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Exponential propagation of large circular DNA by reconstitution of a chromosome-replication cycle

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ABSTRACT

Propagation of genetic information is a fundamental property of living organisms. *Escherichia coli* has a 4.6 Mb circular chromosome with a replication origin, *oriC*. While the *oriC* replication has been reconstituted *in vitro* more than 30 years ago, continuous repetition of the replication cycle has not yet been achieved. Here, we reconstituted the entire replication cycle with 14 purified enzymes (25 polypeptides) that catalyze initiation at *oriC*, bidirectional fork progression, Okazaki-fragment maturation and decatenation of the replicated circular products. Because decatenation provides covalently closed supercoiled monomers that are competent for the next round of replication initiation, the replication cycle repeats autonomously and continuously in an isothermal condition. This replication-cycle reaction (RCR) propagates ∼10 kb circular DNA exponentially as intact covalently closed molecules, even from a single DNA molecule, with a doubling time of ∼8 min and extremely high fidelity. Very large DNA up to 0.2 Mb is successfully propagated within 3 h. We further demonstrate a cell-free cloning in which RCR selectively propagates circular molecules constructed by a multi-fragment assembly reaction. Our results define the minimum element necessary for the repetition of the chromosome-replication cycle, and also provide a powerful *in vitro* tool to generate large circular DNA molecules without relying on conventional biological cloning.

INTRODUCTION

In vitro reconstitution of cellular processes with purified components is not only a powerful approach for understanding living systems, but also sometimes leads to breakthrough applications in biotechnology (1–3). For example, the polymerase chain reaction (PCR), which reconstitutes DNA synthesis, enables rapid and efficient amplification of DNA several thousand base pairs long. The advent of synthetic biology has increased the demand for methods to produce and amplify longer DNA molecules (4,5). Emerging technologies have enabled the creation of large DNA molecules up to the size of a whole bacterial genome (6,7), resulting in the production of artificial bacterial cells controlled by synthetic genomes (8–10). Whole-genome synthesis relies on propagation in living host cells, which is time-consuming and labor-intensive. Living cells achieve propagation of genomic DNA through sequential repetition of the process, consisting of initiation, elongation, termination, separation of fully replicated molecules and reinitiation (Figure 1).

Escherichia coli has a 4.6 Mb circular chromosome with a unique origin of replication, *oriC* (11). More than 30 years ago, replication from *oriC* was reconstituted *in vitro* with purified proteins and supercoiled mini-chromosomes (*oriC*-carrying plasmids; Figure 1) (12). DnaA initiates replication by unwinding the *oriC* duplex in the presence of integration host factor (IHF) (13,14). DnaB helicase, recruited by DnaC, then expands the single-stranded region, onto which replisome components, including single-stranded DNA binding protein (SSB), DnaG primase, and the DNA polymerase III holoenzyme (Pol III HE) assemble (15,16). Pol III HE consists of the DnaN clamp, the clamp–loader complex, and the core polymerase, and it synthesizes the leading and lagging DNA strands concurrently (17). Two replisomes proceed bidirectionally toward the chromosome terminus region (18), with DNA gyrase relieving topological stress ahead of the forks (19). In the *in vitro oriC* replication system, these enzymes and the *oriC* sequence are required for DNA synthesis (12,20,21). Bidirectional DNA synthesis from *oriC* has also been demonstrated *in vitro* (15,17–19,22,23). Furthermore, a series of enzyme reactions to complete the *oriC* replication, maturate Okazaki-fragment synthesis and decatenate the daughter DNA molecules has been reconstituted as follows. Enzymes for Okazaki-fragment maturation (RNase H, Pol I and lig-

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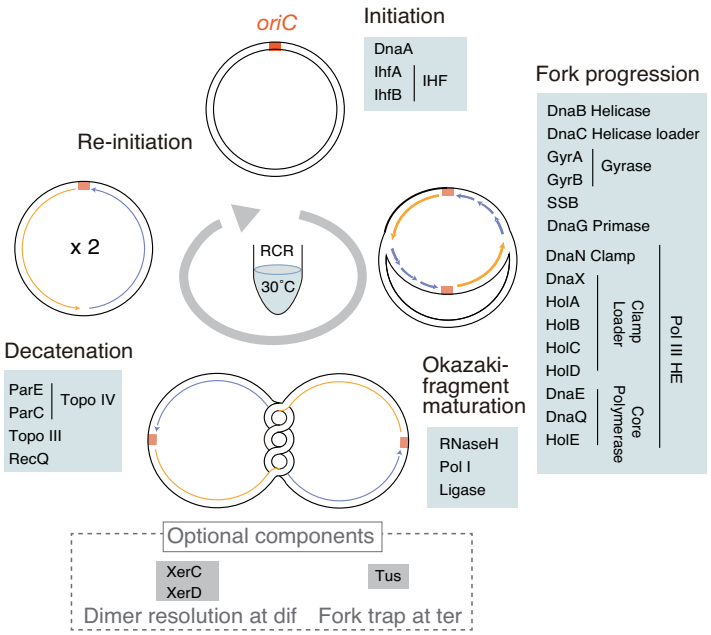


Figure 1. Schematic representation of the RCR reconstituted with purified proteins.

ase) produce covalently closed daughter DNAs (24), which are interlinked catenanes that require Topo IV decatenation activity for release (25). Topo III, in combination with RecQ helicase, is an alternative decatenase that resolves converging replication forks (26,27). After decatenation, DNA gyrase introduces negative supercoils into the DNA molecules. Negative supercoiling is a prerequisite for duplex unwinding at *oriC*, and thus the linear form is known to be inactive in the *oriC* replication reaction (24). In this study, we have reconstituted the *E. coli* chromosome replication cycle using purified components, providing a powerful enzymatic tool for the propagation of large DNA molecules *in vitro*.

