LETTER

doi:10.1038/nature09929

A system for the continuous directed evolution of biomolecules

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how to select

Laboratory evolution has generated many biomolecules with desired properties, but a single round of mutation, gene expression, screening or selection, and replication typically requires days or longer with frequent human intervention[1]. Because evolutionary success is dependent on the total number of rounds performed[2], a means of performing laboratory evolution continuously and rapidly could dramatically enhance its effectiveness[3]. Although researchers have accelerated individual steps in the evolutionary cycle[4–9], the only previous example of continuous directed evolution was the landmark study of Wright and Joyce[10], who continuously evolved RNA ligase ribozymes with an *in vitro* replication cycle that unfortunately cannot be easily adapted to other biomolecules. Here we describe a system that enables the continuous directed evolution of gene-encoded molecules that can be linked to protein production in *Escherichia coli*. During phage-assisted continuous evolution (PACE), evolving genes are transferred from host cell to host cell through a modified bacteriophage life cycle in a manner that is dependent on the activity of interest. Dozens of rounds of evolution can occur in a single day of PACE without human intervention. Using PACE, we evolved T7 RNA polymerase (RNAP) variants that recognize a distinct promoter, initiate transcripts with ATP instead of GTP, and initiate transcripts with CTP. In one example, PACE executed 200 rounds of protein evolution over the course of 8 days. Starting from undetectable activity levels in two of these cases, enzymes with each of the three target activities emerged in less than 1 week of PACE. In all three cases, PACE-evolved polymerase activities exceeded or were comparable to that of the wild-type T7 RNAP on its wild-type promoter, representing improvements of up to several hundred-fold. By greatly accelerating laboratory evolution, PACE may provide solutions to otherwise intractable directed evolution problems and address novel questions about molecular evolution.

We devised a system that exploits the continuous culture and selection of the M13 filamentous bacteriophage[11] (commonly used in phage display[12]) to enable the continuous directed evolution of proteins or nucleic acids. In PACE, *E. coli* host cells continuously flow through a fixed-volume vessel (the 'lagoon') containing a replicating population of phage DNA vectors ('selection phage') encoding the gene(s) of interest (Supplementary Fig. 1).

The average residence time of host cells in the lagoon is less than the time required for *E. coli* replication. As a result, mutations accumulate only in the evolving selection phage population, the only DNA that can replicate faster than the rate of lagoon dilution. The mutation of host cells in the lagoon should therefore have minimal impact on the outcome of the selection over many rounds of phage replication, and mutagenesis conditions are not limited to those that preserve *E. coli* viability.

PACE achieves continuous selection by linking the desired activity to the production of infectious progeny phage containing the evolving gene(s). Phage infection requires protein III (pIII; encoded by gene III), which mediates F pilus binding and host cell entry[13]. Phage lacking pIII are approximately 10^8-fold less infectious than wild-type phage[14]. Crucially, the production of infectious phage scales with increasing levels of pIII over concentrations spanning two orders of magnitude[15].

To couple pIII production to the activity of interest, we deleted gene III from the phage vector and inserted it into an 'accessory plasmid' present in the *E. coli* host cells (see Supplementary Fig. 2 for plasmid maps). The production of pIII from the accessory plasmid is dependent on the activity of the evolving gene(s) on the selection phage. Only phage vectors able to induce sufficient pIII production from the accessory plasmid will propagate and persist in the lagoon (Fig. 1). Because pIII expression level determines the rate of infectious phage production[15], phage encoding genes that result in a higher level of pIII production will infect more host cells than phage encoding less active genes.

Owing to the speed of the phage life cycle (progeny phage production begins approximately 10 min after infection)[16], PACE can mediate many generations of selective phage replication in a single day. We observed activity-dependent phage vectors that tolerate lagoon flow rates up to 3.2 volumes per hour (Supplementary Fig. 3), corresponding to an average of 38 phage generations per 24 h (see the Supplementary Information for an analysis). More conservative flow rates of 2.0–2.5 volumes per hour allow 24–30 generations per day and reduce the risk of complete phage loss (washout) during selections. Multiple lagoons can evolve genes in parallel, with each 100 ml lagoon containing approximately 5×10^{10} host cells selectively replicating active phage variants. Importantly, PACE requires no intervention during evolution and obviates the need to create DNA libraries, transform cells, extract genes or perform DNA cloning steps during each round.

In principle, PACE is capable of evolving any gene that can be linked to pIII production in *E. coli*. Because a wide variety of functions including DNA binding, RNA binding, protein binding, bond-forming catalysis and a variety of enzyme activities have been linked to the expression of a reporter protein[17,18], PACE can be applied to the evolution of many different activities of interest. As examples, we successfully linked protein–protein binding, recombinase activity and RNAP activity to phage infectivity in discrete infection assays by creating variants of the accessory plasmid that associate each of these activities with pIII production (Fig. 2).

PACE applies optimal evolutionary pressure when pIII levels are above the minimal threshold required to prevent phage washout, but below the amount needed to maximize infectious phage production. This window can be shifted by varying the copy number of the accessory plasmid, or by altering the ribosome-binding site (RBS) sequence of gene III to modulate the efficiency with which gene III is expressed (Supplementary Fig. 4).

