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Rationally designed bidirectional promoter improves the evolutionary stability of synthetic genetic circuits

Song Yang[1,*], Sean C. Sleight[2] and Herbert M. Sauro[2]

[1]Genomics Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Mail Stop 84-363, Berkeley, CA 94720, USA and [2]Department of Bioengineering, University of Washington, Seattle, WA 98195, USA

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ABSTRACT

One problem with synthetic genes in genetically engineered organisms is that these foreign DNAs will eventually lose their functions over evolutionary time in absence of selective pressures. This general limitation can restrain the long-term study and industrial application of synthetic genetic circuits. Previous studies have shown that because of their crucial regulatory functions, prokaryotic promoters in synthetic genetic circuits are especially vulnerable to mutations. In this study, we rationally designed robust bidirectional promoters (BDPs), which are self-protected through the complementarity of their overlapping forward and backward promoter sequences on DNA duplex. When the transcription of a target non-essential gene (e.g. green fluorescent protein) was coupled to the transcription of an essential gene (e.g. antibiotic resistance gene) through the BDP, the evolutionary half-time of the gene of interest increases 4–10 times, depending on the strain and experimental conditions used. This design of using BDPs to increase the mutational stability of genetic circuits can be potentially applied to synthetic biology applications in general.

INTRODUCTION

With the recent advances in synthetic biology, scientists are able to manipulate biological organisms to perform specific functions that do not exist in nature by introducing engineered functional genetic circuits into the host genome (1–9). In general, engineered genetic circuits are not essential to the survival of the host cell, and their expression demands an extra metabolic load onto the system (10). Therefore, without any positive selective pressure, synthetic organisms will be outcompeted in the population by faster growing non-functional mutants that have inactivated the expression and function of the heterologous genes. It has been experimentally and theoretically confirmed that genes not under selective pressure will eventually lose their functions (11–16), and their lack of robustness remains a problem for synthetic biology approaches.

A synthetic genetic circuit in a bacteria host is typically composed of one or several promoters, ribosomal RNA-binding sites, terminators and the target genes of interest (14). Among the various parts of the genetic circuits, promoters are found to be most vulnerable to loss-of-function mutations (e.g. insertions, deletions of the entire promoter, deletions of repeated sequences within the promoter and point mutations) (17). Therefore, protecting the promoter from mutation is crucial to enhance the evolutionary stability of synthetic genetic circuits. In this work, we report a novel design of a robust genetic circuit controlled by a bidirectional promoter (BDP).

Typically one strand of DNA encodes functional units, such as coding regions or regulatory regions; the complementary strand does not necessarily contain any information. If the sequence of the promoter region can be designed such that its complementary strand also carries a function that is essential for the survival of the host, the promoter will then be coupled with and protected by its complementary sequence. When the complementary strand of a promoter is designed to encode a promoter, this engineered bidirectional promoter can regulate gene expression in both directions: a target gene of interest in the forward direction and an essential gene, e.g. an antibiotic gene, in the reverse direction. In this case, mutations that occur in the BDP region may, in principle, affect (or turn off) the expression of both the target gene and the essential gene concurrently. Thus, the viability of a non-functional mutant in the population will decrease and be selected against. In other words, the target gene of interest is protected by the essential gene through their shared BDP.

Indeed, overlapping functional units encoded in complementary strands exist in nature (18). In the genomes

*To whom correspondence should be addressed. Tel: +1 510 486 7442; Fax: +1 510 486 5614; Email: songyang@lbl.gov

of many organisms, adjacent promoters on opposite strands can be close to or even overlap with each other. In this way, the same *trans*-regulatory element can co-regulate the expression of multiple genes or operons in the opposite direction, which are usually in the same pathway and functionally related (18). The co-regulation function of natural BDPs is achieved by the proximity of the two promoters on different strands so that they can be controlled by one regulatory element, e.g. a repressor. One example shows that a point mutation within a natural BDP region can cause reduced expression of the genes on both sides (19).