MATERIALS AND METHODS

Replication-cycle reaction (RCR)

A 5 × replication-cycle reaction (RCR) buffer (100 mM Tris-HCl pH8.0, 750 mM potassium glutamate, 50 mM ammonium sulfate, 50 mM $Mg(OAc)_2$, 40 mM dithiothreitol (DTT), 20 mM creatine phosphate, 5 mM each NTP, 0.5 mM each dNTP, 0.25 mg/ml yeast tRNA, and 1.25 mM NAD^+) and a 4 × Enzyme mix (2 mg/ml bovine serum albumin, 0.08 mg/ml creatine kinase, 0.1 mM adenosine triphosphate (ATP), 1.6 μM SSB_4, 160 nM IHF_2, 1.6 μM DnaG, 160 nM $DnaN_2$, 20 nM Pol III*, 80 nM $DnaB_6C_6$, 400 nM DnaA, 40 nM RNaseH, 200 nM ligase, 200 nM Pol I, 200 nM gyrase ($GyrA_2B_2$), 20 nM Topo IV ($ParC_2E_2$), 200 nM Topo III and 200 nM RecQ) were prepared for the standard reaction, unless otherwise noted. The reaction mixture (final 10 μl, unless otherwise noted) included 5 × RCR buffer (2 μl) and 4 × Enzyme mix (2.5 μl) and was assembled on ice. After addition of *oriC*-containing circular DNA, the reaction was incubated at 30°C for the indicated times. [α-^{32}P]dATP was included at 40–100 cpm/pmol of total deoxynucleotides when indicated. The reaction was stopped by addition of an equal volume of 2 × Stop buffer (50 mM Tris–HCl pH8.0, 50 mM ethylenediaminetetraacetic acid, 0.2% sodium dodecyl sulphate, 0.1 mg/ml proteinase K, 10% glycerol and 0.2% bromophenol blue), and further incubated at 37°C for 30 min followed by phenol–chloroform extraction. An aliquot (2 μl) was analyzed by 0.5% agarose-gel electrophoresis followed by SYBR Green I staining (Molecular Probes) or by phosphor imaging. The images were acquired with a Typhoon FLA 9500 (GE Healthcare). The 4 × Enzyme mix can be stored at −80°C after rapid freezing with liquid nitrogen and its activity is retained even after five freeze-thaw cycles. Protein purification is described in Supplementary Methods.

Measurement of [α-^{32}P]dATP-incorporation

An aliquot of the RCR sample was treated with 10% trichloroacetic acid. Nucleotide incorporation into DNA was measured by liquid-scintillation counting of acid-insoluble material retained on Whatman GF/C glass microfiber filters.

Quantification of the number of circular DNA molecules by transformation into *E. coli*

RCR was performed with *oriC* circular DNA templates containing a kanamycin-resistance cassette. An aliquot (1 μl) of the RCR sample was directly subjected to chemical transformation into *E. coli* strain DH5α. The RCR sample was diluted if necessary. Kanamycin (25 μg/ml)-resistant colonies were grown at 37°C overnight on LB-agar plates and counted. To deduce the number of circular DNA molecules from the colony number, 1 ng (10^8 molecules) of pOri8 circular DNA was used for each transformation experiment as a quantitative standard, which typically gave ∼10 000 colonies.

Measurement of the rate of replication errors

Replication errors were detected through *lacZ*$^{-}$ mutations generated during RCR using *oriC* circular DNA containing an intact *lacZ* gene. The RCR product was transformed into *E. coli* strain NM554 [F^{-} *araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL* (Str^{R}) *hsdR2 (rK*$^{-}$ *mK*$^{+}$*) mcrA mcrB1 recA13*]. Blue and white colonies on LB-agar plates containing kanamycin (25 μg/ml), X-Gal (40 μg/ml), and IPTG (0.12 mM) were counted. The propagation value in each passage was determined from the total colony number before and after propagation, which indicated the number of population doublings. In the error-prone reaction, the number of population doublings was estimated to be 20 from the band intensity of the gel (Supplementary Figure S6) and by comparison with the error-free reaction; this was because the possibility of errors in the kanamycin-resistance gene and/or the *parABC* cassette made it difficult to measure the doublings precisely from the colony number. The error rate per base per cycle *(E)* was calculated as described previously (28) using the following equation:

$$F = \left(\frac{1 + e^{-1000E}}{2}\right)^{m-1}$$

In this equation, *F* is the fraction of colonies that are blue and *m* is the number of population doublings. The number of error-detectable sites (i.e. non-silent target sites) in the 3 kb *lacZ* gene was estimated to be 1000.

DNA assembly for cell-free cloning

A 2 × modified Gibson Assembly (mGA) mix (200 mM Tris–HCl pH7.5, 20 mM $MgCl_2$, 0.4 mM each dNTP, 20 mM DTT, 10% PEG8000, 2 mM NAD^+, 16 mU/μl T5 exonuclease, 50 mU/μl Phusion DNA polymerase) was prepared in-house, with some modifications as described previously (29). The reaction (final volume 5 μl) consisted of 2 × mGA mix (2.5 μl) and the indicated amounts of DNA fragments, and was assembled on ice and incubated at 50°C for 1 h. DNA fragments were PCR-amplified with PrimeSTAR HS DNA polymerase (Takara Bio) and purified with the Wizard SV PCR Clean Up System (Promega). A 1 kb *oriC* fragment was amplified with primers SUE654/SUE656 using the *E. coli* K-12 MG1655 genome as a template. A 3.3 kb *lacZ* fragment including the *lac* promoter was amplified with primers SUE594/SUE655 using the MG1655 genome as a template. The 4.6 kb *parABC-Km* cassette was amplified with primers SUE635/SUE637 using pETcocoKm as a template. Primer sequences are listed in Supplementary Table S1. The assembly products before or after RCR were analyzed following transformation of *E. coli* strain NM554.