We constructed an arabinose-inducible mutagenesis plasmid that elevates the error rate during DNA replication in the lagoon by suppressing proofreading[19] and enhancing error-prone lesion bypass (Supplementary Information)[20]. Full induction increased the observed mutagenesis rate by approximately 100-fold, inducing all possible transitions and transversions (Supplementary Fig. 5). This enhanced

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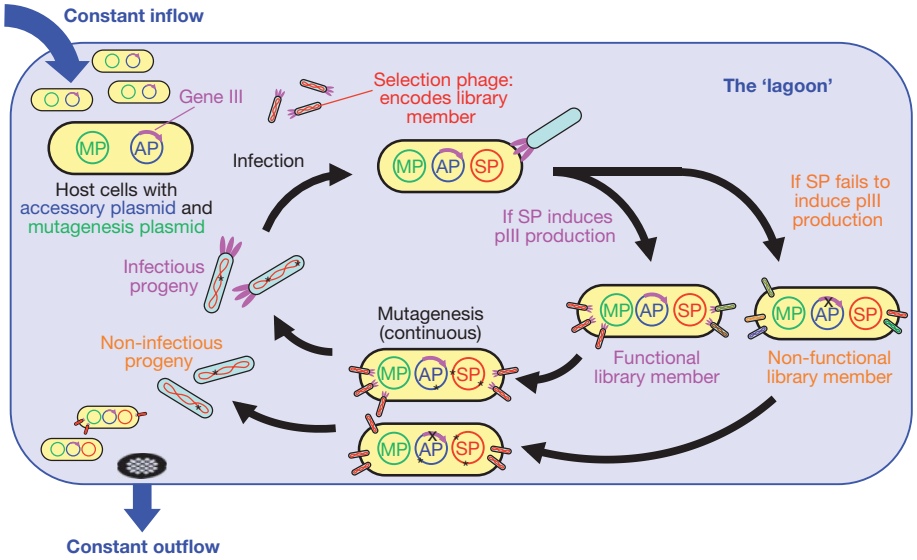

Figure 1 | Overview of the PACE system. PACE in a single lagoon. Host cells continuously flow through a lagoon, where they are infected with selection phage (SP) encoding library members. Functional library members induce production of pIII from the accessory plasmid (AP) and release progeny capable of infecting new host cells, whereas non-functional library members do not. Increased mutagenesis is triggered through induction of the mutagenesis plasmid (MP). Host cells flow out of the lagoon on average faster than they can replicate, confining the accumulation of mutations to replicating phage.

mutation rate is sufficient to sample all possible single and double mutants of a given phage-encoded sequence in each generation (Supplementary Information), in principle enabling single-mutation fitness valleys to be traversed during PACE.

Bacteriophage T7 RNAP is widely used to transcribe RNA *in vitro* and in cells. T7 RNAP is highly specific for its promoter sequence (TAATACGACTCACTATA), and exhibits virtually no detectable activity on the consensus promoter of the related bacteriophage T3 (AATTAACCCTCACTAAA, differences underlined)[21,22]. Despite decades of study and several attempts to engineer the specificity of T7 RNAP towards other promoters[22,23] including that of T3, a mutant T7 RNAP capable of recognizing the T3 promoter has not been previously reported.

To remove potential interference from evolutionary improvements to the phage vector rather than to T7 RNAP, we propagated a selection phage expressing wild-type T7 RNAP for 3 days on host cells containing an accessory plasmid with the wild-type T7 promoter driving gIII expression. A single plaque presumed to represent vector-optimized

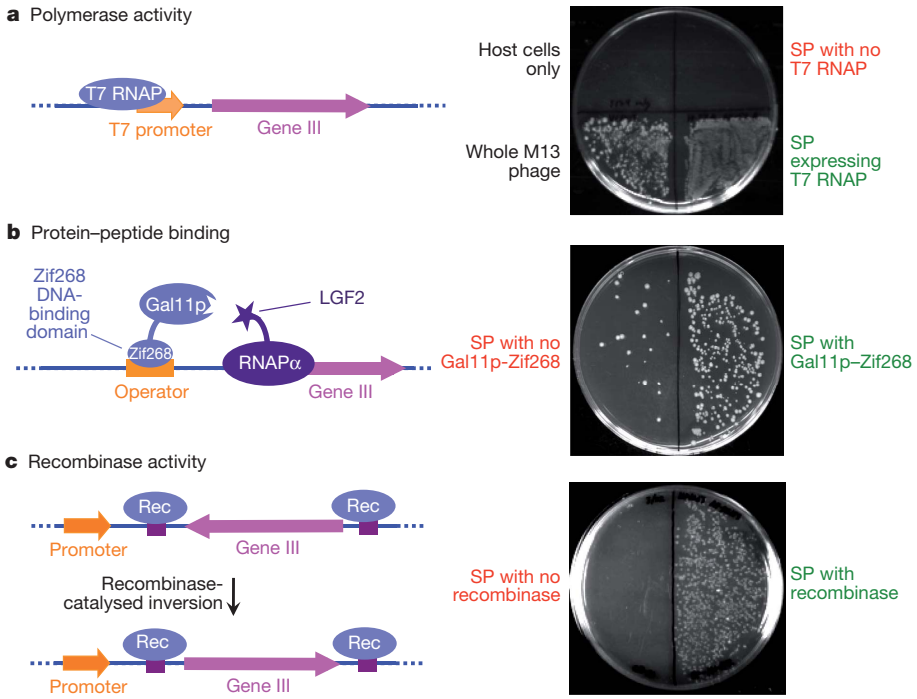

Figure 2 | Linkage of three protein activities to pIII production and phage infectivity using three distinct accessory plasmids. *E. coli* cells containing accessory plasmids encoding conditionally expressed gene III (left) and selection phage were combined with recipient cells. Phage production resulted in colonies with antibiotic resistances conferred by the phage and the recipient cells (right). See Methods for details. **a**, RNAP activity leads to gene III expression and infection comparable to wild-type phage, whereas selection phage lacking T7 RNAP do not infect. **b**, Protein–protein interaction between a Gal11p domain tethered to a Zif268 DNA-binding domain and an LGF2a domain fused to RNAP leads to gene III expression and infection. **c**, Recombinase-catalysed gene inversion induces gene III expression and infection.

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selection phage contained a single mutation (P314T) in T7 RNAP. We confirmed that the activity of the P314T mutant does not significantly differ from that of wild-type T7 RNAP (Supplementary Fig. 6).

