Exploiting spatial dimensions to enable parallelized continuous directed evolution

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Abstract

Current strategies to improve the throughput of continuous directed evolution technologies often involve complex mechanical fluid-controlling system or robotic platforms, which limits their popularization and application in general laboratories. Inspired by our previous study on bacterial range expansion, in this study, we report a system termed SPACE for rapid and extensively parallelizable evolution of biomolecules by introducing spatial dimensions into the landmark phage-assisted continuous evolution system. Specifically, M13 phages and chemotactic Escherichia coli cells were closely inoculated onto a semisolid agar. The phages came into contact with the expanding front of the bacterial range, and then comigrated with the bacteria. This system leverages competition over space, wherein evolutionary progress is closely associated with the production of spatial patterns, allowing the emergence of improved or new protein functions. In a prototypical problem, SPACE remarkably simplified the process and evolved the promoter recognition of T7 RNA polymerase (RNAP) to a library of 96 random sequences in parallel. These results establish SPACE as a simple, easy to implement, and massively parallelizable platform for continuous directed evolution in general laboratories.

Keywords: bacteriophage; directed evolution; range expansion; spatial competition; virus spreading

Subject Categories: Methods & Resources; Microbiology, Virology & Host Pathogen Interaction

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Introduction

Directed evolution mimics natural evolution and typically proceeds with iterative rounds of genotype diversification and selection for desired phenotype activity (Bloom & Arnold, 2009). The steps required in the library construction and selection/screening cycles of conventional directed evolution methods are generally labor-intensive and time-consuming. In order to improve the efficiency and reduce manual labor, researchers are exploring to bring in sophisticated mechanical instruments such as microfluidic/millifluidic systems (Agresti et al., 2010; Fallah-Araghi et al., 2012; Wong et al., 2018) and automated robotic platforms (Pham et al., 2017; Piatkevich et al., 2018; Chory et al., 2021). Continuous directed evolution methods, on the other hand, employ delicate biological designs to enable autonomous cycles of mutant library construction and selection by coupling gene functions of interest to the fitness of replicating organisms (Esvelt et al., 2011; Crook et al., 2016; Ravikumar et al., 2018; English et al., 2019), leading to rapid optimization of biomolecules with little human intervention required. One representative of these methods, phage-assisted continuous evolution (PACE) has been applied to evolve a wide range of protein functions such as the specificity of RNA polymerase, TALEN, and Cas9, target specificity and drug resistance of proteases, activity and target compatibility of base editors, and improved soluble expression of proteins (Esvelt et al., 2011; Carlson et al., 2014; Dickinson et al., 2014; Hubbard et al., 2015; Badran et al., 2016; Bryson et al., 2017; Packer et al., 2017; Hu et al., 2018; Wang et al., 2018; Thuronyi et al., 2019; Richter et al., 2020; Blum et al., 2021). It links the desired property of biomolecules to phage propagation to enable rapid rounds of evolution, and utilizes a chemostat-like apparatus (upper panel, Fig 1A) to constantly supply both uninfected host bacterial cells and a continuously diluted environment for selection. Although the PACE system has been brilliantly designed, the complexity of continuous culturing apparatus and requisite process control make it challenging to perform continuous directed evolution in a highly parallelized form (d’Oelsnitz & Ellington, 2018) unless it is facilitated with robotic platforms (DeBenedictis et al., 2022). This limits its use for important application tasks such as evolution toward multiple targets and in different conditions, or high experimental replication to map evolutionary trajectories (Harms & Thornton, 2013) in general
laboratories not equipped with sophisticated microfluidic or robotic instruments. The once-humble agar plate is increasingly seen as a useful platform that can address questions not amenable to study by standard “well-mixed” liquid culture (Baym et al., 2016; Bosshard et al., 2017; Fraebel et al., 2017; Ni et al., 2017; Shih et al., 2018; Liu et al., 2019). For instance, it has recently been utilized to study the evolution of antibiotic resistance (Baym et al., 2016) and the colonization strategies of bacterial range expansion (Liu et al., 2019). In such studies, bacterial cells are typically inoculated at the center or edge of the semisolid agar plate. The subsequent range expansion of bacteria is led by a propagating front of growing cells (blue circle in lower panel of Fig 1A) moving outward toward the uncolonized territory, while cells with lower motility are left behind to grow until the nutrients are exhausted (Cremer et al., 2019). The steadily advancing front and associated growing wake thus provide a “moving chemostat” that harbors exponentially growing fresh cells (Koster et al., 2012; Cremer et al., 2019), with spontaneous separation from old cells whose growth and motility slow dramatically as nutrients are depleted (lower panel, Fig 1A).

In this study, we sought to develop a new method that combines the advantages of spatial range expansion and PACE. Compared with conventional methods where evolution plays out only on the temporal dimension, bringing in spatial dimensions enables visualization, separation of different evolutionary events, and straightforward operation without requirements for special culturing or monitoring equipment.
Results

We began by inoculating M13 phages, which conduct chronic infections without lysing or severely damaging their host cells, (purple dot in Fig 1B) in front of a motile bacterial inoculum. A substantial fraction of the expanding cells in the front encountered phages and got infected, resulting in a slowdown of their subsequent growth (Appendix Fig S1). Progeny phages were then produced and carried forward along the expansion route by infecting neighboring fresh cells. This combination of cell migration and repeated phage infection cycles was expected to result in the formation of a visible fan-shaped infection zone with lower cell density than the uninfected regions (Li et al, 2020) (Fig 1B). Experiments showed that a suspension of exponentially growing Escherichia coli FM15 cells (Appendix Table S1), when inoculated at the center of an 8.5-cm Petri dish containing 10 ml of LB medium and 0.25% agar, formed a uniform bacterial lawn after overnight incubation (first row in Fig 1C). In contrast, inoculating bacteria at the center and $10^8$ PFU of M13 phages 1 cm away from the center of an identical semisolid agar plate led to formation of a dark (low cell density) fan-shaped pattern in the midst of a white (high cell density) bacterial lawn after overnight incubation (second row in Fig 1C). The fan-shaped pattern of low cell density area was stable for days until the agar dried up.

To gain a quantitative understanding of the patterning process, we developed a mathematical model, RESIR (Range Expansion with Susceptible Infected Recovered kinetics) model derived from previous models (Kermack & McKendrick, 1927; Cremer et al, 2019), based on the characterized properties of the bacteria–phage interaction (Fig EV1A, Appendix Fig S2, Materials and Methods). In our model, numerical simulations with realistic parameter values (Appendix Table S2) recaptured the fan-shaped pattern as the experiments (bottom two rows in Fig 1C). The saturated cell density in this fan-shaped region is lower than that of uninfected region because the nutrient is partially consumed by phage production and is hence less available for supporting bacterial growth (Appendix Fig S3), thereby yielding a visible low cell density region. The development of the fan-shaped pattern was mainly driven by the expansion in the radial direction, supplemented by the extension in the lateral direction (Fig EV1B).

One prediction of the model was that, at the boundaries of the fan, uninfected cells migrating side by side with infected cells continuously served as fresh hosts for progeny phages during the range expansion, resulting in a moderately higher phage titer at the sideward edge than in the central region of the fan-shaped infection zone (Fig 1C and D). To verify this, we visualized the phage-infected bacteria by introducing a fluorescence gene accompanied with phage infection. Specifically, we constructed a reporter phage M13-GFP harboring a “superfolder” variant of green fluorescent protein (gfp) gene located downstream of gene IV in the wild-type M13 phage genome. The infection of M13-GFP phage introduces the gfp gene into the host bacteria. Thus, the bacteria infected by M13-GFP phage could be visualized by the fluorescent signal which reflects the expression level of phage genes locally (Fig 1E). As shown in Fig 1F and G, the brightest fluorescent signals in the semisolid agar plate, which suggested the highest cumulative expression of genes in the phage genome including gfp, overlapped with the sideward edge of the infection zone, consistent with the model simulation (Fig 1D).

The model simulation also predicted that the size of the fan-shaped pattern increased with the phage production rate (Fig EV1C). To test the proportionality predicted by the model, we borrowed one key design in PACE, an activity-dependent phage propagation module located in an accessory plasmid (Esvelt et al, 2011), which constructs a linkage between the function of the biomolecule to be evolved and phage propagation via the activation of gIII expression. It is known that the production of M13 phage scales with increasing levels of its minor coat protein pIII (encoded by gIII) over concentrations spanning two orders of magnitude (Rakonjac & Model, 1998). We used this design to vary the phage production rate by altering the expression level of gIII. Specifically, a selection phage (Esvelt et al, 2011) was constructed by replacing the intrinsic gIII of the M13 genome with the wild-type T7 RNA polymerase (RNAP) gene, while a copy of gIII was inserted into an accessory plasmid (Esvelt et al, 2011) in the host E. coli cell (Fig EV2A). The expression of gIII was put under the control of a library of 17 T7 promoter variants (Appendix Table S3), on which wild-type T7 RNAP exhibits different levels of activities ranging from 0.005 to 85% of the activity of wild-type T7 RNAP on the T7 promoter (Fig EV2B). When a high-copy-number accessory plasmid was used (Materials and Methods, Appendix Table S4), the area sizes of the fan-shaped pattern steadily increased with increasing expression levels of gIII, saturating at an activity level approximately 1.7% of the wild-type T7 RNAP activity on the T7 promoter (Fig EV2C). To distinguish more finely between high expression levels, a low-copy-number accessory plasmid was employed (Materials and Methods, Appendix Table S4). The combined utilization of the high- and low-copy-number accessory plasmids provided a measurement range of expression activity spanning 4 orders of magnitude (Fig EV2D). These results suggested that the area of the fan-shaped pattern could be used as a straightforward assessment of the activity of interest. We first used the high-copy-number accessory plasmid unless noted otherwise.

