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Efficient *in vivo* Directed Evolution in *E. coli* using a Gibson Assembly-Adapted EvolvR System

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Abstract

Genetic diversity is vital for species adaptation and evolution, enhancing resilience to environmental changes and improving desirable traits. Directed evolution simulates natural selection in labs to engineer proteins and microorganisms, utilizing iterative cycles of genetic variation to achieve desirable characteristics. EvolvR is a system that can continuously diversify all nucleotides during an adjustable window at user-defined locations. It is achieved by mutagenesis using engineered DNA polymerases directed to the target site via CRISPR-directed nickases. Although the typical plasmid assembly and gRNA insertion method is Golden Gate cloning, the aim of this study was to set up EvolvR according to our equipment and conditions. The gRNA targeting *rpsE* in *E. coli* DH5α was selected from a previous study. Specific primers that included the gRNA sequence and provided homology on one side were inserted into the EvolvR plasmid using the Gibson assembly method. The constructed plasmid was chemically transformed into *E. coli* DH5α. Bacterial resistance was evaluated by colony counting on culture media containing 50, 100, and 500 µl/ml of spectinomycin. Results showed that the number of *E. coli* DH5α cells in an antibiotic-free medium was 11×10^8 CFU.mL^{-1}, while no growth was observed at any antibiotic concentration. The non-induced EvolvR cells did not grow in a medium containing 100 and 500 µg. mL^{-1} spectinomycin, but grew at 20×10^6 CFU.mL^{-1} in a medium with 50 µg. mL^{-1} antibiotic. Induction of the EvolvR system resulted in a dramatic increase in spectinomycin-resistant mutants, yielding up to 4×10^8 CFU.mL^{-1} on 100 µg·mL^{-1} spectinomycin and a resistance frequency order of magnitude higher than previously reported. Our findings validate Gibson assembly as a robust and accessible alternative to Golden Gate for constructing EvolvR systems and emphasize the high efficacy achievable with a strategically targeted single gRNA.

1. Introduction

Genetic diversity is essential for the adaptation and evolution of species, serving multiple objectives such as enhancing resilience to environmental changes, improving agricultural traits, and facilitating scientific research (KhokharVoytas et al., 2023). The formal understanding of genetic diversity emerged in the 19th century with Charles Darwin's theory of evolution, which emphasized the importance of diversity in the process of natural selection (Adams, 2024). In the modern era, targeted evolutionary techniques have been developed, allowing scientists to apply natural evolutionary processes in laboratory settings to rapidly generate genetic diversity and select beneficial traits (Mavrommati et al., 2022; Singer et al., 2021). This approach has led to significant advancements in biotechnology, including the development of new enzymes and therapeutic proteins, underscoring the ongoing importance of genetic diversity in both natural ecosystems and human applications (Fatima et al., 2024). Directed evolution is a laboratory technique in biotechnology and microbiology that simulates natural selection to engineer proteins, enzymes, and microorganisms with desirable traits (Wang et al., 2021). This method results in biological entities with enhanced characteristics or functions, developed through iterative cycles of genetic diversity, selection, and amplification (Chavhan et al., 2024; Saini et al., 2020). The first step in directed evolution includes generating a library of variable genes, which can be achieved through random mutagenesis or DNA shuffling (Currin et al., 2021). This library can contain thousands to millions of variants, thereby increasing the likelihood of identifying a variant with desirable traits (Kumar & Singh, 2013). In the second step, these variants undergo screening to identify those exhibiting favorable characteristics such as increased activity or stability (Vidal et al., 2023). In the final step, the best-performing variants from each round are selected and amplified to serve as templates for subsequent rounds of mutagenesis and selection. This iterative process continues until a satisfactory level of improvement is achieved (Iqbal & Sadaf, 2022). Directed evolution is particularly valuable as it does not require prior knowledge of protein structure or specific mutation effects, making it