This starting selection phage failed to propagate on host cells containing the T3 promoter accessory plasmid. We therefore propagated the phage on cells containing a hybrid T7/T3 promoter accessory plasmid with the T7 promoter base at the important −11 position[21] but all other positions changed to their T3 counterparts. Two initially identical lagoons were evolved in parallel on the hybrid promoter accessory plasmid for 60 h, then on the complete T3 promoter accessory plasmid for 48 h, and finally on a high-stringency, very low-copy T3 promoter accessory plasmid for 84 h (Fig. 3a).

Phage persisted in both lagoons after 8 days of PACE, surviving a net dilution of 10^{167}-fold, the equivalent of 555 phage population doublings and 200 rounds of evolution by the average phage. We isolated, sequenced and characterized phage vectors from each lagoon after 48, 108 and 192 h, observing up to 8, 10 and 11 non-silent mutations in single T7 RNAP genes at each time point.

Protein-encoding regions (without upstream promoter sequences) of evolved mutant T7 RNAP genes were subcloned into assay plasmids that quantitatively link transcriptional activity to β-galactosidase expression in cells[24]. We defined the activity of wild-type T7 RNAP on the T7 promoter to be 100%. The starting T7 RNAP exhibited undetectable (<3%) levels of activity on the T3 promoter in these cell-based assays. The assayed mutants exhibited more than 200% activity after 108 h of PACE, and more than 600% activity after high-stringency PACE at 192 h, improvements of more than 200-fold (Fig. 3b, c). These results collectively establish the ability of PACE to evolve large changes in enzyme activity and specificity very rapidly with minimal intervention by the researcher.

Several evolved T7 RNAP mutants were also purified and assayed *in vitro* using radioactive nucleotide incorporation assays. Purified T7 RNAP mutants exhibited activity levels on the T3 promoter *in vitro* exceeding that of wild-type T7 RNAP on the T7 promoter, representing improvements of up to 89-fold compared with the starting enzyme (Supplementary Fig. 7). These results indicate that PACE resulted in large improvements in substrate binding or catalytic rate. Evolved activity improvements were higher in cells than *in vitro*, suggesting that these enzymes also evolved improvements in features such as expression level, polymerase folding or stability that are specific to the context of the cytoplasm.

Interestingly, the evolutionary dynamics of the two initially identical lagoons differed significantly (Fig. 3d and Supplementary Results). Within 24 h, lagoon 1 acquired a predominant suite of mutations

