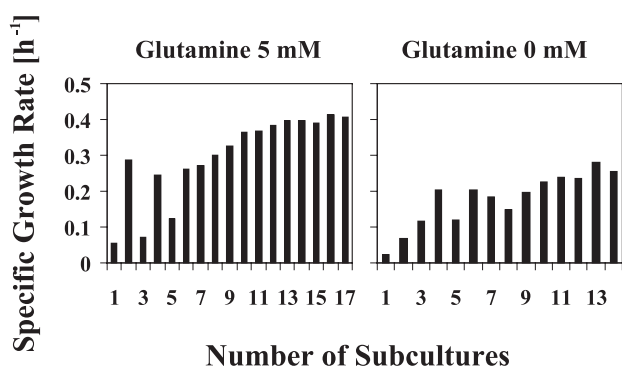


Figure 2 Acclimation of cells by serial-transfer cultures to culture conditions used to start environmental glutamine transition experiments. Specific growth rates of cells were traced at each subculture. *E. coli* strain OSU8 was separately grown at 37°C on medium D with 5 mM glutamine or without glutamine (i.e., 0 mM glutamine) until a plateau level of the specific growth rate of cells was reached.

strain and culture conditions (Fig. 2). We confirmed mutation absence when sequencing the P_{tetA} promoter and *gfp* gene in each of the three colonies that formed the final serial-transfer culture of the two lines grown on medium D with or without 5 mM glutamine.

Among the serial-transfer cultures, we analyzed the cells in the final subculture with a flow cytometer and measured the forward scatter (FS) and GFP fluorescence (FL) signals

(Fig. 3A). The FS and FL signals represent the size²⁵ and GFP content of each cell, respectively. In addition, the GFP concentration in a cell was estimated by dividing the FL by the FS signal (Fig. 3B). We found that each cell had differences in the FS and FL signals, indicating the existence of a variation in cell size and GFP content. Through the serial-transfer cultures, the distribution pattern of the GFP concentration in cells became constant (data not shown). Recent experimental studies have revealed that stochastic effects force cells to show a cell-cell variation in gene expression, even in isogenic populations^{15,26,27}. Thus, cell-cell variation seems to be naturally observed in cultured *E. coli* cells.

Through the acclimation described above, we prepared the cells used for the environmental glutamine transition experiments. The frozen acclimated cell stocks were inoculated and subjected to the serial-transfer cultures. We confirmed that the cells reached a plateau in the specific growth rate, and, at the fifth subculture, the medium was exchanged to either upshift (from 0 mM to 5 mM glutamine) or downshift the glutamine concentration (from 5 mM to 0 mM glutamine). During the serial-transfer cultures, we traced the time-series data of the values from the flow cytometry and the specific growth rate (Fig. 4). After the upshift of the glutamine concentration from 0 mM to 5 mM, the specific growth rate exhibited a gradual increase and reached a plateau at the fourteenth subculture, whereas the GFP concen-

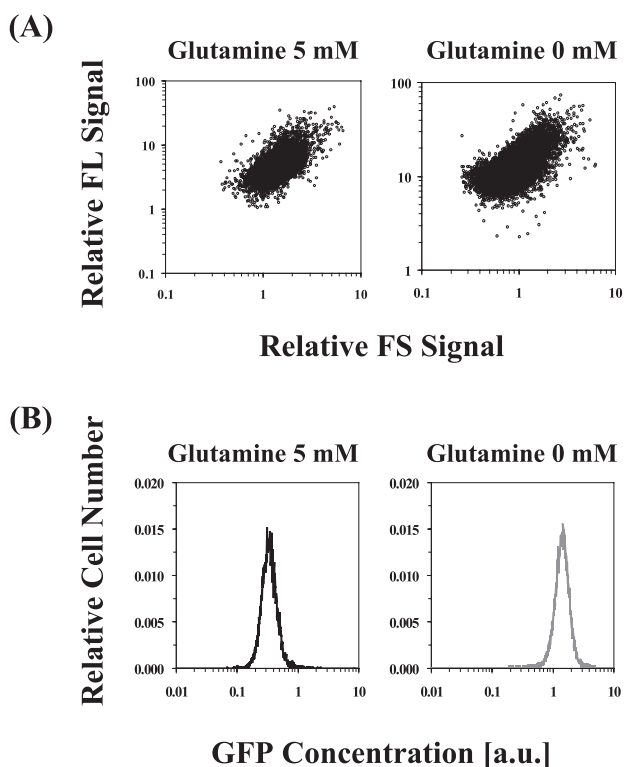


Figure 3 Cell-cell variation of isogenic cells at final serial-transfer subculture grown on medium D with 5 mM glutamine or without glutamine (i.e., 0 mM glutamine). (A) Scattering data with two parameters between FS and FL signals measured by a flow cytometer. (B) The distribution of GFP concentration calculated by dividing the FL by FS signal. GFP concentration values are in arbitrary units (a.u.).