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a versatile tool for various applications in protein engineering and microbial strain improvement (Wang et al., 2023). EvolvR is an advanced technique in genetic engineering, particularly in the directed evolution of genes and strains, which combines CRISPR technology with a high-error-rate polymerase (PolI3M) to facilitate high-rate mutagenesis in specific genomic regions (Halperin et al., 2018). Utilizing a nickase version of Cas9 (nCas9), EvolvR generates single-strand breaks in DNA, thereby enabling the introduction of semi-random mutations within a defined editing window that can extend up to 350 base pairs (Rao et al., 2021). This method achieves a high mutation rate of approximately 10^{-8} mutations per nucleotide per generation, significantly enhancing genetic diversity compared to traditional methods. EvolvR supports continuous diversity over multiple generations, making it particularly useful for applications such as evolving new protein activities, studying cellular responses to environmental changes, and mapping protein-protein interactions (Tou et al., 2020). Additionally, the modular nature of EvolvR allows researchers to customize mutation rates and target multiple genomic sites simultaneously, positioning EvolvR as a powerful tool for synthetic biology, drug development, and the investigation of complex genetic functions (Sadanand, 2018).

Although the EvolvR system is a powerful tool for directed evolution, the insertion of gRNA into the plasmid using the Golden Gate assembly in the original protocol can be a barrier for the broad application of this method due to the limited accessibility and higher price of relevant enzymes. This study aimed to adapt the EvolvR system using the more common and flexible Gibson assembly method. The efficacy of this adapted system was tested by evolving spectinomycin resistance in *E. coli* DH5α by targeting the *rpsE* gene.

2. Materials and Methods

2.1. Culture medium, strain, and plasmid

In this study, Luria Broth (LB) medium was utilized for pre-culture and cultivation throughout all experiments, with its composition consisting of 10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ yeast extract, and 10 $g.L^{-1}$ sodium chloride. The cultures were incubated and shaken at 150 rpm overnight at 37°C. It is important to note that since the strain carried the EvolvR plasmid during the experiments, 50 $\mu g.mL^{-1}$ of kanamycin was also added to the culture medium (Benocci et al., 2018). Additionally, when solid media were required, 1.5% agar was incorporated. The *E. coli* DH5α strain was chosen for its high capability to accept and maintain plasmids (Vatanparast et al., 2024; Wang et al., 2020). The plasmid used in this study is pEvolvR-enCas9-PolI3M-TBD, which can be found under code 113077 on the Addgene website (García-García et al., 2020). This plasmid is classified as a low copy number with an approximate length of 12.7 Kb.

2.2. Gibson Assembly Method

The Gibson assembly method has proven to be an efficient and effective method for plasmid construction (De Munter et al., 2020). It allows the joining of several overlapping DNA pieces in a single isothermal reaction, regardless of the length of the fragments (Avilan, 2023), by utilizing three enzymes:T5 exonuclease, Phusion DNA Polymerase, and Taq DNA Ligase (Valiyev, 2021).