Previous studies have tried to use the co-regulation function of naturally occurring BDPs in synthetic circuits, reviewed in (20), but no attempt has aimed to increase the evolutionary stability of a genetic circuit using BDP. In our design, the engineered gene is detrimental to the host, and the function of the engineered BDP is to prevent mutation. Therefore, not only proximity but also overlap of the forward and backward regulatory regions is required for robust and self-protective BDPs.

MATERIALS AND METHODS

Circuit engineering and use of strains

All circuits were either obtained from the Registry of Standard Biological Parts or engineered using the Clontech In-Fusion PCR Cloning Kit as described in detail previously (21). All circuits are encoded on the pSB1A2 plasmid, a high-copy number plasmid (100–300 plasmids/cell), with an ampicillin resistance gene. Plasmids were transformed into strains via chemical transformation or electroporation. *Escherichia coli* MG1655, multiple deletion strain (MDS42) 'Clean Genome' from Scarab Genomics or MG1655 Z1 (15) was used for inducible expression from LacI-regulated promoters, as these strain overexpress LacI from its chromosome.

The BDPs are derived from the LacI-regulated promoter (R0011) by gradual point mutation. Obtained from the Registry of Standard Biological Parts (14,22), R0011 is an artificial promoter consisting of the −35 and −10 regions from phage lambda with the cI-binding sites replaced with two identical 17-bp lacO1 operator sites, one between the −35 and −10 region and another upstream of the −35 region. This hybrid design makes R0011 a strong promoter, tightly repressed by LacI, and inducible by IPTG in strains that express LacI. It is known that genetic circuits regulated by R0011 quickly lose function (<50 generations) with an evolutionary half-life of ~20 generations, owing to chromosome misalignment between homologous sequences during replication (termed 'replication slippage') (23), or owing to spontaneous direct repeat deletions (24). Because of the fast and well-defined mutation in this promoter, all BDPs in this study were constructed from R0011.

Evolutionary stability measurements

Three individual transformants of each circuit to be tested were grown overnight at 37°C, shaking at 250 r.p.m. in LB + 100 μg/ml ampicillin, and stored in glycerol stock. These freezer stocks were streaked out on LB + 100 μg/ml ampicillin plates and grown overnight at 37°C. Three colonies were chosen from each transformant (nine total colonies) and inoculated into 1.5 ml of LB + 100 μg/ml ampicillin media in Eppendorf deep-well plates sealed with a Thermo Scientific gas-permeable membrane to allow for maximum oxygen diffusion. Where appropriate, the medium was supplemented with 50 μg/ml kanamycin. LacI-regulated circuits under inducible expression in MG1655 Z1 cells were supplemented with 1×10^{-3} M IPTG. After the cultures were grown for 24 h at 37°C, shaking at 250 r.p.m., the cell density (OD_{600}) and fluorescence (excitation wavelength: 485/15, emission wavelength: 516/20) were measured in a Biotek Synergy HT plate reader. Then, the evolved populations were propagated with a serial dilution scheme using a 1:1000 ($\log_2 1000 = 9.97$) dilution, which corresponds to the growth of *E. coli* at ~10 generations per day. This procedure repeats until most of the cultures lost their fluorescence or their fluorescence decreased and arrived to a plateau.

Sequencing results of mutant plasmids

To determine the loss-of-function mutations in replicate-evolved MG1655/MDS42 populations, plasmids were extracted from individual clones with low green fluorescent protein (GFP) fluorescence. Plasmids were polymerase chain reaction amplified using the vector-specific primers, VF2 and VR, and two gene-specific primers, GFP200R and KAN200R, 25-bp primers that are reverse complements of the 200–225 regions of GFP and kanamycin-resistant gene, respectively. For each construct, the plasmids of all nine duplicates were sequenced.