To develop our system of spatial directed evolution, we next sought to modulate the second key design in PACE, the mutagenesis plasmid (Badran & Liu, 2015; Bryson et al, 2017), to match the needs of the applications in semisolid media. The mutagenesis plasmid (MP4) typically used in PACE includes three mutators: DnaQ926, a dominant negative mutant of the delta domain of E. coli DNA polymerase, Dam, DNA adenine methyltransferase, and SeqA, a negative regulator of replication initiation (Badran & Liu, 2015). The expression of these mutator genes is driven by a small molecule inducer arabinose. Differently, for our spatial evolution system, we employed phage shock protein promoter ($P_{pop}$) (Brissette et al, 1990, 1991) to drive the expression of the mutants upon M13 phage infection via a pIV-dependent signaling cascade (Brissette et al, 1991), generating the mutagenesis plasmid MP-s for this study (Appendix Table S4). The stringency of the $P_{pop}$ was confirmed by using E. coli FM15 cells carrying a plasmid with gfp under the control of $P_{pop}$ (Fig 2A). The cells exhibited green fluorescence only in the presence of phage infection (Fig 2B), confirming the stringency of this promoter could prevent undesired induction of mutagenesis in bacteria cells before they came into contact with phages. The mutation rate conferred by MP-s was measured to be comparable to that of MP4, which is $4.4 \times 10^{-7}$ and approximately $5.9 \times 10^{-4}$ substitutions per bp per generation for E. coli and M13 phage, respectively (Badran & Liu, 2015; Materials and Methods).
By introduction of the accessory plasmid carrying the activity-dependent phage propagation module and MP-s carrying the in vivo mutagenesis module into our motile host strain, *E. coli* FM15, we established a prototypic system named as SPACE, standing for Spatial PACE. In SPACE, ancestor selection phages (Esvelt *et al.*, 2011) carrying a wild-type T7 RNAP gene infect bacterial cells, and the expression of mutator genes induced by the infection leads to mutations in the RNAP gene during the replication of phage genome, and then expression of different RNAP variants. Desired RNAP variants with improved activity on the target synthetic promoter activate the expression of *gIII* on an accessory plasmid to produce infectious progeny phages, which in turn infect neighboring susceptible bacterial cells and repeat the process (Fig 2C). In contrast, RNAP variants that do not lead to sufficient production of pIII and infectious progeny result in the formation of typically much smaller fan-shaped pattern or no infection zone at all.

As an initial test of SPACE, T7 RNAP was evolved to recognize a synthetic promoter named 1D8 (Appendix Table S3) with nine bases different from wild-type T7 promoter. A distinct fan-shaped pattern was observed after 20 h development (upper panel of Fig 2D), while no infection zone was formed on the host cells lacking the mutagenesis module (lower panel in Fig 2D). From the side-ward edge of the fan-shaped pattern, five phage clones were purified. Their average transcriptional activity on the promoter 1D8 was 70-fold greater than that of the wild-type T7 RNAP (Fig 2E, Materials and Methods). These results established the ability of the SPACE system to evolve enzyme activities on a single agar plate with minimal efforts.

To better understand the underlying evolutionary process of the SPACE experiment, we extended the RESIR model to describe competitions between two phages with different progeny production rates. The simulated competition results in a spatial separation between the two phages, of which the strong phages with higher progeny production rate dominate outer area near the sideward edge of the infection zone, while the weak phages with lower progeny production rate are confined inside (Fig EV3A and B). To experimentally validate the spatial separation of phages, we competed a phage M13s carrying a red fluorescent protein (*rfp*) gene in wild-type M13 genome against a weaker variant (M13w) carrying a *gfp* gene (Fig 3A). M13s harbored three mutations K184A/R186A/D187A in its *gIII*, which resulted in lower progeny productivity than wild-type M13 (Deng & Perham, 2002). The expression of red or green fluorescent proteins induced by the infection of M13s or M13w could thereby indicate the spatial distribution of these two
phages (Fig 3B). Their spatial abundance patterns were characterized after 20 h of competition assay (Materials and Methods) with an initial titer ratio of 1:1 between M13w and M13s. As expected, M13s (red) dominated the outer area of the fan-shaped infection region along the direction of the sideward edges, while the M13w (green) was localized in the inner area mostly around the initial inoculation spot (Fig EV3C and D).

To more closely reflect actual evolutionary process, in which stronger phages may be generated at very low frequencies, we repeated the competition assay with an M13w: M13s inoculant ratio of 10⁵:1. Both experimental and theoretical results showed that the strong phage M13s could eventually outcompete the weak and take over the population as the phage population spread via its range-expanding host (Fig 3C and D, Appendix Fig S4). And the strong phage is accumulated most efficiently at the sideward edge of the infection zone due to the most active infections occurring there. Thus, during SPACE process, the advantageous phage mutants with high production rates, which usually occur at very low frequencies in an evolutionary process, would be autonomously separated from the weaker and get enriched rapidly at the sideward edge of the infection zone, as illustrated in Fig 3E.

Figure 3. Spatial competition and adaptation of phages during range expansion of their hosts.
A Design of a competition assay between phages with different production rates. M13s carries a red fluorescent protein (rpf) gene in a wild-type M13 genome, while a weaker phage variant M13w carries a green fluorescent protein (gfp) gene. The phage titers of M13s and M13w were 9.6 × 10⁸ and 1.3 × 10⁷ PFU/ml, respectively, after 2.5 h propagation in E. coli FM15 by shaking incubation at 37°C with a multiplicity of infection (MOI) of 0.001.
B Microscopic images of E. coli FM15 cells with or without phage infection. Scale bar represents 1 μm.
C The experimental result shows raw photographs of a representative two-phage competition 20 h after initial inoculation with 10⁵:1 mixture of M13w: M13s at 1 cm away from the center. The simulation result is the outcome of competition between two phages PW and PS with relative production rates of 40 and 100, respectively. Scale bar represents 1 cm.
D Phage titer ratio of M13s to M13w along the central line and the edge of the fan-shaped infection zone in the experimental result in (C). Samples were collected from different positions as shown in the schematic and phage titer was quantified by qPCR (Materials and Methods). Data represent mean values ± s.d. for three independent assays. Two-tailed t-test was used to compare two groups. *P = 0.028.
E Illustration of the phage spatial evolution process during the range expansion of the host bacteria. Mutants with improved activity to produce infectious progeny phages continuously infect neighboring susceptible bacterial cells and get enriched along the sideward edge of the fan-shaped infected zone; meanwhile, weak mutants that do not lead to sufficient production of infectious progeny result in typically much smaller fan-shaped pattern or no visible infection region at all.
Source data are available online for this figure.
We next demonstrated that SPACE is scalable and parallelizable. Ninety-six SPACE experiments were carried out concurrently to evolve T7 RNAP to recognize a library of random T7 promoter variant sequences (Fig 4A). The starting selection phage carrying wild-type T7 RNAP was inoculated onto 96 semisolid agar plates, each of which contained an inoculum of host cells harboring an accessory plasmid containing a random T7 promoter variant (Appendix Table S3) and the mutagenesis plasmid MP-s. Twenty out of the 96 experiments were deemed as successful evolution based on the formation of the fan-shaped pattern, and the evolved phages were isolated and sequenced for mutations in the T7 RNAP mutants (Fig 4B). As the size of the fan-shaped pattern is linked to the activity of RNAP (Fig EV2), we compared the size of the infected region to assess the improvements in enzyme activity, and confirmed that the isolated mutant phages presented larger fan-shaped patterns than the ancestor (Figs 4B and EV4A). The expression activity levels of the evolved RNAP mutants on their target sequences were further measured in vivo based on flow cytometry (Fig EV4B, Appendix Fig S5), and also calculated using the area-to-activity transfer function (given in Fig EV2D). Roughly consistent with the calculation, the fold change in measured activities of the RNAP mutants ranged from 8.1 (A42) to 918.3 (C12) as compared with the activity of wild-type T7 RNAP on the corresponding promoters (Fig EV4B). Several evolved T7 RNAP mutants were further purified and assayed in vitro (Materials and Methods), and the purified RNAP mutants exhibited improvements ranging from 3.4-fold (1G8) to 72.9-fold (1D8), compared with the starting enzyme (Fig 4C). Aside from mutations at frequently reported sites including E222, N748, Q758, D770, and E772 (Ikeda et al, 1993; Raskin et al, 1993; Rong et

![Figure 4. Parallelizable SPACE system.](image)

**A** An illustration of the parallelized SPACE experiments. Each FM15 strain carrying an accessory plasmid with a specific target promoter on it was inoculated on an agar plate to launch the experiments in parallel.

**B** List of library promoter sequences for which SPACE produced improved RNAP variants relative to wild-type RNAP (wild-type T7 promoter sequence is at the top). For 10 selected promoters, improvement of RNAP performance is demonstrated visually on the right, by comparing the size of fan shapes formed by phages carrying corresponding mutant RNAP genes (underlined mutations) with those formed by ancestor (anc) phages carrying the wild-type T7 RNAP gene. The images of a quarter of the agar plate containing a representative fan-shaped pattern are shown.

**C** Fold change in the relative expression activity of the evolved RNAP mutants as compared with the wild-type T7 RNAP, for the 10 selected promoter sequences corresponding to the images in (B). Results were obtained by in vivo transcriptional assay (Materials and Methods). Data represent mean values ± s.d. for three biologically independent assays.

