

Orthogonal DNA replication system accelerates evolution and cell factory construction in *Escherichia coli*

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Directed evolution is a powerful approach for improving catalytic efficiency or changing enzymatic properties of proteins of interest, through iteratively generating diverse protein mutants and selecting for beneficial mutants.¹ During conventional directed evolution, mutations on DNA sequences are introduced *in vitro*, which needs to be further transformed into the competent

cell to obtain mutants with genetic diversity *in vivo*. Therefore, multiple cycles of transformation of DNA sequences with mutations and screening of beneficial mutants are usually labor-intensive and time-consuming, which may also be restricted by inefficient transformation that directly limits mutation library construction and further reduces the possibility of attaining improved

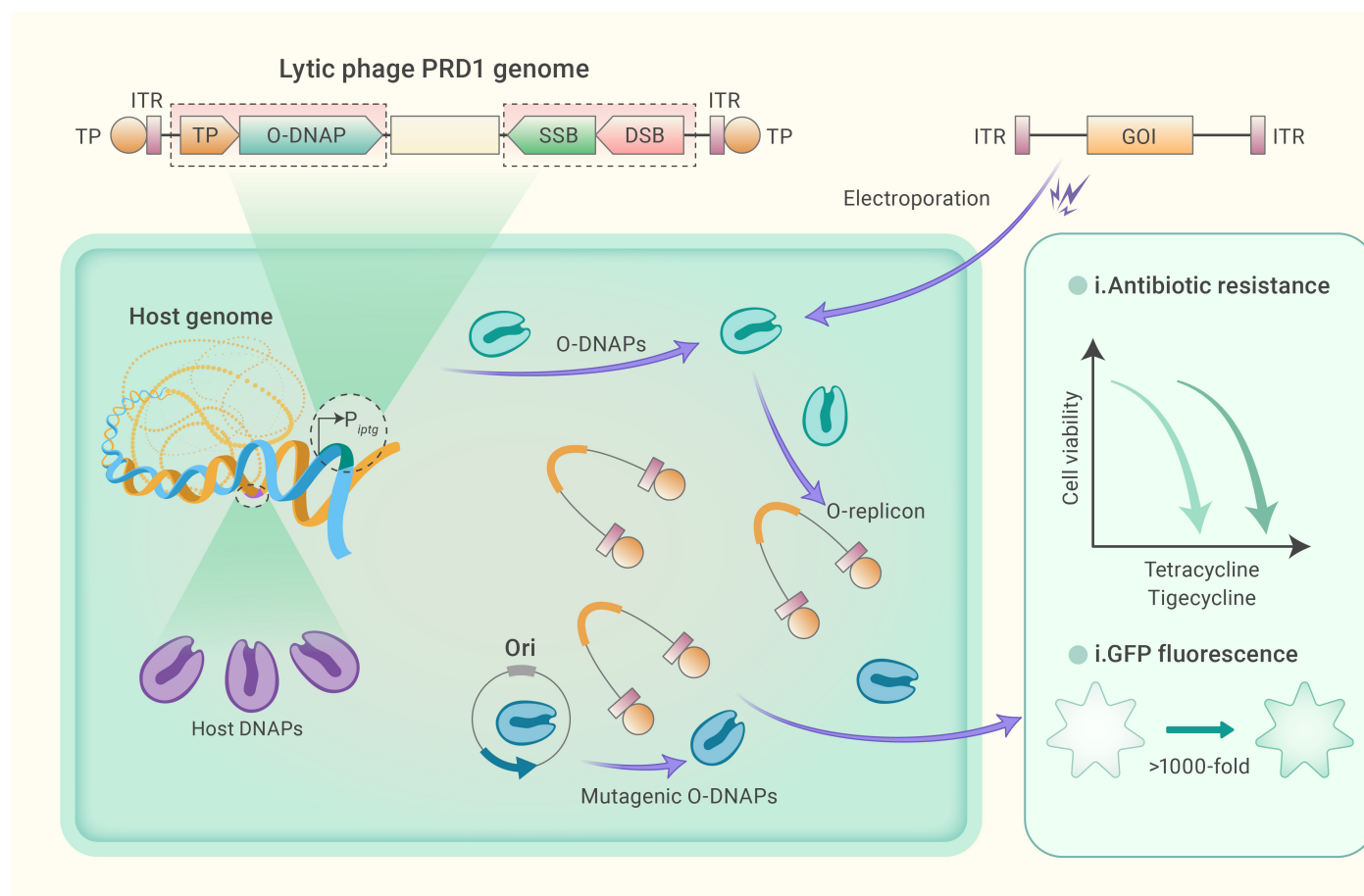


Figure 1. Construction and characterization of the orthogonal replication system in *E. coli* An orthogonal linear plasmid (o-replicon) that does not interfere with the replication of the host genome has been successfully developed in *E. coli*. The genes for the terminal protein (TP), orthogonal DNAP (O-DNAP), double-stranded DNA-binding protein (DSB), and single-stranded DNA-binding protein (SSB) from PRD1 phage are encoded and expressed under the control of an IPTG-inducible promoter in the *E. coli* genome. These proteins interact with the DNA sequence of the heterologous genes of interest (GOIs) containing inverted terminal repeats (ITRs), eventually obtaining an orthogonal replication linear plasmid *in vivo*. Moreover, GOIs can achieve rapid and independent evolution through the continuous action of designed-mutagenic O-DNAPs, such as significantly increasing the cell resistance to antibiotics and the fluorescence intensity of GFP in a short period of continuous evolution.

mutants. To this end, continuous evolution was developed for improving directed evolution by generating genetic mutations *in vivo* and yielding large numbers of mutants. However, two major constraints hinder continuous evolution: 1) random mutation introduction into DNA sequences encoding the proteins of interest for directed evolution and high-fidelity host cell DNA replication interfere with each other, reducing evolution efficiency; 2) the genomic error threshold of mutation cannot be surpassed when evolving the whole genome of the cell.

To facilitate continuous evolution, an orthogonal DNA replications system, OrthoRep composed of linear plasmid and orthogonal DNA polymerase

exclusively for linear plasmid DNA replication, was first developed in the model eukaryotic microorganism yeast *Saccharomyces cerevisiae*. OrthoRep avoids interferences between random mutation of DNA sequences encoding the proteins of interest and high-fidelity chromosome DNA replication.² With an engineered orthogonal DNA polymerase, the mutation rate of OrthoRep surpassed genomic error thresholds, reaching 1×10^{-5} substitutions per base pair (s.p.b.).³ More recently, the first orthogonal DNA replication system in bacteria, BacORep, was developed in *Bacillus thuringiensis*, which expanded using an orthogonal DNA replication system for continuous evolution of gene expression regulatory elements and a metabolic pathway in bacteria for