tration in cells exhibited a gradual decrease and reached a plateau at the fourteenth subculture (Fig. 4A). Among the serial-transfer cultures, the fifth and nineteenth subcultures were evaluated with cell-cell variation (Fig. 5A; Glutamine Upshift). The shape of the distribution of the GFP concentration in cells did not change; rather, its average value shifted. These results indicate that the level of GS expression decreased in response to the increased environmental glutamine concentration, even without regulation by the corresponding transcriptional mechanisms.

On the contrary, after the downshift from 5 mM to 0 mM, the specific growth rate exhibited a rapid decrease to a lower level, and even with a gradual increase at the seventeenth subculture, reached a plateau at a lower level compared with the level at pre-transition, whereas the GFP concentration in cells exhibited a rapid increase from the sixth to ninth subcultures, reaching a plateau at the eighteenth subculture (Fig. 4B). As shown in the upshift experiment, the distribution of the GFP concentration in cells shifted merely by moving its average value (Fig. 5A; Glutamine Downshift). These results indicate that when the cells reached a plateau, the GS expression level increased in response to the decreased environmental glutamine concentration. We repeated the experiments twice, as shown in

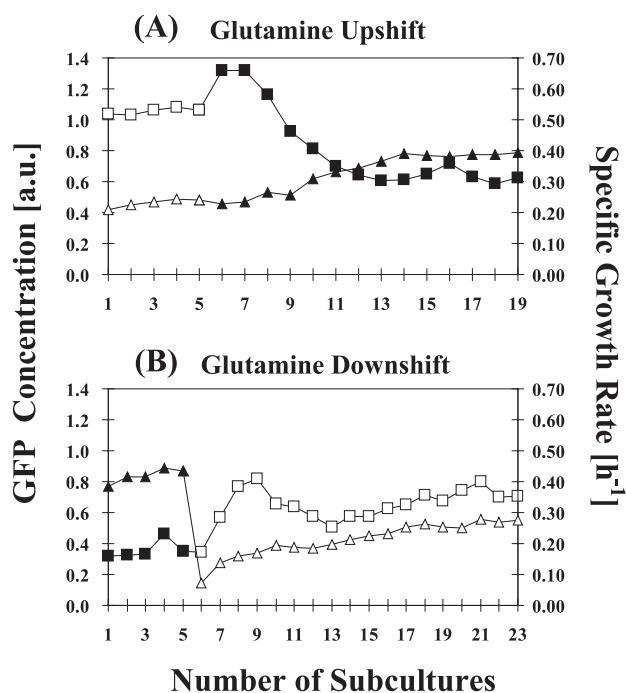


Figure 4 Response behavior of *E. coli* OSU8 to changes in the environmental glutamine concentration. The GFP concentration (squares) and specific growth rate (triangles) of cells were traced during the upshift (A) and downshift (B) of the glutamine concentration. In each experiment, the glutamine concentration was changed in a transition between 0 mM (opened) and 5 mM (closed). GFP concentration values are in arbitrary units (a.u.).

Figs. 4A, 4B, and 5A, and reproducible results were obtained in the direction of the GS expression shift in response to the changing environmental glutamine concentration.

From both the upshift and downshift experiments, the direction of the GS expression shift was inverse against the change in the glutamine concentration (Fig. 5A). In addition, regardless of the direction of the changing glutamine concentration, in each experiment, the GS expression in the 5 mM glutamine culture was lower than that in the 0 mM glutamine culture. Because GS catalyzes the sole reaction of glutamine formation, it seems to require expression patterns in which the glutamine limitation increases GS activity per cell, whereas glutamine sufficiency decreases GS activity per cell. These results suggest that the GS expression shift correlates with compensation for the change in glutamine concentration.