RESULTS

BPD design and characterization

Prokaryotic promoters are characterized by a consensus sequence, the conserved sequence motif in the promoter region. Two hexamers, the −35 region ($T_{82}T_{84}G_{78}A_{65}C_{54}A_{45}$) and the −10 region ($T_{80}A_{95}T_{45}A_{60}A_{50}T_{96}$), as well as the distance between them (16–18 bp), are the most conserved characters (25) (the subscripts denote the percentage occurrence of the most frequently found bases in *E. coli*). Our design rationale is to identify a promoter whose forward −35 and −10 regions happen to be the reverse complementary sequences of the backward −10 and −35 regions. In this ideal case, the forward and backward consensus sequences exactly overlap each other. Any mutation (point mutation, insertion or deletion) in the promoter region on one DNA strand will inevitably cause mutation in the reverse promoter. As a result, in theory, the expression of the engineered gene is protected by the expression of the antibiotic resistance gene or other crucial genes encoded on the other strand through the shared BDP.

Although each base in the consensus sequence is statistically more conserved, the exact consensus sequence is rarely found in nature. Individual promoters usually differ from the consensus sequence by one or more

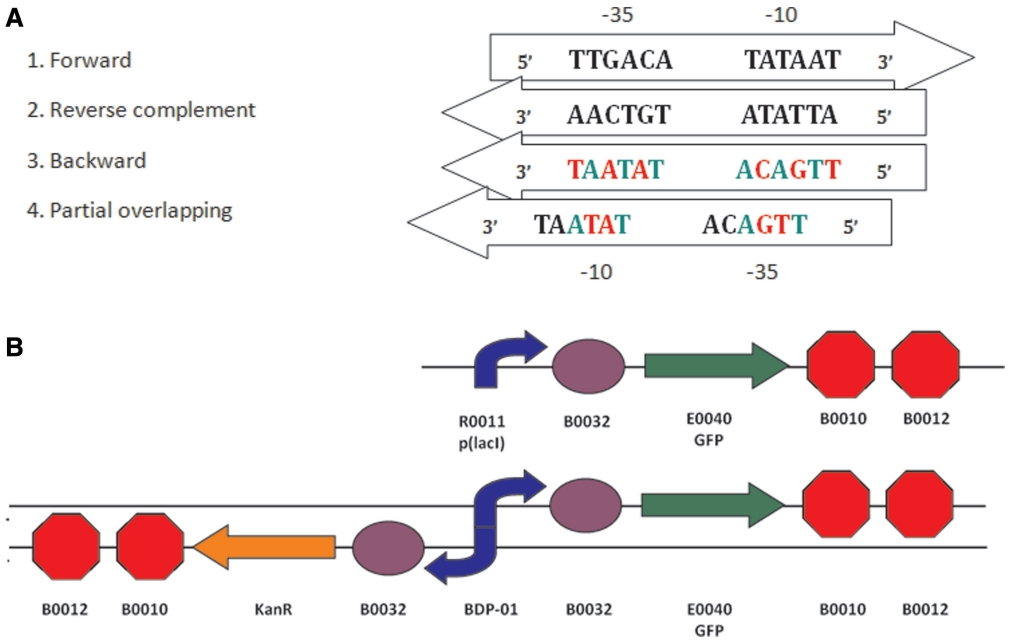

Figure 1. Design of bidirectional promoter by gradually modifying the consensus sequence. (**A**) Four sequences are shown. Sequence 1 is the −35 and −10 regions of the consensus sequence; Sequence 2 is the reverse complement of Sequence 1. Sequence 3 is the promoter sequence identical Sequence 1 but on the opposite strand and reading backwards. Sequence 4 is a partial overlapping promoter that is shifted by two bases. Bases in green represent identical bases between backward (Sequences 3 or 4) and reverse complement sequences (Sequence 2), whereas red color indicates the positions to introduce point mutations to create the BDP. (**B**) Illustration of the genetic constructs, regular promoter (R0011) on top and bidirectional promoter (BDP-01) on the bottom with biological parts on both strands. Curved arrows represent promoters, ovals represent ribosome binding sites, hexagons represent terminators and horizontal arrows represent coding sequences.

positions [up to a 5-bp mismatch has been found (25)]. In general, the more similar the promoter sequence to the consensus sequence, the higher the transcription efficiency of the promoter. The rationale is to find the −35 and −10 sequence pairs whose sequence and complementary sequence are as close to the consensus sequence as possible, ensuring promoter efficiency in both directions. As there are a total of 12 bp and each position can be chosen from four bases, the number of all possible combinations is enormous (see Supplementary Information). Our design strategy is to start from the consensus sequence on one strand and gradually make point mutations to the promoter to achieve BDP functionality.