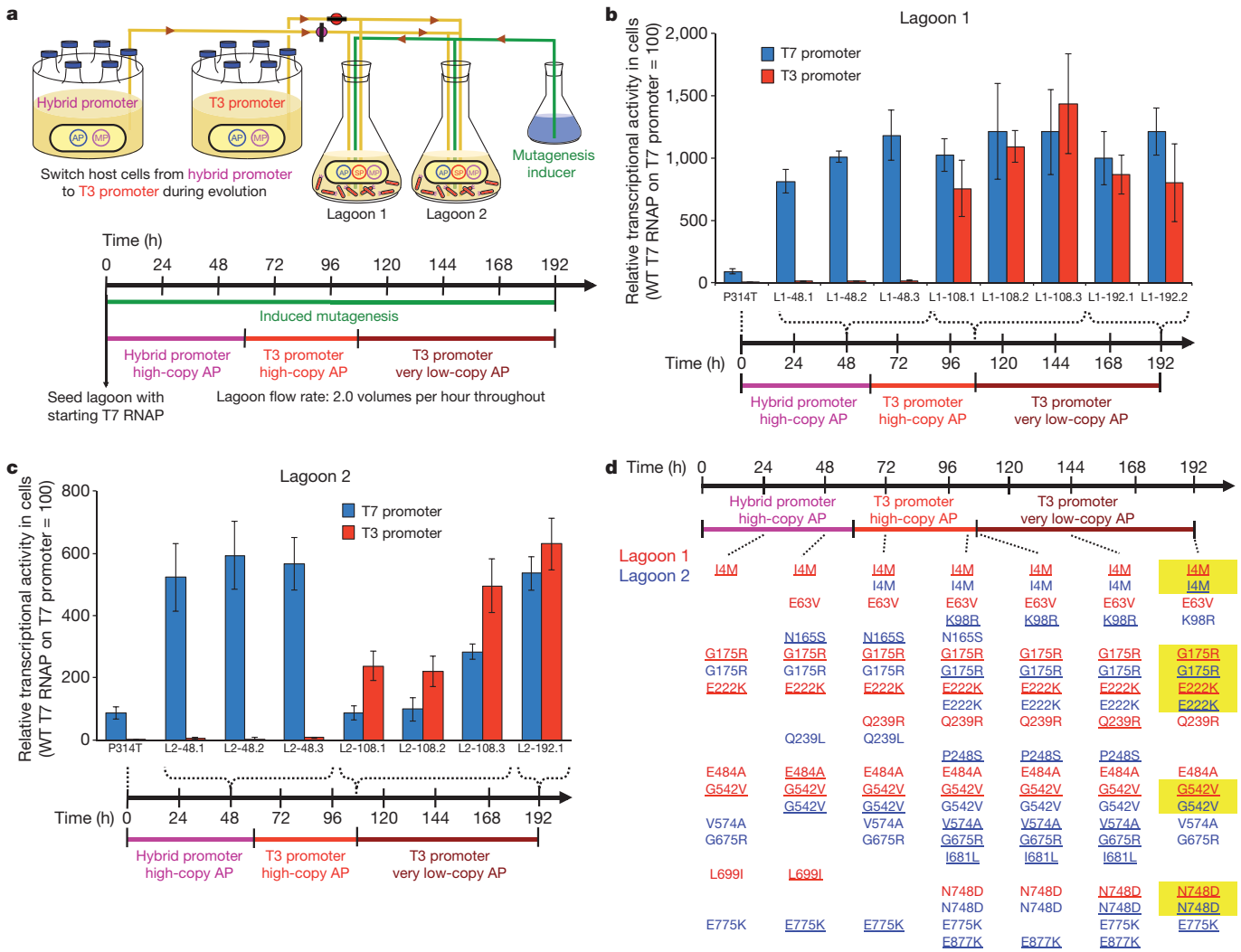

Figure 3 | Continuous evolution of T7 RNAP variants that recognize the T3 promoter. **a**, PACE schedule. **b**, Activity in cells of T7 RNAP variants isolated from lagoon 1 at 48, 108 and 192 h on the T7 and T3 promoters. Transcriptional activity was measured spectrophotometrically by subcloning the protein-encoding regions of the T7 RNAP genes into a construct in which the T7 or T3 promoter drives *lacZ* expression. **c**, Activity in cells of T7 RNAP variants isolated from lagoon 2. Error bars in **b** and **c**, standard deviation of at least three independent assays. **d**, Mutations identified in T7 RNAP clones from lagoons 1 and 2 are shown in red and blue, respectively. Underlined mutations were predominant in that lagoon based on whole-pool sequencing of lagoon aliquots. Mutations highlighted in yellow independently evolved to predominance in both lagoons.

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consisting of I4M, G175R, E222K and G542V and changed little thereafter beyond acquiring N748D, a mutation known to enable recognition of the T3 base at the −11 position[21], after exposure to the full T3 promoter. In contrast, lagoon 2 accessed these mutations more slowly before a different suite of mutations also including N748D became predominant at 108 h, only to be displaced by the same suite of mutations observed in lagoon 1. The presence of several mutations unique to lagoon 2 throughout the experiment suggests that lagoon cross-contamination did not occur. The distinct evolutionary trajectories of the two lagoons before their ultimate convergence upon a common set of mutations highlight the ability of PACE to discover multiple viable pathways to a target activity in parallel experiments. This capability may enable a more in-depth experimental study of protein evolutionary dynamics than can be achieved with conventional directed evolution methods that cannot complete so many rounds of evolution on a practical timescale.

T7 RNAP is highly specific for initiation with GTP[25,26], significantly limiting its usefulness for the *in vitro* transcription of RNAs that begin with other nucleotides. As initiation has been described as a mechanistically challenging step in transcription[27], we next used PACE to evolve T7 RNAP variants capable of initiating transcription with other nucleotides in a template-directed manner. T7 RNAP is known to initiate preferentially with GTP up to several bases downstream of the +1 position if the template is devoid of early guanines in the coding strand[26]. We therefore constructed accessory plasmids in which positions +1 to +6 of the gene III transcript were AAAAAA (iA_6) or CCCCCC (iC_6).

We used PACE to rapidly evolve variants of T7 RNAP capable of initiating with ATP. In view of previous reports indicating varying degrees of initiation of T7 RNAP with ATP[25,26], we propagated starting phage in host cells with a high-copy iA_6 accessory plasmid for 24 h, followed by a 30:70 high-copy:very-low-copy mixture of host cells for 12 h (Fig. 4a). At a dilution rate of 2.5 volumes per hour, phage survived a total dilution of 10^{39}-fold and experienced an average of approximately 45 rounds of evolution.

The wild-type enzyme exhibited undetectable initial activity in cells (<3%) on the iA_6 promoter. All six clones isolated after only 36 h of PACE exhibited at least 170% activity on the iA_6 promoter in cell-based assays, while retaining at least 120% activity on the wild-type promoter (Fig. 4b). Purified variants assayed *in vitro* exhibited activities on the iA_6 promoter matching that of wild-type T7 RNAP on the T7 promoter (Supplementary Fig. 8). RACE analysis of transcripts produced by the most active clone (A6-36.4) confirmed that this enzyme begins transcripts with the template-directed bases on the iA_6, iC_6 and wild-type promoters (Supplementary Fig. 9a). All six characterized clones contained K93T, S397R and S684Y mutations, whereas three of the six also contained S228A (Supplementary Table 2). Residue 397 directly contacts the nascent RNA strand[28], suggesting a role for S397R in allowing efficient initiation of iA_6 transcripts.

We concurrently evolved T7 RNAP to initiate transcripts with CTP (Supplementary Fig. 10a). We observed that wild-type T7 RNAP retains significant activity on the iC_6 promoter (~50%) both in cells and *in vitro* (Supplementary Fig. 10b, c), a surprising observation in view of reports that the enzyme initiates with G at the +2 position if the +1 position is C[25]. Although the high starting activity precluded large improvements, the most active PACE-evolved variants nevertheless exceeded 100% activity on the iC_6 promoter both in cells and *in vitro* (Supplementary Fig. 10b, c and Supplementary Results). RACE analysis of transcripts produced by the most active clone (C6-80.9) confirmed that this enzyme begins transcripts with the template-directed bases (Supplementary Fig. 9b).

The three PACE experiments executed 45–200 rounds of evolution in 1.5–8 days and yielded T7 RNAP variants with activities on their target promoters or templates that exceeded or matched the activity of the wild-type enzyme transcribing the wild-type T7 promoter both in cells and *in vitro*. This degree of improvement is especially significant given that, for two of the evolved activities, the starting polymerase exhibited virtually no detectable activity.

