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Continuous Cell-Free Replication and Evolution of Artificial Genomic DNA in a Compartmentalized Gene Expression System

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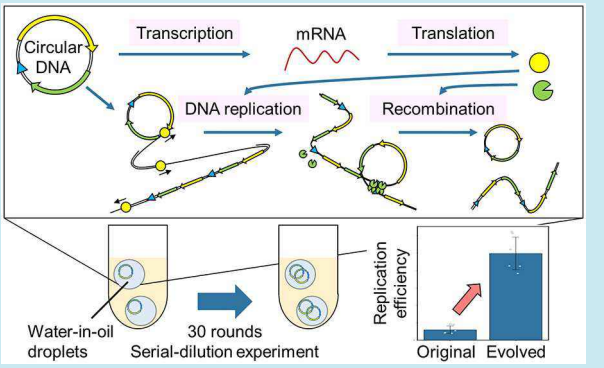
ABSTRACT: In all living organisms, genomic DNA continuously replicates by the proteins encoded in itself and undergoes evolution through many generations of replication. This continuous replication coupled with gene expression and the resultant evolution are fundamental functions of living things, but they have not previously been reconstituted in cell-free systems. In this study, we combined an artificial DNA replication scheme with a reconstituted gene expression system and microcompartmentalization to realize these functions. Circular DNA replicated through rolling-circle replication followed by homologous recombination catalyzed by the proteins, phi29 DNA polymerase, and Cre recombinase expressed from the DNA. We encapsulated the system in microscale water-in-oil droplets and performed serial dilution cycles. Isolated circular DNAs at Round 30 accumulated several common mutations, and the isolated DNA clones exhibited higher replication abilities than the original DNA due to its improved ability as a replication template, increased polymerase activity, and a reduced inhibitory effect of polymerization by the recombinase. The artificial genomic DNA, which continuously replicates using self-encoded proteins and autonomously improves its sequence, provides a useful starting point for the development of more complex artificial cells.



KEYWORDS: *DNA replication, cell-free synthetic biology, artificial cell, Darwinian evolution*

INTRODUCTION

The reconstitution of biological functions *in vitro* is a major challenge in cell-free synthetic biology and artificial cell synthesis, which contributes to the understanding of the underlying principles of living systems and the development of new biotechnologies.[1−11] One of the fundamental functions of living systems that has yet to be fully implemented in an artificial system is evolution. All living organisms use DNA as a genetic information molecule and replicate it by a set of proteins expressed from the genes encoded in the DNA. If replication continues for many generations and mutations are introduced, more adaptive DNA sequences are selected naturally. No such continuous DNA replication by self-encoded proteins and resultant adaptive evolution has yet been realized in cell-free systems.

Presently, various types of isothermal DNA replication systems have been constructed *in vitro*, including natural DNA replication systems of cellular organisms,[12−15] viruses,[16−23] and artificial systems.[24−33] In most cases, DNA replication is performed using purified proteins; in others, some proteins that are expressed from the DNA itself in the same reaction mixture are used,[15,16,33−37] which is more similar to natural organisms. However, these *in vitro* reconstitutions of DNA replication are not sufficient for conducting adaptive evolution, which requires continuous replication for many generations. The adaptive evolution of gene expression-coupled systems also requires compartmentalization or equivalent spatial structures to ensure the linkage between DNA and expressed proteins.[38] Such continuous, compartmentalized, and gene expression-coupled DNA replication remains a challenge.

In this study, we attempted to construct an *in vitro* DNA replication in which DNA molecules continuously replicate by using the proteins encoded within themselves and perform adaptive evolution of the DNA through replication for many generations. We employed an artificial DNA replication scheme that requires only two types of proteins, phi29 DNA polymerase and Cre recombinase. We encapsulated artificial genomic DNA that encoded the two proteins with a reconstituted gene expression system in microsized compartments and then performed gene expression-coupled DNA replication for many generations through a serial dilution cycle.

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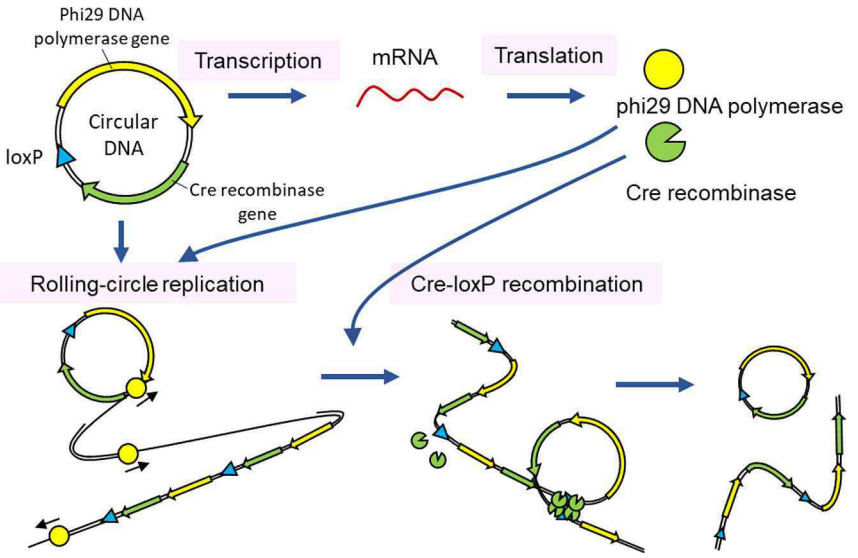

Figure 1. Scheme of transcription and translation-coupled DNA replication and recombination. This system consists of a circular DNA and a customized reconstituted translation system of *E. coli*, including T7 RNA polymerase and dNTP. The circular DNA contains the phi29 DNA polymerase and Cre recombinase genes under the T7 promoter and loxP site for recombination by Cre recombinase. First, phi29 DNA polymerase and Cre recombinase are expressed through transcription and translation. Next, the expressed phi29 DNA polymerase initiates rolling-circle replication to produce a long single-stranded DNA and then synthesizes the complementary strand to produce long double-stranded DNA. Finally, the translated Cre recombinase catalyzes homologous recombination at loxP sites to reproduce circular DNA. The size of the circular DNA product can vary depending on the recombination site. Note that a large fraction of DNA remains in a linear form even after the reaction. Although we did not add any primers in this system, DNA replication proceeded without issue; this is probably because RNAs synthesized by transcription work as primers for DNA polymerization.[36]

