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Built-In Synthetic Gene Circuits in *Escherichia coli* – Methodology and Applications

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1. Introduction

Synthetic approaches are widely employed in the emerging research field of systems and synthetic biology, to learn the living organisms in a physical and systematic manner, such as, cellular dynamics and network interactions. Synthetic gene circuits potentially offer the insights into nature's underlying design principles (Hasty *et al*, 2002), and genetic reconstructions will give better understanding of naturally occurring functions (Sprinzak and Elowitz, 2005). Technical improvements in synthetic biology will provide not only engineering novelty for applications in biotechnology (McDaniel and Weiss, 2005) but also the fundamental understanding of living systems.

It is well-known that a library of the parts comprised in the gene circuits, which can be found in MIT Parts Registry (http://parts.mit.edu/), provides a variety for genetic reconstruction. As well, a new born organization (http://biobricks.org/) provides a platform (BioBrick™ parts) for scientists and engineers to work together. Current pioneer studies provided the successful examples of synthetic circuits working in the living cells, such as, the mutual inhibitory circuits functionally constructed in bacterial cells (Gardner *et al*, 2000), and with newly introduced biological functions (Kashiwagi *et al*, 2006). However, the reported cases generally do not include the vast majority of many failures. After defining a conceptual design as specifying how individual components are connected to accomplish the desired function, the next step is constructing the well-designed foreign circuit in living cells. So far, the strategies for construction of synthetic gene circuits are more of an art form than a well-established engineering discipline, mostly, in a "Plug and Play" manner (Haseltine and Arnold, 2007).

Carriers (vector) used for genetic construction are commonly limited in the plasmid, due to the advantageous of its efficiency and easy manipulation. Successful constructions have been reported to mimic a toggle switch in bacterial cells (Gardner *et al*, 2000), to build a synthetic predator-prey ecosystem (Balagadde *et al*, 2008), to address the dynamical property of positive feedback system (Maeda and Sano, 2006), to study the behaviour of the synthetic circuit under complex conditions: unregulated, repressed, activated, simultaneously repressed and activated (Guido *et al*, 2006). However, noise due to the copy number variation in plasmids is inevitable. As know, copy number variation is an important and widespread component within and

between cell populations. For example, CNV can cause statistically significant changes in concentrations of RNA associated with growth rate changes in bacteria (Klappenbach *et al*, 2000; Stevenson and Schmidt, 2004); as well as, small-scale copy number variation can cause a dramatic, nonlinear change in gene expression from the theoretical study on various genetic modules (network motifs) (Mileyko *et al*, 2008). Thus, low-copy plasmids are utilized for generation of cellular function in the studies of demonstrating that negative auto-regulation speeds the response times of transcription networks (Rosenfeld *et al*, 2002), identifying heuristic rules for programming gene expression with combinatorial promoters (Cox *et al*, 2007), studying the biological networks and produce diverse phenotypes (Guet *et al*, 2002), *etc*. As well, combination of low-copy plasmid and genome has been applied to analyze the multistablity in lactose operon in bacterial (Ozbudak *et al*, 2004), to evaluate the fluctuation in gene regulation at the single cell level (Rosenfeld *et al*, 2005), and to study noise propagation (Pedraza and van Oudenaarden, 2005), and so on. Nevertheless, neither controlling the copy number of plasmid in a living cell nor keeping a constant copy number of plasmid in a growing cell population is easy.

Difficulties in synthetic approaches of genetic constructions are faced, in particular, as the fact that a stable construction is essential for steady phenotypic quantification. Practical methodology is required for the stable maintenance of the synthetic gene circuits in growing cells. As the genome is the most stable genetic circuit in living cells, insertion synthetic circuit into the genome will promise a best solution. Short fragment genome recombination of a reporter gene is widely applied, particularly, such as, the accurate prediction of the behaviour of gene circuits from component properties (Rosenfeld *et al*, 2007), and the study on intrinsic and extrinsic noise in a single cell level (Elowitz *et al*, 2002). It is becoming aware of the importance of genome integration of the synthetic gene networks.

Though the single copy of genome is the best choice for carrying the synthetic circuit stable along with the cell division and propagation, building a complex synthetic circuit, commonly comprised of a few genetic parts, into genome is not an easy job due to the flowing reasons. Inducing these parts into the genome one by one is time consuming, and the frequently repeated genomic construction process can potentially result in unexpected mutagenesis or stress-induced genomic recombination. The modified method introduced here reduces the frequency of recombination, and provides a time-saving approach for efficient synthetic construction on the bacterial genome. The availability of long insertions allows the easy artificial reconstruction of complicated networks on the genome. The examples of synthetic circuits constructing in *Escherichia coli* cells using the refined methods are described in detail. An assortment of synthetic circuits integrated into the genome working as design principles are shown. The switch-like response of the synthetic circuit sensitive to nutritional conditions is specially presented. Constructing synthetic gene circuits integrated in bacterial genome is to form a stable built-in artificial structure, and provides a powerful tool for the studies not only on the field of synthetic and systems biology based on bacteria but also on the applications potential for genetic engineering to achieve metabolic reconstruction.

2. Methodology: Genome-integration of foreign DNA sequences

As the classic methods for genome recombination, a number of general allele replacement methods have been used to inactivate bacterial chromosomal genes (Dabert and Smith, 1997;

Kato *et al*, 1998; Link *et al*, 1997; Posfai *et al*, 1999). These methods all require creating the gene disruption on a suitable plasmid before recombining it onto the chromosome, leading to its complexity in the methodology. A relatively simple method was developed by Wanner's group, a simple and highly efficient method to disrupt chromosomal genes in *Escherichia coli* in which PCR primers provide the homology to the targeted genes (Datsenko and Wanner, 2000). The procedure is based on the Red system that promotes a greatly enhanced rate of recombination over that exhibited by *recBC*, *sbcB*, or *recD* mutants when using linear DNA.