**D** Expression activity of 10 RNAP mutants on T7 promoter and 10 promoter variants measured by in vitro transcriptional assay. Mutants are arranged in the same order as their original target promoters listed on the left side of the heatmap. Amino acid changes of each mutant are listed in Appendix Table S5. Data are normalized so that the activity of wild-type RNAP on the T7 promoter is 100; the mean for three biologically independent replicates is shown (Materials and Methods).

Source data are available online for this figure.
al, 1998; Imburgio et al, 2000; Meyer et al, 2015), some new mutations such as I244V, K286E/R, M219K, and T688P were detected repeatedly. They might play important roles in the recognition of the corresponding artificial promoters, which needs further investigations.

To assess the orthogonality and promoter malleability of the evolved RNAP mutants, we generated an activity map based on the in vivo measurements of 11 promoters against 10 RNAP mutants (Fig 4D, Appendix Fig S6, Appendix Tables S3 and S5). All evolved RNAP mutants retained their activities on the T7 promoter. Besides wild-type T7 RNAP, Mut1 and Mut7 were orthogonal, recognizing only their target sequences (1C12 and 4A2, respectively) and the T7 promoter. Apart from these, off-target crosstalk is consistently observed between different pairs (Fig 4D), which needs further efforts to remove the promiscuity of these RNAPs if they are to be applied in genetic circuit design (Tabor, 2012). The mutation E222K, known to be a specificity broadener (Ikeda et al, 1993), led to nonspecific activity on almost all sequences tested. Aside from changing from glutamic acid to lysine, other mutations at the same site including E222A (mut5) and E222G (mut6) produced mutants with similar off-target activities. H772R (mut8) also showed some nonspecific activity. However, combining E222G and H772R, with one more mutation Q758R, resulted in mut1, which, as described, is highly orthogonal. This implied an underlying epistatic phenomenon. Construction of an exhaustive activity map of RNAP mutants and random sequences would be of interest to deepen understanding of the origin of the orthogonality and promoter malleability. To this end, SPACE can be used to generate abundant mutants and assay the activity without additional infrastructure.

Discussion

Range expansion is a widely observed phenomenon in nature, which involves the movement and successful establishment of natural populations across new territories due to biological invasion, anthropogenetic habitat conversion, or changes of the abiotic and biotic environmental factors (Andow et al, 1990; Walther et al, 2002; Parmesan & Yohe, 2003; Hastings et al, 2004; Excoffer et al, 2009; Frohnofer & Altermatt, 2015; Gandhi et al, 2016; Ochocki & Miller, 2017; Ramirez et al, 2019; Aguirre-Liguori et al, 2021; Liu et al, 2021). Although nonmotile, nonlethal viruses can be easily transmitted via their host species conducting range expansion and therefore spread to broader territories (Jones, 2009; Kareinen et al, 2020). It has been assumed that movements of migrating animal species could enhance the spread of pathogens including zoonotic viruses posing severe threats to human health (Altizer et al, 2011). However, the mechanism by which the range expansion of host species influences the adaptation and evolution of their viruses remains to be elucidated.

It is recognized that range-expanding species experience active evolutionary changes at the expanding front (Deforet et al, 2019; Miller et al, 2020). The fast-dispersing individuals with superior moving ability outrun other individuals of the species and inevitably accumulate at the front, resulting in an evolutionary increase in the moving ability in successive generations. This process has been proposed as a new evolutionary mechanism called “spatial sorting” (Shine et al, 2011; Phillips & Perkins, 2019). Researchers also found that parasite that infects range-expanding hosts will itself be subjected to spatial sorting (Shine et al, 2011), and its improved ability to successfully infect hosts is favored during this evolutionary process (Kelehear et al, 2012). Considering that viruses are obligate parasites and copropagate with their hosts, one could intuitively speculate that viruses that infect a range-expanding host will experience the same evolutionary process with the host, that is, the evolutionary changes of viruses mostly accumulating at the front of host expansion. However, our results in this study have proven otherwise. Unlike the spatial sorting of host species (Deforet et al, 2019; Miller et al, 2020), the active infection of neighboring susceptible host cells leads to the spatial sorting of phages with different progeny productivities perpendicular to the direction of host front propagation. Therefore, other than at the expanding front, bacteriophage genotypes associated with higher phage progeny production rate are significantly accumulated along the sideward edge of the fan-shaped infection zone. It can also serve as a null hypothesis for the prediction of viral spread and evolution during host range expansion for future ecological studies of higher organisms.

Moreover, we applied this ecological insight to develop a SPACE system. Unlike the 17 continuous directed evolution tools comprehensively summarized by a recent review (Morrison et al, 2020), our SPACE system introduces spatial dimensions into continuous directed evolution to streamline protein engineering. This remarkably reduces the complexity of the continuous directed evolution apparatus, and enables direct visual assessment of the progress of the evolution experiments through the link between phage production rate and the size of the infection zone. As to PACE system, its selection stringency has to be optimized from time to time to avoid complete phage washout. To accelerate the selection process, the flow control needs to be closely monitored, either by manually collecting samples from the lagoon and checking the intensity of reporter signals as well as the phage titer, or by using a specialized in-line monitoring instrument (Carlson et al, 2014; Badran et al, 2016), which limits the number of evolution experiments that can be performed in parallel. Moreover, PACE relies solely on the time scale to select for stronger phage mutants based on their faster reproduction in the well-mixed liquid system. In SPACE system, the stronger mutants are spontaneously separated from weaker ones through spatial competition and get most enriched at the rim end of the sideward edge of the fan-shaped infection region. In addition, our model also predicted that the fold-enrichment rate of beneficial mutants provided by the SPACE system is higher than the optimal rate that a chemostat-like liquid continuous culturing system could achieve, regardless of the detailed progeny production rates of the mutants (Appendix Fig S7). These features of SPACE provide convenience to enable evolutionary applications that require extensive parallelization with efficiency, and it is easy to implement in general biology laboratories without expensive automated robotic experimental systems (DeBenedictis et al, 2022).

We have demonstrated that SPACE could improve the in vivo transcriptional activity of T7 RNAP on an artificial promoter by 70-fold after a round of overnight experiment (~20 h), which is comparable to the ~100-fold increase in activity on P7 achieved by PACE at 28 h (Carlson et al, 2014). However, current format of SPACE system still has its drawbacks. For example, the selection stringency cannot be flexibly adjusted within one round of experiment, and the phage generations need to be increased by transferring evolved
phages to a fresh agar plate to start a new run (Appendix Text S1). As a future upgrade, additional relevant factors such as the concentrations of agar and chemotactant in the media could be tuned to change the expansion speed of bacteria (Liu et al., 2019) and hence enable more flexible control over the selection stringency of the system. We expect that the increased expansion speed would result in higher selection stringency, meaning that mutants have to exhibit higher level of improvements in their activity to co-propagate with the range-expanding bacteria and outcompete other mutants (Appendix Fig S8). In addition, an attractive application of SPACE is to chart empirical fitness landscape by extensively parallelized evolution experiments. As the “fossils” will be left behind the expanding front in space, together with the convenient visual readout of competition outcomes, SPACE enables extensive exploration of fitness landscape and together with the convenient visual readout of fitness landscape. Overall, SPACE provides a powerful platform for applied research and fundamental investigation of evolutionary mechanisms.

Materials and Methods

Strains and media

DNA cloning was performed with chemically competent E. coli DH5α cells (TransGen, Beijing). All plaque assays, bacterial migration tests, in vivo transcriptional assays, and SPACE experiments were performed using E. coli FM15 strain. This strain was derived from E. coli MG1655-mCherry (Liu et al., 2019) by the following steps: (i) rendered F+ by conjugation with E. coli K12 ER2738 (NEB); (ii) deletion of the α-fragment of LacZ gene with the λ Red recombinase system. The genotype of the resulting strain FM15 is F' proA + B+ lacIq (lacZ)M15 zsf::Tn10 (TetR)/λ– ilvG– rfb-50 rph-1 attB::KanR Δ(lacZ)M15. Information about bacterial strains used in the study is listed in Appendix Table S1. Cells were cultured in Luria-Bertani medium (LB: 10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract). LB containing 0.25% (w/v) agar (Hankai, Guangdong) was used for SPACE experiments. Antibiotics including chloramphenicol (25 μg/ml), tetracycline (15 μg/ml), and spectinomycin (50 μg/ml) were added where appropriate. For in vivo transcriptional activity measurement, M9 medium (6.78 g/l Na2HPO4, 3 g/l KH2PO4, 1 g/l NH4Cl, 0.5 g/l NaCl) was used, with casamino acid (CAA) and glucose supplemented where necessary.

Cloning and plasmid construction

The vectors were constructed with Gibson assembly (NEB) or ClonExpress II One Step Cloning (Vazyme, Nanjing) kits. PCRs were carried out with PrimeSTAR Max (Takara) or Q5 (NEB) following the manufacturers’ instructions. Accessory plasmids (Esvelt et al., 2011) for T7 RNAP evolution contained, in order, an rrmB terminator, the promoter of interest, a strong RBS 5′-AAGGAGGAAAAATATATAATG-3′ where underlined bases represent the start codon, gIII, either the combination of aidA gene conferring spectinomycin resistance and pUC origin for a high-copy version or bla gene conferring carbenicillin resistance and SC101 origin for a low-copy version. Reporter plasmids were identical to SC101 accessory plasmids except for the replacement of gIII by gfp. T7 RNAP selection phage (Esvelt et al., 2011; Wu et al., 2021) was constructed by replacing all but the last 202 bp of gIII with the gene encoding T7 RNAP in M13K07 (NEB) helper phage and then removing the p15a origin of replication and aph gene to restore the M13 origin of replication. The mutagenesis plasmid (Esvelt et al., 2011; Badran & Liu, 2015) contained dnaQ926, dam, and seqA under control of psp operon. Information about plasmids used in the study is listed in Appendix Table S4.