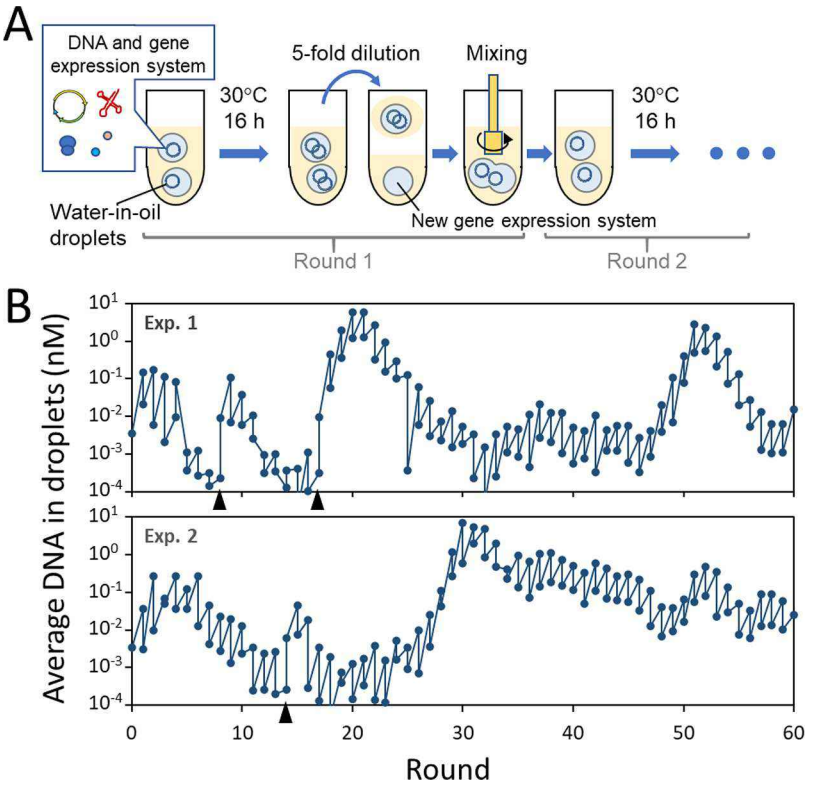

Figure 2. Long-term compartmentalized serial dilution experiment with circular DNA. (A) Experimental procedures. The original circular DNA and the gene expression system were encapsulated in water-in-oil droplets of approximately 0.5−8 μm in diameter (Figure S1) and incubated at 30 °C for 16 h, during which gene expression and DNA replication occurred, as shown in Figure 1. The droplets were diluted 5-fold with new droplets containing the gene expression system and mixed vigorously to induce fusion and division of the droplets. Then, the droplets were used for the next round of reactions. (B) Trajectories of the average DNA concentration in the droplets. The product DNA concentration was measured by qPCR before and after the 30 °C incubation step. At some rounds when the DNA concentration was close to the detection limit (10^{-4} nM), the DNA was amplified by PCR (black arrowhead). The serial dilution experiments were performed twice independently (Exps. 1 and 2).

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The difference of this study from our previous study[32] is the introduction of Cre recombinase gene and continuous replication through a serial dilution cycle. We found that the DNA population accumulated common mutations; the mutated DNA exhibited higher replication abilities than the original DNA due to improvements at several points in the DNA replication processes, indicating that the DNA had undergone adaptive evolution.

RESULTS AND DISCUSSION

Transcription and Translation-Coupled DNA Replication (TTcDR) System. To construct a DNA replication system in which DNA molecules continuously replicate and evolve using proteins encoded by themselves, we employed an artificial DNA replication scheme that requires only two proteins, as originally proposed by Forster and Church[39] and constructed in our previous study.[32] DNA replication in this system requires phi29 DNA replicase, Cre recombinase, and circular DNA as a template (Figure 1). The two proteins are encoded in the circular DNA, and these genes are expressed through transcription and translation in a customized reconstituted translation system of *E. coli*,[40] including T7 RNA polymerase. The expressed phi29 DNA polymerase initiates rolling-circle replication from the circular DNA to synthesize a long single-stranded DNA, which becomes a template for complementary strand synthesis by the polymerase. Then, the expressed Cre recombinase catalyzes the homologous recombination of the loxP site on the long double-stranded DNA to reproduce circular DNAs. In our previous study,[32] Cre recombinase was supplied as a purified protein, but in the current study, it was expressed internally. We did not supply DNA primers in this system because the RNAs synthesized by T7 RNA polymerase are expected to act as primers.[32,33,36]

Long-Term Serial Dilution Experiments. We attempted to conduct the DNA replication scheme shown in Figure 1 for many generations to achieve adaptive evolution of the circular DNA. For this purpose, compartmentalization of the DNA replication system is required to establish a linkage between DNA and expressed proteins. As compartments, we employed water-in-oil droplets of approximately 0.5−8 μm diameter (Figure S1). We encapsulated the original circular DNA (0.01 nM) and the reconstituted gene expression system in the droplets. The DNA concentration corresponds to one DNA molecule per approximately 100 droplets. The droplets were incubated at 30 °C for 16 h to induce gene expression and subsequent DNA replication (Figure 2A). The DNA concentrations before and after the reaction were measured by qPCR. An aliquot of the droplets was then diluted 5-fold with new droplets containing the gene expression system and mixed vigorously to induce fusion and division of the droplets. The resultant droplets were further incubated for the next round of gene expression and DNA replication. When the DNA concentration was close to the detection limit (10^{-4} nM), the whole sequence of the circular DNA was amplified by PCR and circularized for the next round of reactions. During these serial dilution processes, if a mutant DNA that replicates more than the original DNA appears, the mutant DNA should gradually increase its frequency in the population and finally dominate the population through natural selection.

In this serial dilution experiment, point mutations could be introduced in the PCR process [roughly 6×10^{-5} per base, according to a previous report[41]] for the preparation of the initial circular DNA and the amplification steps when the DNA concentration was close to the detection limit. Polymerization by phi29 DNA polymerase also introduces mutations at the rate of 10^{-5} and 10^{-6} per base.[42] Insertion and deletion can be introduced through mishybridization of the single-stranded DNAs produced by rolling-circle replication.