Elegant applications of Wanner's method have been reported, such as, the construction of single-gene knock-out mutants (Baba *et al*, 2006), construction of targeted single copy of lac fusions (Ellermeier *et al*, 2002), produce insertion alleles for about 2,000 genes systematic mutagenesis of *Escherichia coli* genome (Kang *et al*, 2004). Because of the limitation on the insertion length, the optimization on transformation procedure was performed to produce recombinant prophages carrying antibiotic resistance genes (Serra-Moreno *et al*, 2006). Wanner's method is very efficient on deletion mutation, even for quite long genome segments, whereas, insertion is limited within 2-3 Kbs technically. The requirement on constructing complicated networks is facing to the technical problem on the length limitation.

The methodology of genetic construction was recently published as the research article on a new protocol for more efficient integration of larger genetic circuits into the *Escherichia coli* chromosome. Complex synthetic circuits are commonly comprised of a few genetic parts. Inducing these parts into the genome one by one is time consuming, and the frequently repeated genomic construction process can potentially result in unexpected mutagenesis or stress-induced genomic recombination. The refined procedure introduced here shows the availability of the efficient artificial reconstruction of complex networks on the *Escherichia coli* genome, and provides a powerful tool for complex studies and analysis in synthetic and systems biology. Comparison between the genome integrated and the plasmid incorporated genes, reduced cell-to-cell variation was clearly observed in genome format. The method demonstrated that the integrated circuits show more stable gene expression than those on plasmids and so we feel this technique is an essential one for microbiologists to use.

2.1 Refined method

The method has been modified including medium, temperature, transformation, and selection, as described elsewhere (Ying *et al*, 2010). The synthetic sequences need to be wholly constructed on a plasmid in advance. Following PCR amplification and purification of the linear target sequence, transformation (electroporation) for genome replacement is performed, to introduce it into competent cells. To distinguish genomic recombinants from the original plasmid carriers, the target synthetic sequence encodes a different antibiotic resistant gene from the original plasmid. False transformants (*i.e.*, transformed colony) carrying the plasmid grow on both antibiotic plates; genomic recombinants grow only on the plate carrying the antibiotic whose resistant gene is encoded in the circuit, but not the one encoded in the plasmid. Dual antibiotics selection for positive transformants reduces the labour and cost of large-scale screening, and uncovers a high ratio of positive candidates on the colony PCR check. The steps of the refined method are described as follows, along with the schematic illustration of the process (Figure 1).

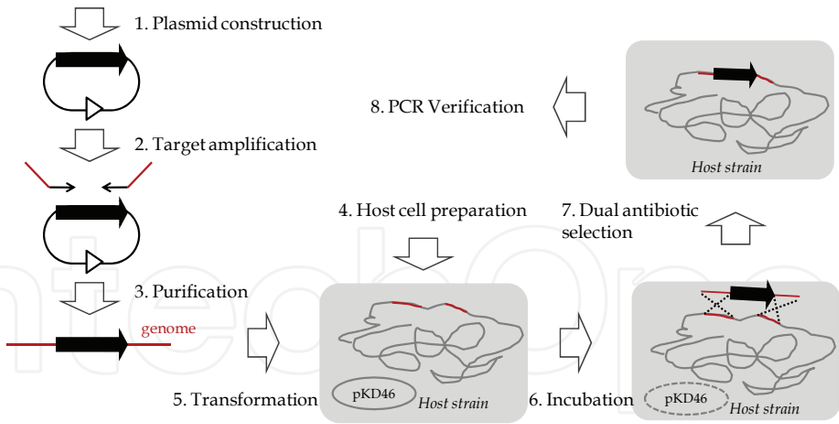

Fig. 1. Scheme of homologous recombination. The numbering steps are corresponding to the listed procedure of the refined method. Modified from the original paper (Ying *et al*, 2010).

- Construction of the synthetic sequence on a plasmid (often containing an Amp^R gene).
- PCR amplification of the target foreign DNA sequence, with the homogenous region corresponding to the recombination site.
- Clean-up (buffer exchange or gel extraction) using commercial kits.
- Digestion by the enzyme *DpnI* at 37˚C for 2 h to remove the trace amount of the original plasmid.
- Clean-up and condensation of the target sequence. Any commercial kit is convenient.
- Transformation to the host strain containing the plasmid of pKD46, encoding the recombinase. Electroporation is crucial.
- Culturing in the rich medium (SOC) with 1 mM of arabinose, at 37˚C for 2 h. Quiet incubation often increases the efficiency of transformation.
- Plating for antibiotic selection, incubation overnight at 37˚C. Once using a slow growth strain, the additional incubation time is required.
- Strike the single colonies onto two plates, each with a different antibiotic, and incubate overnight at 37˚C.
- Selection based on the difference of the clones between the two plates: positive candidates exhibited fast growth on the $Gene^R$ (the antibiotics resistant gene different from Amp^R) plate, and slow or no growth on the Amp^R plate. This dual antibiotics screening on the plates promoted the final positive selection by colony PCR.
- Colony PCR for final confirmation. This step is essential to make sure that no unexpected recombination occurred in genome, particularly repeated homologous recombination have been performed.