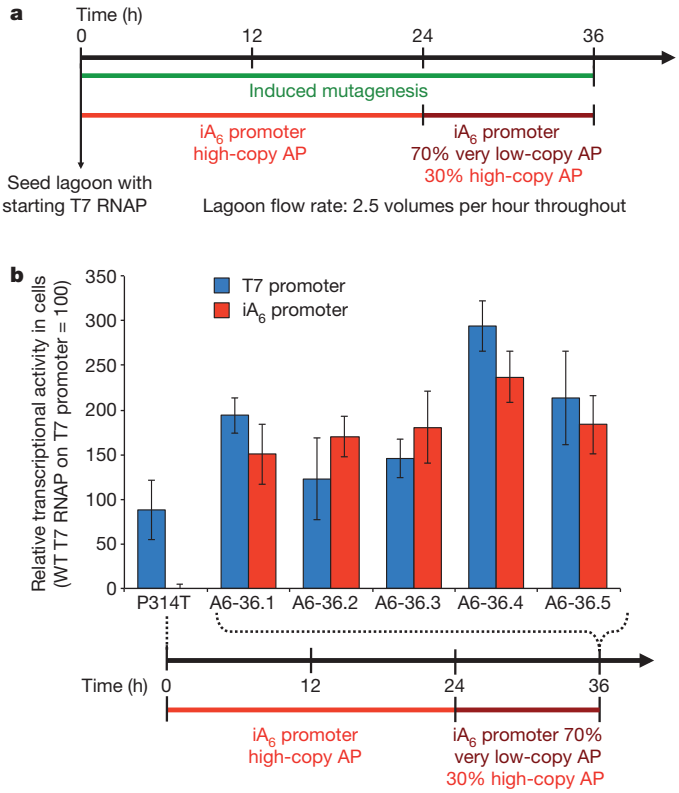

Figure 4 | Continuous evolution of T7 RNAP variants that initiate transcription with A. a, PACE schedule. **b**, Activity in cells of T7 RNAP variants on the T7 and iA_6 promoters isolated after 36 h of PACE. Assays were performed as described in Fig. 3b. Error bars, standard deviation of at least three independent assays.

The evolved A6-36.4 variant of T7 RNAP can initiate transcription from iC_6, iA_6 and wild-type templates in a template-directed manner with efficiencies comparable to that of wild-type T7 RNAP initiating with the wild-type template (Supplementary Fig. 11) and sequence fidelity sufficient to mediate the production of functional pIII and LacZ enzyme. These findings suggest that this enzyme, and possibly other PACE-evolved variants, may represent improved, more general T7 RNAPs for routine *in vitro* and *in vivo* transcription.

The PACE system can be assembled entirely from a modest collection of commercially available equipment (listed in Supplementary Table 3) and does not require the manufacture of any specialized components. The ability to perform dozens of rounds of evolution each day with minimal researcher involvement implies that PACE is particularly well suited to address problems or questions in molecular evolution that require hundreds to thousands of generations, or the execution of many evolution experiments in parallel. More generally, PACE represents the integration and manipulation of many protein and nucleic acid components in a living system to enable the rapid generation of biomolecules with new activities, a significant example and goal of synthetic biology.

METHODS SUMMARY

Discrete infection assays. Donor culture (2 μl) containing accessory plasmid and selection phage were mixed with 198 μl of F^+ recipient cells and incubated for 1.5 h at 37 °C. Dilutions were plated to select for recipient cells containing selection phage.

PACE. *E. coli* host cells with accessory plasmid and mutagenesis plasmid (see Methods) maintained at 5×10^8 cells ml^{-1} were pumped at 2.0, 2.5 or 3.2 volumes per hour into a fixed-volume lagoon seeded with selection phage. Aliquots were taken regularly. Evolved mutants were isolated as individual plaques and subcloned for cell-based or *in vitro* activity assays.

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Transcription assays. Activity in cells was measured using a standard β-galactosidase expression assay[24]. *In vitro* activity was assessed using a standard radioactive nucleotide incorporation assay[29] with His-tag-purified enzyme variants.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 10 November 2010; accepted 11 February 2011.
Published online 10 April 2011.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements This work was supported by National Institutes of Health/National Institute of General Medical Sciences R01 GM065400 and by HHMI. K.M.E. acknowledges graduate research fellowships from the Hertz Foundation and the National Science Foundation. J.C.C. was supported by the Harvard Chemical Biology Graduate Program. We thank B. Dorr for assistance with phage generation modelling, E. Curtis for suggestions and V. D'Souza for plasmid pT7-911Q.

Author Contributions K.M.E., J.C.C. and D.R.L. designed the experiments. K.M.E. designed and built the PACE apparatus. K.M.E. and J.C.C. performed the experiments. All authors analysed the data and wrote the manuscript.

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METHODS

General methods. All equipment, reagents, suppliers and relevant catalogue numbers are detailed in Supplementary Table 3. All PCR reactions were performed with HotStart Phusion II polymerase. Water was purified using a MilliQ water purification system (Millipore).

DNA cloning. All vectors were constructed by isothermal assembly cloning.[30] Isothermal assembly buffer (5×) contained 3 ml 1 M Tris-HCl pH 7.5, 300 µl 1 M $MgCl_2$, 600 µl 10 mM deoxynucleotide triphosphates, 300 µl 1 M dithiothreitol, 1.5 g PEG-8000, 20 mg NAD, and H_2O to 6 ml. Individual 320 µl aliquots were frozen at −20 °C. Isothermal assembly master mix was prepared by mixing 320 µl 5× buffer with 1 µl T5 exonuclease, 20 µl Phusion polymerase, 160 µl Taq DNA ligase, and H_2O to 700 µl. Individual 15 µl aliquots in PCR tubes were frozen at −20 °C. DNA fragments to be assembled were PCR-amplified using oligonucleotide primers designed to ensure between 30 and 40 base pairs (bp) of overlap homology with each adjacent fragment. DpnI was added directly to the PCR reactions to remove template DNA, followed by PCR cleanup with MinElute columns according to the manufacturer's protocol. Fragments were assembled by mixing equimolar amounts totalling 5 µl with 15 µl isothermal assembly master mix and incubating at 50 °C for 1 h. Assembly mixtures were directly transformed into NEB Turbo competent cells by heat shock or purified by MinElute columns as described before electroporation.

Plasmids. T7 RNAP-dependent accessory plasmids contained, in order, a strong *rrnB* terminator, the promoter of interest, a desired RBS, gene III, the *bla* gene conferring carbenicillin resistance, and either the pUC or SC101 origin of replication. For selection stringency assays, RBS A = 5′-AAGGAGGTAACTCATAG TG-3′, RBS B = 5′-AAGGAAATAACTCATAGTG-3′ and RBS C = 5′-AAG AAAATAACTCATAGTG-3′, where underlined bases represent the start codon of gene III. Reporter plasmids were identical to SC101 accessory plasmids except for the replacement of gene III by full-length *lacZ*. T7 RNAP selection phage was constructed by replacing all but the last 180 bp of gene III with the gene encoding T7 RNAP in VCSM13 helper phage. The mutagenesis plasmid consisted of dnaQ926, umuD′, umuC and recA730 under control of the araC operon. Expression plasmids used for quantification assays consisted of the cloD13 origin of replication, aadA and the wild-type gene III promoter and RBS driving expression of the evolved T7 RNAP variant. All plasmids used in this work are described in Supplementary Table 1. Vector maps of representative plasmids are shown in Supplementary Fig. 2.