We repeated the serial dilution cycle for 60 rounds twice independently (Exps. 1 and 2) and plotted the average DNA concentration in the droplets before and after the incubation step (Figure 2B). The initial DNA population was the same for both experiments, which means initial mutational repertoire is identical. In Exp. 1, the DNA concentration gradually decreased to nearly the detection limit (10^{-4} nM) at Round 9; therefore, we PCR-amplified the DNA (indicated with arrowheads). The DNA concentration increased at first but decreased again, and we amplified the DNA at Round 18. Afterward, the DNA concentrations were maintained over the detection limit until Round 60 by repeating up−down cycles. Similarly, in Exp. 2, the DNA concentration initially decreased and was recovered by PCR at Round 15 but then exhibited a sharp increase followed by a gradual decrease until Round 60 while maintaining a concentration higher than the detection limit. These results suggest that the replication ability of the DNA was improved during the serial dilution processes and became continuously replicable without PCR amplification.

Isolation of Mutant DNAs. To analyze the sequences, we PCR-amplified the whole sequence of the circular DNA genes at Rounds 30, 51, and 60 and inserted the PCR fragments into a plasmid for cloning. We isolated 17 and 18 clones at Round 30 for Exps. 1 and 2, respectively, but failed to amplify DNA fragments at Rounds 51 and 60 for unknown reasons (discussed later).

Point mutations are shown in Figure S2. Each clone contains 4.3 and 7.4 point mutations on average for Exps. 1 and 2, respectively. Some of the mutations were commonly found in most of the isolated clones for each Exp. 1 and 2 (for example, G3361A for Exp. 1 and G2650A for Exp. 2), suggesting that these mutations increased their frequencies in the population due to their beneficial effects on replication. We also found various types of insertions upstream of the polymerase gene (Figure S3). Most of these insertions contained T7 promoters, as described in the next section. To understand the relationships among these sequences, we constructed a phylogenetic tree (Figure S4). The clones in Exps. 1 and 2 formed their own clades, indicating that the DNA accumulated different mutations in the two experiments.

Another DNA Replication Scheme That Requires Only One Type of Protein. In addition, we performed a serial dilution experiment using another DNA replication scheme, repetitive sequence replication, which we found in a previous study.[33] Continuous DNA replication in this system requires only phi29 DNA polymerase (*i.e.*, the recombinase is not needed) and linear DNA that consists of a repeating sequence as a template. The polymerase is repeatedly encoded in the linear DNA and is expressed in the TTcDR mixture (Figure S5A). The expressed phi29 DNA polymerase initiates DNA synthesis from within the linear DNA to produce single-stranded linear DNAs, which hybridize with one another in various positions in the repetitive sequence, and the complementary strands are synthesized to reproduce linear repetitive DNA.

We performed a serial dilution experiment for 44 rounds using the same method as for the circular DNAs and measured

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DNA concentrations by qPCR (Figure S5B). We amplified the DNA by PCR when the concentrations were close to the detection limits (Rounds 6, 15, 21, and 27). In contrast to Circular DNA Exps. 1 and 2, as shown in Figure 2B, the DNA concentrations tended to decrease even in the later rounds. Sequence analysis of nine clones at Round 30 showed that there were no common point mutations among the clones (Figure S5C), and only one type of insertion was found in five out of nine clones (Figure S5D). These results indicate that adaptive evolution seldom occurred for the repetitive linear DNA, which may be caused by differences in the replication schemes, as discussed later.

DNA Replication Abilities of Mutant DNAs. To investigate the DNA replication abilities of the isolated clones, we chose five and four clones that contain most of the common mutations from Exps. 1 and 2, respectively. Each circular DNA (0.05 nM) was incubated in the reaction mixture for transcription and translation-coupled DNA replication (TTcDR mixture) for 16 h at 30 °C, and replicated DNA was measured by qPCR (Figure 3A). Most of the isolated clones represented greater replication than the original DNA. A time-course experiment for one of the clones (E1−14, Evo2) exhibited faster replication for E1−14 than the original DNA (Figure S6). These results indicate that most of the mutants isolated at Round 30 acquired higher replication abilities. We renamed the top two clones in each Exp. 1 and 2 "Evo1−4" and used them for further investigation.

Next, we examined whether circular DNA was reproduced during replication. We conducted gene expression-coupled DNA replication, initiated with a higher circular DNA concentration (1 nM), for 16 h at 30 °C. With this initial DNA concentration, the final DNA concentrations became similar among all clones due to substrate (dNTP) limitations (Figure S7). After degrading linear DNAs with exonucleases, we conducted agarose gel electrophoresis to detect the remaining circular DNA (Figure 3B). Bands corresponding to single circular DNAs, seen as multiple bands due to different topologies,[43] were detected relatively clearly for Evo2 and faintly for the original Evo1 and Evo4, indicating that circular DNAs were reproduced for these isolated clones. We predominantly detected the circular DNA of the minimal size although larger circular DNA can be produced depending on the recombination sites. A possible reason is that larger circular DNAs further undergo intramolecular recombination between multiple loxP sites in itself, which finally produces the minimal circular DNA. The minimal circular DNA, which contains only one loxP site, can be a larger circular DNA when recombined with other circular DNAs, but such an intermolecular reaction is probably unlikely at the low minimal circular DNA concentration of this experiment.

We summarized all mutations introduced into Evo1−4 on a schematic DNA map (Figure 4). Nonsynonymous mutations were introduced in both phi29 DNA polymerase and Cre recombinase, especially for Evo3 and Evo4, suggesting functional changes in these proteins. Moreover, 1−3 copies of the T7 RNA promoter were inserted upstream of the original T7 promoter with variable numbers and directions. The possible effects of these insertions are discussed later in this paper.

Biochemical Analysis of the Four Isolated Clones. Efficiency of the circular DNA replication shown in Figure 1 depends on several abilities of the circular DNA molecule and its encoded proteins, including the ability of the DNA to be used as a template by the polymerase, the rolling-circle replication ability of the encoded phi29 DNA polymerase, the recombination ability of the encoded Cre recombinase, and the interaction between the polymerase and the recombinase. In this section, we investigated which abilities contribute to the improved DNA replication of Evo1−4.