2.2 High efficiency of recombination

Synthetic DNA sequences of various lengths (1 — 10 Kbs) have been inserted into the different sites on genome, such as, *intC*, *argG*, *glnA*, *leuB*, *ilvE*, *hisC* and *galK*. Comparatively short insertions result in accurate genome replacement. In contrast, longer insertions generally lead to fewer transformants and a worse outcome (*i.e.*, fewer positive colonies); nevertheless, usually there are still sufficient transformants for further selection (Table 1). Genome location (gene site) dependent efficiency of homologous recombination was noticed (unpublished data). The site of *galK* always gave the best score of successful recombination, regardless of the length of inserted sequences. The efficiency of successful recombination,

based on the positive ratio of the colony PCR, depended on the insertion length. Previous studies provide myriad examples of short DNA fragment recombinations (Elowitz *et al*, 2002), but none of long ones. The ability of this methodology to successfully recombine a foreign DNA fragment of 9 to 10 Kbs indicates that it is possible to construct a complex synthetic gene circuit of a considerable length onto the bacterial genome.

Structure of Insertion	Length (Kbs)	[1]Transformants (per plate)	[2]Positive ratio (colony PCR)
$Gene^R$ (cat) for deletion	<1.5	hundreds	~100%
Gene A-$Gene^R$	2-3	hundreds	70-100%
Gene A-Gene B-$Gene^R$	3-4	20-100	70-100%
Gene A-Gene B-Gene C-$Gene^R$	4-5	20-100	50-80%
Gene A-Gene B-…-$Gene^R$	9-10	10-30	30-50%

Table 1. Recombination efficiency. Genes A, B, C, etc. represent an assortment of genes incorporated into the synthetic circuits. $Gene^R$ indicates the antibiotic resistance gene. [1]Number of clones grown on an agar plate plated with 300 µL of SOC transformation mixture. [2]Ratio of the number of positive clones to the total number of clones applied in the colony PCR test, for final confirmation. Modified from the original paper (Ying *et al*, 2010).

In addition, the homologous sequences of various lengths have been evaluated for the transformation efficiency at the identical genome location. In general, a 100-bp overlap, which can be easily generated by PCR amplification, could enable reasonably high genome recombination efficiency. Thought longer homologous sequences are supposed to give higher recombination efficiency, our test showed that length dependency was only present when the homologous sequence ranged from 50 to 150 bps, as an overlap of 300 to 500 bps decreased the number of transformants (Ying *et al*, 2010). We assume that the accessibility of the secondary structure, caused by the complex conformation of genome, for annealing possibly plays a role in successful recombination. According to the reported studies, genome integration has been generally carried out for 1 or 2 genes for each recombination step. If a relatively large fragment comprising 4 to 8 genes, four cycles of genome recombination procedure are needed. The reduced repetition of homologous recombination procedure of this refined protocol allows long DNA segments exchange at once. It greatly saves time and labour, and is supposed to contribute to the microbiological engineering.

2.3 Reduced cell-to-cell variation

Copy number variation in genetic materials can cause large variation within and between cell populations. As reported, it caused significant changes in RNA concentrations associated with growth rate changes (Klappenbach *et al*, 2000; Stevenson *et al*, 2004), as well as dramatic differences in gene expression compared to theory in various genetic modules (network motifs) (Mileyko *et al*, 2008). It is why combination of low-copy plasmids and genomes is currently applied in the studies on fluctuations in living cells (Ozbudak *et al*, 2004; Rosenfeld *et al*, 2005).

The relation between the copy number of the plasmid and the cell-to-cell variation in gene expression has been evaluated by an assortment of experiments using the flow cytometry, as

previously reported (Ito *et al*, 2009). The results showed that the copy number of the plasmid was correlated to the protein abundance, which indicated that the variable in DNA copy number contributed to the cell-to-cell variation in gene expression. The copy number issue is important to be considered, when discussing the property of the target gene and its expression fluctuation. Genomic recombination seems to be the best choice for the studies on biological noise and phenotypic fluctuation, as the genome is a stable carrier of the constant copy number.

The comparison between the plasmid and the genome clearly verified this statement. Identical synthetic sequence, comprising a reporter gene *gfp* (green fluorescence protein, GFP) and an antibiotic resistant gene kan^R, has been transformed into the host *Escherichia coli* cells, either in a low-copy plasmid (~ 20 copies per cell) or onto the genome. Once both cell types were induced to display similar green fluorescent intensities (*i.e.*, the same averaged concentration of GFP), the variance of the cellular GFP concentration obtained from plasmid carriers was much larger than that from genomic carriers (Ying *et al*, 2010). It demonstrates that the variety of gene expression levels among the cells carrying the synthetic sequence in plasmid format is much larger than that in the genomic format. Using a very low-copy plasmid (< 5 copies per cell) reduced the cell-to-cell variation but still showed larger variance than the genome format (unpublished data). It greatly suggests that it is noise in the gene copy number that increases the variation in a genetically identical population. In addition, less heterogeneous in the transformant phenotypes and longer generations were often observed in genome-integrated carriers than those of plasmid insertion (personal communication). Taken together, construction of a synthetic gene circuit on the genome will greatly improve the stability of the genetic structure itself and reduce the biological noise from copy number variation.

3. Applications: Synthetic gene circuits work as design principles

An applicable protocol for solid and efficient genome recombination is described above. An assortment of well-designed synthetic circuits built-in *Escherichia coli* cells are going to be introduced as successful applications. Precise reconstruction of synthetic gene circuits to mimic gene expression and regulation is generally employed in a plasmid with a chromosomal reporter gene (Blake *et al*). Using the modified method, we successful integrated a variety of gene circuits into the bacterial genome. First of all, a relatively long genetic loop of mutual inhibitory structure displayed a strong hysteric expression pattern was successfully constructed, which was assumed to be resulted from the decreased fluctuation due to the genome recombination. Applying the mutual inhibitory design, a bistable dual function genetic switch was built in the genome. Selective expression of the genome integrated genetic switch sensitive to the environmental transition was clearly observed, which suggested that the synthetic circuits could show physiological activity in living cells and play an important role in population adaptation.