In this study, gRNA-A, designed by Halperin and colleagues in 2018, was selected to target the *rpsE* gene of the *E. coli* DH5α to induce mutations conferring resistance to the antibiotic spectinomycin. This gRNA targeted 119 nucleotides from the beginning of the *rpsE* gene (Table 1). Four primers suitable for Gibson assembly with overlapping regions of 28 and 30 bp were designed using SnapGene software version 3.2.2. Two primers were selected that contained overlapping regions corresponding to the gRNA of interest (Table 1). Using these primers, the EvolvR plasmid was amplified into two fragments of 5.2 kb and 6.5 kb, with PCRs conducted using the Spidi™ Pfu DNA polymerase kit (Parstous, C101101) (Figure 1) and visualized by agarose gel electrophoresis against a 1kb molecular marker (SinaClon), due to the observation of non-specific bands in the amplified fragments. The annealing temperature for fragment 6.5 kb was set at 60 °C, with 30 PCR cycles and an annealing time of 45 seconds. For fragment 5.2 kb, the annealing temperature was adjusted to 67 °C, using the same number of PCR cycles (30 cycles) and an identical annealing time of 45 seconds. Gel extraction was performed on the PCR products, followed by calculation of the required amounts for each fragment based on the concentration read using a Nanodrop, as per the BioLabs instructions. The concentrations of the larger and smaller fragments were measured using a Nanodrop at 20 and 36 ng/µl, respectively. For the Gibson Assembly reaction, the larger fragment was assumed to be the vector, and the smaller fragment was assumed to be the insert. According to the Gibson assembly protocol and due to the similar size of the fragments, 80 ng of the larger fragment and 180 ng of the smaller fragment were added to the microtube to ensure a vector-to-insert molar ratio of 1:2. Finally, the reaction volume was adjusted to 20 µl by adding 10 µl of the Gibson reagents (ReNAP Magic Cloning Kit, ReNAP Biotech). Each microtube was incubated at 50 °C for 1 hour using a thermocycler. This method was based on calculations from the biocalculator.com website and the standard principles of Gibson Assembly. The Gibson reaction product was then chemically transformed into the target strain by the heat-shock method, and the presence of the assembled plasmid was confirmed using primers listed in Table 2 and visualized in Figure 2. The assay was performed with both positive and negative controls to ensure the reliability of the results. The inner circle demonstrates pEvolvR with its different components. The outer circle illustrates fragments A (6.5 kb) and B (5.2 kb), represented in yellow and blue, respectively, for the large and small fragments. The insertion of gRNA would remove the black fragment in the outer circle that contains the GFP cassette (bright green arrow). GFP was used as a marker gene in the plasmid construct. *E. coli* colonies carrying the plasmid exhibited a faint green coloration. Also, the primer annealing sites for fragment amplification, gRNA insertion, and plasmid assembly are indicated in the outer circle. The primers for gRNA insertion are represented in purple, while copper shows primers for the homology region in each fragment.

Table 1. gRNA and Primers for the Gibson AssemblyMethod, Inserting gRNA into an EvolvR Plasmid

Primers & gRNA	Sequence (5' to 3')
gRNA-A	GCTCTGACTGTAGTTGGCGA
RgRNA-A	tctaaaacTCGCCAACTACAGTCAGAGCgctagcattgtaccaagggctgagctagctataaacg*
FHR-A	gccgcattcaagtacttcgacaccaccat
FgRNA-B	GCTCTGACTGTAGTTGGCGAgttttagagctagaaatagcaagttaaaataagg*
RHR-B	gatggtggtgtcgaagtacttgaat

Note. The gRNA-A sequence is shown in capital letters. The lower-case letters annealed to the plasmid backbone for reconstruction of the EvolvR plasmid by Gibson assembly.

Table 2. Primers used for Molecular Verification of the Assembled Plasmid

Primers	Sequence (5' to 3')
RgRNA-A	tctaaaacTCGCCAACTACAGTCAGAGCgctagcattgtaccaagggctgagctagctataaacg
pGEX3	GGAGCTGCATGTGTCAGAGG