Figure 1A shows the design of the BDP, where Sequence 1 represents the forward −35 and −10 regions of the consensus sequence and Sequence 2 is the reverse complement of Sequence 1. To have the same promoter sequence from Sequence 1 on the opposite strand, consensus sequence is reversed to create Sequence 3. In other words, Sequences 1 and 3 are identical sequences, but read in opposite directions (TTGACA_17bp_TATAAT). However, to obtain the BDP, Sequences 2 and 3 must match each other. Comparison of Sequences 2 and 3 indicates that among the 12 positions of the consensus sequence, 6 are already identical (labeled by green), so only six point mutations are required to make them match completely (labeled by red). The six variable sites allow 4^6 possible combinations, still an enormous search space.

In this prove-of-principle work, its impossible to enumerate all possible sequence combinations, so the experiment began with the top candidates, where the designed sequences carry minimum total number of mutations from the consensus sequence (six mutations) and equally distribute the mutations onto the forward and backward strands (three each). The sequences of the forward and backward promoters do not have to be identical. There are totally 14 promoter sequences that meet the criteria above (Supplementary Data). We tested their BDP function by constructing red fluorescent protein (RFP) upstream and GFP downstream of the promoter. Unfortunately, the fluorescence measurement showed that none of constructs were able to transcribe bidirectionally and produce both red and green fluorescence (Supplementary Data).

Besides the ideal design of complete overlapping of forward and backward promoters, a more practical partial overlapping design was then tested. Less stringent and robust to point mutations than the complete overlapping design, but easier to implement, the partial overlapping design will in theory maintain promoter function while minimizing mutation and recombination. Sequence 4 illustrates this partial overlapping design by shifting the entire backward sequence (Sequence 3) by two positions to the left. In this design, only four positions need to be mutated (instead of six). Other designs of forward-shifted and reverse-shifted promoters are described in Supplementary Table S1 in the Supplementary Information. If the sequence is shifted by more than 2 bp, it will increase the chance of an inactivating mutation on the opposite strand. In this partial overlapping design, one BDP was identified and experimentally confirmed through the gradual point mutation procedure. The sequence of the −10 and −35 regions of BDP-01 is