3.1 Mutual inhibitory construct behaved as design principles

Mutual inhibitory structure has been successfully constructed in bacterial cells on the plasmids (Gardner *et al*, 2000; Kashiwagi *et al*, 2006), and the pretty work of this structure in genome format was firstly reported using the refined method (Ying *et al*, 2010). As shown in Figure 2A, the mutual inhibitory structure, which was integrated into the genome and showed the expected features, was finally acquired using the method described here. The *Escherichia coli* DH1 cells were used as hosts for the construct. To prevent disturbance of the

expression from the promoter P_{lac} by the endogenous level of LacI protein, the native lacI gene and the related genes lacY and lacZ were deleted from the genome. The reporter genes, *gfp* and *rfp*, were employed as two different visible phenotypes and were used for quantitative evaluation of the protein levels of the two repressor proteins, TetR and LacI. The two antibiotic resistant genes were used as selection markers during genetic construction. According to the design principle, when the two expression units (*i.e.*, *gfp-lacI-kan*^{R} and *rfp-tetR-cat*^{R}), which are regulated by the promoters P_{lac} and P_{tet}, respectively, are expressed, they will inhibit each other, and the cells will show a "red" or "green" phenotype. The *gfp-lacI-kan*^{R} unit is highly induced ("green" phenotype) when the chemical inducer, doxycycline (Dox), is added. Similarly, the *rfp-tetR-cat*^{R} unit is greatly induced ("red" phenotype) by isopropyl β-D-1-thiogalactopyranoside (IPTG).

The engineered *Escherichia coli* cells carrying this mutual inhibitory structure (Figure 2A) displayed either green or red fluorescent intensity (Figure 2B), representing the two discrete phenotypes of highly induced expression of either unit. As known, the positive feedback structure could accelerate the expression of any of the two expression units occasionally showing slightly higher expression level, while the mutual inhibitory structure would suppress expression of the other unit, leading to a fixation effect of the expressed unit (Gardner *et al*, 2000; Kashiwagi *et al*, 2006; Ozbudak *et al*, 2004). The discrete expression of the two units verified the bistable nature, involved in the mutual inhibitory structure, as designed features.

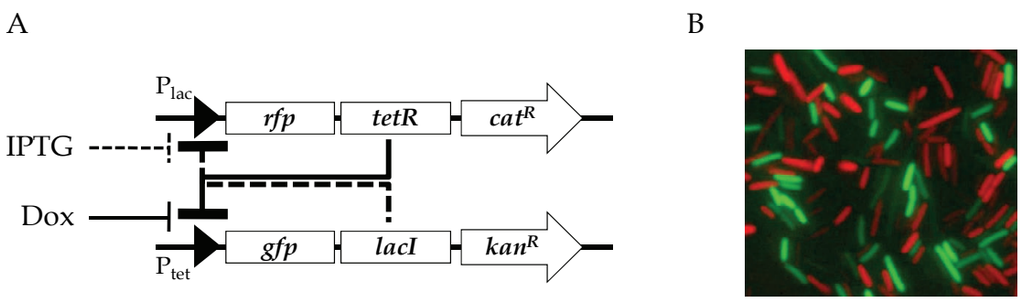

Fig. 2. *Escherichia coli* cells carrying the mutual inhibitory circuit. A. Genetic construction of the mutual inhibitory structure. B. Immerged image of the *Escherichia coli* cells observed under fluorescent microscope. Cells carrying the synthetic circuit as illustrated in A were grown in the minimal medium with induced conditions.

Following preliminary incubation in a doxycycline-free or doxycycline-supplemented (40 nM) medium, *Escherichia coli* cells carrying the mutual inhibitory structure were cultured in various concentrations of doxycycline (*i.e.*, 0, 5, 10, 15, 20, 30 and 40 nM) to induce the expression of *gfp-lacI-kan*^{R}, the "green" unit. Due to the different initial states, that is, the "green" or "red" induced expression level, the *gfp* expression levels varied even under the same condition, for instance, in the presence of 5 nM doxycycline, cells with high expression of the "red" unit at preincubation (doxycycline-free condition) showed an induced expression of *rfp* after incubation (Figure 3, upper panel), while those with high expression of "green" unit in preincubation (doxycycline-supplemented condition) showed an induced expression of *gfp* after incubation (Figure 3, bottom panel). The hysteresis in gene expression was clearly observed in the engineered *Escherichia coli* cells, under the inductions of 5 — 15 nM doxycycline, and kept for several days undergoing serial transfer, equal to approximate 30 to 50 generations. It indicated that the applicable synthetic approach of building genome integrated gene circuits successfully presenting the designed principles.

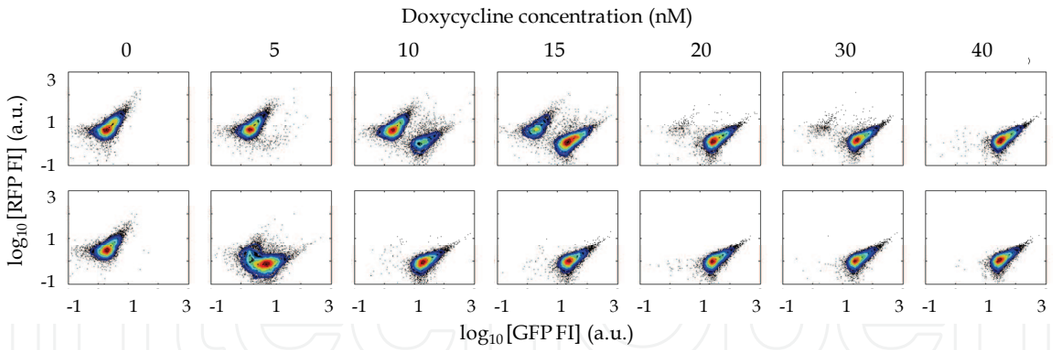

Fig. 3. Memory effect of the mutual inhibitory structure. Cells populations growing under the different induced conditions were measured using flow cytometry (FC500, Beckman). Each dot spot represents a single cell. Every 10 000 cells were collected and shown in the density maps here. GFP FI and RFP FI represent the green and red fluorescence intensity, respectively.