Bacterial strains. All DNA cloning was performed with Mach1 cells or NEB Turbo cells. Early discrete infection assays and PACE experiments were performed with PirPlus DH10βF′DOT cells. Plaque assays and PACE experiments with T7 RNAP were performed with using *E. coli* S109 cells derived from DH10B by replacement of the proBA locus with the *pir116* allele, as previously described.[31] To our knowledge, this modification was not required for PACE experiments with T7 RNAP. Similarly, the *lacI* cassette was deleted from the F plasmid and from the chromosome to enable mutagenesis assays. S109 cells were rendered F^+ by conjugation with ER2738. The complete genotype of the resulting strain is F′proA+B+ Δ(lacIZY) zzf::Tn10(TetR)/ endA1 recA1 galE15 galK16 nupGrpsLΔlacIZYA araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) proBA::pir116 λ^-.

Discrete infection assays. Discrete (non-continuous) infectivity assays were performed by co-transforming phage bearing antibiotic resistance genes with the appropriate accessory plasmid into competent cells to generate phage donors. For two-hybrid and recombinase experiments, phage-producing cells contained gIII-deleted helper phage, accessory plasmid and selection phagemid. The recombinase used was Hin, a member of the serine resolvase family. Colonies were picked and grown overnight in 2× YT media with both antibiotics. Donor cells (2 µl) were mixed with 198 µL F^+ recipient cells in mid-exponential phase containing an antibiotic resistance gene not found in the donor. Mixtures were incubated at 37 °C for 1.5 h and 20 µl of serial dilutions were spread on plates containing the donor and the recipient antibiotics. Infection was quantified by the number of resulting colonies after incubation at 37 °C overnight. For plaque assays, phage DNA was transformed into electrocompetent cells containing the appropriate accessory plasmid and recovered for 1 h at 37 °C, or simply isolated from a lagoon. Serial dilutions were mixed with 300 µl F^+ recipient cells grown to exponential phase in 14 ml Falcon culture tubes and incubated at 37 °C for 15 min. Top agar (3 ml) (7 g l^{-1} from Luria–Bertani (LB) broth base) at 50 °C was added to each tube, briefly vortexed and poured onto minimal agar plates incubated at 37 °C for 8 h or overnight to generate plaques.

Turbidostat assembly. Assembly followed the schematic shown in Supplementary Fig. 1c. Turbidostats were constructed from BioProbe flasks on magnetic stir plates. Each flask was equipped with a TruCell2 cell density meter held in a GL32 probe holder with compression fitting. GL45 and GL32 septa pierced with needles transferred media to and from the turbidostat through an 8-channel peristaltic pump with Tygon tubing. A needle set at the desired turbidostat volume level pumped excess cells to the waste container. A 0.2 µm filter attached to a 14-gauge needle piercing the septum vented the turbidostat vessel. A two-way valve controlled media flow to the turbidostat, connected such that a closed valve state returned the media to source. The valve opened and closed in response to TruCell2 4–20 mA output processed by a digital panel meter programmed with the desired set point. Panel meters were unlocked according to the instruction manual and programmed to the following settings: Input dc_A, Setup 30_ 10, Config 00000, Filtr 11009, dec.pt ddd.dd, lo in 00.400, lo rd 004.00, hi in 02.000, hi rd 020.00, Alset 00036, deu1h 000.01, deu2h 000.01. The digital panel meter and valve were connected with a solderless breadboard according to the diagram shown in Supplementary Fig. 12. Lagoons consisted of 100 ml Pyrex bottles with GL45 septa pierced with needles for fluid delivery, a 0.2 µm filter-terminated vent line and a magnetic stir bar. Excess lagoon volume was continually pumped to waste through a waste needle set at the desired lagoon volume.

Media preparation. Each 20 l media carboy received 140 g anhydrous potassium phosphate dibasic, 40 g potassium phosphate monobasic, 20 g ammonium sulphate, and 20 ml Tween 80 in 20 l H_2O. Carboys were loosely capped with Polyvent filling/ venting closures with an autoclavable 0.2 µm filter fastened to the venting port. Media was autoclaved until visibly boiling (typically 120 min at 30 pounds per square inch (p.s.i.), 121 °C) and allowed to cool overnight. Media supplement was prepared from 90 g glucose, 10 g sodium citrate, 0.25 g anhydrous magnesium sulphate, 10 g casamino acids, 0.15 g tetracycline-HCl, 0.6 g carbenicillin, 0.6 g spectinomycin and 0.5 g L-leucine, dissolved in 500 ml H_2O, and filtered with a Nalgene 500 ml filtration unit. Media supplement (500 ml) was added to each carboy under conditions that minimized the risk of media contamination (in the case of the reported experiments, immediately after 1 h of germicidal ultraviolet irradiation).

Sterilization and cell culture. The autoclavable components of a turbidostat apparatus include the BioProbe flask, TruCell2 probe, needles, vent filter and tubing. All such components were autoclaved fully assembled except for tubing, which was connected while hot. Upon equilibrating to ambient temperature, the peristaltic pump responsible for media addition and waste removal was started with the valve opened until the desired volume was reached. The TruCell2 probe was connected to its transmitter and zeroed. Turbidostats were seeded with 100 µl of an overnight culture of host cells. Turbidostats and lagoon cultures were grown at 37 °C.