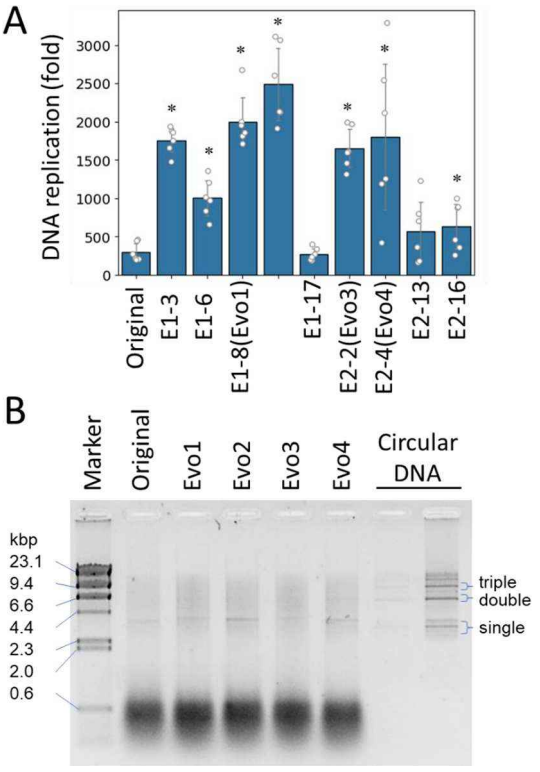

Figure 3. Transcription and translation-coupled DNA replication assay of isolated circular DNA clones at Round 30. (A) The DNA replication of the isolated circular DNAs. Each circular DNA (0.05 nM) was incubated in a TTcDR mixture at 30 °C for 16 h. The DNA concentration was measured by qPCR before and after replication, and the number of replications (fold) was calculated. The error bars represent standard deviations of six independent experiments. Asterisks represent a statistically significant difference ($p < 0.05$) from the original DNA. (B) Detection of circular DNAs. Each circular DNA (1 nM) was incubated in a TTcDR mixture at 30 °C for 16 h. The mixtures were treated with RNase and DNA exonuclease to degrade RNAs and linear DNAs, respectively. Note that, with this initial DNA concentration, the DNA replication stopped due to substrate limitation, and thus, the DNA concentration after the incubation was similar for all clones (Figure S7). The resultant circular DNAs were analyzed by 1% agarose gel electrophoresis. As a control, the original circular DNA was applied in two different amounts (circular DNA). In the left lane, the same amount of the initial circular DNA before the reaction was applied. The bands corresponding to single, double, and triple unit sizes are indicated.

First, we compared the replication ability of phi29 DNA polymerase encoded in each isolated clone. We prepared DNA fragments containing the phi29 DNA polymerase gene encoded in each isolated clone (original and Evo1−4) under the same 5′-untranslated sequence containing a ribosome binding site (RBS) and T7 promoter. Each DNA fragment was incubated in the TTcDR mixture with a template circular DNA (pUC19, 0.05 nM) at 30 °C for 16 h to induce rolling-circle replication of pUC19 by the expressed polymerase (Figure 5A). The replicated DNA concentration was measured by qPCR (Figure 5B). Compared to the original DNA, the DNA

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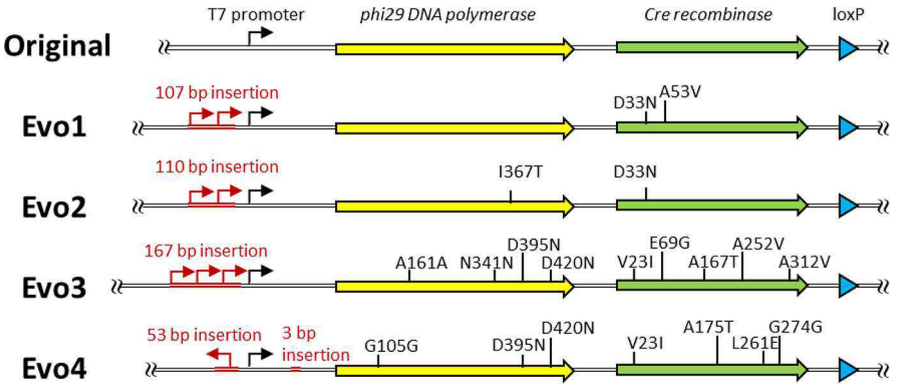

Figure 4. Schematic representation of the mutations in the isolated clones. All point mutations and insertions are indicated. The insertions are shown in red. Two synonymous mutations (G105G and G274G) are also shown. The Phi29 DNA polymerase gene used here is the three-residue shorter variant that starts from "MPRK. . ." instead of "MKHKP. . .".

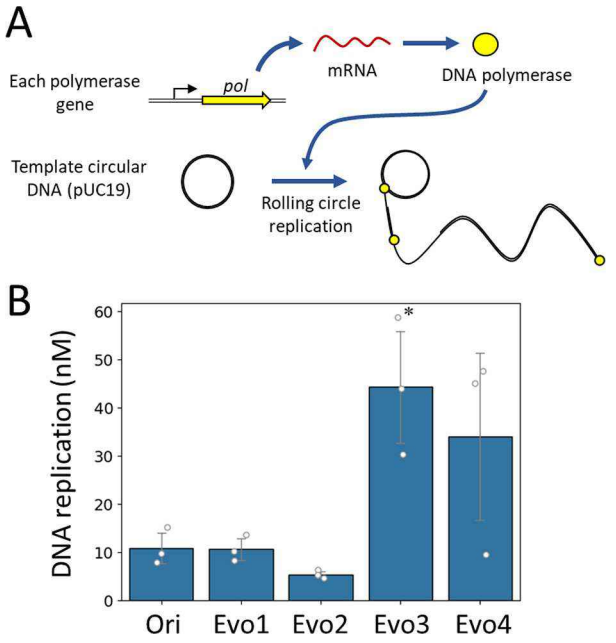

Figure 5. DNA polymerase activity assay. (A) Assay procedure. DNA fragments (0.5 nM) that contained the phi29 DNA polymerase gene encoded in each isolated clone under the same 5′-untranslated sequence containing a ribosome binding site and T7 promoter were incubated in the TTcDR mixture with a template circular DNA (pUC19, 0.05 nM) for 30 °C for 16 h to induce rolling-circle replication of pUC19 by the expressed polymerase. (B) Average DNA replication. The DNA concentration after incubation was measured by qPCR and evaluated as DNA replication after subtracting the DNA concentration of a negative control experiment from which the polymerase gene was omitted. The error bars represent standard deviations of three independent experiments. Asterisks represent a statistically significant difference ($p < 0.05$) from the original DNA.

replication levels of Evo1−4 varied: it was similar for Evo1, lower for Evo2, and approximately 3 times higher for Evo3 and Evo4, indicating that the rolling-circle replication ability of the encoded polymerase increased in the clones in Exp. 2 (Evo3 and Evo4) but not in the clones in Exp. 1 (Evo1 and Evo2).