The success of the synthetic design was due to the easy genomic construction method. Construction of a simple toggle switch on a plasmid and transformation into bacterial cells was previously reported (Gardner *et al*, 2000). A genomic version of the similar toggle switch is introduced here. It could be steadily maintained along with propagation and cell division. Note that, to optimize this structure (*i.e.*, for strong hysteresis of gene expression), the "green" unit (*i.e.*, *gfp-lacI-kan*^{R}) was fixed and the regulatory region of the "red" one (*i.e.*, *rfp-tetR-cat*^{R}) was flexible, by using various operators or promoters. As differences in the sequence of promoters and the number of operators could strongly influence the binding affinity of the repressor proteins, regardless of the identical promoter, the operator sequences have been adjusted to amend the expression level of the repressors. The promoters and operators have been optimized for the enhanced binding affinity with the repressors, strong hysteresis (memory effect) was clearly observed in this construction. Owing to the greatly reduced copy number of the genome integrated synthetic construct, the changes in binding affinity of operator and repressor were clearly observed. The final construct of the satisfying promoter and operator sequences was decided by means of the "Plug and Play" strategy (Haseltine *et al*, 2007). Thus, optimization of the genetic construct in genome becomes practical, as the modification of the method reduces the steps and increases the efficiency of genome recombination.

3.2 Synthetic switch sensitive to environmental transitions

An assortment of synthetic circuits of varied genetic designs has been successfully constructed into the *Escherichia coli* genome (Kashiwagi *et al*, 2009; Ying *et al*, 2010). Experimental investigation demonstrated that these synthetic circuits were functional in living cells and could survive cells from starvation (Tsuru *et al*, 2011; Shimizu *et al*, in revision). Among these constructs, a relatively complex circuit of mutual inhibitory structure (Matsumoto *et al*, 2011) is introduced here.

3.2.1 Design principles

As described, bistability can be easily introduced into the genetic design of the synthetic circuit to produce two discrete stable states, "red" and "green", as shown in Figure 2B. Once the biological functions, for instance, which are crucial for cell growth, are introduced to the synthetic circuit, the two stable states will represent two phenotypes of physiological

activities. For instance, two additional genes, *geneA* and *geneB*, can be inserted into the two expression units, "red" and "green", as shown in Figure 4. The *geneA* and *geneB* encode the proteins (*e.g.*, enzymes) that catalyze the biological reactions promoting the two independent physiological pathways (Figure 4, Function A and Function B), respectively. Switching between the two expression units is supposed to have selective activation of the two pathways, resulting in growth recovery of cell population and/or causing improved production of target proteins.

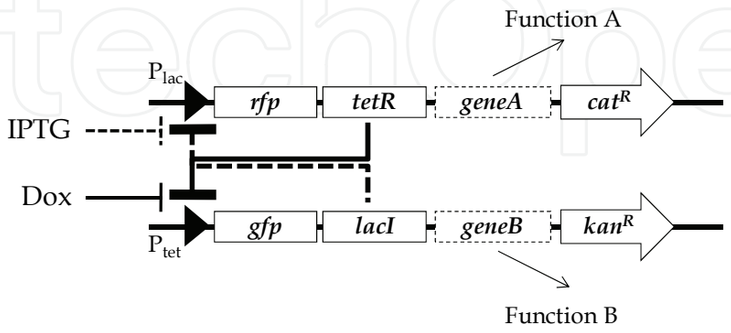

Fig. 4. Synthetic circuit designed for physiological functions. Dual functions are introduced in the mutual inhibitory structure. Induced expression of *geneA* and *geneB* represent the activation of Function A and Function B (*e.g.*, amino acid biosynthesis), respectively.

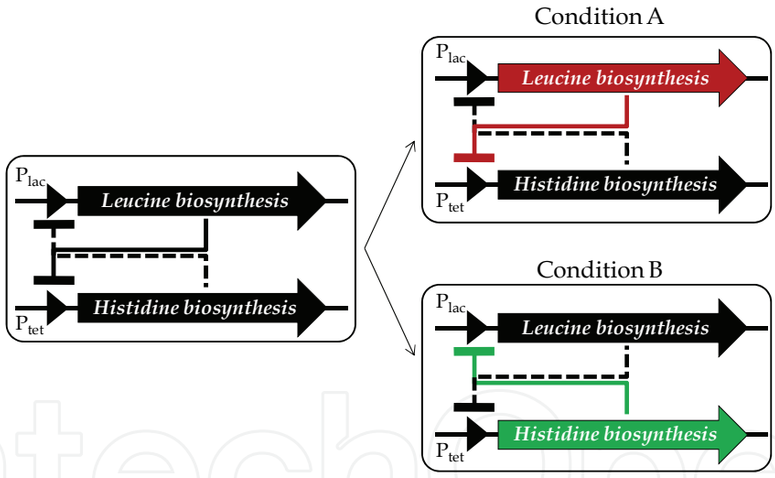

Fig. 5. Discrete phenotypes designed for the synthetic circuit. Induced expression of either "red" or "green" unit is supposed to be decided by the growing environment, either condition A (*e.g.*, leucine-free medium) or condition B (*e.g.*, histidine-free medium). Selectively induced expression of the two units is shown in red or green.

