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Plasmid replication based on the T7 origin of replication requires a T7 RNAP variant and inactivation of ribonuclease H

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ABSTRACT

T7 RNA polymerase (RNAP) is a valuable tool in biotechnology, basic research and synthetic biology due to its robust, efficient and selective transcription of genes. Here, we expand the scope of T7 RNAP to include plasmid replication. We present a novel type of plasmid, termed T7 *ori* plasmids that replicate, in an engineered *Escherichia coli,* with a T7 phage origin as the sole origin of replication. We find that while the T7 replication proteins; T7 DNA polymerase, T7 single-stranded binding proteins and T7 helicase-primase are dispensable for replication, T7 RNAP is required, although dependent on a T7 RNAP variant with reduced activity. We also find that T7 RNAP-dependent replication of T7 *ori* plasmids requires the inactivation of cellular ribonuclease H. We show that the system is portable among different plasmid architectures and ribonuclease H-inactivated *E. coli* strains. Finally, we find that the copy number of T7 *ori* plasmids can be tuned based on the induction level of RNAP. Altogether, this study assists in the choice of an optimal genetic tool by providing a novel plasmid that requires T7 RNAP for replication.

INTRODUCTION

RNA polymerase from the T7 phage (T7 RNAP) is a compact single-subunit enzyme and a highly active driver of transcription, with RNA synthesis several times as fast as *Escherichia coli* RNAP (1). It initiates with high specificity from a short cognate sequence known as the T7 promoter (P_{T7}) (2–4) and the enzyme functions robustly under a range of conditions including *in vitro* (5), and in different prokaryotic (3,6–9) and eukaryotic cells (10). It can also be used as a fusion protein to reprogram promoter sequence specificity, to co-localize additional activities to transcription initiation sites (11–13), or as a split protein to enable optical switching (14). These properties have given it an important role in biotechnology, basic research and synthetic biology, in *in vivo* as well as *in vitro* settings, and most prominently for gene overexpression (3,15). In fact, T7 RNAP can drive transcription so strongly that overexpression becomes toxic or leads to a depletion of cellular resources (3,16). Other advances include the development of an error-prone T7 RNAP version (17), T7 RNAP variants with reduced toxic effects (18), T7 RNAP variants with orthogonal promoter specificities (4,18–20), split T7 RNAP versions that retain *in vivo* activity and are useful for e.g. the creation of AND gates (15,21,22), and allocation of cellular resources (23).

The T7 phage genome replication starts from the primary origins, ϕ1.1A and ϕ1.1B, but replication can also be initiated at secondary origins (e.g. ϕOR and ϕ6.5) if the primary origins are deleted (24). Early *in vitro* studies demonstrated the initiation of DNA synthesis in plasmids containing a T7 primary origin using purified T7 phage enzymes (25,26). An essential element of every T7 origin of replication (T7 *ori*) is a promoter dependent on T7 RNAP and the promoter obtained from origin ϕ10 is present in extensively used plasmid series (27).

The T7 replisome is relatively simple and consists of only four different replication proteins (T7 DNA polymerase (T7 DNAP), T7 helicase-primase, T7 ssDNA-binding protein, and *E. coli* thioredoxin (trx)) (28). *In vitro* studies with purified T7 proteins indicate that T7 RNAP and T7 DNAP are essential for the initiation of DNA replication (25). Furthermore, DNA replication is stimulated in the presence of the T7 helicase-primase (T7 HelPrim) (26). Small minicircular DNA was synthesized to study the coordination and the characteristics of the T7 replisome *in vitro* (29), and replisome assembly could be artificially initiated (30,31). However, although the T7 replisome has now been resolved at

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high resolution (32), plasmid replication *in vivo* based solely on T7-derived functions remains elusive, despite additional studies with T7 *ori*s *in vivo*, e.g. to investigate the initiation of T7 phage replication (24) or the role of T7 RNAP and T7 lysozyme during T7 phage infection (33). Importantly, these studies relied on additional *ori*s on the same plasmid for replication in the absence of phage infection.

Here, we expand the scope of T7 RNAP further by engineering a T7 *ori* plasmid that replicates in an engineered *E. coli* host without any known additional plasmid *ori* and without concomitant phage infection. We start from a strain with the genes encoding the key elements of the T7 phage replisome (25,34) and find that, in a cell that lacks ribonuclease H, the only T7-derived requirement for plasmid replication is T7 RNAP.

MATERIALS AND METHODS

Strains and media

All *E. coli* strains (Supplementary Table S1) were grown in LB Miller broth as the standard growth medium. If not otherwise indicated, cells were incubated and cultivated at 37°C at 200 rpm in a 50 mm orbital shaker in shake flasks or cultivation tubes. Antibiotics and supplements were added, if appropriate, to the following concentrations: kanamycin (Kan, 50 μg ml^{-1}) chloramphenicol (Cm, 34 μg ml^{-1}), carbenicillin (Carb, 100 μg ml^{-1}), rifampicin (Rif, 100 μg ml^{-1}) and isopropylthiogalactoside (IPTG, 0.1 mM). Determination of the doubling time of strains took place, if not indicated otherwise, under standard growth condition in cultivation tubes. The doubling time of each culture was measured in exponential growth phase with at least three separate precultures.