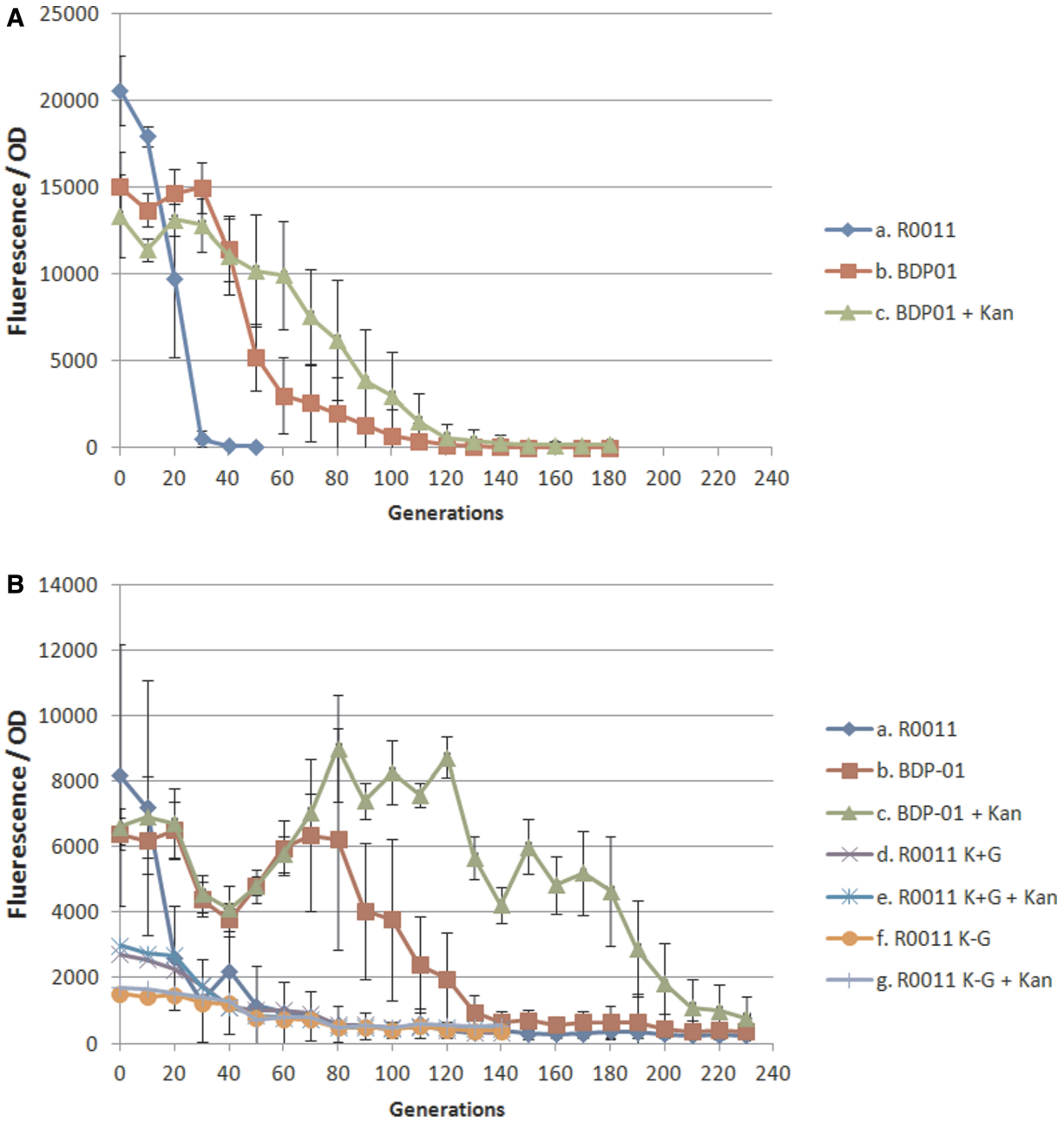

Figure 2. Performance stability of genetic circuits in *E. coli* MG1655 strain (**A**) and clean genome MDS strain (**B**). In the MG1655 strain, three circuits are studied: regular promoter R0011 as control (a), the circuit with BDP-01 and kanamycin resistance gene encoded in the backward direction of BDP-01 in the culture without kanamycin (b) and with kanamycin (c). Construct b is a negative control for c, where the absence of kanamycin in the media puts no evolutionary pressure on KanR, so the bidirectional promoter is free from evolutionary changes and acts as a regular promoter. In the MDS strain, in addition to the three circuits tested in the MG1655 strain, GFP–kanR fusion protein (d,e, R0011 K + G) and polycistronic design (f,g, R0011 K-G) are compared.

as follows: atTTGACA_15bp_tgTCAAAT (lowercase denotes the 2 bp required by the backward promoter on the other strand).

Evolutionary stability dynamics and loss-of-function mutations of genetic circuits in *E. coli* MG1655

To examine whether our designed BDP does improve the mutational robustness of genetic circuits, the evolutionary stability dynamics of circuits with the BDP (BDP-01) versus unidirectional promoter (R0011) were initially measured and compared. These circuits are encoded on the same high-copy number plasmid conferring ampicillin resistance and use of GFP as the target gene, but the circuit with the BDP-01 promoter controls expression of kanamycin resistance gene in the reverse direction, whereas R0011 does not. R0011 contains two identical 17-bp lac operator sites. Derived from R0011, BDP01

and R0011 differ only in the −35 and −10 region, but share the double operator sites.