synthetic biology.⁴ However, an orthogonal DNA replication system is lacking in *Escherichia coli*, which is the most widely used microbial chassis cell in molecular biology and synthetic biology.

Tian et al. report establishing a synthetic orthogonal replication system enables accelerated evolution in *E. coli* (EcORep) that demonstrates efficient continuous evolution of gene expression regulatory elements and an antibiotic resistance gene *in vivo* (Figure 1).⁵ Specifically, the DNA replication machinery of PRD1, a lytic phage for *E. coli*, was adopted and redesigned for building a linear plasmid that orthogonally replicated in *E. coli*. Subsequently, *E. coli* stably harbors the linear plasmid for 300 generations, which is favorable for gene expression and evolution. Moreover, the copy numbers of the linear plasmid can be tuned from 2.5 to 1166 copies per cell, which holds both desired low copies for evolution and high copies for gene overexpression for recombinant protein expression. Next, by engineering orthogonal DNA polymerases for linear plasmid replication, the maximal mutation rate reached 7.6×10^{-6} s.p.b.. Finally, applicability of EcORep was demonstrated by evolving green fluorescence protein (GFP) gene for enhanced fluorescence intensity and tetracycline resistance gene for improved tetracycline resistance, which both shortened the evolution period from months to a couple of days, demonstrating the highly efficient evolution process.

One of the most important breakthroughs of EcORep's establishment is the *de novo* design and building of a synthetic linear plasmid in a microorganism that does not harbor any linear DNA replication machinery and mechanism. This opens the avenue for *de novo* design and building of synthetic linear plasmids that orthogonally replicate in other industrially or environmentally important microorganisms, such as *Corynebacterium glutamicum*, and *Pseudomonas putida*. Through the design-build-test cycle of orthogonal DNA replications system demonstrated during EcORep development, novel orthogonal DNA replication systems are expected to be built.

Noticeably, EcORep is more than a continuous replication system, which also demonstrates its great potential for recombinant protein production and metabolic pathway construction for biochemical-producing cell factory development. The high copy numbers of the linear plasmid of EcORep

reaches 1166 copies per cell in combination with highly stable plasmid retention for 300 generations constitute the two most important traits for recombinant protein expression. Therefore, pharmaceutically and industrially important proteins can be potentially produced in an efficient manner by the EcORep system. Moreover, synthetic gene cluster can be inserted into linear plasmids for metabolic pathway construction and evolution, which may contribute to the biochemical-producing cell factory. In summary, the synthetic orthogonal replication system in *E. coli* (EcORep) provides powerful tools for accelerating continuous evolution and cell factory construction through a synthetic biology approach.

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DECLARATION OF INTERESTS

The authors declare no competing interests.