Molecular biology and strain constructions

DNA manipulation was done following standard procedures as described previously (35). Suppliers of enzymes, oligonucleotides, and kits are listed in Supplementary Table S2 and were used as described by the manufacturer unless otherwise mentioned. Oligonucleotides were purchased desalted and a complete list of them is provided in Supplementary Table S3. A standard PCR protocol was applied unless mentioned otherwise (60 s 94°C; 20−33 times, 94°C 20 s, 60°C 30 s, −0.3°C cycle^{-1}, 72°C 90 s; 72°C 600 s). Isothermal assembly was performed as described previously (36). The required parts were amplified by PCR and subsequently subjected to a *DpnI* digest directly in the polymerase buffer, if appropriate. The primers were designed with an overlap of 18–25 nucleotides. Gene deletions were performed as described by Datsenko & Wanner (37). The correctness of all introduced knock-outs was verified by PCR and Sanger sequencing. Where suitable, strains with single deletions were also selected from the Keio collection of single gene deletions (38). If the kanamycin resistance gene in these strains had to be removed, this was done as previously described (37).

Strain JM101T7 containing the different T7 genes was constructed by homologous recombination using the pKO3 integration vector (39). The T7 replication genes were amplified by PCR from wild-type T7 phage and cloned behind the P*tac*-promoter of pKQV4 (40). We selected the *tau* and the *prp* operon of *E. coli* JM101 as integration spots since the proteins originally encoded in these regions are not required for growth under laboratory conditions. Chromosomal regions were amplified by PCR and cloned. The T7 genes were re-excised with promoter and inserted into the different plasmid-based *tau* and *prp* genes: the gene for T7 DNAP into *prpE*, the gene for T7 HelPrim into *tauA*, the gene for the ssDNA binding protein into *tauC*, and the gene for the T7 RNAP into *prpR*. From there, the constructs with T7 genes inside *prp* and *tau* genes were transferred to pKO3 for introduction into *E. coli* JM101 (details of these delivery plasmids are summarized in Supplementary Table S4 and the full sequence in Supplementary Table S5). The function of the different transferred genes was tested immediately after construction of the respective strain in two different ways. First, correct introduction of the T7 genes was confirmed by PCR amplification of the corresponding regions of the chromosome. Second, the activity of the introduced T7 proteins was assayed in complementation assays using mutant T7 phages. Since F' plasmid containing strains (such as JM101) can only be productively infected by T7 phages by overexpression of the non-toxic phage T3 1.2 allele we introduced plasmid pK173 into the different constructed strains as described earlier (41) and infected the constructed strains successfully with phages carrying inactive genes for T7 DNAP, RNAP, ssDNA binding protein, or hel/primase, respectively. We subsequently discovered that JM101T7 developed some spontaneous mutations and termed the resulting strain JM101T7m ('mutations').

Strain JM101repA was constructed by amplifying *repA* including its native promoter sequence from plasmid pCKO1, transfer of *repA* to pUC18 and then application of the lambda InCh method for chromosomal insertion as described earlier (42).

Digital droplet PCR

The plasmid copy number (PCN) as the average plasmid copy number per chromosome was measured by quantification of the targets *cat* and *uidA* in *E. coli*. The PCN was calculated as the ratio of the *cat*/*uidA* concentration in a duplex reaction setup for the simultaneous detection of a plasmid-located target (*cat*) and a chromosome reference (*uidA*). Primers and probes were designed with Primer3Plus (29) according to the recommendation of the manufacturer of the BioRad QX100 system (Bio-Rad, USA). The probes were modified at the 3′ end with a quencher (Black hole quencher, BHQ-1) and at the 5′ end with one of the fluorophores carboxyfluorescein (FAM) or Yakima-Yellow for *cat* and *uidA*, respectively. The primers and probes used for ddPCR are summarized in Supplementary Table S3.

To conduct a single reaction for the ddPCR, an aliquot of 20 μl of sample was prepared according to the Bio-Rad QX100 manufacturer's instruction: 10 μl 2× ddPCR Supermix, 2 μl primer and probes (primers to a final concentration of 900 nM each, probes to a final concentration of 250 nM, and 8 μl of DNA sample). A mastermix containing all ingredients except the bacterial sample was allowed to equilibrate to room temperature and 12 μl of it was added to one DNA sample. For the heat-treated sample, overnight

cultures were diluted to 500–1500 cells μl^{-1}, distributed in eight-well strips and frozen at −20°C. Subsequently, the samples were thawed on ice, heated at 95°C for 20 min and immediately put on ice again. Droplets of the samples were generated in the droplet generator of the QX100 system (Bio-Rad, USA) according to the manufacturer's protocol and then transferred to a 96-well PCR plate (twin.tec, Eppendorf, Germany). Then, the PCR reaction was performed in a Eppendorf mastercycler nexus GSX1 with the following protocol: 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 60 s, followed by 98°C for 10 min. The signals of FAM and Yakima Yellow in the droplets were detected simultaneously with the QX100 droplet reader (Bio-Rad).

Characterization of fluorescent strains

For each strain, at least three separate cultivations were started to determine the *in vivo* fluorescence caused by expression of the gene for GFP and incubated at 37°C and 250 rpm overnight (deep well plates containing 1 ml of LB per well, System Duetz, EnzyScreen, Heemstede, Netherlands). Afterwards, 200 µl of sample was analyzed in parallel in Greiner 96-well plates in a Victor plate reader (excitation 485 nm, emission 530 nm, Perkin Elmer, USA). The fluorescence signal was further corrected for the fluorescence of the medium and normalized to cell density (OD_{600}).