In addition, we used OSU7 (a progenitor strain of strain OSU8) to demonstrate how the GS expression shifted in response to the changing glutamine concentration. Compared with strain OSU8, the difference in these strains is the sequence of the GS gene (i.e., GS-Y397H for OSU7 and GS-N219D/Y397H for OSU8). When the environmental glutamine concentration changed, the distribution of the GFP concentration in cells shifted. As observed for OSU8, during both the upshift and downshift experiments, the direction of the GS expression shift was also inverse against

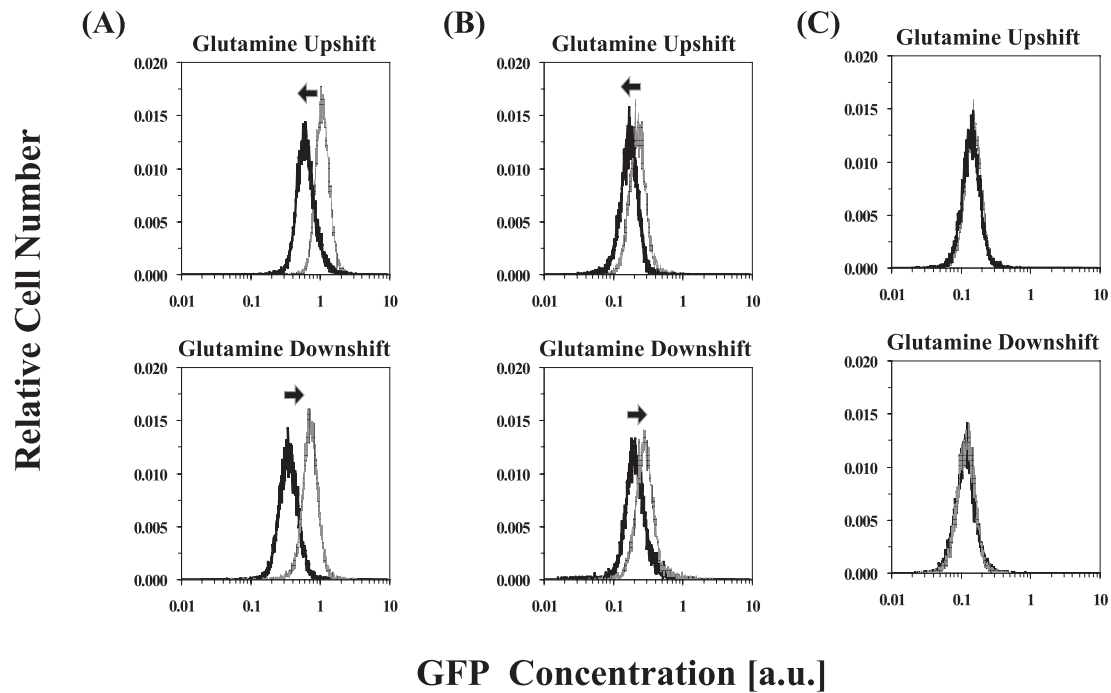


Figure 5 Shift in the GFP concentration distribution observed during the upshift and downshift of the glutamine concentration. Strains OSU8 (A), OSU7 (B), and OSU9 (C) were cultured for serial-transfer cultures. In each experiment, the glutamine concentration was changed in a transition between 0 mM (gray curve) and 5 mM (black curve). Arrows indicate the direction of the distribution shift. The GFP concentration values are in arbitrary units (a.u.).

the change in the glutamine concentration (Fig. 5B), although the degree of the shift was much smaller than in OSU8 (Fig. 5B). At the same time, the cell growth rate hardly changed (data not shown).

As a control experiment, we constructed another strain (*E. coli* OSU9) and repeated experiments similar to those described above. Compared with strains OSU8 and OSU7, the specific difference is that the cassette containing the P_{tetA} promoter and the GFP gene is located at a different site. When the environmental glutamine concentration was changed, the distribution of the GFP concentration in the cells apparently did not shift (Fig. 5C). These results indicate that the GFP concentration shift does not correlate with the specific characteristics of the transcriptional expression from the P_{tetA} promoter and the GFP expression.