Such synthetic gene circuit was finally designed as described elsewhere (Matsumoto *et al*, 2011) and constructed as follows (Figure 4 and Figure 5). The two expression units, "red" and "green", representing a dual-function synthetic switch, were built in the *Escherichia coli* genome by homologous recombination as described in 2.1. The "red" unit contained three genes, *rfp*, *tetR* and *leuB*, encoding a red fluorescent protein (RFP), a repressor protein (*i.e.*, TetR) for blocking expression of the "green" unit (the promoter P_{tet}) and an enzyme contributing to leucine biosynthesis, respectively. The "green" unit consisted of three genes, *gfp*, *lacI* and *hisC*, encoding a green fluorescent protein (GFP), a repressor protein (*i.e.*, LacI)

inhibiting expression of the "red" unit (the promoter P_{lac}) and an enzyme involved in histidine biosynthesis, respectively. That is, the *geneA* and *geneB* were replaced by *leuB* and *hisC*, and leucine and histidine biosynthesis represented the Function A and B, respectively (Figure 4). By the way, to oblige the cells to use the genes, *hisC* and *leuB*, within the synthetic switch, the native regulation of *Leu* and *His* operons was disturbed by removing *leuB* and *hisC* from their native chromosomal locations. Thus, the expression of *leuB* and *hisC* only inside the "red" and "green" units were reported by the red and green fluorescence, respectively. Thus, a synthetic switch based on a mutual inhibitory structure showing two discrete physiological states, the induced leucine (red) and histidine (green) productions, was constructed as designed (details in Matsumoto *et al*, 2011).

Rewiring of the stress-stringent genes (*i.e.*, *leuB* and *hisC*) to the synthetic circuit allows us not only to investigate the unknown survival strategy in living systems but also to search the possibility of metabolism reconstitution. As genome recombination promises a stable genetic carrier, this synthetic dual-function circuit can be applied to mimic cellular behaviour. According to the design principle, the *Escherichia coli* cells carrying this synthetic switch are able to show two different phenotypes, "red" and "green", representing the induced expression of *leuB* and *hisC*, and related to two physiological functions, *i.e.*, leucine and histidine biosynthesis, respectively (Figure 5). Bistability, resulting from the mutual inhibitory structure, was assumed to confer the "memory effect" on the cells carrying this structure, as shown in Figure 3, where the same promoter and repressor cassette is used. That is, the cells are thought to be able to show two distinct phenotypes under identical culture conditions due to the diverse histories (induction) of gene expression.

Here, two diverse biological functions leucine and histidine biosynthesis are designed, both of which result in a fitness recovery depending on the external conditions (Figure 5). For instance, it is the cells only showing an induced expression level of the "red" unit, which contacting the gene *leuB* essential for leucine biosynthesis that could survive under leucine-depleted conditions (*i.e.*, Condition A). In general, *Escherichia coli* cells use the *Leu* operon and *His* operon to respond to starvation (Keller and Calvo, 1979; Searles *et al*, 1983; Wessler and Calvo, 1981). Depletion of leucine will lead to the induced expression of structural genes in the *Leu* operon; similarly, histidine depletion will cause an increase in expression of proteins encoded within the *His* operon (Henkin and Yanofsky, 2002; Keller *et al*, 1979). *leuB* and *hisC*, which are located within the *Leu* and *His* operons (Gama-Castro *et al*, 2008), are responsible for leucine and histidine biosynthesis, respectively. Rewiring these stringent starvation genes to the synthetic circuit not only introduces physiological activities to the synthetic design but also disturbs the original native regulation.

3.2.2 Experimental investigation

Firstly, whether the constructed synthetic circuit was functional was examined. The addition of IPTG induced the expression of the "red" unit that comprising *leuB* greatly improved the cell growth under the leucine-depleted conditions; similarly, the addition of doxycycline (Dox) induced the expression of *hisC* within the "green" unit, and allowed the cells to grow in histidine-depleted conditions (Table 2). Obviously, the selective full induction of the two expression units enabled the cells to grow under starved conditions. These results verified the following points: 1) the rewired genes, *leuB* and *hisC*, were biologically active, regardless of the chromosomal replacement; 2) the repressor-promoter interactions involved in the mutual inhibitory structure were strong; 3) the synthetic circuit was genetically stable and functional in the living cells.

Cell growth

	No add.	+IPTG	+Dox
+Leu	0.24	0.07	0.25
+His	0.10	0.35	0.10
Both AA	0.37	0.40	0.40

Gene Expression

	No add.	+IPTG	+Dox
+Leu	hisC	leuB	hisC
+His	hisC	leuB	hisC
Both AA	hisC	leuB	hisC

Table 2. Cell growth and gene expression under varied conditions. +Leu, +His and Both AA indicate the addition of leucine, histidine and both amino acids, respectively. +IPTG, +Dox and No add. indicate the addition of IPTG, doxycycline, and in the absence of inducers, respectively. Cell growth is shown in the growth rate (h^{-1}), and gene expression is marked as the induced gene name in red or green, indicating the fluorescence of the cell population.

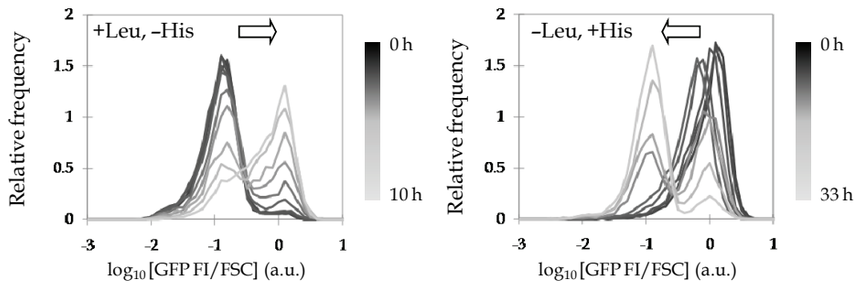

Fig. 6. Population shift in response to environmental transition. The distributions of newly formed populations (from 0 to 10 h or 33 h) are shown from light to dark grey, respectively. Green fluorescence intensity (GFP FI) and forward scattering (FSC) represent the abundances of GFP expressed in single cells and the relative cell size, respectively. GFP FI/FSC indicates the expression level of the "green" unit (*i.e.*, *hisC*) in cells. The figures are partially modified from the original paper (Matsumoto *et al,* 2011).