Whole genome sequencing

Genomic DNA was isolated out of overnight cultures with the GeneElute Bacterial Genomic Kit (Sigma Aldrich) and sequenced on an Illumina MiSeq platform (Illumina RTA Version: 1.18.54, Sequencer: GFB MiSeq (Yoda), Run type: paired-end). The resulting fastq files were further analyzed for single-nucleotide mutations, insertions and deletions, and large deletions using the computational pipeline breseq (43). Raw data is provided at the Sequence Read Archive under Project PRJNA735647.

Fluctuation assay

Measurement of the spontaneous error rates on the chromosome were based on a fluctuation assay. The strains of interest were inoculated in LB supplemented with the appropriate antibiotic and grown overnight (37°C, 250 rpm). A mastermix of diluted culture with an approximate concentration of 5000 cells ml^{-1} was prepared from each overnight culture in LB (supplemented with antibiotic if appropriate). A minimum of 12 parallel cultures of 200 µl of each mastermix were filled in a 96-deep well block and grown again overnight (37°C, 250 rpm). Upon reaching stationary phase, the whole volume in a minimum of 12 wells was spread on LB agar plates containing 100 $\mu g\ ml^{-1}$ rifampicin. An appropriate dilution of 3 wells were spread on non-selective medium to determine the final cell concentration (N_t). The non-selective and the rifampicin plates were incubated at 37°C and total CFU counts were determined after incubation of 21 and 23 h, respectively. The relative mutation rate was calculated via Luria-Delbrueck fluctuation analysis by using the FALCOR web tool (44).

RESULTS

Constructing a plasmid depending on the T7 ΦOR *ori* for replication

In order to develop a plasmid that could replicate in *E. coli* using only a T7 *ori*, specifically ΦOR, a test platform was put in place (Figure 1A). This platform aimed to separate plasmid assembly in a cloning strain from testing of the function of the T7 *ori* in a testing strain. To create a conditional origin of replication we took advantage of the pSC101 core origin which requires RepA for replication (45). For this purpose, we placed *repA* onto the chromosome of *E. coli* JM101 resulting in strain JM101repA. Correspondingly, test plasmids contained the core pSC101 origin, but not *repA* thereby creating a plasmid that requires RepA in trans, such as provided by the JM101repA strain. Similarly, T7 *ori* test plasmids were constructed with a T7 ori and the core pSC101 *ori*. Therefore, we could assemble plasmids with the core pSC101 *ori* in JM101repA and confirm T7 *ori* function as the sole functioning origin by transforming *repA*-free strains, in which the core pSC101 *ori* is non-functional and therefore does not lead to transformants. For the *repA*-free testing strain, we used JM101T7m, a strain with key elements of the T7 replication machinery (namely the T7 genes for RNAP, DNAP, HelPrim and ssDNAbp, each under the control of a P*tac* promoter) integrated on the chromosome (28,34). We found that the transformation rate of JM101T7m with the T7 test plasmid, pTestOR, was extremely low and therefore, we used a transformant of JM101T7m cured of the T7 *ori* plasmid, termed JM101T7c ('cured'). JM101T7c exhibited a transformation rate for T7 test plasmids similar to transformation with pCKO1 which contains *repA* and the pSC101 *ori*. The exact nature of the genetic makeup of JM101T7m and JM101T7c will be discussed later.

We hypothesized that replication of a plasmid with a given T7 *ori* in a strain in which RepA was not produced indicated that the T7 *ori* was the sole functioning origin. Once confirmed in the testing strain, the core pSC101 *ori* sequence was removed in order to confirm the functionality of the T7 *ori*; plasmids that were successfully replicated were deemed T7 *ori* plasmids (Figure 1B). Following this strategy, pTestOR, containing a chloramphenicol resistance gene, and both the core pSC101 *ori* and the T7 *ori* was assembled and tested in JM101T7c, JM101 and JM101repA (Figure 1C). As expected, replication of pTestOR, in strains lacking the T7 replication machinery, was dependent on RepA as evidenced by the presence of colony forming units in JM101repA, but not in JM101. Transformation of JM101T7c with pTestOR was successful, suggesting that a functional replication system dependent on the T7 machinery had been assembled in this strain. Next, the core pSC101 *ori* present in pTestOR was removed yielding plasmid pT7-OR. pT7-OR was subsequently used to transform JM101T7c, JM101 and JM101repA, and transformants could only be obtained from JM101T7c, from which pT7-OR was successfully re-isolated. Accordingly, we categorized JM101 and JM101repA as T7-*ori*-rejecting strains and JM101T7c as T7-*ori*-supporting. From these experiments