'Pop-out' preparation of *oriC*-containing circular DNA

pOri8 (9.5 kb), pOri80 (85 kb), pOri200 (205 kb), pMSR227 (205 kb) and pOriDif (12 kb) were prepared by a pop-out method. λ Red recombination was carried out in *E. coli* K-12 MG1655 strain containing pKD46, as described previously (30), except that MH005, instead of MG1655, was used for pMSR227. The *Km* cassette (1.2 kb) was PCR-amplified from pUC4K with primers SUE351/SUE352 for pOri8. The *parABC-Km* cassettes (4.8 kb) were PCR-amplified from pETcocoKm with primers SUE409/SUE410 for pOri80 and SUE411/SUE412 for pOri200 and pMSR227. The *Km-oriC* cassette (2.1 kb) was PCR-amplified from pETcocoKmOri with primers SUE507/SUE509 for pOriDif. Primer sequences are listed in Supplementary Table S1. These cassettes contain 40 bp homologous arms targeting sites 4 kb upstream and downstream of the chromosomal *oriC* site (for pOri8), 40 kb upstream and downstream of *oriC* (for pOri80), 100 kb upstream and downstream of *oriC* (for pOri200 and pMSR227) and 4.2 kb upstream and 6 kb downstream of the chromosomal *dif* site (for pOriDif). After electroporation, kanamycin-resistant colonies were selected and successful generation of the *oriC*-containing plasmids was verified through colony PCR. The plasmids were then partially purified and used for transformation of *E. coli* strains DH5α (for pOri8), HST08 (Takara Bio) (for pOri80, pOri200 and pMSR227) or DH10B (Life Technologies) (for pOriDif). Transformants were selected for large-scale purification of the plasmids using the QIAfilter plasmid kit (Qiagen) (for pOri8 and pOriDif) or NucleoBond Xtra BAC Kit (Takara Bio) (for pOri80, pOri200 and pMSR227). In the MH005 strain, a MG1655 derivative, the *dnaN* gene was replaced with a *mCherry-dnaN* fusion at its endogenous locus.

Other DNA constructs

To construct M13ms9 (8 kb), the 0.42 kb *oriC* fragment was PCR-amplified from the MG1655 genome using primers SUE260/SUE261, followed by NcoI–NsiI digestion and cloning into a vector prepared from M13mp18 by PCR with primers SUE226/SUE227, followed by NcoI–NsiI digestion. To construct M13ms10 (8 kb), a 0.25 kb fragment containing the chromosomal *terB* region and a second inverted *terB* sequence was PCR-amplified from the MG1655 genome using primers SUE236/SUE238, followed by NheI–NsiI digestion and cloning into the XbaI–PstI sites of M13ms9. Duplex, replicative forms of phage were prepared in *E. coli* JM109 strain and purified using the QIAfilter plasmid kit (Qiagen).

pPKOZ (8.9 kb) was constructed using the cell-free cloning method described in Figure 6. After transformation of DH5α, the plasmid was purified with the QIAfilter plasmid kit. To construct pETcocoKm (11.3 kb), the 1.2 bp *Km* fragment was PCR-amplified from pUC4k using primers SUE296/SUE375, digested with PciI–PstI, and cloned into the PciI–NsiI sites of pETcoco-2 (Novagen). To construct pETcocoKmOri, a 1 kb *oriC* fragment was PCR-amplified from the MG1655 genome using primers SUE505/SUE506 and cloned into the NheI–AatII sites of pETcocoKm. Primer sequences are listed in Supplementary Table S1.

RESULTS

Repetition of replication cycle by the presence of decatenases

The *in vitro* reconstitution system for the bidirectional fork progression initiated from *oriC* has been constructed previously using at least 18 individual proteins and super-

coiled *oriC* plasmids (Figure 1, Initiation, Fork progression) (12,15,17–23). Further addition of RNaseH, Pol I and ligase to this system leads to Okazaki fragment maturation (Figure 1, Okazaki fragment maturation) (24). The resultant interlinked circular DNA molecules (catenanes) are the target for decatenation enzymes (Figure 1, Decatenation) (25–27). We here speculated that successful completion of decatenation might lead to the re-initiation of replication and thus the autonomous repetition of replication cycle (Figure 1, Re-initiation), since the decatenation provides negatively supercoiled monomers competent for initiation at *oriC* in the presence of gyrase, which is topologically identical to the input template molecules (24). To test this hypothesis, a replication-cycle reaction (RCR) was established with an 8 kb supercoiled circular DNA containing *oriC* as the template (Figure 1), and the effect of decatenation by Topo IV or Topo III–RecQ was assessed. In the absence of decatenation enzymes, slow-migrating products were observed as reported previously (22), including catenated intermediates and large concatemers from a rolling-circle type of over-replication (some of which were trapped in the well of the gel) (Figure 2A). When Topo IV or Topo III–RecQ were included in the reaction, supercoiled DNA monomers were efficiently generated (25,26). In the presence of Topo III–RecQ, further stimulation of the supercoiled DNA production by Topo IV was only slight (5 nM Topo IV, Supplementary Figure S1). Although Topo III alone could provide the decatenation activity and thus generate the supercoils, RecQ stimulated the Topo III decatenation activity (Figure 2C) (26,27). By the presence of either Topo IV or Topo III–RecQ, total DNA synthesis, which was 20- to 40-fold over input DNA in the absence of decatenation enzymes, was stimulated up to 80- and 100-fold over input DNA, respectively (Total DNA synthesis, Figure 2B), and in addition, the levels of supercoiled products relative to the level of the input supercoiled template were increased up to 7- and 25-fold, respectively (Supercoiled products, Figure 2B). These observations supported the hypothesis that decatenation of replicated daughter DNA molecules promotes successful repetition of the replication cycle. We further demonstrated the requirement of proteins in RCR (Supplementary Figure S2), in which also the Topo IV-dependent stimulation of the total DNA synthesis in the Topo III–RecQ absent condition was confirmed (panel B).