Second, we compared the template ability of each isolated clone to be replicated using commercially available phi29 DNA polymerase (Thermo Fisher Scientific). We incubated each circular isolated clone (original and Evo1−4) with the same purified phi29 DNA polymerase in the TTcDR mixture, including streptomycin to avoid internal gene expression, for 16 h at 30 °C (Figure 6A) and then measured replicated DNA

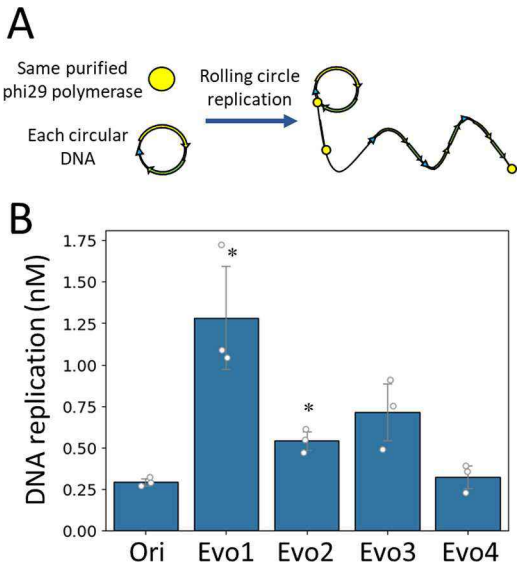

Figure 6. Template activity assay. (A) Assay procedure. Circular DNA of each clone (0.05 nM) was incubated for 16 h at 30 °C in the TTcDR mixture containing the same purified phi29 DNA polymerase (1 U/μL) and 30 μg/μL streptomycin to avoid internal gene expression. (B) Average DNA replication. The DNA concentration after incubation was measured by qPCR and evaluated as DNA replication after subtracting the DNA concentration of the negative control experiment from which the purified phi29 polymerase was omitted. The error bars represent standard deviations of three independent experiments. Asterisks represent a statistically significant difference ($p < 0.05$) from the original DNA.

concentration by qPCR (Figure 6B). Most of the evolved clones (Evo1−3) exhibited higher replication levels than the original clone, indicating that the template ability to be replicated was improved in these clones. The inhibition of translation with streptomycin was confirmed using a reporter gene (Figure S8).

Third, we compared the recombination ability of Cre recombinase encoded in each isolated clone. We prepared DNA fragments that each contained Cre recombinase gene under the same RBS and T7 promoters. Each DNA fragment was incubated in the TTcDR mixture for 16 h at 30 °C with

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two recombination template DNAs (10 nM) containing loxP sites (AB or CD fragments) to induce loxP-dependent recombination by each expressed Cre recombinase (Figure 7A). The concentration of the recombined DNA was measured

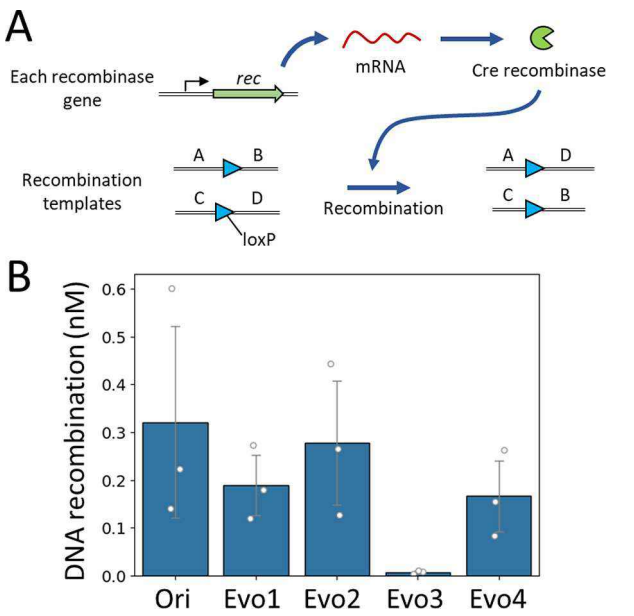

Figure 7. Cre recombinase activity assay. (A) Assay procedure. A DNA fragment (0.025 nM) that contained a Cre recombinase gene of each isolated clone under the same RBS and T7 promoter was incubated in the TTcDR mixture for 16 h at 30 °C with two recombination template DNAs containing loxP sites (AB or CD fragments, 10 nM). (B) Average concentration of the recombination product. The recombination product (AD fragment) was measured by qPCR with specific primers. The error bars represent standard deviations of three independent experiments.

by qPCR using primers that are specific to the recombined "AD fragment" (Figure 7B). The recombined DNA for the evolved clones (Evo1−4) was similar to the original DNA for Evo2, slightly decreased for Evo1 and Evo4, and significantly decreased for Evo3, indicating that the recombination ability was not improved in these clones. The decreased recombinase activity of the Evo3 clone seems to be unreasonable because the serial dilution experiment without Cre recombinase did not continue for many rounds (Figure S5B). One possible explanation is that the decreased activity might be caused by mutations introduced just before Round 30 before they are negatively selected.

Fourth, we compared the inhibitory effect of Cre recombinase on DNA polymerization using phi29 DNA polymerase. In our previous study, we found that Cre recombinase significantly inhibits rolling-circle replication by the polymerase,[32] one of the most serious problems in our DNA replication system. To examine whether the inhibitory effect is weakened by the introduced mutations, we expressed the original phi29 DNA polymerase and the Cre recombinase of each isolated clone together and conducted rolling-circle replication of a template DNA containing a loxP site (Figure 8A). As a control, we performed the same experiment without the DNA encoding Cre recombinase. The replicated DNA was measured by qPCR (Figure 8B). For the original Cre recombinase (Ori), the replication was significantly decreased compared to that without Cre recombinase (w/o Cre), confirming the inhibitory effect of Cre recombinase, while for all isolated clones (Evo1−4), the DNA replications were 3−5 times greater than the original Cre recombinase, indicating that the inhibitory effects of the Cre recombinase encoded in the isolated clones were smaller than the original recombinase.