Subsequently, whether the synthetic circuit comprised of the rewired genes can be used by the *Escherichia coli* cells as a functional genetic switch in response to environmental transition was investigated. Under the hysteretic conditions, exponentially growing cells were transferred to the fresh media without the essential amino acid, leucine or histidine, for growth. Temporal changes in population dynamics were analyzed using flow cytometry, as described somewhere else (Matsumoto *et al*, in submission). When the "red" cell population of repressed expression of *hisC* was transferred to histidine-free conditions, the composition of the cell population changed gradually. More and more "green" cells of induced expression of *hisC* were born within the population, resulted in the population transition from "red" to "green" (Figure 6, left). Similarly, once the "green" cell population of repressed expression of *leuB* was transferred to leucine-free conditions, the "red" cells of induced expression of *leuB* were born and the fast growth allowed the "red" cells to take over the whole population, leading to the distribution shift from "green" to "red" (Figure 6, right). Note that in the rich medium, both "red" and "green" populations kept their distributions as initial expression level. Taken together, the *Escherichia coli* cells carrying this synthetic circuit formed the new population in accordance with the nutritional status.

Furthermore, the relation between cell growth and expression of the synthetic circuit was evaluated. The temporal trajectory of the relation between cell growth and relative

expression level in the cell population showed that the growth rate decreased significantly under conditions of leucine or histidine depletion but recovered gradually, accompanied by a gradual increase in *leuB* ("red" unit) or *hisC* ("green" unit) expression level. The growth recovery and population transition due to histidine depletion were faster than those due to leucine depletion. The "green" unit (*hisC*) was much easier to induce than the "red" unit (*leuB*), possibly due to slight leakage of gene expression from P_{tet}, diverse essentiality in amino acid requirement, or as yet unknown synchronised expression changes in other related genes, *etc*. Further applications using other genes and amino acids are required to determine the universality of the capacity of the synthetic circuit to respond to external perturbations and to function as genetic switch sensitive to surroundings.

The experiments demonstrated that bacterial cells carrying this synthetic circuit formed diverse populations in response to the nutritional conditions and survived under conditions of nutrient depletion. A genome-integrated dual-function synthetic circuit sensitive to an environmental transition was successfully acquired. It strongly suggested that the synthetic design of proto-operons sensitive to external perturbations is practical for native cells. In summary, the method of genetic engineering and the application studies introduced here provides an efficient constructive approach for the studies or analysis in bacterial systems biology. Here, the genes involved in physiological functions responsive to external changes are introduced into the two expression units, the selective expression of the two units in cells can be considered a synthetic operon contributing to survival and/or adaptation.

4. Conclusion

A modified method for the integration of complicated genetic circuits into the *Escherichia coli* genome is introduced. The methodology provides an efficient synthetic approach for the dynamic and stochastic study of genetic networks. Linear artificial sequences as long as ~ 9 Kbps can be easily integrated into the bacterial genome at one time. The applications clearly showed accurate phenotypic behavior of the genome-integrated synthetic gene circuits corresponding to the design principle, which confirmed that the improved method allows the efficient construction of a single copy of a complicated genetic circuit in cells. As the genome recombination generally minimizes the copy number noise in the genetic circuit, it allows the precise design and interpretation of the cellular network. The availability of long-fragment insertions allows the easy reconstruction of complicated networks on the genome, and provides a powerful tool for synthetic and systems biology. Furthermore, the *Escherichia coli* cells carrying the synthetic circuit showed selective expression pattern in accordance with the environmental conditions, demonstrated the successful application of the genome-integrated synthetic circuit in cells. The applications owing to the simplified protocol demonstrated that the synthetic construct in the genome could be physiologically functional and sensitive to environmental transition. Synthetic approaches not only leads to the technical evolution for industrial use, but also can be employed to observe novel phenomena in living organisms. Further applications and improvement may bring us the completely synthetic genome functionally works in protocells.

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6. References

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2: 2006 0008.

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L (2008) A synthetic Escherichia coli predator-prey ecosystem. *Mol Syst Biol* 4: 187.

Blake WJ, M KA, Cantor CR, Collins JJ (2003) Noise in eukaryotic gene expression. *Nature* 422: 633-637.

Cox RS, 3rd, Surette MG, Elowitz MB (2007) Programming gene expression with combinatorial promoters. *Mol Syst Biol* 3: 145.

Dabert P, Smith GR (1997) Gene replacement with linear DNA fragments in wild-type Escherichia coli: enhancement by Chi sites. *Genetics* 145: 877-889.

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* 97: 6640-6645.

Ellermeier CD, Janakiraman A, Slauch JM (2002) Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. *Gene* 290: 153-161.

Elowitz MB, Levine AJ, Siggia ED, Swain PS (2002) Stochastic gene expression in a single cell. *Science* 297: 1183-1186.

Gama-Castro S, Jimenez-Jacinto V, Peralta-Gil M, Santos-Zavaleta A, Penaloza-Spinola MI, Contreras-Moreira B, Segura-Salazar J, Muniz-Rascado L, Martinez-Flores I, Salgado H, Bonavides-Martinez C, Abreu-Goodger C, Rodriguez-Penagos C, Miranda-Rios J, Morett E, Merino E, Huerta AM, Trevino-Quintanilla L, Collado-Vides J (2008) RegulonDB (version 6.0): gene regulation model of Escherichia coli K-12 beyond transcription, active (experimental) annotated promoters and Textpresso navigation. *Nucleic Acids Res* 36: D120-124.