Figure 2A shows that the evolutionary half-life of the original R0011 circuit (a) is ~20 generations, when the GFP fluorescence/OD level drops to half of the initial value. When this promoter is replaced by BDP-01, but with no kanamycin in the growth media (b), this circuit has an evolutionary half-life of ~45 generations. As BDP-01 does not have a protective function without the antibiotic, there are at least two possible reasons for the improved life span. One reason might be the lowered expression of the gene, which on average is predicted to improve evolutionary half-life (15). The second reason is the 2-bp shorter repeated lacO regions in BDP-01 relative to R0011, which presumably lower the replication slippage rate. When kanamycin was added to the media (c), the half-life of the same BDP-01 circuit increased to ~80 generations. The comparison of the BDP-01 circuit with and without kanamycin shows that the rationally designed BDP-01 promoter does provide a protective function that increases the evolutionary stability of genetic circuit.

Sequence analysis of loss-of-function plasmids in evolved populations demonstrates the reason for this protective function (Table 1 and Figure 3). In both the original R0011 circuit and BDP circuit propagated without kanamycin, replication slippage took place in all samples, resulting in deletion of one lacO sequence and the −35 region. In contrast, no deletions were found in the BDP circuit propagated with kanamycin. All mutations in the BDP kanamycin-evolved populations are caused by insertion sequence (IS) elements (26,27) that insert into downstream of the BDP-01 region, disrupting the expression of GFP and leaving KanR expression intact. Interestingly, among the 13 identified IS insertions, 6 are IS2 elements that insert right before the last 2 bp of the forward −10 region, which is right upstream of the backward −35 region. IS2 elements insert into an AT-rich region (AA-IS2-AT), which is the hotspot for IS2 insertion. Therefore, although these insertions took place in the BDP region, disrupting the forward promoter but not the reverse promoter, the expression of kanamycin resistance gene is not affected.

Evolutionary stability dynamics and loss-of-function mutations of genetic circuits in *E. coli* MDS42 ('Clean Genome' strain)

Because IS insertions are the major cause of mutations in the BDP-01 circuits, next, we obtained cell lines devoid of