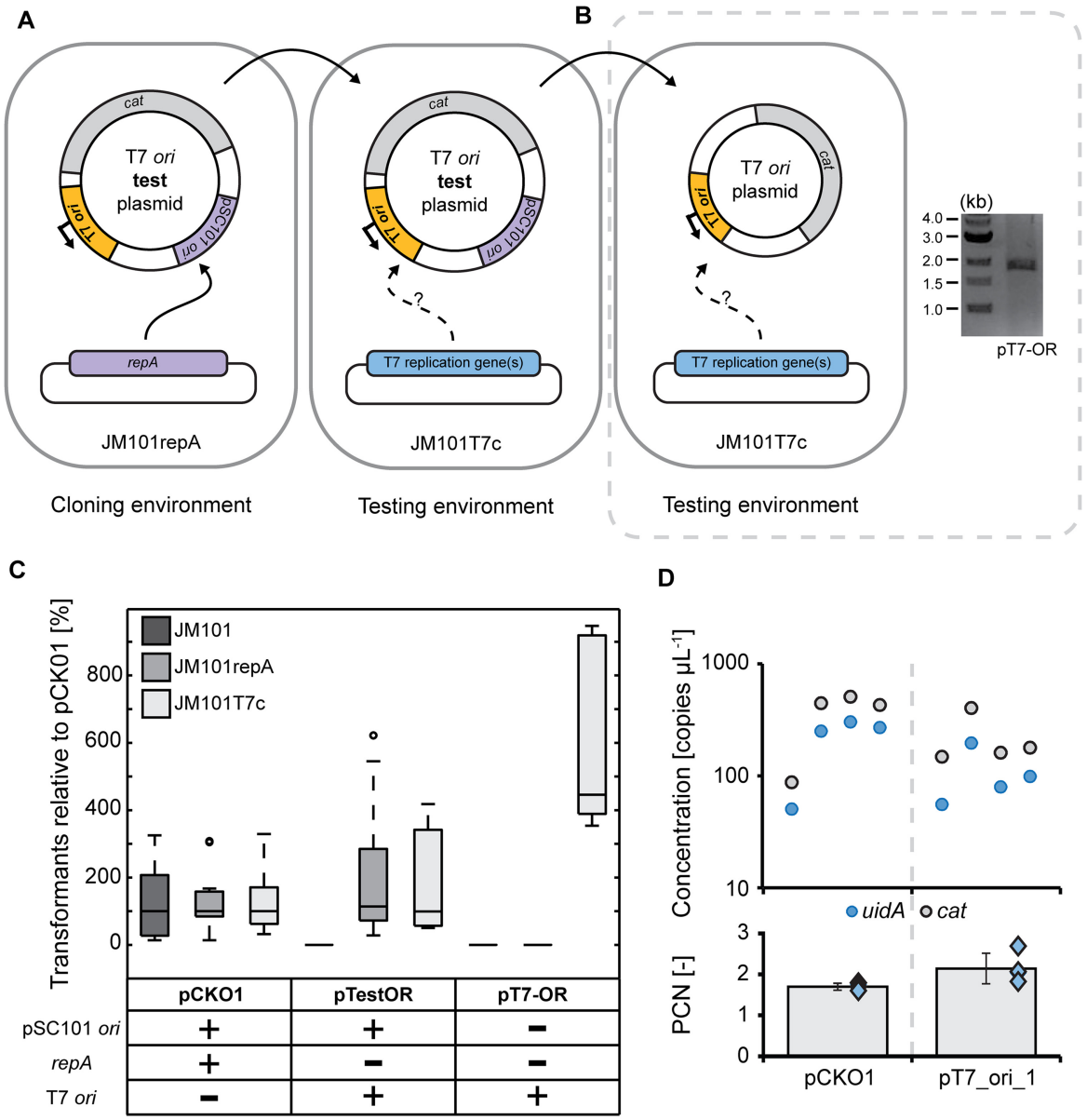

Figure 1. Construction of a T7 *ori* plasmid. (**A**) Left: Cloning environment: Plasmids containing the T7 *ori* and a core pSC101 *ori* lacking *repA* (T7 *ori* test plasmids) are assembled and used to transform a strain which expresses *repA* from the chromosome and allows use of the core pSC101 *ori*. Right: Testing environment: T7 ori test plasmid or T7 ori plasmids are transformed into a strain without repA but containing T7 phage replication genes. The dashed arrow is to indicate that the chromosomally integrated features are/may be important for plasmid replication. (**B**) Confirmation of replication of a T7 *ori* plasmid (i.e. without cores pSC101 *ori*, e.g. pT7-OR) in testing environment. Panel on the very right: Stained agarose gel of a restriction digest of pT7-OR isolated from JM101T7c (*Eco*RI, expected band size: 1818 bp). (**C**) Transformation efficiencies for reference plasmid pCKO1 (containing a complete pSC101 *ori*, set to 100%), T7 *ori* test plasmid (pTestOR), and T7 *ori* plasmid (pT7-OR) in standard and cloning and testing environment strains. CFUs for all plasmids were determined from at least three separate experiments and normalized to the number of CFUs observed with pCKO1. (**D**) PCN determination by ddPCR for T7 *ori* plasmid pT7_ori_1 (described in main text) and pCKO1 in JM101T7c. The PCN was calculated as the ratio of plasmid target concentration (*cat*, grey dot) and chromosomal target concentration (*uidA*, blue dot). Dots/diamonds and bars indicate replicate and average values, respectively.

we concluded that the T7 *ori* plasmid can be replicated in an *E. coli* strain and this process likely depended on some or all of the integrated T7 genes. To the best of our knowledge, this is the first report of a plasmid whose replication depends on a T7 *ori* and can replicate in *E. coli* without phage infection.

Characterization of T7 *ori* plasmids

In order to identify which cis-elements of T7 *ori* plasmids were important for successful replication, we assembled a range of variants of plasmid pTestOR in the cloning strain JM101repA and tested their replication in JM101T7c (Supplementary Figure S1). First, we removed the T7 *ori* from

pTestOR and found that while we could obtain JM101repA transformants, we could not isolate transformants from JM101T7c, confirming that replication is indeed dependent on the T7 ori. We also found that replication in JM101T7c was dependent on the T7 promoter and the sequences that surround the T7 *ori*. However, functional replication was independent from orientation of the antibiotic resistance gene on the plasmid, excluding the possibility that the corresponding constitutive promoter somehow played a role.