Gardner TS, Cantor CR, Collins JJ (2000) Construction of a genetic toggle switch in Escherichia coli. *Nature* 403: 339-342.

Guet CC, Elowitz MB, Hsing W, Leibler S (2002) Combinatorial synthesis of genetic networks. *Science* 296: 1466-1470.

Guido NJ, Wang X, Adalsteinsson D, McMillen D, Hasty J, Cantor CR, Elston TC, Collins JJ (2006) A bottom-up approach to gene regulation. *Nature* 439: 856-860.

Haseltine EL, Arnold FH (2007) Synthetic gene circuits: design with directed evolution. *Annu Rev Biophys Biomol Struct* 36: 1-19.

Hasty J, McMillen D, Collins JJ (2002) Engineered gene circuits. *Nature* 420: 224-230.

Henkin TM, Yanofsky C (2002) Regulation by transcription attenuation in bacteria: how RNA provides instructions for transcription termination/antitermination decisions. *Bioessays* 24: 700-707.

Ito Y, Toyota H, Kaneko K, Yomo T (2009) How selection affects phenotypic fluctuation. *Mol Syst Biol* 5: 264.

Kang Y, Durfee T, Glasner JD, Qiu Y, Frisch D, Winterberg KM, Blattner FR (2004) Systematic mutagenesis of the Escherichia coli genome. *J Bacteriol* 186: 4921-4930.

Kashiwagi A, Sakurai T, Tsuru S, Ying BW, Mori K, Yomo T (2009) Construction of Escherichia coli gene expression level perturbation collection. *Metab Eng* 11: 56-63.

Kashiwagi A, Urabe I, Kaneko K, Yomo T (2006) Adaptive response of a gene network to environmental changes by fitness-induced attractor selection. *PLoS ONE* 1: e49.

Kato C, Ohmiya R, Mizuno T (1998) A rapid method for disrupting genes in the Escherichia coli genome. *Biosci Biotechnol Biochem* 62: 1826-1829.

Keller EB, Calvo JM (1979) Alternative secondary structures of leader RNAs and the regulation of the trp, phe, his, thr, and leu operons. *Proc Natl Acad Sci U S A* 76: 6186-6190.

Klappenbach JA, Dunbar JM, Schmidt TM (2000) rRNA operon copy number reflects ecological strategies of bacteria. *Appl Environ Microbiol* 66: 1328-1333.

Link AJ, Phillips D, Church GM (1997) Methods for generating precise deletions and insertions in the genome of wild-type Escherichia coli: application to open reading frame characterization. *J Bacteriol* 179: 6228-6237.

Maeda YT, Sano M (2006) Regulatory dynamics of synthetic gene networks with positive feedback. *J Mol Biol* 359: 1107-1124.

Matsumoto Y, Ito Y, Tsuru S, Ying BW, Yomo T (2011) Bacterial cells carrying synthetic dual-function operon survived starvation. *J Biomed Biotechnol*, in press.

McDaniel R, Weiss R (2005) Advances in synthetic biology: on the path from prototypes to applications. *Curr Opin Biotechnol* 16: 476-483.

Mileyko Y, Joh RI, Weitz JS (2008) Small-scale copy number variation and large-scale changes in gene expression. *Proc Natl Acad Sci U S A*.

Ozbudak EM, Thattai M, Lim HN, Shraiman BI, Van Oudenaarden A (2004) Multistability in the lactose utilization network of Escherichia coli. *Nature* 427: 737-740.

Pedraza JM, van Oudenaarden A (2005) Noise propagation in gene networks. *Science* 307: 1965-1969.

Posfai G, Kolisnychenko V, Bereczki Z, Blattner FR (1999) Markerless gene replacement in Escherichia coli stimulated by a double-strand break in the chromosome. *Nucleic Acids Res* 27: 4409-4415.

Rosenfeld N, Elowitz MB, Alon U (2002) Negative autoregulation speeds the response times of transcription networks. *J Mol Biol* 323: 785-793.

Rosenfeld N, Young JW, Alon U, Swain PS, Elowitz MB (2005) Gene regulation at the single-cell level. *Science* 307: 1962-1965.

Rosenfeld N, Young JW, Alon U, Swain PS, Elowitz MB (2007) Accurate prediction of gene feedback circuit behavior from component properties. *Mol Syst Biol* 3: 143.

Searles LL, Wessler SR, Calvo JM (1983) Transcription attenuation is the major mechanism by which the leu operon of Salmonella typhimurium is controlled. *J Mol Biol* 163: 377-394.

Serra-Moreno R, Acosta S, Hernalsteens JP, Jofre J, Muniesa M (2006) Use of the lambda Red recombinase system to produce recombinant prophages carrying antibiotic resistance genes. *BMC Mol Biol* 7: 31.

Sprinzak D, Elowitz MB (2005) Reconstruction of genetic circuits. *Nature* 438: 443-448.

Stevenson BS, Schmidt TM (2004) Life history implications of rRNA gene copy number in Escherichia coli. *Appl Environ Microbiol* 70: 6670-6677.

Tsuru S, Yasuda N, Murakami Y, Ushioda J, Kashiwagi A, Suzuki S, Mori K, Ying BW, Yomo T (2011) Adaptation by stochastic switching of a monostable genetic circuit in Escherichia coli. *Mol Syst Biol* 7: 493.

Wessler SR, Calvo JM (1981) Control of leu operon expression in Escherichia coli by a transcription attenuation mechanism. *J Mol Biol* 149: 579-597.

Ying BW, Ito Y, Shimizu Y, Yomo T (2010) Refined method for the genomic integration of complex synthetic circuits. *J Biosci Bioeng* 110: 529-536.

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