Repression of the multimer products

Bidirectional replication in the reconstituted *oriC* replication system has been well established previously (15,17–19,22,23). It is, however, known that an unidirectional rolling-circle type replication occurs from *oriC* in the case of a low concentration of DnaG primase (<10 nM) and produces multimer DNA byproducts (17,23). Although RCR includes a high enough concentration of DnaG (400 nM) to ensure bidirectional replication, multimer products were still observed even in the presence of both Topo IV and Topo III–RecQ. A rolling-circle type replication is also known to arise via a template switching mechanism due to inadequate replication termination upon collision of bidirectional replication forks (22). To address whether multimer products in RCR are due to the inadequate termination of bidirectional forks, the replication termination system operating at the chromosomal terminus was integrated into RCR (Figure 1, optional components). Tus binds to the *ter* DNA sequence and the *ter*-Tus complex arrests replication forks in an orientation-dependent manner (31). Two inverted *ter* sites were placed opposite to *oriC* in the circular DNA template to block forks from excess progression beyond where most forks collide and thus to repress the rolling-circle type replication (22). As expected, inclusion of Tus in RCR efficiently repressed accumulation of multimer products (Supplementary Figure S3A). In contrast, the amplification level of supercoiled monomer products was only slightly repressed, confirming that two bidirectional replication forks progress successfully from *oriC* to each *ter* site. Furthermore, we examined the XerCD-*dif* system which operates also at the chromosomal terminus (Figure 1, optional components). In this system, a site-specific recombinase complex consisting of XerC and XerD resolves catenanes by the chromosome dimer resolution mechanism at the *dif* site (32). Inclusion of a *dif* site opposite to *oriC*, and addition of XerCD to the RCR, also significantly reduced multimer products (Supplementary Figure S3B). This result suggests that a considerable level of the catenated intermediates of the replication cycle is included in multimer products. In contrast to Tus, XerCD did not completely eliminate large concatemers (which remained trapped in the gel wells), which is consistent with the proposed activity of XerCD in decatenation and that of Tus in preventing rolling-circle type over-replication. Although both the Tus-*ter* and XerCD-*dif* systems enable efficient repression of multimer product formation, these systems were excluded from subsequent RCR experiments because of their specific requirements for *cis*-acting DNA elements.

Exponential propagation of circular DNA molecules

DNA propagation by RCR was evaluated with a limited amount of circular DNA template. Titration of the template demonstrated that even a single molecule of 9.5 kb circular DNA in the 10 μl reaction (10^{-9} ng/μl) was efficiently propagated within 3 h (Figure 3A). Quantification of total DNA product, either with an intercalating-dye method or by colony counting following transformation into *E. coli*, revealed $>10^{10}$-fold propagation from the single DNA molecule (Supplementary Figure S4). The transformation method had high sensitivity, detecting as few as $\sim10^4$ molecules per μl ($\sim10^{-4}$ ng/μl), and enabled measurement of DNA production, which showed exponential propagation with a doubling time of ∼8 min (Figure 3B). When the DNA concentration reached $\sim10^{10}$ molecules per μl (∼100 ng/μl), the RCR plateaued, probably due to depletion of enzymes and/or nucleotide pools. Serial passage of plateaued samples directly into the fresh RCR mixtures using 10^6-fold dilutions enabled further repetition of the replication cycle for many generations (Figure 3C and D). Through the 10th serial passage (∼200 total population doublings), supercoiled monomers were produced constantly without significant accumulation of multimer products which include catenanes as replication cycle intermediates and concatemers as unfavorable rolling-circle replication products. This observation excludes the possibility that