Cell density calibration. Serial dilution plating was used to generate a calibration curve to determine TruCell2 output and panel meter setting corresponding to the desired cell density. For these experiments, both panel meter alarms were programmed to open the valve at 6.80 mA. Cells were pumped from the turbidostat to the lagoons by peristaltic pumps with silicone (platinum) two-stop tubing. Calibration curves relating pump speed in revolutions per minute to volumetric flow rate were determined experimentally with a timer and graduated cylinder for each tubing size.

PACE experiments. Turbidostats and lagoons were assembled as described above. Upon the turbidostat reaching the desired set point of 6.80 mA (corresponding to 5×10^8 cells ml^{-1} in our hands), lagoons were connected to turbidostats, waste needles were set at the desired volume and lagoon pumps were set to a flow rate corresponding to the desired dilution rate. Each lagoon was seeded with 100 µl of an overnight culture producing selection phage. To induce elevated mutagenesis, 10% filter-sterilized L-arabinose was delivered by a separate peristaltic pump to each lagoon requiring enhanced mutagenesis to a final concentration of 1%. Lagoon aliquots were taken by sampling lagoon waste lines at the luer lock just after the peristaltic pump. Individual clones were isolated by plaque assay or amplified by PCR, assembled into a T7 RNAP activity assay plasmid, and transformed into cells containing a *lacZ* reporter plasmid. Active clones were picked by blue/white screening.

Selection phage optimization. T7 RNAP was subcloned into VCSM13 helper phage encoding kanamycin resistance, generating HP-T7RNAP A, used in the discrete infection assay shown in Fig. 2a. To ensure that improvements to the phage genome did not interfere with the evolution of T7 RNAP, HP-T7RNAP A was propagated in a lagoon fed by S109 host cells containing AP-T7 A and DP-QUR and supplemented with arabinose for 72 h at 2.0 volumes per hour. Individual plaques were isolated and their T7 RNAP genes sequenced. One plaque contained only a single point mutation in T7 RNAP, P314T, and was chosen as the preoptimized selection phage for T7 RNAP evolution. Sequencing of the rest of the selection phage revealed numerous changes relative to the parental VCSM13. Notably, the entire p15a-Kan^R cassette inserted into the intergenic region to create VCSM13 had been perfectly deleted to reconstitute the wild-type M13 intergenic region. Other changes included N79S, F286S and I360T mutations in gIV, a K249R mutation in gII, three silent mutations back to the corresponding M13 base, two other silent mutations and the deletion of one thymine residue in the terminator before gIII, possibly increasing the expression of T7 RNAP. These regional patterns of variation parallel those observed by Husimi in more extensive filamentous phage evolution

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experiments[11]. This evolved phage, designated SP-T7RNAP P314T, was used as the starting selection phage for all subsequent PACE experiments.

Mutagenesis assays. The *lacI* gene was cloned into VCSM13 between the p15a origin and kan^r to generate VCSM13-lacI. A turbidostat was grown to a set point equivalent to 5×10^8 cells ml^{-1} with S109 cells containing the mutagenesis plasmid. Lagoons were seeded with 10 µl VCSM13-lacI and run at 2.5 volumes per hour for 3 h. One lagoon was supplemented with 10% L-arabinose to a final concentration of 1%, whereas the other received H_2O. Aliquots were removed after 3 h and each was used to infect a 100-fold greater volume of recipient cell culture of S109 cells containing a reporter plasmid conferring carbenicillin and spectinomycin resistance with a *lacI* binding site (*lacO*) capable of repressing spectinomycin resistance. Mixtures were incubated for 1.5 h at 37 °C, spread on 2× YT plates containing spectinomycin, kanamycin and carbenicillin, and incubated at 37 °C overnight. Colonies were counted for induced and uninduced lagoons to estimate the fold increase in mutagenesis. Seventy-two colonies were sequenced to determine the frequencies of all transitions and transversions within mutated *lacI* genes. The results are shown in Supplementary Fig. 5. All sequenced colonies contained at least one mutation capable of inactivating repressor function.

Cell-based T7 RNAP activity assays. Overnight cultures of S109 cells grown in 2× YT containing reporter plasmid and expression vector were diluted fourfold in fresh 2× YT media. Diluted culture (20 µl) was mixed with 80 µl Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) in Falcon Microtest 96-well OptiLux assay plates and the absorbance at 595 nm was measured using a Spectra M5 plate reader. Methylumbelliferyl-β-(D)-galactopyranoside (25 µl of 1 mg ml^{-1}) was added to each well and the time recorded. Plates were incubated at 30 °C and fluorescence was measured at 360/460 nm on a Spectra M5 plate reader. Plates were measured at multiple time points to avoid saturation of the spectrophotometer or consumption of the substrate, depending on the activity level of the T7 RNAP enzyme being assayed. MUG fluorescence units were calculated as previously described[24]. The activity level of wild-type T7 RNAP on the T7 promoter was defined as 100%; activities greater than 3% were considered significantly above the background level of this assay.

T7 RNAP protein purification. T7 RNAP variants were cloned into pT7-911Q, a His-tagged T7 RNAP expression vector.[32] Overnight cultures grown at 30 °C were diluted 1:500 in LB broth containing 50 µg ml^{-1} carbenicillin and 2% glucose. Upon reaching absorbance $A_{600} \approx 0.5$, cultures were centrifuged at 4,000*g* for 5 min and re-suspended in LB broth with 0.4 mM IPTG and 50 µg ml^{-1} carbenicillin. Cultures were grown for 4 h at 30 °C, spun at 8,000*g* for 6 min, and the pellet was frozen overnight. Binding buffer consisted of 50 mM Tris, 300 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol and 10 mM imidazole at pH 8.0. Wash buffer consisted of 50 mM Tris, 800 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol and 20 mM imidazole at pH 8.0. Elution buffer was equivalent to wash buffer with 500 mM imidazole. Pellets from 25 ml culture were resuspended in 1 ml wash buffer and cells were lysed by sonication while kept on ice using a Misonix CL4 sonicator at maximal microtip power for 45 s in 1 s bursts. Cell debris was spun down at 20,000*g* for 15 min at 4 °C. Ni-NTA spin columns were equilibrated with 500 µl binding buffer and spun at 800*g* for 2 min. Lysate supernatant was loaded onto each column and spun at 300*g* for 5 min. Columns were washed twice with 500 µl wash buffer, spinning at 800*g* for each, then eluted twice with 250 µl elution buffer. Proteins were dialysed into 20 mM Tris, 100 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM DTT, pH 8.0, and concentrated using Amicon Ultra-0.5 30K concentration columns.