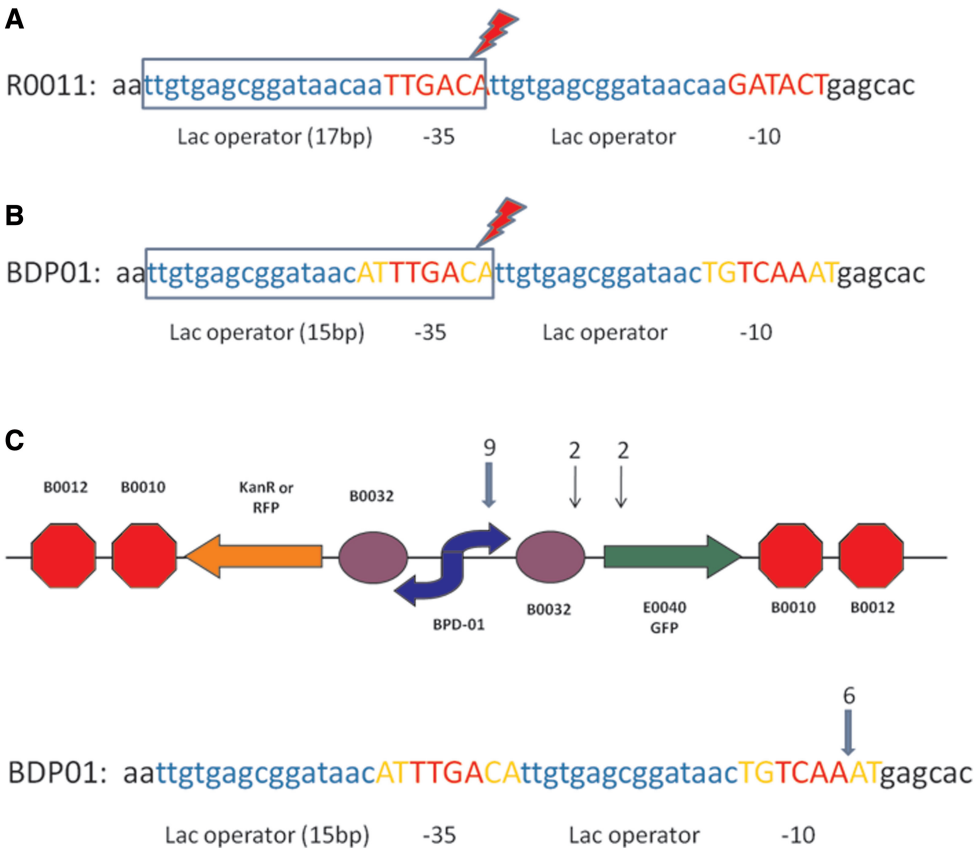

Figure 3. Mutation map of loss-of-function mutations in evolved MG1655 populations as shown In Table 1. (**A**) and (**B**), there is a deletion of one Lac operator and the −35 region due to homologous recombination (replication slippage) in the original R0011 circuit and the BDP01 circuit without kanamycin in growth media. (**C**), No replication slippage was found. Instead, among the 13 recognizable insertions, 4 cases are located in the forward GFP coding region and the ribosomal biding site, and among the 9 cases in the promoter region 6 cases are located exactly at the boundary of the start position of the reverse promoter −35 region. The BDP is colored in red (complete overlapping of forward and reverse strain) and yellow (partial overlapping).

Table 1. The sequencing results of the loss-of-function mutations in evolved MG1655 populations

	Promoter	Reverse gene	Forward gene	Strain	Media	1	2	3	4	5	6	7	8	9
a	R0011	N/A	GFP	MG1655	Amp									
b	BDP-1	KanR	GFP	MG1655	Amp									
c	BDP-1	KanR	GFP	MG1655	Amp + Kan	IS2	IS2	IS2	N/A	IS2	N/A	IS5	IS2	IS2

For each replicate-evolved population, cells highlighted with red indicate that mutations are because of deletion by replication slippage; orange cells indicate that mutations are because of insertion by IS elements. Cells without highlight indicate sequencing results that are vague and indiscernible. N/A = not applicable.

IS elements to test whether this strain increases evolutionary stability of the BDP-01 circuits. The 'Clean Genome' *E. coli* MDS42 strain (multiple deletion strain) (28) by Scarab Genomics (http://www.scarabgenomics.com/) is a synthetic strain that has 15% of the genome deleted from *E. coli* K12, including non-essential genes, recombinogenic or mobile DNA (including IS elements),and cryptic virulent genes. Figure 2B shows the evolutionary dynamics of various circuits propagated in MDS42. Overall, the expression level of GFP is lower in this strain relative to MG1655. The R0011 circuit had an evolutionary half-life of ~18 generations, about the same as when propagated in MG1655. The BDP-01 circuit grown without kanamycin in the media has an evolutionary half-life of ~105 generations, more than double of that in MG1655. The BDP-01 circuit grown with kanamycin media has an evolutionary half-life of ~185 generations, also more than double compared with MG1655. The kanamycin-evolved BDP-01 circuit has about 10 times the life span of the original R0011 circuit in the MDS42 strain.