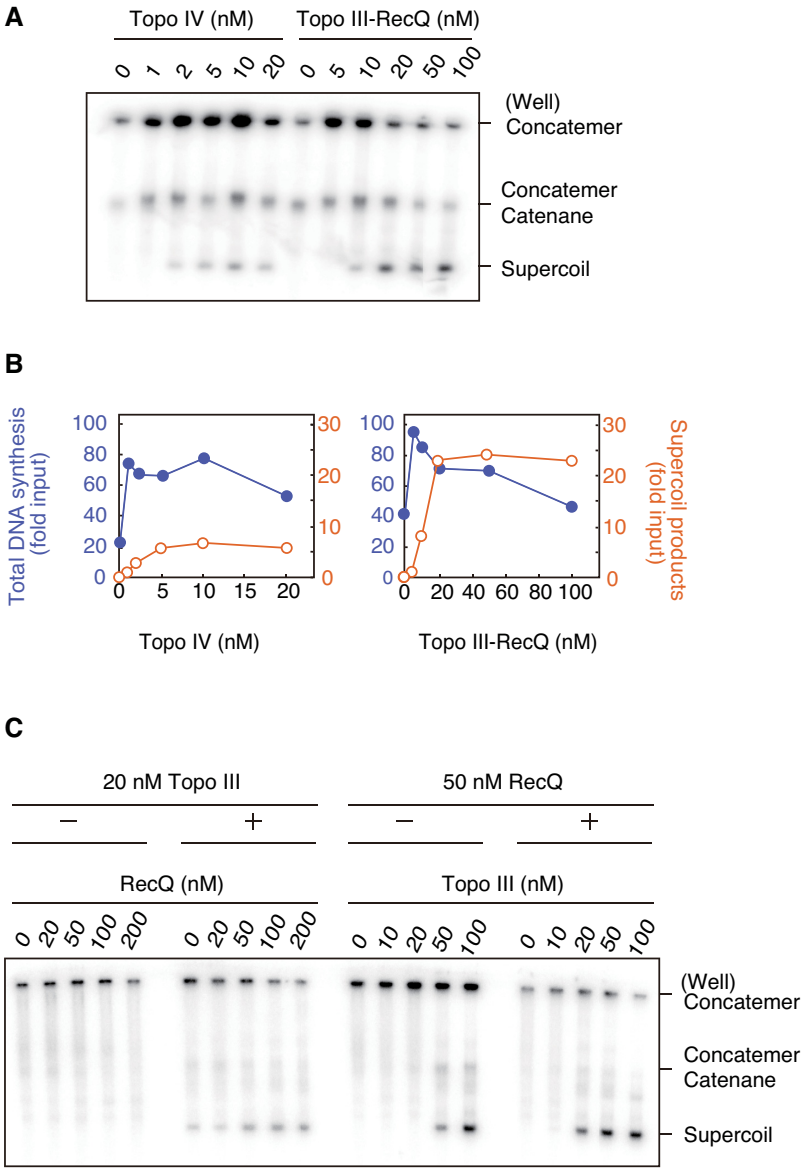
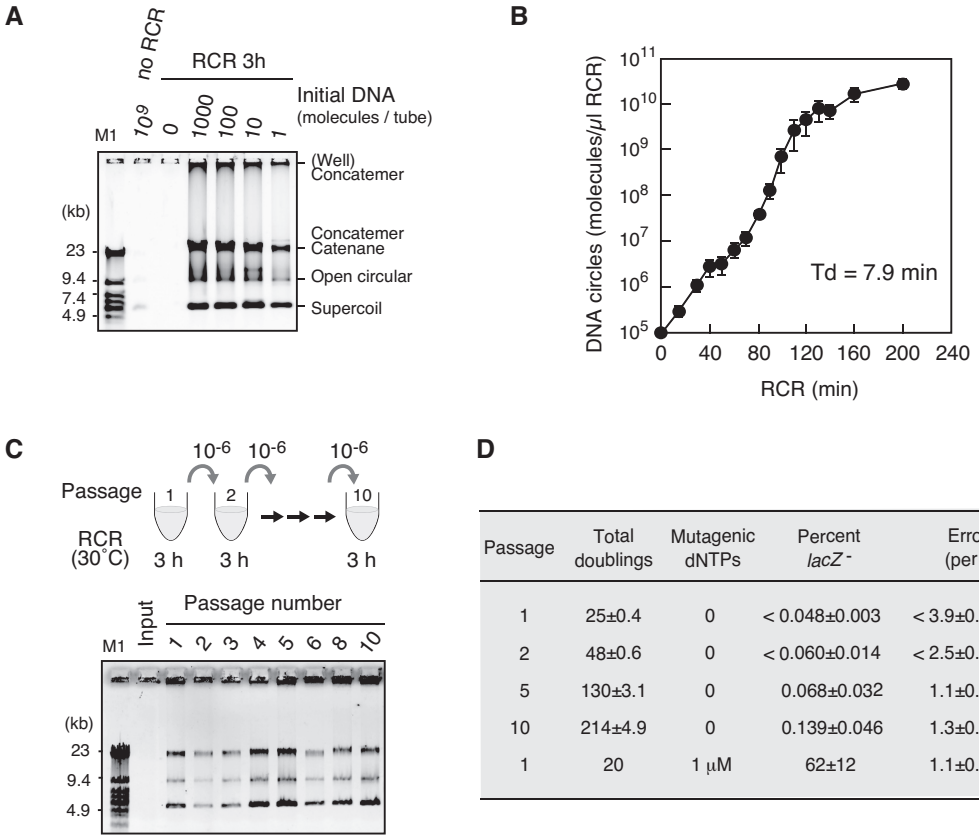


Figure 2. Replication reaction of *oriC*-circular DNA in the presence of decatenation enzymes. (**A**) *oriC*-containing supercoiled DNA (M13ms10, 150 pM as supercoiled circular DNA, 24 pmol as nucleotides in 10 μl reaction) was incubated in RCR mix (containing [α-^{32}P]dATP) at 30°C for 1 h in the presence or absence of either Topo IV or Topo III–RecQ. DNA products were analyzed by agarose-gel electrophoresis. (**B**) The amount of total DNA synthesis. dNTP incorporation was quantified and values were normalized to the amount of input supercoiled template as nucleotide (fold input, closed circles). The ratio of supercoiled:total products was determined from the band intensities in the gel image, enabling calculation of the amount of supercoiled product normalized to input supercoiled template from the amount of total DNA synthesis (fold input, open circles). (**C**) RecQ stimulates Topo III decatenation activity in RCR. *oriC*-containing circular DNA (M13ms10, 150 pM) was incubated in the RCR mixture, excluding Topo IV, at 30°C for 20 min in the presence of the indicated concentrations of Topo III and/or RecQ. [α-^{32}P]dATP-incorporated DNA was then detected. The compositions of the RCR mixture are listed in Supplementary Table S4.

a continuous rolling-circle type replication occurs in preference to the replication from *oriC* during the reaction. We further assessed whether over-initiation at *oriC* and resulting multi-fork replication occur in RCR, since RCR does not include negative regulation system for *oriC* initiation (33). qPCR determination of ori/ter ratio of RCR product did not detects the over-initiation (Supplementary Figure S5), which is reasonable considering that the replication completes quite rapidly after initiation in the case of plasmid-sized DNA according to the fork speed (0.5–0.6 kb/s *in vitro*) (34). This observation is also in agreement with the previous report that initiation is rate-limiting and elongation is rapid in the reconstituted *oriC* replication system (24).