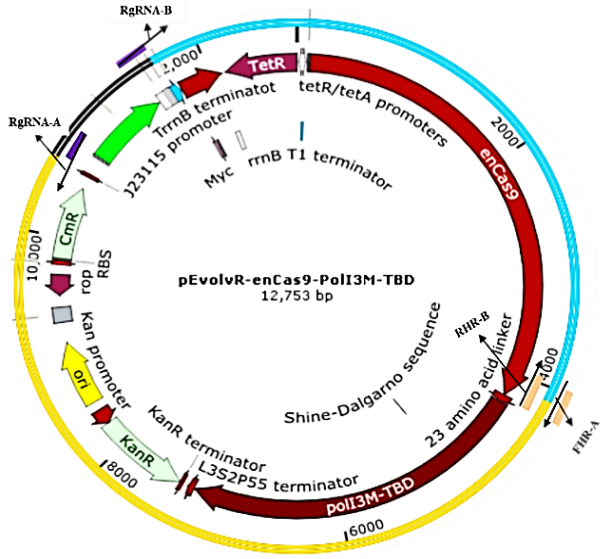

Figure 1. pEvolvR and Gibson Assembly Method

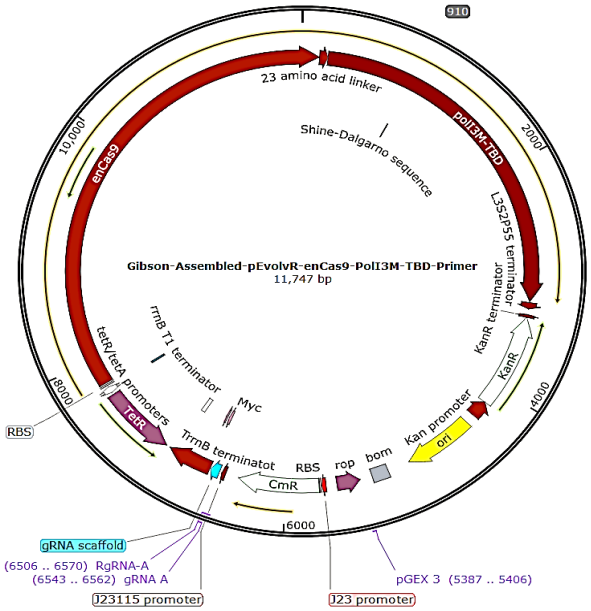

Figure 2. The Assembled Plasmid and Primers used for Molecular Verification (**Note.** The molecular confirmation of the plasmid was performed using the primers pGEX 3 and RgRNA-A.)

2.3. Directed evolution experiments

The control strain, *E. coli* DH5α transformed with the original pEvolvR plasmid (lacking the *rpsE*-targeting gRNA), and the experimental strain, which contained the newly constructed pEvolvR-gRNA plasmid, were cultured in LB+Kan medium until an optical density (OD) of 0.5 was reached. Expression of enCas9 and PolI3M-TBD in LB+Kan was induced by 400 ng.ml^{-1} tetracycline (DAYA EXIR) (Bertram et al., 2022).

After 16 hours of incubation at 37 °C and 150 RPM, the OD of the samples reached 2.5 and 2.4, respectively. Each sample was diluted to 10^{-7} using sterile physiological saline (0.89% NaCl) in test tubes. From the 10^{-5} and 10^{-6} dilutions, 100 µl was transferred to solid culture media containing spectinomycin at concentrations of 50, 100, and 500 µg.ml^{-1} respectively. The cultures were incubated for 16 h, and then the viable cells were counted three times. However, for better colony visibility, the medium with 500 µg.ml^{-1} spectinomycin was incubated for a longer period. These dilutions were also transferred to solid LB media without antibiotics to compare growth behavior. All OD values were measured at a wavelength of 600 nm.

3. Results and Discussion

3.1. Assembly of Plasmid Constructs and Their Phenotypic and Molecular Validation

After setting up the PCR reactions, the plasmid was split into two fragments, designated as A and B, with molecular sizes of 6.5 and 5.2 kbp, respectively. This was confirmed by running the PCR product on an electrophoresis gel and examining the resulting bands along with the ladder (Figure 3). Due to the presence of non-specific bands, the volume of the PCR products for both fragments was adjusted to 80-100 µl, and then the relevant bands were extracted from the gel.

The Gibson assembly process was successfully employed to join the two desired DNA fragments,

resulting in the formation of a recombinant construct (Figure 4). Following this, the Gibson assembly product was transformed into *E. coli* DH5α, leading to the emergence of numerous colonies after an incubation period of 16-20 hours. These colonies were subsequently verified through phenotyping and molecular analysis. The superfolder GFP cassette within the pEvolvR-enCas9-PolI3M-TBD plasmid caused a green hue in the bacterial culture medium. The introduction of gRNA-A into this plasmid removes the superfolder GFP cassette, thereby eliminating the green coloration of the medium (Figure 5A). Additionally, *E. coli* DH5α, which harbored the integrated plasmid, was able to grow on culture media supplemented with kanamycin. A confirmatory PCR was conducted using primers listed in Table 2, with the expected size of the resulting PCR product being 1.1 kbp, a finding that was validated by analyzing the resulting band alongside a DNA ladder (Figure 5B). The assembled plasmid, along with the region where gRNA-A is located, can be seen in Figure 6. By comparing the resulting band to a DNA ladder, it was confirmed that the assembled plasmid exceeds 10 kbp in length. This finding supports the conclusion that the Gibson assembly process was executed correctly.