The GFP fluorescence level of BDP-01 circuits, propagated with and without kanamycin, first dropped to almost half the maximum magnitude, then rose to the highest level and dropped again to near zero. These dynamics are observed in all nine independently evolved populations with or without kanamycin. This phenomenon probably occurs because there is a physiological adaptation of the MDS42 strain required to maintain this circuit on a high-copy number plasmid. As this strain has a 15% smaller than normal genome, it may take more time to maximize the growth rate, given the extra metabolic load due to the high-copy number plasmid. Sequence analysis of the loss-of-function plasmids showed that no IS insertion was observed in evolved populations, indicating that the use of MDS strain prevents the prevalent IS insertion in MG1655 strains. However, various other types of insertion and recombination-based mutations happened. Besides, despite being rare in MG1655, point mutations are often found in MDS42 strains, possibly indicating that because of the truncated genome, the replication repairing machinery in the clean genome is less efficient than that in the complete genome (Supplementary Information).

In addition, two other control circuits were tested in the MDS42 strain. One circuit expresses a GFP–KanR fusion protein with a glycine–serine linker and another circuit expresses GFP polycistronically linked with KanR. Both circuits were originally engineered in a previous study (15). As shown in Figure 2B, the initial expression levels of these two circuits are much lower than the other circuits. This may be because of generally lower expression differences in this strain, improper folding of GFP in the fusion protein circuit and/or differences in translation rate between GFP and KanR in the polycistronic circuit. Despite their low expression level, both circuits quickly lose function, with and without kanamycin, and have an evolutionary half-life of ~50 generations. This result shows that target gene (GFP) cannot be protected by directly connecting it to an essential gene through fusion or polycistronic link, in accordance of our previous work.

DISCUSSION

In this study, we have designed, constructed and characterized a BDP that expresses GFP in the forward direction and kanamycin resistance gene in the reverse direction. The utility of BDP-01 is demonstrated by the increased evolutionary stability of this circuit when propagated in media with kanamycin. Furthermore, the loss-of-function mutations in the kanamycin-evolved populations only occur in the GFP-expressing component of the circuit, indicating that the BDP-01 promoter expressing kanamycin resistance is essential for survival. The design of BDP sequence and genetic circuit controlled by BDP is independent of target gene of interest; this design can be generally applied to other synthetic genetic circuit.

It is clear that the BDP-01 circuit has an improved evolutionary stability when propagated in kanamycin versus without kanamycin, compared with the control KanR-expressing circuits that do not show a difference in stability. In a previous study (15), it was found that both the GFP–KanR fusion protein and polycistronic circuits lost function at almost the same rate, whether propagated in kanamycin or not, consistent with this study. Therefore, the difference between these KanR-expressing control circuits and the BDP-01 circuit is not because of the different promoters in use, R0011 in this study, and the R0010 promoter (more mutationally robust because of the absence of repeated operator sequences) in the previous study. The major difference is the design of the circuit. Although the metabolic load for BDP circuit and control circuits is similar, both GFP–KanR fusion protein and polycistronic circuits produce long RNA products (and long proteins for the fusion version), which affect the transcription and translation rate and the biological function of the final protein product. In contrast, the BDP design keeps the original RNA and protein product of the target gene (GFP) and the

protection gene (kanamycin resistance), so their normal biological function is maintained.

Another interesting result is that the GFP expression levels in MDS42 are ~40% lower than in MG1655 on average between circuits. Previous work has demonstrated that the expression level and evolutionary half-life for two different genetic circuits are negatively correlated; on average, the higher the expression level, the shorter the half-life (15). However, this observation is based on comparing different circuits in the same strain. The increase from 80 to 185 generations in the MG1655 and MDS42 strains, respectively, for the kanamycin-evolved BDP-01 circuit, is likely not because of the decrease in expression level, as the evolutionary half-life of the original R0011 circuit did not change (~20 generations in both MG1655 and MDS42 strains).

As can be learned from the clean genome evolution experiment, the evolutionary half-life of our designed synthetic circuit increases when IS elements are eliminated, but eventually other mutations, such as point mutations, insertions/deletions and recombination, will happen, and the designed circuit will be outcompeted. Step by step, we try to eliminate the most probable mutations, but evolution will pick the next feasible mutations and eventually take over. It is an everlasting procedure for synthetic biology to elongate the life span of synthetic circuits.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–5.

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