Fidelity of DNA propagation in RCR

The genome replication apparatus of *E. coli* has extremely high fidelity, and replication errors generated during *in vitro oriC* replication reactions are hard to detect (35). Successive rounds of RCR in the passage experiments used here enabled detection of accumulated errors through *lacZ*$^{-}$ mu-



Passage	Total doublings	Mutagenic dNTPs	Percent *lacZ*⁻	Error rate (per base)
1	25±0.4	0	< 0.048±0.003	< 3.9±0.31 x 10^{-8}
2	48±0.6	0	< 0.060±0.014	< 2.5±0.6 x 10^{-8}
5	130±3.1	0	0.068±0.032	1.1±0.52 x 10^{-8}
10	214±4.9	0	0.139±0.046	1.3±0.46 x 10^{-8}
1	20	1 μM	62±12	1.1±0.37 x 10^{-4}

Figure 3. Continuous repetition of the RCR. (**A**) The indicated amount of pOri8 (9.5 kb) was incubated in the RCR mixture for 3 h. Aliquots (1 μl) were detected by agarose-gel electrophoresis and SYBR Green staining. The input DNA (10^9 molecules in 10 μl, 1 ng/μl) was also detected ('no RCR'). Size-marker fragments (M1) were derived from phage λ DNA. (**B**) pOri8 (10^5 molecules/μl) was incubated in the RCR mixture at 30°C. Aliquots (1 μl) were taken at the indicated times and the number of DNA circles was quantified using the transformation method. The RCR mixture was preincubated at 30°C for 15 min before the addition of template DNA. The values from three independent reactions are shown with the error bars (standard error of the mean). (**C**) pPKOZ (10^{-5} ng, 8.9 kb) was incubated in the RCR mixture for 3 h (passage 1). The sample was then diluted 10^6-fold in the fresh RCR mixture and further incubated for 3 h (passage 2). This sequence was repeated for a total of 10 incubations. At each passage, aliquots were analyzed. Size-marker fragments (M1) were derived from phage λ DNA. DNA was visualized by staining with SYBR Green I. (**D**) The total doublings were deduced using the transformation method. Error rates per base per replication cycle were deduced by blue–white determination of the *lacZ* status (28). Mutagenic dNTPs, when included, consisted of 1 μM each of 8-oxo-GTP and dPTP. The transformation analysis and the blue–white determination were performed twice and the mean values were shown with the ranges.

tations, affecting blue-white identification of transformant colonies (Figure 3D). The error rate per cycle was calculated using an equation used in a previous PCR-fidelity assay (28). This calculation gave a value of $\sim 1.2 \times 10^{-8}$ per base per replication cycle, which is comparable to the error rate in *E. coli* cells lacking *mutL* mismatch-repair gene function (36) and $\sim 10^4$-fold lower than the error rate of *Taq* DNA polymerase (28). To know the sequence of mutant variants generated in RCR, 15 independent plasmids isolated from the *lacZ*$^-$ colonies in the 10th passage sample were analyzed by DNA sequencing. All of these plasmids carry a nonsense or frameshift mutation in the *lacZ* gene, and no other mutation was detected in the 3 kb gene regions. (Supplementary Table S2). The error rate of RCR increased in the presence of mutagenic nucleotide analogs (37) (Figure 3D and Supplementary Figure S6), demonstrating error-prone RCR could be used as a random mutagenesis technique.

Monoclonal propagation of circular DNA by limiting-dilution

The ability of RCR to proceed from a single template molecule enabled testing of whether it could propagate a monoclonal population of circular DNA stochastically from a mixed population by limiting dilution. We mixed two different sized DNA species, pOri8 (9.5 kb) and pOriDif (12 kb), and diluted the mixture extremely, expecting that only one of them could be present in a single tube. With the mixed population containing 15 molecules each per reaction as estimated copy number, both DNA species were still propagated together (Figure 4, samples 1–3). When this mixed template population was diluted 10-fold further, only one DNA species was propagated per reaction (Figure 4, samples 4–7). This confirms the observation that RCR propagates DNA from almost single molecule level, and highlights the possibility that RCR could be used as a cell-free technique to generate monoclonal DNA circles from a mixed population.

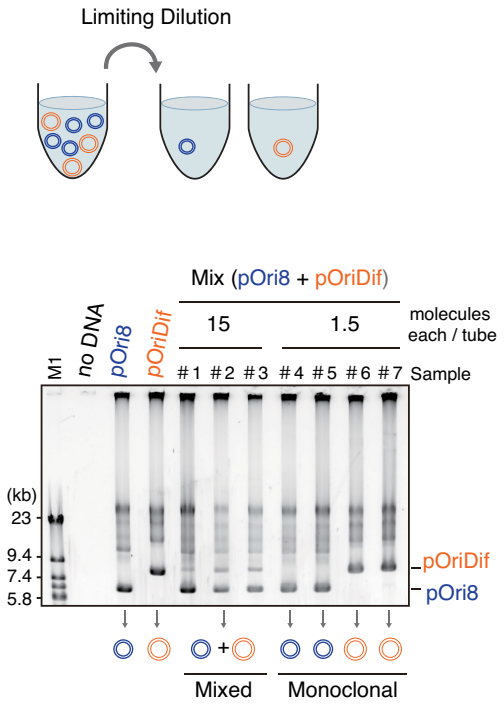


Figure 4. Monoclonal propagation of DNA by limiting-dilution. Mixtures of pOri8 (9.5 kb) and pOriDif (12 kb) after limiting dilution (15 molecules of each in samples 1–3, an average of 1.5 molecules of each in samples 4–7) in the RCR mixture (10 μl) were incubated for 6 h. pOri8 and pOriDif were also individually propagated. Size-marker fragments (M1) were derived from phage λ DNA. DNA was visualized by staining with SYBR Green I.