Expansion of DNA Sequence. One of the possible applications of the circular DNA that was isolated is the introduction of more genes and its use as the genome for an artificial cell. To examine the capability of the isolated clones to expand the encoding sequence, we introduced additional DNA sequences into each circular DNA (3.3 kbp) and compared their replication abilities. We introduced a pUC19-derived plasmid sequence (2.5 kbp) including the *bla* gene between the loxP site and T7 promoter and incubated it in the TTcDR reaction mixture for 16 h at 30 °C for replication. Replication of the original circular DNA and Evo4 decreased significantly (Figure 9) compared to replication without the additional sequence (Figure 3A), while replication of Evo1−3 was similar to that without the additional sequence, indicating that these evolved clones have the potential to effectively replicate when additional genes are introduced.

CONCLUSION

In this study, we demonstrated the continuous replication and evolution of DNA through a compartmentalized serial dilution cycle. The circular DNA replication scheme requires two proteins, phi29 DNA polymerase and Cre recombinase, both of which are expressed by the DNA itself (Figure 1). We succeeded in continuous DNA replication for 60 rounds of the serial dilution cycle, corresponding to 140 generations (*i.e.*, 2^{140} replications) (Figure 2B). Furthermore, the DNA population at Round 30 accumulated common mutations (Figure S2) and exhibited higher replication abilities (Figure 3), demonstrating that the DNA underwent adaptive evolution. We also demonstrated that some of the evolved DNA replicated efficiently even after expanding the DNA sequences (Figure 9), implying that the DNA evolved here can be used as a basis to develop more complex artificial cells.

Biochemical analyses showed that the isolated DNA clones improved their replication abilities in different ways, depending on the two independent serial dilution experiments. Evo1 and Evo2, isolated from Exp. 1, increased the template ability (Figure 6) and decreased the inhibitory effect of Cre recombinase (Figure 8). Evo3 and Evo4, isolated from Exp. 2, increased DNA polymerase activity (Figure 5) and decreased the inhibitory effect of Cre recombinase (Figure 8), whereas template activity was increased by Evo3 alone (Figure 6). These clones consistently accumulated different mutation sets depending on the experiment (Figure 4). The only common mutation between the experiments was the insertion of additional T7 promoters upstream of the original T7 promoter in the same direction in Evo1−3 (Figure 4). Previously, we reported the possibility that RNA produced by T7 RNA polymerase functions as a primer for phi29 DNA polymerase.[36] The insertion of multiple promoters may contribute to the improved template activities of these clones (Figure 6), although the reason for the insertion of the T7 promoter in the opposite direction in Evo4 is unknown.

We failed in continuous replication of the repetitive linear DNA scheme (Figure S5A,B) and found that no common point mutations accumulated in the population at Round 30 (Figure S5C). These results suggest that the adaptive evolution

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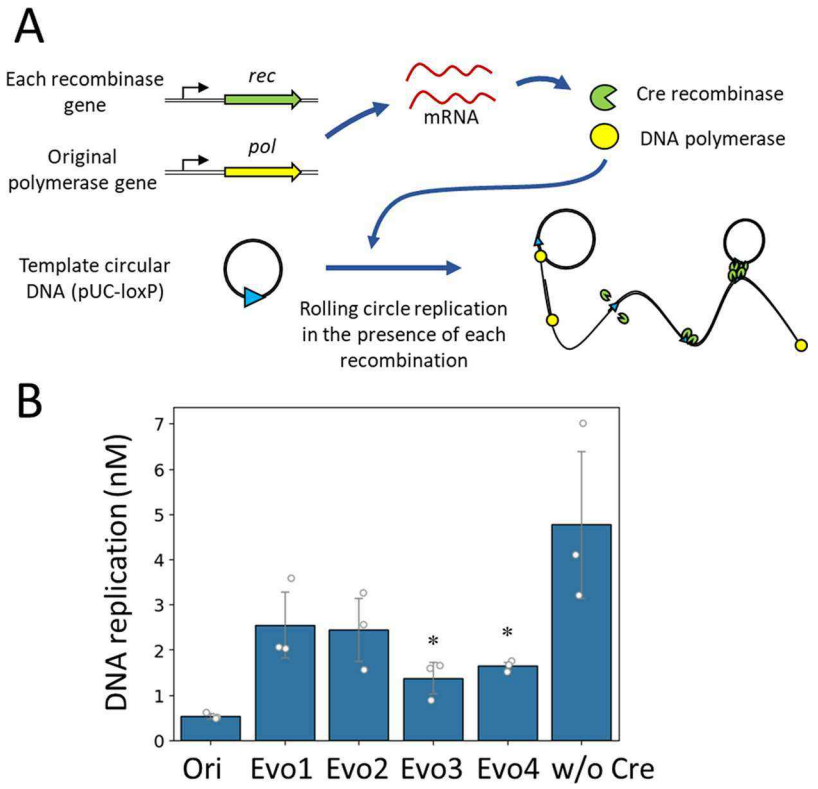

Figure 8. Assay of the inhibitory effect of Cre recombinase on DNA polymerization. (A) Assay procedure. A DNA fragment (0.5 nM) that encodes a Cre recombinase of each isolated clone under the same promoter and RBS and another DNA fragment (0.5 nM) encoding the original phi29 DNA polymerase gene were incubated for 16 h at 30 °C in the TTcDR mixture with the template circular DNA (pUC-loxP, 0.05 nM) containing a loxP site. As a control experiment, the same procedure was conducted without the DNA fragment encoding Cre recombinase (w/o Cre). (B) Average DNA replication. The DNA concentration after incubation was measured by qPCR and evaluated as DNA replication after subtracting the DNA concentration of the negative control experiment from which the polymerase gene was omitted. The error bars represent standard deviations of three independent experiments. Asterisks represent a statistically significant difference ($p < 0.05$) from the original DNA.