***In vitro* T7 RNAP activity assays.** Purified T7 RNAP variant concentrations were determined by Bradford assay and then by Coomassie stain on a 4–12% NuPage gel. Templates were prepared by PCR amplification of 150-bp fragments of the reporter plasmids used for *in vivo* assays including the promoter and the start of the *lacZ* gene. Templates were purified by MinElute spin column. Transcription reactions were performed in 1× RNAP buffer consisting of 40 mM Tris-HCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine pH 7.9 with 1 mM ribonucleotide tri-phosphates, 1 ng template DNA, purified polymerase variant and 2 mCi [α-^{32}P]ATP. Reactions were incubated at 37 °C for 20 min, mixed with an equivalent volume of loading dye consisting of 7 M urea, 178 mM Tris-Cl, 178 mM H_3BO_3, 4 mM EDTA and 0.002% bromophenol blue, then electrophoresed on Criterion 5% or 10% TBE-urea denaturing gels. RNAs were transcribed from double-stranded templates of sequence 5′-TAATACGACTCACTATAGGGAGAGCCACCACCACCACCACCACCA-3′, 5′-TAATACGACTCACTATACCCCCCGCCACCACCACCACCACCACCA-3′, and 5′-TAATACGACTCACTATAAAAAAAGCCACCACCACCACCACCACCA-3′ (the +1 base is underlined). To remove differences in specific radioactivity from [α-^{32}P]ATP incorporation in iA_6, wild-type and iC_6 transcripts arising from the differing number of A nucleotides in their first six bases, transcripts were digested with T1 RNase to remove the first seven nucleotides. This digestion step also enabled all iA_6 transcription products to enter the gel completely, which did not always occur for iA_6 products of either wild-type or evolved polymerases. Transcripts were electrophoresed on Criterion 15% TBE-urea gels, exposed to phosphor screens, and imaged on a Typhoon Trio phosphorimager. Bands corresponding to transcription products were quantified with ImageJ software.

RACE analysis. *In vitro* transcription reactions with purified T7 RNAP variants were performed as described above but without the addition of any radioactive nucleotide using polymerase variant C6-80.9 on the iC_6 and wild-type templates, and using polymerase variant A4-36.4 on the iA_6, iC_6 and wild-type templates. DNA oligonucleotides of sequence 5′-TAATACGACTCACTATACCC-3′ and 5′-CCCACCCAAAAAAAAAAAAAAAGGGGGGTATAGTGAGTCGTATTA-3′ formed the iC_6 template, 5′-TAATACGACTCACTATAAAA-3′ and 5′-CCCACCCAAAAAAAAAAAAAAATTTTTTTATAGTGAGTCGTATTA-3′ formed the iA_6 template and 5′-TAATACGACTCACTATAGGG-3′ and 5′-CCCACCCAAAAAAAAAAAAAATCTCCCTATAGTGAGTCGTATTA-3′ formed the wild-type template. Each 200 µl transcription reaction was treated with 2 µl calf intestinal phosphatase for 1 h at 37 °C, extracted with phenol-chloroform twice to remove enzymes and precipitated with ethanol. Pellets were resuspended in 1× DNase Turbo buffer with 5 µl DNase in a total volume of 100 µl, incubated for 2 h at 37 °C, extracted with phenol-chloroform twice and precipitated with ethanol. Purified transcript (5 µl) was mixed with 1 µl T4 polynucleotide kinase (in 1× polynucleotide kinase buffer) and incubated at 37 °C for 1 h. Treated RNA (1 µl) was ligated to 30 ng RNA adaptor of sequence 5′-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3′ with T4 RNA ligase in 1× RNA ligase buffer at 37 °C for 1 h. Ligated RNA (1 µl) was reverse transcribed by mixing with 1 mM deoxynucleotide triphosphates, and a complementary DNA primer of sequence 5′-CCCCCAAACCCCCAAAAAAAAAAACCCACCCAAAAAAAAAAAA-3′ at a final concentration of 5 µM at 65 °C for 15 min followed by 10 U $µl^{-1}$ SuperScript III reverse transcriptase in 1× reverse transcriptase buffer, 5 mM $MgCl_2$ and 10 mM dithiothreitol at 50 °C for 1 h. Enzymes were denatured at 85 °C for 5 min followed by DNA amplification with sequential PCR reactions using primers 5′-CGATCCGAACGCAGCATTTACGCTGATGGCGATGAATGAACACTG-3′ and 5′-CCCCCAAACCCCCAAAAAAAAAACCCACCCAAAAAAAAAAAA-3′, digestion with MlyI and HinfI in NEBuffer 4 to cleave sequences containing the promoter, and PCR amplification with 5′-GCTAGTTATTGCTCAGCGGAATAACGATCCGAACGCAGCATTTAC-3′ and 5′-GCTAGTTATTGCTCAGCGGAAAAAAAAAAAAACCCCCAAACCCCC-3′. PCR products were cloned into the backbone of plasmid AP-T7 P amplified with primers 5′-CGGATCGTTATTCCGCTGAGCAATAACTAGCAGAGCAAAAGGCCAGC-3′ and 5′-GGGGGTTTGGGGGTTTTTTTTTTTTTCCGGCCTTGTCGGCCTTAC-3′ by isothermal assembly cloning. Individual colonies were picked and sequenced using a primer of sequence 5′-CAGGAAGGCAAAATGCCG-3′.

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