In vitro propagation of 200 kb DNA as covalently closed circles

We also assessed the ability of RCR to propagate large, circular DNA molecules. *Escherichia coli* genome regions including *oriC* were retrieved by a 'pop-out' method involving the *parABC-Km* cassette (4.8 kb) and λ Red recombination (30) (Figure 5A), yielding circular DNA (85 kb or 205 kb) inherited in host cells as an auto-replicative *oriC* plasmid (38). In the case of plasmids larger than several dozen kb, the supercoiled forms migrate more slowly than the linear forms on agarose gels, can be separated according to their size differences beyond the size separation limit of the linear forms, and the circular forms migrate even more slowly than the supercoiled forms (39,40). The supercoiled molecules were seen in the DNA samples isolated from *E. coli* (RCR-, Figure 5B). After RCR, propagation of these large DNA circles as supercoiled molecules were observed (RCR+, Figure 5B). Substantial production of the linear forms is most likely due to double-strand break of some of circular molecules during RCR because large DNA molecules tend to be sheared. The circular and catenane products are likely to be trapped in the well in this analysis (39). We also demonstrated DnaA requirement for the 200 kb propagation in RCR (Supplementary Figure S7). In this experiment, we observed small byproducts during a longer reaction time (18 h), which may be generated due to collapse or stalling of replication forks.

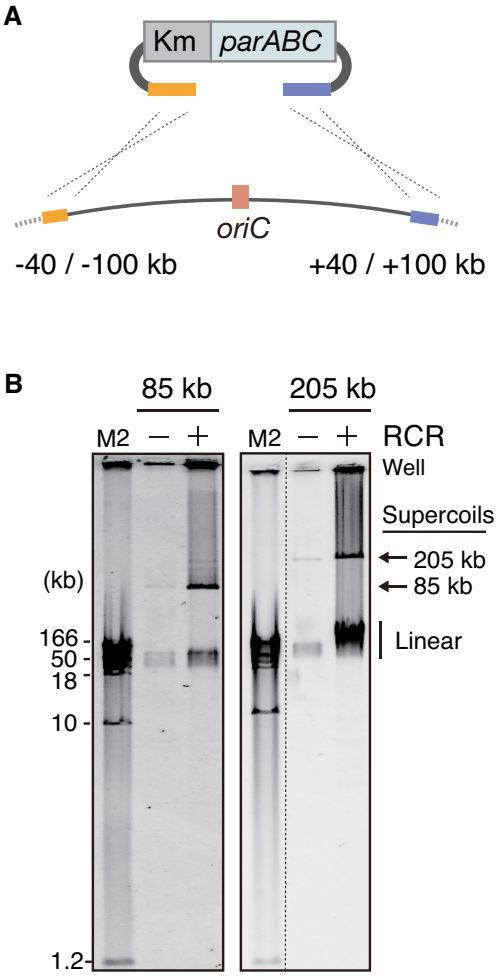


Figure 5. Propagation of large, circular DNA in RCR. (**A**) 'Pop-out' method for generating large, *oriC*-containing circular DNAs. An 80 or 200 kb chromosomal region containing *oriC* was retrieved via the *parABC-Km* cassette in cells expressing the λ Red genes. The structure of the isolated 205 kb DNA was verified by restriction enzyme analysis (Supplementary Figure S8). (**B**) A total of 85 kb (pOri80, 15 pM) or 205 kb (pOri200, 5 pM) *oriC*-containing circular DNA was incubated in the RCR mixture at 30°C for 3 h. Samples before (−) or after (+) RCR were analyzed by agarose-gel electrophoresis and SYBR Green staining. Size-marker fragments (M2) were derived from T7 DNA.

Cell-free cloning using RCR

Recent advances in DNA-assembly techniques, including Gibson Assembly, enable one-step seamless ligation of multiple overlapping fragments *in vitro* (4,29). We tested whether RCR can replace living host cells for the propagation of circular products generated by Gibson Assembly. The 4.6 kb *parABC-Km* cassette, a 3.3 kb *lacZ* fragment and a 1 kb *oriC* fragment were designed with 60 bp overlapping ends to enable generation of a circular DNA (Figure 6). After an assembly reaction at 50°C for 1 h, an aliquot (1 μl) was transferred to the RCR mixture (total 10 μl) and further incubated at 30°C for 3 h. DNA products, including supercoiled forms, were efficiently propagated (Figure 6). Propagation was dependent on the assembly reaction, which is consistent with the proposal that RCR requires a circular template but not a linear template. Transforma-

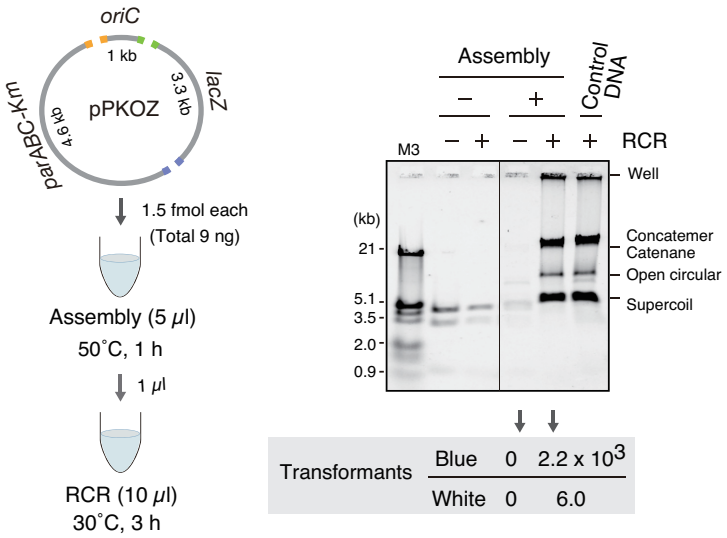


Figure 6. Two-step cell-free cloning using RCR. Each of three PCR fragments (1 kb *oriC*, 3.3 kb *lacZ* and 4.6 kb *parABC-Km;* 1.5 fmol) containing 60 bp overlaps at both ends were incubated in modified Gibson Assembly mixture (5 μl) at 50°C for 1 h. Aliquots (1 μl) were then incubated in the RCR mixture (10 μl) at 30°C for 3 h. Samples without assembly and/or RCR were analyzed as negative controls (−). pPKOZ was propagated by RCR ('Control DNA'). The assembly samples before or after RCR were also analyzed by *E. coli* transformation and blue-white determination. Size-marker fragments (M3) were derived from phage λ DNA.