Construction of promoter libraries

A target promoter library was constructed by altering bases of the original T7 promoter at positions from −17 to −1 relative to the transcriptional start site. In principle, this library should contain (417−1) variants, which is not a reasonable size to subject to biological process. Therefore, several rules were applied to narrow down the sequences to those which are more likely to act as promoters: (i) G + C content within 40–60%; (ii) no complicated secondary structure nor self-complementary structure; (iii) no consecutive GC regions; (iv) classifiable according to sequence similarity to T7 promoter. After this step, the size of the promoter library was reduced to approximately 2000. Ninety-six promoters were randomly selected from this library for the SPACE experiments. The sequences of these promoters are listed Appendix Table S3. These promoters were inserted upstream of gIII in the accessory plasmids.

Measurement of bacterial growth

The cells were grown according to the following protocol before assaying their growth curve. First, three single colonies of FM15 or FM15 carrying low-copy accessory plasmid with T7 promoter were picked from freshly streaked LB agar plates and grown overnight in 2 ml LB broth with appropriate antibiotics (tetracycline 15 μg/ml, carbenicillin 50 μg/ml) in Falcon tubes at 37°C with shaking (200 rpm). The overnight culture was then 1,000-fold diluted into prewarmed LB broth in Falcon tubes. After around 3-h shaking incubation when its optical density at 600 nm (OD600) reached 0.1−0.2, the diluted culture was again 20-fold diluted into prewarmed LB media in 100-ml flask and incubated for another 2 h. Then the OD600 was measured with a Thermo Genesyts 105 ultraviolet spectrophotometer and a proper volume of the culture was inoculated into 100 ml prewarmed LB to make an initial OD600 of around 0.01 in 500-ml flasks. The flasks were then incubated in a Warm-bath Shaker (ZHICHU) maintained at 37°C and 200 rpm. When the OD600 reached 0.1, bacterial suspensions were diluted to make OD600 0.01 and different quantities of M13 or SP-T7 phages were added into each flask to obtain final titers of 107 or 108 PFU/ml. The shaking incubation continued and samples were taken for measurement of phage titer and cell number every 10 min. The phage titers were calculated by double-layer plating plaque assay or by qPCR in a CFX Connect Real-Time System (BIO-RAD) using TB Green Premix Ex Taq™ II (Tli RNaseH Plus) (Takara) and primer sets: fg2 5′-GCTGACGACCAACCATTTCCAG-3′ and rg2 5′-AAGCAAACTCCAACAGG TGTCCTGTTTT-3′ for T7 phages; or SG2f 5′-CTTTCCTTGATGTCCTGTTT-3′ and SG2r 5′-AAGCAAACTCCAACAGG TGTCCTGTTTT-3′ for red fluorescence marker M13 phage. The cell numbers were assayed using a CytoFLEX flow cytometer (Beckman), and the gating strategy for this analysis was illustrated in Appendix Fig S5.
**Competition assay**

GFP or RFP gene following a constitutive promoter J23100 was inserted into the phage genome downstream of gIV. Phages with different fluorescence genes were paired and mixed at titers ranging from $10^3$ to $10^5$ PFU/ml. Phage mixtures were made with different ratios dependent on experimental designs. FM15 cell suspensions at exponential phase (OD$_{600}$ ≈ 0.2) and 2-μl aliquots of the phage mixtures were inoculated onto soft agar. After 18–20 h incubation at 37°C, soft agar plates were inspected by fluorescence microscopy. When necessary, 2 μl of the soft agar containing bacteria and phages was aspirated with a pipettor from different positions at the center or the edge of the fan-shape infection zone, added to 998 μl fresh LB broth, and mixed by vortex at low speed. This liquid sample was then filtered through a 0.22-μm pore size PES syringe filter to remove bacteria, and subjected to quantification of phage particles by qPCR.

**Single-cell fluorescence imaging**

Fluorescence signals induced by M13s or M13w were measured after overnight co-culture of FM15 cells and phages in LB. For stringency test of psp promoter, FM15 cells carrying plasmid with/out gfp downstream of P$_{psp}$ were cultured until mid-log phase and subjected to M13 phage infection at a multiplicity of infection (MOI) of approximately 10 for 2 h. Cells were imaged using a Nikon Ti-E microscope equipped with a Plan Apo λ 100× Oil Ph3 DM objective (N.A. = 1.4) and an Andor Zyla 4.2 sCMOS camera. A 1% agarose pad with 0.9% NaCl was used to immobilize the cells. After cell immobilization, images were acquired within 5 min at room temperature (RT). Fluorescent images were taken with an EGFP filter (49002; ET470/40×, ET525/50 m) or an RsRed filter (49005; ET545/30×, ET620/60 m), and a 40-ms exposure time.

**Plate image capturing and processing**

Plates were imaged using a Canon EOS 60D digital camera with a Canon EFS 18–135 mm lens and an exposure setting of f11, 1/500 s, ISO3200. The agar plates were illuminated by a white LED ring light with the diameter of 36 and 16 cm below (Liu et al., 2019). The area of agar plates was measured with ImageJ software (ver. 1.52a). For each plate, the region within a circle concentric to the plate bottom with radius 3.5 cm was used for area measurement. Fluorescent images of agar plates with fan shapes were imaged using a Nikon Ti-E microscope and a Plan Fluor 4× PhL DL objective (N.A. = 0.1). Each picture of a complete plate was obtained by stitching $27 \times 27$ evenly divided square fields of view taken with the “ND processing” function of the NIS-Elements AR software (ver. 4.50.00). The green fluorescence images were taken with an EGFP filter (49002; ET470/40×, ET525/50 m), and the red fluorescence with an RsRed filter (49005; ET545/30×, ET620/60 m).

**Mutation rate measurement**

M13mp18 (Messing, 1983) (NEB), a phage vector containing betagalactosidase (LacZ) alpha gene (507 bp) downstream of gIV in the M13 genome, was used for the modified lacZ inactivation assay. Overnight cultures of E. coli FM15 without mutagenesis plasmid, with P$_{psp}$-driven mutagenesis plasmid MP-s constructed in this study, and with MP4 (Badran & Liu, 2015) used in FACE were inoculated into 2 ml LB media supplemented with antibiotics where appropriate, and cultured at 37°C until log phase (OD$_{600}$ ≈ 0.2). Then approximately 20 PFU of M13mp18 was added to each bacterial culture. For induction of MP4, 25 mM (final concentration) of arabinose was added. The culture was continued to allow phages to propagate for 7–8 h. Supernatants containing progeny phages were collected after centrifugation and filtered through a 0.22 μm pore-size PES syringe filter. These samples were serially diluted, mixed with 200 μl log-phase culture of FM15 without mutagenesis plasmid, and applied to double-layered method with top agar containing 0.04% Bluo-Gal (Sangon, Shanghai) and 15 μg/ml tetracycline. The number of white or light blue plaques (lacZα− phenotype) and the total plaque number were counted and used as a measure of mutation frequency. After 24 h propagation on E. coli FM15 cells without mutagenesis plasmids, the testing phage vector did not produce any lacZα− inactive mutant plaques, while 3.9 and 3.1% of mutant plaques were produced by phages propagated on FM15 carrying MP-s and MP4 (Badran & Liu, 2015), respectively.

**Range expansion-based continuous evolution**

Soft LB agar was freshly prepared in 8.5-cm Petri dishes before each SPACE experiment. FM15 cells carrying both accessory plasmid and mutagenesis plasmid were cultured until its OD$_{600}$ ≈ 0.2, and 2-μl aliquots of the cell suspension were inoculated at the center of the agar plates. Two microliters of selection phages with a titer of approximately $5 \times 10^5$ PFU/ml were inoculated 1 cm away from the center of the soft agar. The inoculated plates were incubated at 37°C for 18–20 h, which was typically the duration of bacterial growth and phage propagation required for the formation of clear fan-shaped infection zone. After the incubation, 5 μl of the soft agar containing bacteria and phages was aspirated with a pipettor from the end of each edge of the fan shape, added to 495 μl fresh LB broth, and mixed by vortex at low speed. This liquid sample was then filtered through a 0.22-μm pore size PES syringe filter to remove bacteria, and stored at −20°C before use.

**In vivo transcriptional activity measurement**

FM15 cells were grown according to the following protocol before assaying their fluorescence. First, cells were inoculated from three single colonies on LB agar plates and grown overnight in 2 ml LB or M9 supplemented with 1% (w/v)CAA and 0.4% (w/v)glucose with 15 μg/ml tetracycline and 50 μg/ml carbenicillin in Falcon tubes at 37°C with shaking (200 rpm). Then, the overnight cultures of E. coli FM15 carrying low-copy reporter plasmids containing gll downstream of T7 promoter or T7 promoter variants were diluted 1,000-fold in prewarmed M9 supplemented with 1% CAA and 0.4% glucose in Falcon tubes. After approximately 3 h, once the diluted cultures reached an OD$_{600}$ of 0.1–0.2, the cultures were diluted 20-fold with prewarmed media in Falcon tubes again and incubated for another 2 h. OD$_{600}$ was measured with Thermo Genesys 10S ultraviolet spectrophotometer and cultures were diluted with prewarmed media to OD$_{600}$ of 0.02 then divided into a 96-well plate. Selection phages carrying wild-type or mutant RNAp genes were prepared by adjusting phage titers to approximately $10^{10}$ PFU/ml, and 20-μl
aliquots were also added to make up a total volume of 200 μl per well. The plate was then incubated at 37°C in a Digital Thermostatic Shaker (AOSHENG) maintained at 37°C and 1,000 rpm. Finally, after the 2-h incubation, a 10–20 μl sample of each culture was transferred to a new plate containing 180–190 μl PBS buffer and 2 mg/ml kanamycin to stop protein expression. For fluorescent protein maturation, all the samples were then incubated at 37°C in a Digital Thermostatic Shaker (AOSHENG) maintained at 37°C and 1,000 rpm for another 30 min. The fluorescence distribution of particles in each sample was assayed using a CytoFLEX flow cytometer (Beckman) with appropriate voltage settings (SSC: 500, FSC: 500, FITC: 2000); each distribution contained no < 50,000 events and was gated by the forward and side scattering using CytExpert (v2.2). The gating strategy for this analysis was illustrated in Appendix Fig S5. The intensity of fluorescence of each sample was calculated and normalized by the value of the wild-type T7 RNAP and T7 promoter pair.