Discussion

In this study, we observed the characteristics of basal gene expression of GS in response to environmental glutamine changes. We found that without the corresponding transcriptional regulation, GS expression was able to respond to the changing environmental glutamine concentration, and that the shifting direction of the GS expression was inverse against the change in the glutamine concentration. For growth, *E. coli* requires an adequate supply of glutamine supplemented from environmental nutrients or via internal production by GS. The shifting direction of the GS

expression level exhibited similar patterns to the direction that compensates for environmental glutamine changes, suggesting that the shift in the GS expression correlated with buffering environmental nutritional perturbation. This shifting the expression level was shown in both strains OSU8 and OSU7, but not in OSU9. These results suggest that gene expressions from the P_{tetA} promoter do not always exhibit the responsive buffering ability, which seems to be an inherent property of basal GS expression. In addition, the difference between OSU7 and OSU8 is just the sequence of the GS gene (i.e., GS-Y397H for OSU7 and GS-N219D/Y397H for OSU8), which suggests that the difference in the shifting degree of GS expression between these strains was attributed to the specific GS characteristics (for example, the enzymatic activity of GS). Using the present approach, one may attempt to investigate the responsive buffering ability on a genomic scale by covering most of the organism's genes. It is important to assess whether the responsive buffering ability is shown in other genes.

In explaining this responsive buffering ability, it might be plausible that the GS expression shift was caused by dilution through cell division; in other words, the protein concentration in a cell seemed to decrease/increase as the speed of cell division increased/decreased (i.e., the specific growth rate). However, during the serial-transfer cultures from the sixth to ninth subculture at the downshifting of the glutamine concentration, the GFP concentration increased as the specific growth rate increased (Fig. 4B). This result

indicates that the increase/decrease of GS expression is not always explained by the decrease/increase of the speed of cell division. Also, we confirmed mutation absence when we sequenced the P_{tetA} promoter region in each of the three colonies from the nineteenth subculture in the upshift experiment and the twenty-third subculture in the downshift experiment, suggesting that the majority of the cell population had no mutation on the P_{tetA} promoter region. Hence, the possibility is very slim that the shift in the GFP concentration shown in Fig. 5A, where the shift was observed for the majority of the population, is caused by mutation. In addition, we conducted the upshift and downshift experiments twice and obtained reproducible results. These results suggest that the GS expression shift was not caused by mutations.

Concerning a plausible explanation of the mechanism for the responsive buffering ability, there seems to be two scenarios: one is a mechanism occurring at population level, and the other at a single-cell level. The former is derived from the suggestive data of previous studies that inherent variation of a cell population could involve selectable traits²⁸. In this case, at one extreme, cells could be rendered extremely sensitive to stress, and, at the other end, they could be very tolerant, and the survivors would lead to the regeneration of the next distribution. The latter mechanism could allow each cell in a culture population to adjust its GS expression level. In this case, because the strains used in this study do not carry the corresponding transcriptional regulations, the promoters, and the ribosome binding sites derived from the *glnALG* operon, the shift in GS expression may be regulated by other molecules described above (for example, by controlling the degradation of RNA or protein etc²⁹.) In any case, in our future work, we will reveal which mechanisms the responsive buffering ability have adopted by analyzing the simultaneous, real-time measurement of single-cell fluorescence using optical well arrays and time-lapse microscopy³⁰.

Because the responsive buffering ability is facilitated by a simple design in which the enzymatic gene is expressed only from an unrelated plain promoter with particular environmental nutrient conditions, we expected this property to be performable in a case where the promoter is still not highly developed, so that it works before acquiring decorated systems. Accounting for these ideas, this property may be useful for adjusting its gene expression to catalyze unknown chemicals that cells encounter at an early evolutionary stage^{31,32} or to balance gene dosage when regulatory regions are deleted³³. Note that at the change in glutamine concentration, gene expression from the P_{tetA} promoter exhibits a lower level in the shifting degree of the expression level and needs a longer time to attain a plateau than that from the wild-type promoter with *glnALG* operator regulations. These results suggest that the rational regulations of the optimal performance of gene expression require not only basal gene expression but also transcriptional regula-

tions. For an in-depth understanding of living systems, we must study the naturally inherent properties of interactive systems from various standpoints^{34,35}.