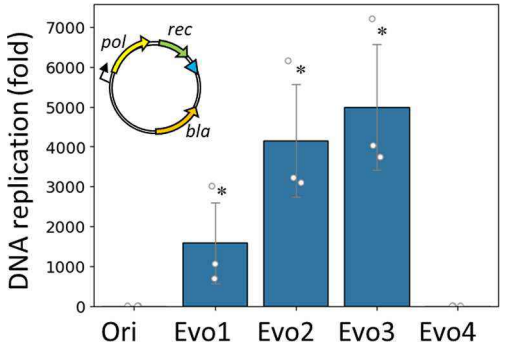

Figure 9. Self-replication assay of expanded circular DNAs. An additional DNA sequence (2.5 kbp) encoding the *bla* gene, derived from a plasmid pUC19, was inserted into each circular DNA (0.05 nM, 3.3 kbp) and incubated in the TTcDR mixture at 30 °C for 16 h. The DNA concentration was measured by qPCR before and after replication, and the number of replications (fold) was calculated. The error bars represent standard deviations of three independent experiments. Asterisks represent a statistically significant difference ($p < 0.05$) from the original DNA.

of repetitive DNA is more difficult to implement than that of circular DNA. Such difficulty in evolution is theoretically reasonable because a single linear repetitive DNA, approximately 23−50 kbp,[33] should contain more than seven copies of the polymerase gene. Such large numbers of genes would average out the effect of mutations introduced into one of the genes. This averaging effect would slow down both the increase and decrease in the frequencies of beneficial and deleterious mutations. In a previous study, we proposed repetitive DNA replication as one of the possible ancient DNA replication schemes, although ancient organisms that contained repetitive DNA sequences would suffer from slow evolution. This problem might be a driving force for the development of genomic DNA that contains a single copy of any given genes.

Circular DNA constituted only a small fraction in the DNA population (estimated at approximately 0.05% from the band intensity in Figure 3B and the total replicated DNA amount in Figure S7), which means that most of the DNA exists as a large linear DNA during replication. This result may be reasonable because a large linear DNA, a repeat of the sequence of the circular DNA, can be produced constitutively from the circular DNA and also replicates by itself through the repetitive sequence replication as illustrated in Figure S5A. Therefore, in our experimental setup, large repetitive DNAs and circular DNAs should coexist and be interconverted. The balance between the linear and circular DNAs would depend on both the recombination activity and the replication activity of the large linear DNA. Further serial dilution experiments at a lower DNA concentration may produce DNA mutants that produce circular DNAs more efficiently because replication of the large linear DNA requires higher DNA concentrations.[33] Although there is room for improvement in the circularization efficiency, the continuous and evolvable DNA replication we demon-

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strated in this study would be a useful tool for artificial cell synthesis.

An obstacle for further serial dilution experiments is the difficulty in PCR amplification of the circular DNA during the cloning process. We successfully PCR-amplified the whole sequence at Round 30 but failed at Round 60 at which only smaller DNA was amplified. Presently, the reason for this is unknown, but it might be due to the appearance of a repetitive DNA sequence consisting of a small DNA unit, which replicates in a "parasitic" manner using DNA polymerase expressed from other DNAs. To circumvent this problem, we are planning to repeat short-term (*e.g.*, less than 30 rounds) serial dilution experiments before the appearance of parasitic DNA.

One of the major challenges in cell-free synthetic biology or artificial cell synthesis is the construction of a reproducible artificial system. Recently, scientists have attempted to regenerate transcription and translation factors[34,35,44−49] or other important cellular functions[50−57] through gene expression in cell-free systems to achieve reproducible artificial systems. However, the expression of these genes has not previously been coupled with DNA replication, which is an essential function of a reproducible system. The continuously replicable and evolvable DNA replication system reported here can be a basis for an artificial genomic DNA that encodes all genes required for a regenerative artificial cell.

METHODS

Circular DNA Preparation. The DNA used as the initial template for the serial dilution experiment was prepared as follows. We first PCR-amplified DNA fragments encoding phi29 DNA polymerase, Cre recombinase, and loxP sequence using KOD FX (Toyobo, Japan), primers 1 and 2, and the plasmid (pUC-phi29DNAPevo56_loxP_cre; the full sequence is shown in Supporting Information) as a template. The plasmid was constructed by inserting the Cre recombinase gene into another plasmid (pUC-clone6), as previously reported.[32] The PCR product was purified using a QIAquick PCR Purification Kit (QIAGEN). All DNA purification procedures were performed using the kit. The purified DNA fragment was digested with 0.6 U/μL SphI (TaKaRa, Japan) in the reaction mixture for 1 h at 37 °C; after purification, it was self-ligated with 17.5 U/μL T4 DNA ligase (TaKaRa) at 16 °C overnight, followed by purification. The original and evolved circular DNAs used for the biochemical assay were prepared as described above using each plasmid containing the cloned sequence; primers 9 and 10 for PCR amplification; 0.6 U/μL *Bgl*II (TaKaRa, Japan) for digestion. The sequences of the primers used in this study are listed in Table S1.

Transcription and Translation-Coupled DNA Replication (TTcDR) Mixture. The TTcDR mixture used contained all factors required for DNA replication, transcription, and translation based on the reconstituted translation system of *E. coli*,[40] as described previously,[32] except that the concentrations of dNTP and magnesium acetate were 0.6 and 10.5 mM, respectively. The complete composition is presented in Table S2.

Serial Dilution Experiment. The TTcDR mixture (10 μL) containing the original circular DNA (0.01 nM) was mixed vigorously in a 1 mL buffer-saturated oil phase using a homogenizer (Kinematica POLYTRON PT 1300 D) with a disposable shaft (Kinematica Typ: PT-DA 07/2 SYN-E082) at 16 krpm for 1 min on ice to prepare water-in-oil droplets (a microscope image is shown in Figure S1). The buffer-saturated oil phase was prepared as follows according to a previous study.[41] First, mineral oil (Sigma-Aldrich) was mixed with 2% (w/v) Span 80 and then with 3% (w/v) Tween 80. The oil was immediately mixed vigorously with a 1/20 volume of a saturation buffer, which omits all macromolecules (DNA, RNA, and proteins), dNTP, NTP, and magnesium acetate from the TTcDR mixture. After incubation at 37 °C for 10 min, the mixture was centrifuged at 20k*g* for 5 min, and the upper clear oil phase was used as the buffer-saturated oil phase. The droplets were incubated at 30 °C for 16 h for gene expression and replication. The droplets (200 μL) were then mixed with 8 μL of the TTcDR mixture omitting DNA and 800 μL of the buffer-saturated oil phase, followed by mixing with the homogenizer for 1 min at 16k rpm on ice. The resultant droplets were incubated at 30 °C for 16 h for the next round of reaction. Before and after incubation, an aliquot of the droplets was diluted 100-fold with 1 mM EDTA (pH 8.0) to measure the DNA concentration by quantitative PCR (qPCR) (TB Green Premix Ex *Taq*II, TaKaRa) using primers 3 and 4. When the DNA concentration was below the detection limit (10^{-4} nM), the DNA was amplified as follows: the droplets were collected by centrifugation at 20k*g* for 5 min and diluted 10-fold with distilled water. The DNA was PCR-amplified using primers 1 and 2 and subjected to size selection using an E-Gel system (E-Gel CloneWell II, Thermo Fisher Scientific). The extracted DNA fragment (3076 bp) was PCR-amplified again, as described above. After purification, the DNA was digested with SphI, self-ligated by T4 DNA ligase, and used for the next round of reaction.