**T7 RNAP purification**

T7 RNAP and mutants were cloned into isopropylthio-β-galactoside (IPTG) inducible expression vector, pQE82L, and transformed into E. coli BL21 competent cells. Clones were cultured in 5 ml LB with carbenicillin (50 μg/ml) overnight and transferred into 200 ml fresh LB with carbenicillin and grown until OD₆₀₀ ≈ 1.2. Cells were then induced by IPTG (final conc. 0.5 mM) and incubated for another 3 h in a shaking incubator at 30°C. For cell lysis, the bacterial suspension was divided into 50-ml aliquots and centrifuged at the maximum speed at 4°C for 15 min. With supernatants removed carefully, pellets were resuspended in equal volume of lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 0.5 mg/ml lysozyme, 0.5 mM dithiothreitol, DTT), and stored in freezer at −80°C immediately. After the samples were completely frozen (approximately 30 min), they were thawed on ice for 1 h, and this freeze-thaw cycle was repeated twice. The supernatants of bacterial lysates were collected by centrifugation and sterilized with 0.45 μM imidazole, and then eluted with 5 ml elution buffer containing 20 μM imidazole, and then eluted with 5 ml elution buffer containing 20 and 50 μM imidazole. Elutes were collected with 1.5-ml microtubes. Proteins were dialyzed in 100 mM NaCl, 50 mM Tris–HCl, 0.1 mM DTT, 0.1 mM EDTA, 50% v/v glycerol, pH 8.0, and stored at −20°C.

**In vitro transcriptional assay**

The concentrations of purified T7 RNAP and mutants were determined by Bradford assay and then by Coomassie stain on a 10% SDS-PAGE gel. A 1.8-kb DNA fragment containing each promoter variant was amplified by PCR from the corresponding accessory plasmid. PCR products were purified with a QIAquick PCR purification kit, digested with DpnI to remove the template plasmids, and purified again. The purified amplicons were used as templates of the in vitro transcriptional assay. Transcription reactions with volume of 10 μl consisted of 40 mM Tris–HCl (pH 7.9), 6 mM MgCl₂, 2 mM spermidine, 10 mM of DTT, 200 μM of ribonucleotide triphosphates, 0.3 μl of RNase inhibitor, 0.2 μl of pyrophosphatase, 1 μM of T7 RNAP or mutant, 20 ng/ml template, and DEPC-treated water. Reactions were incubated at 37°C for 1 h, mixed with an equivalent volume of RNA loading dye consisting of 95% formamide, 0.02% SDS, 0.02% bromophenol blue, 0.01% xylene cyanol, and 0.5 mM EDTA, and then electrophoresed on 2% agarose gels. Gels were stained with 1 μg/ml ethidium bromide and viewed on a UV transilluminator. Bands corresponding to transcription products were quantified with ImageJ software.

**Model for range expansion with susceptible infected recovered kinetics**

Based on classic assumptions (Kermack & McKendrick, 1927; Bailey, 1957; Anderson & May, 1991; Busenberg & Cooke, 1993; Capasso, 1993; Hethcote, 2000; Keeling & Rohani, 2007), the host population could be categorized into three classes: the susceptible cells S, the infective cells I, and the recovered cells R. Susceptible cells enter into the infective compartment after catching an illness or virus and then into the recovered class as a consequence of recovery. Presumably, an individual who recovers from the illness has perpetual immunity thereafter. The model on the bases of these hypotheses is referred to as the SIR model, which is a classical and simple model to explain the rapid rise and fall in the number of infected patients observed in epidemics such as the plague (London 1665–1666, Bombay 1906) and cholera (London 1865).

Unlike most phages, the infection of M13 phage is chronic. During the process, infected host cells are not killed, and progeny phages are continuously produced and extruded through the cell membrane as the infected cells continue to grow at a lowered rate (Marvin & Hohn, 1969; Smeal et al., 2017a, 2017b) until they become recovered (Appendix Fig S1). Recovered cells grow as fast as the susceptible ones, and they produce progeny phages at a much lower level as compared to the freshly infected cells (Appendix Fig S1). These features make it suitable to describe M13 phage infection process with a modified SIR model (Fig EV1, Appendix Fig S2). And different from models developed for other phages with a lytic life cycle, our model does not include a term of latent phase. This is because the initial period of ~10 min before the progeny phages start to be continuously produced (Marvin & Hohn, 1969; Ploss & Kuhn, 2010) is a negligible short time scale compared to the whole process of bacteria-phage copropagation (> 10 h) in our system.

For E. coli grown in semisolid agar plate, which allows the bacterial cells to swim, the spatiotemporal dynamics is governed by two elements: cell motility and cell growth, that is, diffusion, chemotaxis, and cell growth. To gain a quantitative insight on the spatial bacteria–phage copropagation process, we developed a RESIR (Range Expansion with Susceptible Infected Recovered kinetics) model based on navigated range expansion model of bacterial population (Cremer et al., 2019) and modified SIR model of phage infection. The motility and chemotaxis of bacteria are represented by the Keller-Segel-type diffusion and advective terms widely used in the literature (Keller & Segel, 1971a, 1971b; Fu et al., 2018; Cremer et al., 2019). The spatiotemporal dynamics of bacterial cell density (susceptible bacteria S, infected bacteria I, recovered bacteria R), concentrations of the main nutrient (n) and chemoattractant (a), and the number of M13 phage particles (p) are described as follows:
of phage, Yn is the yield nutrient consumption. Growth limitations caused by nutrient availability (Murray, 2002), and follows a Monod relation

\[
\frac{\partial S}{\partial t} = \mu \nabla^2 S - \chi \nabla \cdot (\nabla f) + \lambda(n) S - \kappa(p) \lambda(n) p S - \eta_1 \lambda(n) I - \kappa(p) \lambda(n) p S - \theta_1 I
\]  

(1)

\[
\frac{\partial I}{\partial t} = \mu \nabla^2 I - \chi \nabla \cdot (\nabla f) + \eta_1 \lambda(n) I + \kappa(p) \lambda(n) p S - \theta_1 I
\]  

(2)

\[
\frac{\partial R}{\partial t} = \mu \nabla^2 R - \chi \nabla \cdot (\nabla f) + \beta_1 \lambda(n) R + \theta_1 I
\]  

(3)

\[
\frac{\partial m}{\partial t} = D_m \nabla^2 n - \lambda(n)(S + I + R)/Y_n
\]  

(4)

\[
\frac{\partial a}{\partial t} = D_a \nabla^2 a - \gamma(a)(S + I + R)
\]  

(5)

\[
\frac{\partial p}{\partial t} = \alpha((1 - \eta_1)\lambda(n) I + (1 - \beta_1)\lambda(n) R)
\]  

(6)

Herein, \(\mu\) and \(\chi\) are the effective diffusion and chemotactic coefficient of bacteria, respectively; \(D_m\) and \(D_a\) are diffusion coefficients of the nutrient and chemoattractant, respectively. \(\eta_1\) and \(\beta_1\) are the growth suspension rate of infected bacteria and recovered bacteria, \(\theta_1\) is the recovery rate of infected bacteria, \(\alpha\) is the production rate of phage, \(Y_n\) is the yield nutrient consumption.

Chemotactic movement of bacteria depends on the concentration of the local attractant as follows (Liu et al., 2019)

\[
f = \log \frac{1 + \frac{S}{K_2}}{1 + \frac{S}{K_1}}
\]  

(7)

where \(K_1\) and \(K_2\) are the lower and upper Weber offset of attractant sensing.

Bacterial cell growth is described with consideration of the growth limitations caused by nutrient availability (Murray, 2002), and follows a Monod relation

\[
\lambda(n) = \frac{\lambda_0 n}{n + \lambda_k}
\]  

(8)

with the Monod constant \(\lambda_k\) and the growth rate \(\lambda_0\).

Similarly, phage infection efficiency is described by a Monod relation

\[
\kappa(p) = \frac{\kappa_0 p}{p + p_k}
\]  

(9)

with the Monod constant \(p_k\) and the phage infection efficiency \(\kappa_0\).

In the same way, chemoattractant uptake is described by a Monod relation (Fu et al., 2018; Cremer et al., 2019):

\[
\gamma(a) = \frac{\gamma_0 a}{a + a_k}
\]  

(10)

with the Monod constant \(a_k\) and the uptake rate of chemoattractant \(\gamma_0\).

**Simulations**

The equations of the above bacteria–phage interaction models were integrated in MATLAB (R2019a) using second-order-centered differences for the spatial derivatives (mesh size 100 \(\mu\m\)) and an explicit fourth-order Runge–Kutta routine for temporal integration (time step 1 s) in a Cartesian coordinate system. The parameters used in this study are summarized in Appendix Table S2.