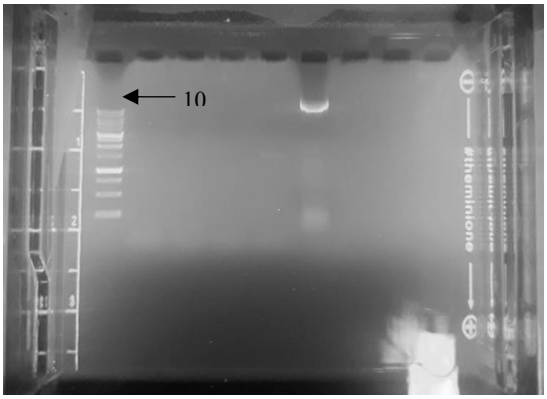

Figure 4. The Plasmid Obtained from the Gibson Assembly

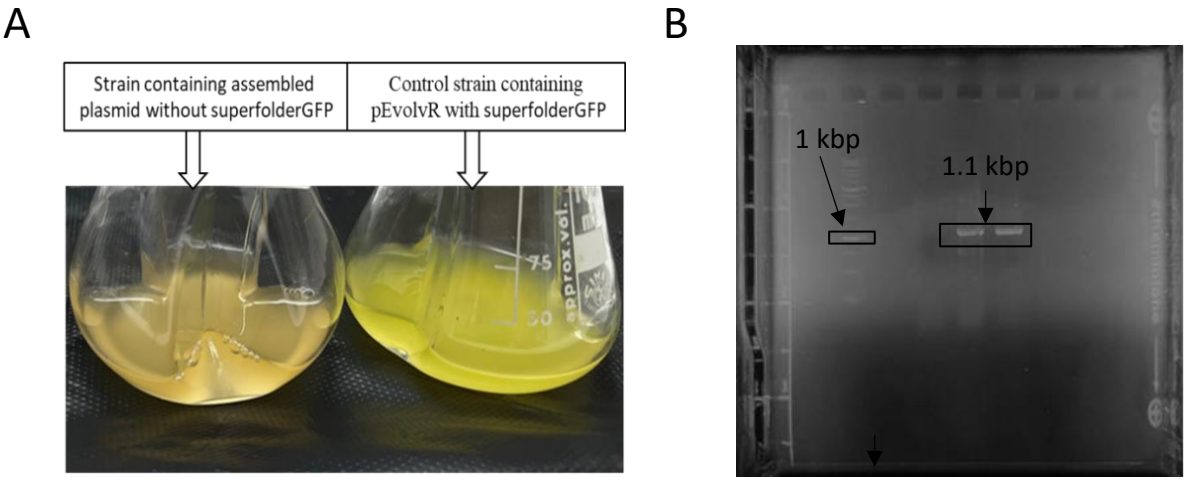

Figure 5. Phenotypic and Molecular Confirmation of the Assembled Plasmid (**Note.** The comparison of the two flasks demonstrates how (A) superfolderGFP deletion changes the culture medium color from green to cream, and (B) verifies the correct insertion of gRNA-A into a plasmid by observing a 1.1 kbp band.)

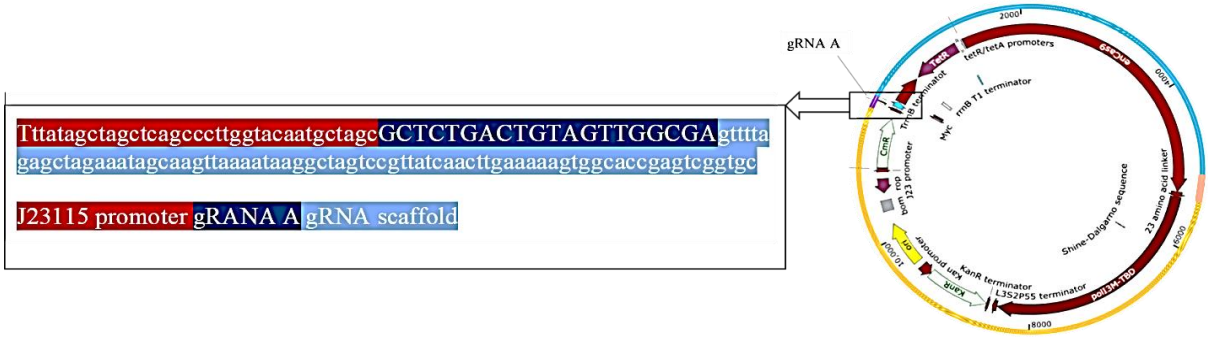

Figure 6. Schematic of the gRNA Expression Cassette in the Final Assembled Plasmid (**Note.** The gRNA sequence and location between the promoter and scaffold are shown in this figure.)