tion analysis revealed that RCR propagated the circular assemblies by >2000-fold, and 99.7% of colonies were *lacZ*-positive. Some of the *lacZ*-negative colonies might have resulted from errors introduced during the assembly reaction (29).

DISCUSSION

We have developed here a biochemical system that propagates large circular DNA molecules through an autonomous repetition of a chromosome replication cycle. Propagation of genetic information is a fundamental biological process that is common to all living organisms and likely started before the origin of life itself. Our successful reconstruction of such a process opens the way for the bottom-up approach toward a self-replicating system composed of biological molecules. While the *oriC* replication has been reconstituted *in vitro* more than 30 years ago, continuous repetition of the replication cycle has not yet been achieved. In a previous attempt to complete the *oriC* replication, only about one-fifth of template molecules were replicated, and of those, only one-third were completed (24). It has been therefore thought that other factors may be required for the efficient completion of replication and the following repetition of the replication cycle. However, we have for the first time established a reconstituted system which enables the repetitive reaction cycle of the *oriC* replication using 25 individual proteins which have been well characterized. This reconstituted system defines the minimum set of enzymes required to repeat a complete chromosome replication cycle. It has been demonstrated that the rolling-circle type of over-replication is induced in the *oriC* replication system upon including the Okazaki fragment maturation system (22,24). Our RCR system successfully represses the rolling-circle type replication by the decatenation system combining Topo IV and Topo III–RecQ, which leads to production of supercoiled monomer molecules that are structurally identical to the input templates. Since Topo IV has a supercoil relaxation activity, excess Topo IV is more inhibitory to DNA replication in RCR in the gyrase limiting condition (25 nM gyrase, Supplementary Figure S2B) in contrast to the excess gyrase condition (150 nM gyrase, Figure 2B, left). This observation supports the notion that negative supercoiling introduced by gyrase is important for re-initiation of replication and also for progression of DNA replication in RCR. Bidirectional replication from *oriC* has been previously shown in the *oriC* replication system (15,17–19,22,23), and our experiment using the Tus-*ter* system located opposite of *oriC* confirmed the bidirectional replication in RCR. We have further successfully established a reaction condition enabling to start the *oriC* replication cycle from a single DNA molecules in 10 μl reaction, which allows us to propagate the *oriC* DNA circles over many generations in a single reaction.

In vivo, the RCR enzymes are involved in replication of the 4.6 Mb *E. coli* genome, but the *in vitro* size limit of RCR is still under investigation because of the difficulty in preparing intact circular DNA >200 kb *in vitro* without mechanical shearing. When a lower amount of the 200 kb template (0.5 pM) was propagated with a long time (18 h) incubation, small byproducts were generated particularly in the presence of a higher concentration of DnaA, probably due to collapse or stall of replication forks (Supplementary Figure S7). It might be required to integrate fork stabilization mechanisms and/or the initiation regulation mechanisms to propagate stably larger DNAs.

RCR has several advantageous features to amplify very large DNA molecules in contrast to PCR or RCA (rolling circle amplification) (41). PCR and RCA require DNA denaturation for primer annealing, which often causes damage to the large DNA template. Monoclonal amplification

(Figure 4) has been also reported previously in RCA (42), which uses single stranded DNA as a template for the RCA amplification, while the amplification efficiency for double-strand DNA would be much lower. Single strand nicks or gaps in the template DNA are detrimental to the PCR/RCA amplification, RCR could, in contrast, repair these damaged DNA by ligase and the gap filling polymerase (Pol I) included in the reaction. In agreement with this, RCR was able to amplify the DNA molecules prepared by the modified Gibson Assembly reaction excluding ligase (Figure 6). RCR also has a 10- to 100-higher fidelity (10^{-8} error per base) than that of the bacteriophage phi29 DNA polymerase used in RCA (43). The phi29 DNA polymerase replicates the 19.3 kb phi29 DNA with a rate of ∼0.05 kb/s (41). The advantage of RCR can be attributed to its *in vivo* function, in which the multi-enzyme system replicates the 4.6 Mb *E. coli* chromosome with a rate of ∼1 kb/s by two bidirectional forks from *oriC* (34). Other biochemical systems that are associated with the *E. coli* chromosome replication would be integratable into RCR. For example, the mismatch repair system in *E. coli*, which has also been reconstituted with purified proteins (44), may be useful to further increase the replication fidelity of RCR. In contrast to RCA, which produces concatemer, RCR produces monomer supercoils that is structurally identical to the input template molecules. This property allows us to continue the RCR amplification over many generations simply by serial passage into the fresh reaction mixture, in which the circular DNA molecules are propagated vigorously as if they were proliferating cells in culture medium.

Conventional DNA cloning using living hosts requires a plasmid vector, transformation, culturing and plasmid purification. These procedures are time-consuming and difficult to automate. In addition, some sequences are toxic or unstable in host cells. There is also a size limit for DNA to be able to introduce into host cells. RCR provides a cell-free cloning tool that overcomes these constraints and it could be a valuable platform for the creation of large DNA constructs, such as synthetic genomes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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