For the serial dilution experiment with linear repetitive DNA (Figure S5), we first prepared circular DNA, which contained only the phi29 DNA polymerase gene, as described above, using primers 1 and 2 and pUC-clone6 as a template for PCR. We initiated the serial dilution cycle with this circular DNA, which produced linear repetitive DNA through rolling-circle replication at Round 1 and the linear repetitive DNA replicates in the later rounds.

DNA Cloning at Round 30. The droplets at Round 30 were collected by centrifugation at 20k*g* for 5 min and diluted 10-fold with distilled water. The DNA was PCR-amplified with KOD FX polymerase and primers 5 and 6. The PCR product was purified and ligated to a vector DNA fragment, prepared by PCR with primers 7 and 8 using pUC19 as a template, with the In-Fusion Cloning Kit (Takara, Japan). The ligated fragments were introduced into *E. coli* strain DH5alpha. The *E. coli* was cultured in Luria−Bertani medium containing 50 μg/mL ampicillin at 30 °C, and plasmids were purified using the QIAGEN miniprep kit. We also PCR-amplified the original circular DNA sequence and inserted it into the plasmid (pUC-DNAPcre-HO_original; the full sequence is shown in the Supporting Information) using the same method for comparison. These plasmids were used for circular DNA preparation as PCR templates, as described above. To prepare the expanded circular DNAs and because Cre recombinase is not under the T7 promoter in the plasmids, we further reordered the genes in the plasmids as follows: We first prepared the circular DNA from the plasmids as described above and then conducted PCR using each of the circular DNAs as a template with primers 20 and 21. The PCR fragments were ligated with a vector fragment and PCR-amplified by the In-Fusion Cloning Kit (Takara, Japan) using pUC19 as a template with primers 7 and 8.

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Transcription and Translation-Coupled DNA Replication Assay. Each circular DNA molecule (0.05 nM) was incubated in the TTcDR mixture at 30 °C for 16 h. The DNA concentration was measured by qPCR using primers 3 and 4. To detect circular DNA (Figure 3B), the circular DNA was increased to 1 nM and treated as follows: the mixtures after the reaction were diluted 50-fold with 1 U/μL exonuclease V (New England BioLabs) in solution (1 mM ATP, 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9) and incubated at 37 °C for 4 h to degrade linear DNAs. Then, RNase A (QIAGEN) was added to 8.3 ng/μL and incubated at 37 °C for 30 min. After purification and concentration, the remaining circular DNA was subjected to 1% agarose gel electrophoresis with control circular DNA prepared by self-ligation as described above. DNA was stained with a fluorescent dye (SAFELOCK Green, Fujifilm, Japan).

Polymerase Activity Assay. The coding regions of each phi29 DNA polymerase (the original and Evo1−4) were PCR-amplified using primers 11 and 12 and then further amplified by PCR using primers 12 and 13. Through these PCRs, the same ribosome binding site (RBS) and T7 promoter were attached to all genes. Each of the DNA fragments (0.5 nM) was incubated at 30 °C for 16 h in the TTcDR mixture with pUC19 (0.05 nM), and the amplified pUC19 concentration was measured by qPCR using primers 14 and 15.

Template Activity Assay. Each circular DNA (0.05 nM) was incubated at 30 °C for 16 h in a TTcDR mixture containing 1 U/μL purified phi29 DNA polymerase (EquiPhi29 DNA polymerase, Thermo Fisher) and 30 ng/μL streptomycin to avoid polymerase expression. The DNA concentration was measured by qPCR using primers 3 and 4.

Recombination Activity Assay. The coding regions of Cre recombinase in each circular DNA (the original and Evo1−4) were PCR-amplified using primers 22 and 23 and ligated to a vector fragment and PCR-amplified from pET21 using primers 24 and 25 with the In-Fusion Cloning Kit (Takara, Japan). Each plasmid (0.025 nM) was incubated at 30 °C for 16 h in the TTcDR mixture with recombination templates (10 nM AB and CD fragments) prepared by PCR using the plasmid containing the original circular DNA sequence as a template with primers 26 and 27 for the AB fragment (1394 bp) or primers 28 and 29 for the CD fragment (907 bp). The recombined DNA concentration was measured by qPCR using primers 16 and 17.

Inhibition Assay by Cre Recombinase. The DNA fragment containing the original phi29 DNA polymerase was prepared as described in the Polymerase Activity Assay section. The DNA fragments encoding each Cre recombinase under the same RBS and T7 promoters were prepared by the same method using primers 18 and 19 in the first PCR and primers 13 and 19 in the second PCR. Both DNA fragments containing the original polymerase (0.5 nM) and each of the Cre recombinases (0.5 nM) were mixed with template circular DNA (pUC-loxP, 0.5 nM), which contained a loxP sequence in pUC19, and subsequently incubated at 30 °C for 16 h in the TTcDR mixture. The DNA concentration was measured by qPCR using primers 14 and 15 and corrected by subtracting the value of the negative control experiment in which the DNA fragment containing phi29 DNA polymerase was omitted. The template plasmid, pUC-loxP, was prepared by ligating PCR fragments amplified with primers 30 and 31 using pUC-DNAPcre-HO_original as a template after *Bgl*II digestion.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00430.

Differential interference contrast microscopy; list of point mutations; list of insertions in the isolated clones; phylogenetic tree; long term compartmentalized serial dilution experiment of a linear repetitive DNA; time course data; DNA concentration before the electrophoresis; the effect of streptomycin on translation; primer sequences; composition of the TTcDR mixture; DNA sequences (PDF)

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Author Contributions

H.O. planned and performed all experiments and wrote the manuscript. N.I. planned the experiments and wrote the manuscript.

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Notes

The authors declare no competing financial interest.

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its Supporting Information. Raw data are available on request from the corresponding author.

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