Boundary conditions obey zero diffusive (\(\partial_t \Phi = 0, \partial_r \Phi = 0\), where \(\Phi = S, I, R, n, a, p\)). The simulation starts with a locally restricted susceptible cell density \(S^{\text{init}}\), in the radial distance \(r_0 (r < 2 \text{ mm})\) from the center of the simulation region, \(S^{\text{init}} = S_0 e^{-\frac{r^2}{\Phi}}\) \((\Phi = 0.2 \text{ OD}_{600}\) and \(r_0 = 1 \text{ mm}\)), and the initial phage density at the inoculated site is \(p^{\text{init}} = p_0 e^{-\frac{r^2}{\Phi}}\), where \(r_0 = 1 \text{ mm}\), and \(p_0 = 1 \text{ a.u.}\). The initial nutrient and attractant concentrations are homogeneously distributed with the concentration \(n_0 = 30 \text{ mM}\) and \(a_0 = 60 \text{ \mu M}\).

**Competition simulation**

We further extended the RESIR model to the phage competitive model that can describe the competition between two phages, in which the phages were assumed to infect with different progeny phage production rates, despite the same infection efficiency. The phage competition infection efficiency is described as

\[
\kappa(p) = \frac{\kappa_0 (p_1 + p_2)}{(p_1 + p_2) + p_k}
\]  

(11)

The boundary conditions are the same as the former model, that is, all terms obey zero diffusive flux. The bacteria cell, nutrition, and chemoattractant initial conditions are the same as the former model, except for the phage. For initial conditions of the phage, the unequal (or equal) initial uniform mixture of two phages is located at the same position and the same initial density profile as the former model, that is, \(p_1^{\text{init}} = p_0 e^{-\frac{r^2}{\Phi}}\) and \(p_2^{\text{init}} = C p_0 e^{-\frac{r^2}{\Phi}}\), \(C\) representing the ratio of strong and weak phages in the initial mixture.

We further considered the possibility that resistant bacterial cells immune to phage infection at all times exist in the population. Experimental results showed that ~90% of the bacterial cells exhibit fluorescence signals 10 h after infection by a reporter phage carrying red fluorescent protein gene in its genome, and this percentage could maintain over an extended culturing period (Appendix Fig S9A). Subsequent transfer of such preinfected (recovered) cells into fresh medium and inoculation of another reporter phage carrying green fluorescent protein gene did not produce any cells exhibiting green fluorescence signals (Appendix Fig S9B). These results suggested that at least 90% of bacterial cells were infected and then became resistant, and this percentage could be maintained over time. The rest 10% were possibly immune to phage infection for unknown reasons even before exposure to phages. Nevertheless, even when we assume that 10% of the bacterial cells are indeed resistant to phage infection at the beginning, the conclusions of the model simulated phage spatial competition are not changed (Appendix Fig S10).

**Data availability**

Major data for figures have been included in Source Data. Modeling and analysis code have been submitted to GitHub with a link https://github.com/YiZhangsiat/RESIR-model.
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Author contributions

Ting Wei: Data curation; investigation; methodology; writing – original draft; writing – review and editing. Wangsheng Lai: Data curation; investigation; methodology; writing – original draft. Qian Chen: Data curation; investigation; methodology; writing – original draft. Yi Zhang: Software; methodology; writing – original draft. Chenjian Sun: Data curation; investigation; writing – original draft. Xiong He: Writing – review and editing. Guoping Zhao: Writing – review and editing. Xiongfeng Fu: Software; supervision; methodology; writing – review and editing. Chenli Liu: Conceptualization; supervision; methodology; writing – original draft; project administration; writing – review and editing.

Disclosure and competing interests statement

The authors have filed a provisional patent application on the SPACE system and related improvements.

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Figure EV1. Kinetic model of the interaction between bacteria and phage.

A The bacterial populations are classified into three categories: susceptible, infected, and recovered bacteria. Infected bacteria are converted from susceptible bacteria by phage infection, and eventually become recovered bacteria. These three bacterial populations all proliferate by consuming the nutrition in the semisolid culture media. In our experimental system, motile bacteria expand their range into unoccupied territories by diffusion and chemotaxis. In the meantime, the nonmotile phages are transmitted by their host bacteria, and their titer depends on the level of infectious progeny phage production by infected and recovered bacteria. The details of this model are described in Materials and Methods and Appendix Table S2.

B The spatiotemporal dynamics of phage infection in the radial direction (①) and the lateral direction (②) of the fan-shaped infection region are distinct from each other. In the radial direction, the expansion of infection range is driven by a “hitchhiking effect” (Ping et al., 2020), that is, phages are transported by the bacteria at the moving front. The bacterial population at the front experiences an active infection process involving the emergence of infected and recovered cells, the annihilation of susceptible cells, and eventually all cells become recovered cells, maintaining a balance between cell growth at the front and back diffusion (Cremer et al., 2019) (③). Differently, along the sideward edge, the expansion of infection range is driven by a “relay effect” of infected bacteria in the lateral direction, that is, infected bacteria from the infected region invade into the uninfected region, in which they produce phages continuously encountering and infecting susceptible bacterial cells. This cycle repeats and shows a relay-like effect, effectively generating a moving boundary between the infected and uninfected regions, which is eventually presented as the sideward edge (②).

C Model prediction showing that the size of the fan-shaped pattern is positively correlated with the phage production rate.

Source data are available online for this figure.
Figure EV2. Relationship between the area of the fan-shaped pattern and the gIII expression level.

A. An activity-dependent phage propagation cassette on accessory plasmid. Expression level of gIII is under the control of a library of synthetic promoter variants. Selection phage carries a wild-type T7 RNA polymerase (RNAP) gene in place of its gIII. The T7 RNAP exhibits different transcriptional activities on different promoter variants.

B. Relative expression levels of 17 synthetic promoter variants (sequences shown in Appendix Table S3) were determined by in vivo transcriptional activity assay (Materials and Methods). Error bars represent s.d. of three biologically independent assays.

C. Photographs of a quarter of the semisolid agar plates with typical patterns obtained for bacteria carrying accessory plasmids containing representative promoters with different expression activities in (B). Scale bar represents 1 cm.

D. Relationship between the area of the fan-shaped pattern and the gIII expression level. Data represent mean ± s.d. for at least three biological replicates. Fitting lines are generated with functions $y = 10 + 600x/(0.8 + x)$ and $y = 700x/(12 + x)$ for high- and low-copy accessory plasmids, respectively.

Source data are available online for this figure.
Figure EV3. Competition between weak and strong phages with an initial titer ratio of 1:1.
A Simulated kymograph of weak (PW) and strong (PS) phage titers in the bacterial expanding front as shown by the red dashed arc line in the schematic with an initial titer ratio of 1:1. The production rates of PW and PS are set as 40 and 100, respectively.
B The experimental result shows raw photographs of a representative two-phage competition assay after initial inoculation with 1:1 mixture of M13s and M13w at 1 cm away from the center. The simulation result is the outcome of competition between two phages with relative production rates as those in panel (A). Scale bar represents 1 cm.
C Profiles of the fluorescence intensity along the central radial line and the sideward edge of the fan-shaped infection zone in the experimental result in (B). The relative intensities were obtained by dividing the detected values with the maximum value of red or green fluorescence intensity, respectively.
D Plots of the simulated phage-titer profiles of PW and PS after 24-h competition.
Source data are available online for this figure.

Figure EV4. Improved expression activity of RNAP mutants.
A Improvement in RNAP recognition of 10 selected synthetic promoters as shown in Fig 4B is demonstrated visually, by comparing the area size of fan shapes formed by phages carrying corresponding mutant RNAP genes with those carrying the wild-type T7 RNAP gene (Anc.). Escherichia coli FM15 cells carrying low-copy accessory plasmids were used. The images of a quarter of the agar plate containing a representative fan-shaped pattern are shown.
B Fold changes in the relative expression activity of RNAP mutants on their corresponding target promoters. The activity of the mutants was measured either by the in vivo transcriptional assay based on flow cytometry (Materials and Methods) or converted from the area of fan-shaped pattern using the transfer function in Fig EV2D. Data represent mean values ± s.d. for three biological replicates.
Source data are available online for this figure.
Exploiting spatial dimensions to enable parallelized continuous directed evolution

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Calculation of phage generation time of SPACE system

We estimated the phage generation time using the same model proposed for PACE (Esvelt et al., 2011). This model is based on an assumption that lagoons, in which the phage-assisted evolution takes place, operate in a steady state that the total phage and cell concentration does not change over time. The average phage generation time in this model is equal to $\frac{2}{\text{dilution rate}}$ (Esvelt et al., 2011). In SPACE, the bacterial propagating front composed of exponentially growing and steadily advancing cells provides a constant flow of fresh host. Once infected by the phages, bacterial cells are able to migrate and carrying along progeny phages. It is analogous to a lagoon. Those left behind the propagating front are considered as outflow of the lagoon. Therefore, the dilution rate in SPACE system should be equal to (cell expansion speed /front width).

The cell expansion speed measured from time-lapsed images (Fig 1C) is approximately 3.89 mm/h, and the front width is 1.10-1.41 mm, so the dilution rate of SPACE under this condition is 2.76-3.54 h$^{-1}$, comparable with the dilution rate of PACE (2.5-3.2 h$^{-1}$) (Esvelt et al., 2011). In the meantime, the effective duration of the evolution in a single run is calculated by dividing the distance from the phage inoculation spot (10 mm from the center) to the edge of the agar plate with cell expansion speed, which is 8.35 h for an 8.5-cm plate, and 16.71 h for a 15-cm plate. Thus, a single run using an 8.5-cm agar plate corresponds to approximately 12-15 phage generations. Longer effective duration provided by a 15-cm agar plate allows 23-30 generations in a single run over night. More phage generations could be achieved by simply transferring to a new agar plate and start another run.
Appendix Figure S1 - Growth curves of *E. coli* and phage production after infection.