3.1.1. Induction of Plasmid Expression and Observation of Outcomes

The control for the induction step was *E. coli* DH5α carrying the pEvolvR-enCas9-PolI3M-TBD plasmid without gRNA-A.

The number of colonies of *E. coli* DH5α carrying the pEvolvR-enCas9-PolI3M-TBD plasmid without gRNA-A was 7×10^8 CFU.mL^{-1} in LB medium without antibiotics, and the number of colonies for cells carrying the pEvolvR-enCas9-PolI3M-TBD plasmid inserted by gRNA-A was 11×10^8 CFU.mL^{-1} in the same medium (Figure 7).

After induction with tetracycline some spectinomycin resistance colonies appeared in the agar, this number decreased as the antibiotic concentration increased from 50 to 500 µg.mL^{-1}. The results are presented in Table 3 and Figure 8 and 9.

These findings underscore the differential growth responses of *E. coli* DH5α under varying antibiotic concentrations and confirm the successful induction and selection of plasmid-bearing cells.

The ratio of resistant colonies to total viable colonies was calculated to better demonstrate the cells' resistance to different antibiotic concentrations (Table 4).

The purpose of this work was to count the number of viable cells to determine the ratio of resistant cells. It should be noted that only at this stage was an antibiotic-free culture medium used for counting the number of viable cells.

Table 3. The Number of Viable Cells for the Control and Induced Strains at Different Antibiotic Concentrations

Spectinomycin Concentration (µg. mL^{-1})	Colony count (CFU.mL^{-1})	
	Control	After induction
0	7×10^8	1.1×10^9
50	2×10^7	1.2×10^9
100	0	4×10^8
500	0	40

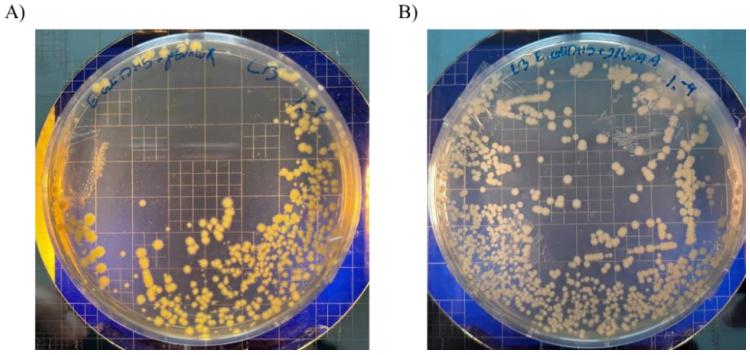

Figure 7. The Colony Counts Recorded for the (A) Control Strain and (B) the Experimental Strain.