The function of the T7 ori plasmid pT7-OR was further characterized and optimized in view of its stability and copy number, as well as the growth behaviour of a strain carrying it. We repeatedly observed 'loss' of pT7-OR (and also pTestOR) with retention of antibiotic resistance, presumably due to recombination of the plasmid into the genome of the testing strain. As such, we removed two regions of pT7-OR that could allow for homologous recombination into the endogenous genome of *E. coli* (46). There were two regions of 48 bp and 162 bp sequences that were homologous to *lacI* and *lacZ* genes and their removal led to plasmid pT7-OR_s ('smaller'). However, although it was more stable than pT7-OR, we noticed that even pT7-OR_s tended to be lost after 40 to 50 generations (Supplementary Figure S2). As such, we cultivated JM101T7c [pT7-OR_s] for more than 50 generations and isolated a mutated version of pT7-OR_s. This new plasmid exhibited two mutations relative to pT7-OR_s (change of G_{167}GTCCTTAAG to G_{167}-TCCT—G and a C to G exchange at position 429) and allowed proliferation of the T7 *ori* plasmid without any addition of IPTG. The doubling time of 39±4 min, was reduced 10% relative to cultivation with pT7-OR_s (Supplementary Figure S3). The plasmid could be repeatedly propagated, without acquiring any further mutations, in JM101T7c for more than 80 generations in rich medium with antibiotic selection. As this was the first T7 *ori* plasmid consistently and stably replicated in *E. coli*, we termed it pT7_ori_1 and used it for subsequent studies.

Next, we set up a digital droplet PCR (ddPCR) protocol to determine the plasmid copy number (PCN) for pT7_ori_1 and pCKO1 (pSC101 *ori*) in JM101T7c overnight cultures under standard cultivation conditions (Figure 1D). We found that pT7_ori_1 had a PCN of around two, comparable to the PCN observed for pCKO1. These results were in line with our densitometric analysis of purified plasmid DNA of pT7_ori_1 and pCKO1 (data not shown) and comparable to a previous study of plasmid using the complete pSC101 *ori* at approx. 3.4 using ddPCR as an analysis method (47).

The importance of T7-derived functions for replication of T7 *ori* plasmids in JM101T7c

After generating pT7_ori_1, a stably replicating plasmid dependent only on a T7 *ori*, we investigated which elements were essential in *trans* for replication. Specifically, we wanted to examine whether all four T7 genes that had been integrated into the chromosome of JM101 were required. We sequenced the T7 genes on the chromosome of JM101T7c and found that all of the T7 genes had acquired spontaneous mutations. The gene for T7 RNAP carried a mutation leading to the amino acid exchange Q737P in the enzyme domain close to the promoter recognition loop (2). The T7 DNAP exhibited the mutations R219S in the DNA synthesis domain and F334L at the end of the region interacting with thioredoxin. The gene for ssDNAbp was disrupted by a stop codon at position 111, leaving more than half of the codons untranslated. Finally, HelPrim carried the mutation R487S located within the helicase domain. In summary, ssDNAbp likely lost its function, and all the other genes acquired mutations in regions that encode functionally important regions of the gene products, raising the possibility that the gene products were changed in their activity or no longer functional. To determine when these mutations arose, we sequenced the parental strain, JM101T7m, and found that it carried the identical T7 gene mutations as JM101T7c, suggesting that even in the absence of a T7 *ori* plasmid, the T7 genes may create a burden for *E. coli*.

To clarify the functional importance of the mutated versions of the T7-derived genes, we constructed single knock-out derivatives (Figure 2A). For each test, we first ensured that the strains, JM101T7c and each of the single knock-out strains, was transformation-competent by transforming pCKO1. We then transformed JM101T7c and each of the single knock-out strains with pT7_ori_1, examined the number of CFUs after overnight growth, and, where appropriate, purified the plasmid to confirm successful replication. Interestingly, the only strain that did not allow replication of pT7_ori_1 was JM101T7c*Δrnap*. Replication of pT7_ori_1 in JM101T7c*Δrnap* was restored when the gene for T7 RNAP-Q737P was expressed from a support plasmid, pT7RNAP-Q737P (with repA and a pSC101*ori*) under the control of P*tac* (the same promoter that drives expression from the chromosomal copy of T7 RNAP-Q737P in JM101T7c), but not when the support plasmid contained wild-type T7 RNAP. This suggests that the only essential T7 function that is required for propagation of a T7 *ori* plasmid in JM101T7c is T7 RNAP-Q737P and, consequently, that the *E. coli* replication machinery can replace the functions of T7 DNAP, HelPrim and ssDNAbp during T7 *ori* plasmid replication in JM101T7c.

We next examined the importance of the Q737P mutation in T7 RNAP. First, we restored the chromosomal copy of the mutated gene to the wild-type sequence, resulting in the strain JM101T7c_RNAPwt. Following the approach outlined previously, we could not obtain colonies after transformation of this strain with pT7_ori_1, suggesting that the mutation was indeed crucial for the successful replication of pT7_ori_1.