Materials and methods

Plasmids and bacterial strains

We constructed a plasmid, pGAG-2, which is a derivative of pIGGStetak', with the reporter cassette sequentially containing the Kan^r marker, P_{tetA} promoter, *gfpuv5* gene, and *glnA100*, which is a GS variant gene. The Kan^r marker was obtained from pHSG299 (Takara), and the P_{tetA} promoter from pASK-IBA3 (Sigma-Genosys). The *gfruv5* gene, previously prepared³⁶, encodes a GFP variant. The *glnA100*, a derivative of the previously prepared *gls-h* gene³⁷, has a mutation of T4A, C5G, and T1192C, encoding the GS-Y397H.

The *Escherichia coli* strains used in this study were OSU7, OSU8, and OSU9, all derivatives of the *E. coli* strain DH1³⁸. The *E. coli* strain OSU7 was constructed by replacing the partial region of the *glnALG* operon, including the entire operator and promoter regions and the *glnA* gene (at the start coordinate of 4054648 and end coordinate of 4056343 from the sequenced genome³⁹), with the reporter cassette on pGAG-2 using homologous recombination⁴⁰. The *E. coli* strain OSU8 was constructed in the same way as strain OSU7 except for the presence of the introduced gene for GS, *glnA101*, instead of *glnA100* (more detail below). The *E. coli* strain OSU9 was constructed by replacing the entire *intA* coding region with the reporter cassette, except for the *glnA100* gene on pGAG-2, using homologous recombination⁴⁰.

GS Mutants

We obtained a mutant gene for GS, *glnA101*, in the following way. Error-prone PCR was applied for the mutagenesis of *glnA100* with ΔTth DNA polymerase⁴¹, and the primers 5'-CTGTTTCGGGCCAGAACCTGAATTCTTCC TG-3' and 5'-GCCAAAACCGATGTTTCGGTGATAACGG CTC-3'. PCR fragments were digested with *EcoRV* and *BglII* and ligated into the reporter cassette on the pGAG-2 pre-digested with the same restriction enzymes. The resultant reporter cassettes were introduced by replacing the partial region of the *glnALG* operon, including the entire operator and promoter regions and the *glnA* gene, using homologous recombination⁴⁰. An aliquot of 0.5 ml from a total of a 1 ml recombination mixture was plated on an LB agar plate⁴² containing 25 mg/l kanamycin; thirteen colonies were obtained. Each isolated strain was grown separately at 37°C on medium D (0.1 M L-glutamate, 4 g/l glucose, 10.5 g/l K₂HPO₄, 4.5 g/l KH₂PO₄, 50 mg/l MgSO₄·7H₂O, 5 mg/l thiamine HCl, and 25 mg/l kanamycin) and their growth curves were measured. Among the strains capable of growing on medium D, the *E. coli* strain OSU8 was selected as it exhibited the lowest growth rate among all

strains; its gene for GS is called *glnA101*. The sequence of the *glnA101* gene is identical to that of the *glnA100* gene except for the additional T513C and A658G mutation, encoding the GS-N219D/Y397H.

Serial-transfer cultures

Serial-transfer cultures consist of successively repeated subcultures. Fifty μl of cells were inoculated from the frozen stock and subjected to the serial-transfer cultures. At each subculture, cells were grown in 5 ml of medium D with 5 mM L-glutamine or without glutamine at 37°C, shaken at 170 rpm, and transferred into fresh medium. The number of cells in each subculture was measured with a particle counter (SYSMEX SD-2000). During the serial-transfer cultures, the number of cells was cultivated up to the range of 1.0×10^7 – 1.0×10^8 per ml. The initial and final cell densities of each subculture were measured and the specific growth rate of cells in each subculture was calculated by assuming logarithmic cell growth. The serial-transfer cultures were repeated until the specific growth rate of cells reached a plateau, and, if necessary, the final subculture was stock-frozen (–80°C).

Flow cytometer

All expression data were collected using a COULTER®EPICS®ELITE flow cytometer with a 488-nm argon excitation laser and bandpass filter at 525 ± 25 nm for GFP fluorescence. For each culture, 20,000 events were collected. The dynamic range of the flow cytometer was confirmed using commercialized beads with a known amount of fluorescent dye (CLONTECH). All flow data were converted to a text format using WinMDI Version 2.8. To eliminate particles smaller in size and weaker in fluorescence than cells, we analyzed cells using an appropriate gate in a forward and light-scattering space.

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