A Growth curves of uninfected, freshly infected, and pre-infected (recovered) bacteria. *E. coli* FM15 cells were cultured in LB broth. Bacteria are FM15 cells without phage inoculation; Bacteria + phage are cells with $10^8$ PFU/ml of M13 phages added at the 0 time point. Pre-infected (recovered) bacteria are cells co-cultured overnight with phages, washed to remove phages in the supernatant, and transferred to fresh LB broth. For all three groups, the initial OD$_{600}$ of the bacterial suspension was adjusted to 0.01.

B Phage titer in the supernatant of collected samples quantified by qPCR.

C Phage number per cell calculated by dividing phage titer with the cell density quantified by flow cytometry. Data represent mean values ± s.d. for three biological replicates.
Appendix Figure S2 - Model simulation of phage infection process.
A Growth curves of *E. coli* FM15 cells in liquid culture without phage, or with $10^7$ PFU/ml phages added at the beginning of the experiment.
B Model simulated bacterial growth curve without phage, or with an initial phage titer of 0.01 a.u. in a well-mixed system.
Appendix Figure S3 - Time-lapse plots of the simulated relative bacterial cell-density and nutrient profiles along an arc line across the infected region with a fixed radius.

A Schematic of a simulated fan shape formed after 20 h of bacterial range expansion. Yellow filled circle represents bacterial lawn. The fan-shaped infected zone is signified by purple color.

B Bacterial cell density and nutrient concentration values obtained from an arc line with a fixed radius across the fan shape as shown in (A). The model parameters used were identical to those used in the bottom two rows of Fig 1C. The fixed radius was set at 25 mm from the center of the agar plate.
Appendix Figure S4 - Competition between weak and strong phages with an initial titer ratio of $10^5:1$.

A, B Profiles of the fluorescence intensity along the central line and the edge of the fan-shaped infection zone in the experimental result in Fig 3C. The relative intensities were obtained by dividing the detected values with the maximum value of red or green fluorescence intensity, respectively.

C, D Simulated profiles of phage titer of the weak phage (PW) and the strong phage (PS) along the central line and the edge of the fan-shaped infection zone after competition as shown in Fig 3C.
Appendix Figure S5 - Gating strategy used in flow cytometry analysis.
A-D Gating strategy used in cell counting. The DAPI-positive particles in P1 in panel (A) were regarded as bacterial cells. The FSC-H/SSC-H plots of populations inside and outside of P1 are shown in panels (B) and (C), respectively. The FSC-H/SSC-H plot of the staining buffer (0.9 % NaCl with 1 μg/ml DAPI) is shown in panel (D).

E-I Gating strategy used in the measurement of in vivo transcriptional activity. Particles in P1 in panel (E) were regarded as bacterial cells, and the population formed by these particles is shown in panel (F). The FSC-H/SSC-H plot of phage-infected bacterial cells is shown in panel (G). (H) For the measurement of in vivo transcriptional activity, V1L covering 99.95% of the population in an uninfected bacterial sample based on the distribution of FITC-H signal values was set to determine the background fluorescence. This gate was applied to phage-infected samples of the same bacterium, and particles falling in V1R as shown in panel (I) were used to measure the transcriptional activity.
(Fig S6, Part 1)
| Mut6 | Mut7 | Mut8 | Mut9 | Mut10 |
|------|------|------|------|-------|
| ![Graph](image1.png) | ![Graph](image2.png) | ![Graph](image3.png) | ![Graph](image4.png) | ![Graph](image5.png) |
| ![Graph](image6.png) | ![Graph](image7.png) | ![Graph](image8.png) | ![Graph](image9.png) | ![Graph](image10.png) |
| ![Graph](image11.png) | ![Graph](image12.png) | ![Graph](image13.png) | ![Graph](image14.png) | ![Graph](image15.png) |
| ![Graph](image16.png) | ![Graph](image17.png) | ![Graph](image18.png) | ![Graph](image19.png) | ![Graph](image20.png) |
| ![Graph](image21.png) | ![Graph](image22.png) | ![Graph](image23.png) | ![Graph](image24.png) | ![Graph](image25.png) |
| ![Graph](image26.png) | ![Graph](image27.png) | ![Graph](image28.png) | ![Graph](image29.png) | ![Graph](image30.png) |
| ![Graph](image31.png) | ![Graph](image32.png) | ![Graph](image33.png) | ![Graph](image34.png) | ![Graph](image35.png) |
| ![Graph](image36.png) | ![Graph](image37.png) | ![Graph](image38.png) | ![Graph](image39.png) | ![Graph](image40.png) |
| ![Graph](image41.png) | ![Graph](image42.png) | ![Graph](image43.png) | ![Graph](image44.png) | ![Graph](image45.png) |

(Fig S6, Part 2)
Appendix Figure S6 - Histograms obtained for the measurement of in vivo transcriptional activity based on flow cytometry.

Results from one out of three replicated assays to measure the expression activity of the wild-type and 10 mutant RNAPs on T7 promoter and 10 promoter variants in Fig 4D are shown in this figure. Results from the other two replicates are included in the Source Data.
Appendix Figure S7 - Enrichment of PS by SPACE system and chemostat-based competition system by model simulation.

A, B Simulated fold change of PS/(PS+PW) and PS accumulation rate in SPACE and a chemostat-based competition system with a significant difference in the strength of PS and PW. Initial PW: PS is 10^5:1. Production rates of PW and PS are 40 and 100, respectively. Fold change along the sideward edge of infection zone under a SPACE condition the same as in Fig 3 is compared with that in well-mixed chemostat-based competition system using different dilution rate (DR, volumes of the liquid system exchanged by continuous flow per hour). Data for liquid system in (B) are obtained by calculating the slope of curves on logarithmic scale with varied DR as shown in (A).

C, D Simulated fold change of PS/(PS+PW) and PS accumulation rate in SPACE and a chemostat-based competition system with a mild difference in the strength of PS and PW. Initial PW: PS is 10^5:1. Production rates of PW and PS are 90 and 100, respectively.
Appendix Figure S8 - Simulated competition of five phage strains with different bacterial expansion speed. Production rates of Phage 1-5 are 80, 85, 90, 95, and 100, respective. The initial mixture of phage inoculant consists of the five strains, each composing 20% of the whole population. Graphs show the fold change of the relative abundance of each phage at the bacterial expanding front near the edge as compared to that at the initial phage spot 3 h and 4 h after phage infection.
Appendix Figure S9 - Ratio of bacterial cells with fluorescent signals after infection by reporter phages.

A Time course of the percentage of cells exhibiting red fluorescence signals after overnight pre-infection by marker phages. *E. coli* FM15 cells in LB broth were infected by M13s used in Fig 3, the reporter M13 phage with a red fluorescent protein gene in its genome, for 10 hours. These pre-infected cells were washed to remove supernatant phages, and 1:500 transferred to fresh LB broth to continue culturing with fluorescence measurements at different time points.

B Time course of the percentage of cells exhibiting green fluorescence signals after M13-GFP infection of pre-infected and uninfected fresh bacterial cells. Pre-infected cells were prepared as in (A).

The ratio of fluorescent cells was measured by flow cytometry using PE and FITC channels for red and green fluorescence, respectively. Data represent mean values ± s.d. for three biological replicates.
Appendix Figure S10 - Simulated competition of two phages with a host bacterial population containing resistant cells.

A, B The outcome and fold enrichment of the strong phage in simulated competition assay with an initial of weak phage (PW): strong phage (PS) at $10^5$:1. The production rates of PW and PS are 40 and 100, respectively. In the host bacterial population, 10% of cells resistant to phage infection all the time was assumed for this simulation. In panel (B), the fold enrichment along the central radial line and sideward edge of infection zone is compared between two cases: bacterial population containing 0%, or 10% of cells resistant to phage infection.
### Appendix Table S1. Bacterial strains used in this study.

| Strain name               | Genotype and remarks                                                                 | Reference or source          |
|---------------------------|--------------------------------------------------------------------------------------|------------------------------|
| E. coli MG1655-mCherry    | $F\lambda-\text{ilvG-rfb-50 rph-1 attB::mCherry (AmpR)}$                           | Ref. (Liu et al., 2019)      |
| E. coli ER2738            | $F'\text{proA+B+ lacF}\Delta(lacZ)M15\text{zzf::Tn10 (TetR)}/\text{fhuA2 glnV}\Delta(lac-proAB)\text{thi-1}\Delta(hsdS-mcrB5}$ | NEB                          |
| E. coli DH5α              | $F\varphi80d\text{laczDA15}\Delta(lacZYA-argF)\text{U169 endA1 recA1 hsdR17(\text{r}, \text{m}) supE44\lambda- thi-1 gyrA96 relA1 phoA}$ | TransGen Biotech, Beijing    |
| E. coli BL21              | $F\text{dcm ompT hsdS(\text{r}, \text{m}) gal [malB']}\text{K-12 (}\lambda\text{S)}$ | TransGen Biotech, Beijing    |
| E. coli FMG1655-mCherry   | $F'\text{proA+B+ lacF}\Delta(lacZ)M15\text{zzf::Tn10 (TetR)}/\lambda-\text{ilvG-rfb-50 rph-1 attB::mCherry (AmpR)}$ | This study                   |
| E. coli FMG1655-mCherry Δ(lacZ)M15 | $F'\text{proA+B+ lacF}\Delta(lacZ)M15\text{zzf::Tn10 (TetR)}/\lambda-\text{ilvG-rfb-50 rph-1 attB::mCherry (AmpR)}\Delta(lacZ)M15$ | This study                   |
| E. coli FM15              | $F'\text{proA+B+ lacF}\Delta(lacZ)M15\text{zzf::Tn10 (TetR)}/\lambda-\text{ilvG-rfb-50 rph-1 attB::KanR \Delta(lacZ)M15}$ | This study                   |