Next, in order to explore possible effects of the Q737P substitution, we constructed plasmid pEGC that contains the gene for green fluorescent protein (GFP) under the control of a T7/lacO fusion promoter (Figure 2B). The T7 promoter part is identical to the T7 *ori* promoter used in pT7_ori_1. For clarity JM101T7c will be referred to as JM101T7c_RNAP-Q737P when discussing the effects of the RNAP mutations and the strain variant in which the mutated gene of the T7 RNAP had been exchanged for the wildtype gene as JM101T7c_RNAPwt. JM101T7c_RNAPwt and JM101T7c_RNAP-Q737P were transformed with pEGC and GFP fluorescence was measured (Figure 2B). GFP fluorescence was lower in JM101T7c_RNAP-Q737P than for JM101T7c_RNAPwt.

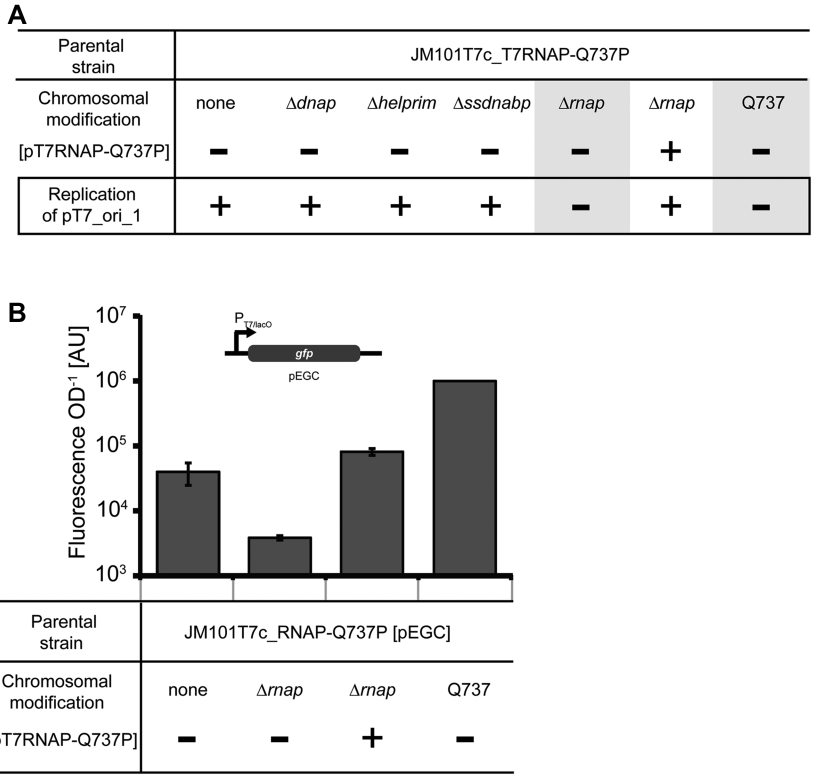

Figure 2. Importance of T7-derived genes in JM101T7c for replication of T7 ori plasmids. (**A**) Essentiality of phage-derived genes for replication of T7 ori plasmids. Transformation of JM101T7c and its deletion variants with the T7 ori plasmid pT7_ori_1 was attempted into a series of knock-out strains based on JM101T7c. In one case, the recipient strain contained the support plasmid pT7 RNAP-Q737P from which a variant T7 RNAP (penultimate entry). In another case (final entry), the mutated gene for the T7 RNAP in JM101T7c has been replaced with the wild-type gene (T7 RNAP Q737). Successful transformation and plasmid recovery is indicated by '+' in the last line. (**B**) Effect of the Q737P mutation on expression from T7 RNAP. Set-up and results of functional analysis of T7 RNAP Q737P and T7 RNAP. Fluorescence values were normalized to optical density.

We also reconstituted the Q737P mutation on the RNAP support plasmid pT7RNAP-Q737P in a JM101T7c *Δrnap* strain and observed lower GFP fluorescence than strains carrying the gene for the wild-type T7 RNAP (Figure 2B). The decreased fluorescence indicates that T7 RNAP-Q737P is produced at a lower level, has altered or reduced activity, or has a decreased half-life relative to wild-type T7 RNAP. Overall, this highlights a correlation between a change in RNA polymerase activity and the ability to successfully replicate a T7 *ori* plasmid, but does imply a mechanistic relationship.

Identifying further essential T7 functions for the replication of T7 *ori* plasmids

Unexpectedly, replication of T7 *ori* plasmids in *E. coli* seemed to require only one T7-derived function. This put into question our original assumptions, based on reported *in vitro* experiments (25,26,48), of the requirements for T7 *ori*-based replication *in vivo*. Additionally, we wanted to determine the reason for the increase in transformation efficiency of JM101T7c compared to its parental strain JM101T7m when transformed with T7 *ori* plasmids. To this end, we sequenced the genomes of JM101T7c and JM101T7m in order to determine whether relevant cellular functions had changed in JM101T7c (Supplementary Figure S4). Whole genome analysis revealed 14 point mutations relative to JM101T7m and that no additional mutations were present in the T7 genes (for a complete list of mutations, see Supplementary Table S6). To identify which mutation(s) led to replication of T7 *ori* plasmids, two new T7-*ori*-supporting strains (JM101T7c2, JM101T7c3) were independently generated from JM101T7m. These were created by transforming JM101T7m with a T7 *ori* plasmid and then curing the plasmid from successful transformants, in a similar fashion as the creation of JM101T7c. Interestingly, obtaining the whole genome sequence of the two new strains and subsequent comparison of the sequence of all four strains revealed the gene *rnhA* as the single common mutated host gene: two strains showed single point mutations leading to amino acid residue exchanges (G38D in strain JM101T7c2 and Q72P in strain JM101T7c3), and one strain contained a large insertion element that interrupted the gene (JM101T7c), while the gene was without mutation in JM101T7m. We concluded that the different mutations most likely led to a reduction or a complete loss in the activity of the protein encoded by *rnhA*, ribonuclease H (RNase HI).