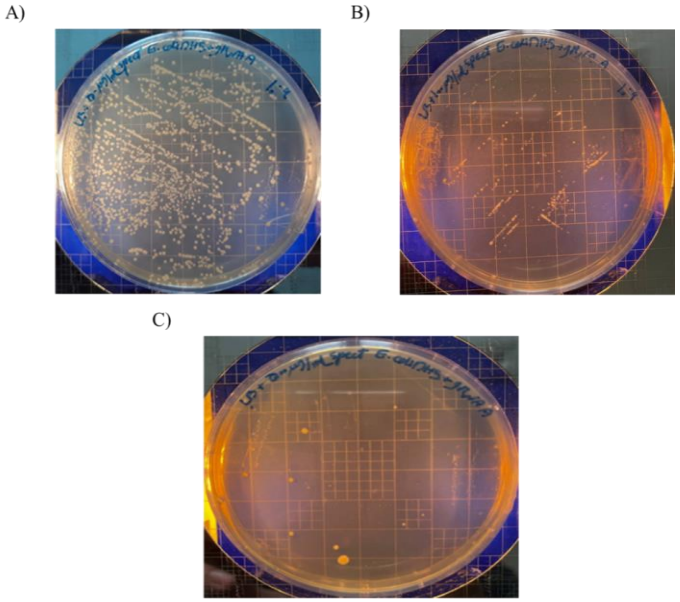

Figure 8. The Number of Colonies Counted for the Strain Carrying the Plasmid pEvolvR-enCas9-PolI3M-TBD gRNA-A (**Note.** The number of colonies resistant to 50 µg.mL^{-1} spectinomycin is 12×10^8 CFU.mL^{-1} (A), 100 µg.mL^{-1} spectinomycin is 4×10^8 CFU.mL^{-1} (B), and 500 µg.mL^{-1} spectinomycin is 40 (C).)

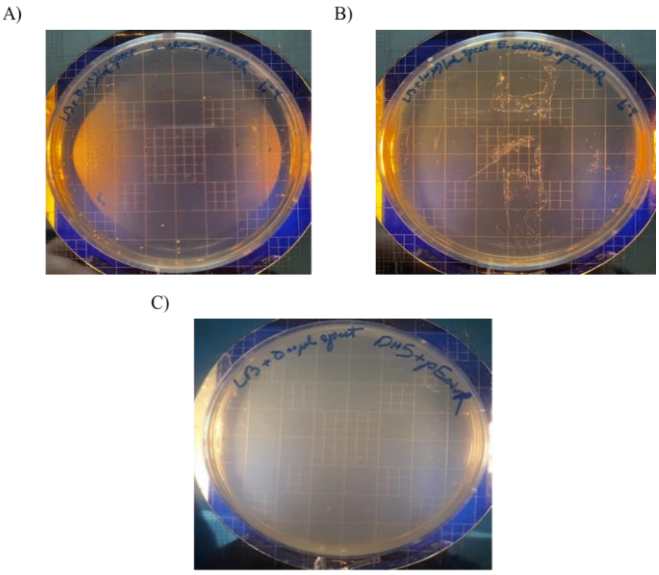

Figure 9. Number of Colonies Counted for the Strain Carrying the Plasmid pEvolvR-enCas9-PolI3M-TBD without gRNA (**Note.** The number of colonies resistant to 50 µg.ml^{-1} spectinomycin was 20×10^{6} (A), but no colonies were resistant to 100 µg.ml^{-1} spectinomycin and 500 µg.ml^{-1} spectinomycin (B & C).)

Table 4. The Ratio of Resistant Cells to Viable Cells in the Control and Induced Strains

Spectinomycin Conc. (µg. mL^{-1})	pEvolvR (Induced Control)	pEvolvR-gRNA (Induced)
50	0.028571429	~1
100	0	0.363636364
500	0	3.63636E-08