### Appendix Table S2. Model parameter

| Parameter                        | Symbol | Value                  | Reference or parameter variation |
|----------------------------------|--------|------------------------|----------------------------------|
| Effective diffusion coefficient  | $\mu$  | $60 \mu m^2/s$         | Ref. (Liu et al., 2019)          |
| Chemotactic coefficient          | $\chi$ | $410 \mu m^2/s$        | Fit to match expansion speed for expansion in reference condition. |
| Diffusion of nutrient            | $D_n$  | $800 \mu m^2/s$        | Ref. (Cremer et al., 2016)       |
| Diffusion of attractant          | $D_s$  | $800 \mu m^2/s$        | Ref. (Cremer et al., 2016)       |
| Growth rate                      | $\lambda_0$ | $2.08 h^{-1}$       | Average growth rate in LB medium. |
| Yield nutrient consumption       | $Y_n$  | $0.064\text{ OD}_{600}/\text{mM}$ | Ref. (Liu et al., 2019)          |
| Monod constant nutrient uptake   | $n_k$  | $0.4 \text{ mM}$       | Defined by fitting the growth curve of E. coli. Appendix Figure S2 |
| Lower Weber offset attractant sensing | $K_1$ | $3.5 \mu M$ | Ref. (Yang et al., 2015) |
| Upper Weber offset attractant sensing | $K_2$ | $1000 \mu M$ | Ref. (Yang et al., 2015) |
| Uptake rate chemoattractant      | $g_0$  | $9 \mu M/(\text{min} \cdot \text{OD}_{600})$ | Ref. (Fu et al, 2018)            |
| Monod constant attractant uptake | $s_k$  | $1 \mu M$              | Ref. (Schellenberg & Furlong, 1977) |
| Phage production rate            | $\alpha$ | $0.1 \sim 100 \text{ a.u.}/\text{OD}_{600}$ | Production rate varied to match the resultant fan shape size at different levels of $\text{gIII}$ expression |
Infection ability of phage

Monod constant phage uptake

Growth coefficient of infected cell

Growth coefficient of recovered cell

Recovered coefficient of infected cell

Initial concentration of nutrients

Initial concentration of attractant

| Number | Sequence |
|--------|----------|
| 1A1    | ACCACAGTGACCTACAGGGAGA |
| 1A2    | TAATACGAGTCCTACAGGGAGA |
| 1A3    | TCATACGAGTACCTACAGGGAGA |
| 1A4    | ATCGGAGTAAACGAGAAGGGAGA |
| 1A5    | AACTACCTCTTCTAGCCGGAGA |
| 1A6    | GTTCGGGATTATGCCGGAGA |
| 1A7    | GAATGCACACCTCCATAGGGAGA |
| 1A8    | TAATACGACACACCATAAGGGAGA |
| 1A9    | GGATATGTACCTCAGAAAGGGAGA |
| 1A10   | ACGCAGAACAGTACTAGGGGAGA |
| 1A11   | CCTAGTCTTTACGAGATGGGAGA |
| 1A12   | AAGTACCCCTTACCCCTGGAGA |
| 1B1    | TAAGACGAAACACGATGGGAGA |
| 1B2    | TAATACGACGACAGATGGGAGA |
| 1B3    | TATAGCTAGGACGAAAGGGAGA |
| 1B4    | TAATACGATGACGAAAGGGAGA |
| 1B5    | TAATACGCTCTCAGGGGAGA |
| 1B6    | AAACGCCACTCACTAGGGGAGA |
| 1B7    | AAGCGGCACTCAATGAGGGGAGA |
| 1B8    | TAATCCTCCTCAATAGGGGAGA |
| 1B9    | GGGAGAATTAACGGGAGGGAGA |
| 1B10   | GAATGCAACTCAGGAAAGGGAGA |
| 1B11   | AGTAAACCTCGTTACAGGGGAGA |
|   | DNA Sequence                   |
|---|-------------------------------|
| 1B12 | GTTAGAGCAACCATTCTGGGAGA     |
| 1C7  | TAGTACGCCCCCACTATAGGGGA      |
| 1C8  | ACATACGATTCAACGCGGGGAGA     |
| 1C9  | AGGACTCTGAGTACCTGGGGAGA     |
| 1C10 | TATCGAGCTCCCACAAGGGGAGA     |
| 1C11 | ATGAAGCGTCACTAGGGGAGA       |
| 1C12 | TAAATACGACTTACATAGGGAGA     |
| 1D1  | TTAGAATTCCGTTGACAGGGGAGA    |
| 1D2  | CAAAGACTTCTGACGATAGGGGAGA   |
| 1D3  | TTAGAGCTTCTCGTTACAGGGGAGA   |
| 1D4  | CGGGTGATATAGGTCAGGGGAGA     |
| 1D5  | TTAGTGAGGCTTACATAGGGGAGA    |
| 1D6  | TGGTGAGCTTACATAGGGGAGA      |
| 1D7  | TTTAATAACTTCTGCGAGGGGAGA    |
| 1D8  | TGTATAGCTTCTGTTACAGGGGAGA   |
| 1D9  | GCTAGAATTCGGTTGACAGGGGAGA   |
| 1D10 | GTATAAGTATTCGGCAGGGGAGA     |
| 1D11 | TTGGCCACTTTGAGACTAGGGGAGA   |
| 1D12 | AAATACGACTTACATAGGGGAGA     |
| 1E1  | GAAGACGTCTCTCATATAGGGGAGA   |
| 1E2  | GCTAGGATTACGCTATAGGGGAGA    |
| 1E3  | GCTAGGATTACGCTATAGGGGAGA    |
| 1E4  | GCTAGGATTACGCTATAGGGGAGA    |
| 1E5  | TCCCCTTTGCCGGTTGGGAGA       |
| 1E6  | TCCCCTTTGCCGGTTGGGAGA       |
| 1E7  | TAACACTTCTCACAGATAGGGGAGA   |
| 1E8  | CAATGCTCCTCATACAGGGGAGA     |
| 1E9  | GAAATACGACTTACATAGGGGAGA    |
| 1E10 | GAAATACGACTTACATAGGGGAGA    |
| 1E11 | TCACGCACACACCTATAGGGGAGA    |
| 1E12 | ATCTCCCGTACAGATAGGGGAGA     |
| 1G1  | TCTAGGAAATGCTCCATAGGGGAGA   |
| 1G2  | ATGATCCCGGTAGTCAAGGGGAGA    |
| 1G3  | TAGTACGCGTACATAGGGGAGA      |
| 1G4  | ATTCTGTTCTGATCCGGGGGAGA     |
| 1G5  | CTTCGTGTCCGATTAGGGGAGA      |
| 1G6  | GTACCCCTTTAACTCTGGGAGA      |
| 1G7  | GTACCCCTTTAACTCTGGGAGA      |
| 1G8  | TAAATACGCTCACCACAGGGGAGA    |
| 1G9  | AATTGACCTCACCAGGGGAGA       |
| 1G10 | CGTGTATGTGATACCTGGGAGA      |
| 4A1  | TAATACGCTCACCACAGGGGAGA     |
| 4A2  | GAATGCGACTCCTCATAGGGGAGA    |
| 4A3  | AAATACGACTTACATAGGGGAGA     |
| 4A4  | TAGGACGTAGCCGAATAGGGGAGA    |
| 4A5  | GCTAGGATTAAGCTAAGGGGAGA     |
| 4A6  | TTTACGATTCTGACACGGGAGA      |
| Plasmid name       | Class | Antibiotic resistance | Origin of replication | Promoter        | Genes                                   |
|-------------------|-------|------------------------|-----------------------|-----------------|-----------------------------------------|
| pLAa1~96          | AP    | Spe                    | pUC                   | P$_{T7}$ and variants | gIII                                    |
| pLAasc1~21        | AP    | Carb                   | SC101                 | P$_{T7}$ and variants | gIII                                    |
| pLAa188a          | AP    | Carb                   | SC101                 | P$_{psp}$       | gIII                                   |
| pLM1              | MP-§  | Chl                    | CloDF13               | P$_{psp}$       | pspABCDE-dnaQ926-dam-seqA               |
| pLRp              | RP    | Chl                    | CloDF13               | P$_{psp}$       | pspABCDE-sfGFP                          |
| pLAR1             | ARP    | Spe                    | ColEI                 | P$_{psp}$       | pspABCDE                                |
| pLAr1~21          | RP    | Carb                   | SC101                 | P$_{T7}$ and variants | sfGFP                                   |
| SP-T7             | SP    | f1                     | f1                    | P$_{gIII}$      | T7 RNAP WT                              |
| SP-T7mut          | SP    | f1                     | f1                    | P$_{gIII}$      | T7 RNAP mutant                          |

**Appendix Table S4.** Plasmids used in this study.
Appendix Table S5. Representative mutants obtained from parallelized SPACE of T7 RNAP

| Mutant no. | Amino acid changes            |
|------------|-------------------------------|
| Mut1       | E222G Q758R H772R             |
| Mut2       | E222K                         |
| Mut3       | H772R                         |
| Mut4       | E222A                         |
| Mut5       | E222G                         |
| Mut6       | K206R I244V                   |
| Mut7       | E242K                         |
| Mut8       | E222V D770N                   |
| Mut9       | K206R E222K                   |
| Mut10      | I244V                         |

Appendix References

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