To verify the role of *rnhA* in the replication of T7 *ori* plasmids, we transformed the strain BW25113 and BW25113Δ*rnhA* (Keio collection, (49,50)) with pT7_ori_1 and the RNAP support plasmid, pT7RNAP-Q737P (Figure 3A). Consistent with the previous observations, efficient transformation of pT7_ori_1 was only achieved in the

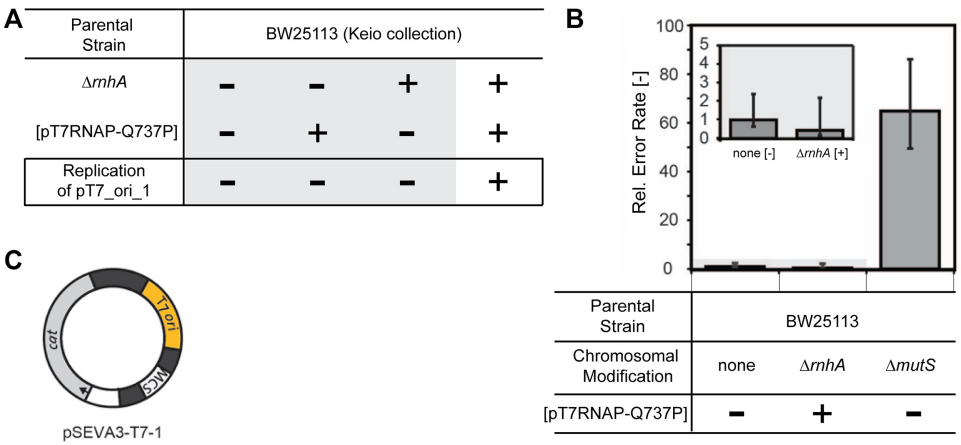

Figure 3. Portability of the T7 ori system. (**A**) Function of the T7 ori system in E. coli BW25113 strain background. Transformation of BW25113 and BW25115ΔrnhA in the presence (+) or absence (-) of pT7RNAP-Q737P with the T7 ori plasmid, pT7_ori_1. (**B**) Comparison of the chromosomal error rate of Keio collection BW25113 wild-type, ΔrnhA [pT7RNAP-Q737P] and ΔmutS. Inset: comparison of the error rates of strains BW25113 and BW25113ΔrnhA [pT7RNAP-Q737P]. The error rates were measured in a fluctuation assay, error bars represent 95% confidence intervals based on at least 12 parallel samples. The calculated error rate of the WT strain was set to 1. (**C**) Transfer of T7 ΦOR ori to a Standard European Vector Architecture (SEVA)-format plasmid, resulting in pSEVA3-T71.

Δ*rnhA* variant of BW25113 and only in the presence of the T7 RNAP Q737P. The successful re-engineering of all elements that are required for replication of a T7 *ori* plasmid in strain BW25113 also confirms that our findings are not specific for a given *E. coli* strain and we have correctly identified all essential functional elements.

Strains that are deficient in *rnhA* display an increased SOS response, which in turn is associated with higher mutation rate (51,52). Since an increased mutation rate in an Δ*rnhA* strain would be undesirable for many synthetic biology or metabolic engineering efforts, we decided to quantify the effect of the *rnhA* deletion on the spontaneous chromosomal mutation rate of BW25113 and set up fluctuation assays for the parental strain BW25113, strain BW25113Δ*rnhA* [pT7RNAP-Q737P], and strain BW25113Δ*mutS* as a control (Figure 3B). The deletion of *mutS* leads to the inactivation of the methyl-directed mismatch repair (MMR) system and increases the error rate significantly (53). No significant elevation of the chromosomal error rate was observed in BW25113Δ*rnhA* [pT7RNAP-Q737P] when compared to BW25113, unlike the chromosomal error rate for BW25113 Δ*mutS* which was increased 65-fold, comparable with previous experiments (53).

Finally, we transferred the T7 *ori* as a new type of origin of replication into the pSEVA standard plasmid scaffold, generating pSEVA3-T7-1 (Figure 3C), which was successfully isolated from JM101T7c. The Standard European Vector Architecture (SEVA) applies to a repository of vectors aimed at facilitating the optimal choice of bioengineering tools by mix-and-match combinations of functional DNA segments (54).

Copy number control of T7 *ori* plasmids with T7 RNAP

Our results demonstrated the involvement of the T7 RNAP in the replication of the T7 *ori* plasmid. Hence, we hypothesized that an increase in T7 RNAP expression might influence the copy number of the T7 *ori* plasmid as long as we do not reach again activity levels that prevent the system from operation (see above). The expression level of T7 RNAP can be altered because the gene encoding the T7 RNAP is under the control of the IPTG-inducible P*tac* promoter. To test this hypothesis, we determined the PCN of the T7 *ori* test plasmid pTestOR in JM101repA and JM101T7c cultures that had been supplemented with different IPTG concentrations (Figure 4A). We selected this plasmid as it presented the possibility to use the core pSC101 *ori* as a comparison. The ddPCR results show that higher copy numbers are indeed found with higher IPTG concentration. Compared to the uninduced case, approx. 3 to 4 times as much plasmid was detected in the presence of 0.1 mM to 0.2 mM IPTG. In contrast, a constant PCN was observed in the JM101repA cloning environment strain in which no T7 RNAP was present and replication starts at the core pSC101 *ori*. A similar effect was observed for pSEVA3-T7-1 in JM101T7c, in which the PCN increased from 1.8 (0 mM IPTG) to 4.9 (0.1 mM IPTG) (Figure 4B), suggesting that T7 *ori* plasmids can be dynamically controlled.