3.2. Discussion on the Improved Outcomes and Optimized Approach Compared to Previous Studies

In this work, Gibson assembly was successfully used to create a functional EvolvR plasmid, and this system generated spectinomycin resistance at a very high frequency. Very few studies have utilized the EvolvR technique for directed evolution experiments. In their pioneering work, Halperin and colleagues employed the EvolvR method to develop resistance in *E. coli* against spectinomycin at concentrations of 10, 100, and 1000 µg.mL^{-1}. In the present study, *E. coli* demonstrated resistance to spectinomycin at concentrations of 50, 100, and 500 µg.mL^{-1}. Aligning with the previous findings, as the concentration of the antibiotic increased, the number of resistant colonies decreased. However, in Halperin et al.'s research, the ratio of resistant cells to viable cells was at best 10^{-7} to 10^{-4}, which is much lower than the results of this study (14). In previous research, resistance to spectinomycin was achieved via simultaneously targeting the *rpsE* gene with five gRNAs; however, this study successfully obtained resistance using only a single gRNA (gRNA-A). Using this gRNA allowed targeting approximately 100 nucleotides from the beginning of the *rpsE* gene, which plays a crucial role in conferring resistance to spectinomycin (Halperin et al., 2018). So, the choice of this gRNA in the present study was purposeful. The observed increase in the number of resistant colonies in this study can be attributed to several factors. When utilizing a single gRNA, all expressed enCas9 and Pol3M-TBD proteins bind to that gRNA and effectively carry out their function. In contrast, employing five gRNAs results in enCas9 and Pol3M-TBD binding to gRNAs that play a lesser role in conferring resistance to spectinomycin. Additionally, the difference in antibiotic quality between this study and that of Halperin et al. (2018) may also account for the discrepancies, as the number of resistant colonies reported by Halperin and colleagues at concentrations of 10, 100, and 1000 µg.mL^{-1} spectinomycin was lower than those obtained in this research. Halperin et al. used the BsmBI enzyme for plasmid integration and gRNA incorporation along with promoters, scaffolds, and terminators in the Golden Gate method. This study introduced gRNA-A into the EvolvR plasmid using Gibson assembly rather than the Golden Gate method. The Gibson assembly method is more accessible and cost-effective than the Golden Gate, which can be prohibitively expensive. Furthermore, Gibson assembly can be executed by designing multiple pairs of primers. According to studies conducted by Bertram et al. in 2022 and Lutz et al. in 1997, the *tetR*/*tetA* promoter can be effectively controlled using anhydrotetracycline, doxycycline, and tetracycline. These inducers bind to TetR, preventing its attachment to the *tetR*/*tetA* promoter, thereby facilitating transcription of the target genes. The studies indicate that concentrations ranging from 25 to 2000 ng. mL^{-1} of these inducers can be utilized to promote this system, with a recommended range of 100 to 200 ng. mL^{-1} for bacterial induction. Furthermore, it has been shown that increasing concentrations of inducers correspondingly enhance the expression of the target genes (Bertram et al., 2022; Lutz & Bujard, 1997). In this research, a concentration of 400 ng. mL^{-1} was employed to successfully induce the expression of enCas9 and Pol3M-TBD genes to compensate for the potentially low quality of the tetracycline antibiotic. In another study conducted

by Long et al. in 2020, modifications were made to the EvolvR plasmid, specifically altering the Ori region from pBR322 to a modified version of pSC101. This modification enabled researchers to eliminate the plasmid at 42 °C and co-transform *E. coli* with another plasmid. In their study on *E. coli*, various gRNAs were individually incorporated into the EvolvR plasmid. In this study, plasmids were assembled using the homologous recombination method using the ClonExpress® II One-Step Cloning Kit. Additionally, a control plasmid without gRNA was utilized in their research, consistent with the current study, which also employed the EvolvR plasmid without gRNA (Long et al., 2020). In their 2022 study, Feng and colleagues successfully modified the EvolvR method for *Mycobacterium tuberculosis*, which has a distinct physiology compared to *E. coli*. They selected DNA polymerase A from *M. tuberculosis* and engineered it to be error-prone, combining it with XCas9, which recognizes the NGN PAM sequence. The gRNA was introduced into *M. tuberculosis* using a separate plasmid. This approach allows for targeted mutagenesis in *M. tuberculosis*, facilitating the exploration of genetic modifications that confer antibiotic resistance (Feng et al., 2022). These findings demonstrate that the EvolvR method with an appropriate design can target various genes in different strains for specific objectives, leading to desirable outcomes.

4. Conclusion

In conclusion, our findings demonstrate that EvolvR plasmid reconstruction and gRNA insertion can be achieved using Gibson assembly without function loss. On the other hand, choosing one gRNA over multiple gRNAs that target the same gene enhances the mutation rate and produces more resistant cells. It emphasizes the careful designation or selection of gRNA as a key factor for EvolvR's successful implementation and the creation of desirable phenotypes in bacteria. In most cases, the desired strains can be selected only after several passages under an appropriate selection pressure. Finally, for better results, another plasmid should be considered for introducing the gRNA, so the EvolvR plasmid is not subjected to any manipulation.

Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Authors' Contributions

YV conducted experiments, GE and MYA conceived and designed the study. MYA designed experiments. YV and MYA contributed to the analysis and interpretation of the data. YV and MYA drafted this manuscript with input from other co-authors. All authors have read and approved the final version of the manuscript.

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