DISCUSSION

T7 RNAP is a widely used workhorse in biotechnology and fundamental biology *in vivo* (22) and *in vitro* (55). Here, we add stable plasmid replication to the list of applications. We introduce a novel replicon that is compatible at least with pSC101-type replicons. In this general context, it is interesting to note that a panel of highly selective orthogonal T7 RNAPs with orthogonal promoter counterparts have been previously described (4). It is likely that these polymerase/promoter pairs—possibly after introducing a mutation akin to RNAP Q737P—would allow for the construction of a small series of T7 *ori* plasmids, all of which should be compatible.

It is important to note that we have not clarified whether reconstitution of a minimal T7 DNA replication system, as

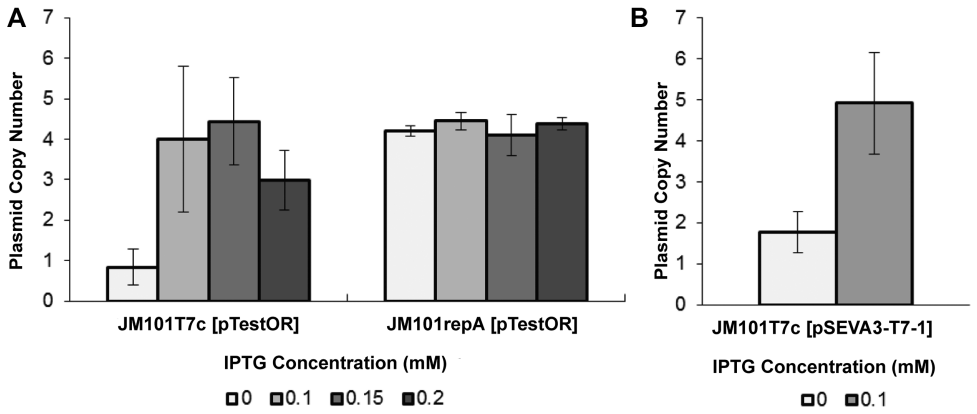

Figure 4. Copy number control of the T7 *ori* plasmid by varying induction of expression of the gene for T7 RNAP. PCN of (**A**) T7 *ori* plasmid pTestOR and (**B**) pSEVA3-T7-1 in JM101T7c as a function of inducer concentration. The measured PCN of pTestOR in JM101repA (using the pSC101 *ori*) is given as a reference.

suggested from *in vitro* studies, is possible *in vivo* in *E. coli* or not, as the T7-derived functions in the strain in which we observed T7 *ori* plasmid replication had mutated. In general, mutation of T7 functions in *E. coli* seem to occur readily, as evidenced by the current work, which points to a requirement to carefully manage onset of expression and expression levels (56). If this could be achieved more fully, successful reconstitution of such a system involving additional T7 functions and not requiring a mutation in *rnhA* cannot be excluded.

Still, we found that replication proceeded through a mechanism independent of these T7-derived functions, but dependent on the inactivation of *E. coli* ribonuclease H. *E. coli* cells deficient in the activity of this enzyme exhibit a mechanism called constitutive stable DNA replication (cSDR), which is defined as the ability to continue chromosomal replication even after a cessation of protein synthesis (57,58). Initiation of cSDR takes place at so-called *oriK*s that are not yet well characterized (59,60) and their definitive locations and their impact on genome stability remains elusive (60). Methods for the identification of *oriKs* often rely on copy number distributions over the chromosome which only provide information about approximate regions and are prone to false positives, for instance in the termination region (59). Replication via cSDR is semiconservative and bidirectional (57). During a transcription step, RNAP generates RNA for the formation of an R-loop. In a strain with a functional ribonuclease H, this R-loop would be recognized by the enzyme and removed, thus preventing further steps. In a strain defective in *rnhA*, the R-loops persist and the 3′ end of the RNA can serve as primer for *E. coli*'s DNAP I, and replication continues along standard procedures using ultimately DNAP III, the main replicative DNAP in *E. coli*. The mechanism of cSDR is very similar to the mechanism of replication start and cSDR has been discussed as an option for priming replication restart even in wild-type *E. coli* (61,62). It is, therefore, tempting to speculate that a very similar mechanism allows for replication of T7 *ori* plasmids, with the T7 RNAP acting to produce an R-loop. Clearly, T7 *ori* plasmids could also support further investigations into cSDR, for example by serving to clone *oriK*s whose exact location and sequences remain unclear so far (59).

Overall, our results add a new facet—plasmid replication—to the broadly used and versatile T7 RNAP. Applications include gene expression, which benefits here from two effects with one inducer: increasing gene dosage as well as increasing transcription of a recombinant gene. As the T7 RNAP has proven to be valuable in many fields (63) the T7 *ori* plasmid suggests many potential applications ranging from basic science to synthetic biology. We expect that this replicon will be put to broad use.

DATA AVAILABILITY

NGS data is provided at the Sequence Read Archive under Project PRJNA735647. Plasmid sequences and annotations can be found at GenBank, accessions numbers MZ395120-MZ395127.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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