CRISPR-guided DNA polymerases enable directed evolution of luciferase

Weizhe Chen, Rongxiao Zhang, Jiushi Wei, Xuyao Song, Jialiang Guo, Xinqi Zou , Zixiang Zhong, Xianghong Wang

Abstract

EvolvR, a system that can continuously diversify all nucleotides within a tunable window length at user-defined site, is an attractive method for continuously directed evolution. Our project attempts to conduct directed evolution of luciferase lux of Marine *Vibrio FG-1* isolated from the Yellow Sea by using EvolvR system, aiming to explore a luciferase mutant with better potential performance and more various colors of the luminescence spectrum. In addition, multiple gRNAs-guided EvolvR system is of great application value. However, due to the problem of genetic instability in the construction process of multiple gRNA, we propose the idea of using ELSA (Extra-long sgRNA Arrays) to help build multiple gRNAs-guided EvolvR system. We envision that a stable multiple gRNAs-guided EvolvR system will provide a new strategy for simultaneous evolution of multiple genes, such as metabolic pathways.

Introduction

EvolvR system, using low-fidelity DNA polymerases variants as well as CRISPR-guided nickase to mutate the target region, enables the diversification of nucleotides within a tunable window at user-desired loci [1]. It is EvolvR system than can achieve continuous diversification of user defined loci, which will contribute to a wide range of ~~basic and~~ biotechnology applications. After the expression of user-designed gRNA, enCas9 is mediated to target and nick the user-defined loci on the genome or plasmid. The pol13M mutant with low fidelity degrades single strand from 5' to 3' from the nick and synthesizes a new strand replacing with a length of about 350bp (Figure 1a). The EvolvR system enable targeted genes to mutate, express, be screened according to phenotype. Recycling these steps can achieve continuous-mutagenesis in vivo. In comparison to other directed evolution techniques for bacteria (MAGE, MMR, error-prone PCR), EvolvR is an ideal continuous directed evolution platform, which can specially evolve user-desired locus with high mutation rates but not require mutation libraries [2,3]. Compared with other directed evolution methods, it can spontaneously mutate target gene in an efficient and tunable way without harming the host bacteria, which is also labor-saving [2, 3]. Recently， the EvolvR system has now been demonstrated to work in eukaryotes, such as yeast [4]. This will broaden the range of applications of EvolvR system and contribute to a wide range of future biotechnology applications [5].

Bacterial luciferase is a flavin-dependent monooxygenase, catalyzing the reaction of O_2 with reduced FMN and a long-chain aliphatic aldehyde, and releasing oxidized FMN, water, and a fatty acid, with concomitant emission of blue-green light [6]. Aliphatic aldehyde, the luciferase substrate of vibrio, is with low toxicity and easy to enter cells，so it is harmless to cells in most cases. In comparison with GFP, luciferase does not require external light source, so the process of luminescence detection does not affect the metabolic activities of cells, which is suitable for long-term studies in living materials, especially light-sensitive tissues like retina. Luciferase can either diffuse or aggregate, which means the expression of luminescence can be employed to accurately locate cells [6].

In our study, the *Vibrio FG-1* isolated from the Yellow Sea was identified and its bioluminescence related genes luciferase *luxA* and *luxB* were isolated. The luminescence system was reconstructed in *Escherichia coli.* By designing gRNA targeting different sites of *luxA*, the CRISPR-mediated EvolvR system is constructed to carry out directed evolution of luciferase α domain, aiming to explore potential luciferase mutants with better performance and various luminescence spectrum. The luciferase lux mutants with different wavelength emission light have the potential to promote the development of the field of optogenetics and biological imaging. EvolvR system has been proven to couple the mutagenesis to a genetic screen of non-selectable phenotype. Shakked O. Halperin et al. have demonstrated this ability by mutating GFP protein. So it is feasible to use EvolvR system to directed evolve vibrio luciferase [1](figure 1b).

In addition, multiple gRNAs-guided EvolvR system is of great potential application value. Co-diversifying a series of genes simultaneously will allow the whole key genes of metabolic pathway to mutate. Optimization of EvolvR system is worthy of consideration. However, the CRISPR/Cas9 system with multiple targeting sites has difficulties in the construction of gRNAs and stably inheritance [7]. EvolvR system, which mainly guided by CRISPR/Cas9, probably faces the same problem. We propose to use ELSA (Extra-long sgRNA Arrays) to design and build the EvolvR system with multiple gRNAs, so as to solve the problem. Also, we put forward an idea that the mutagenesis window length of the EvolvR system can be enlarged by using several gRNAs targeting adjacent locus.

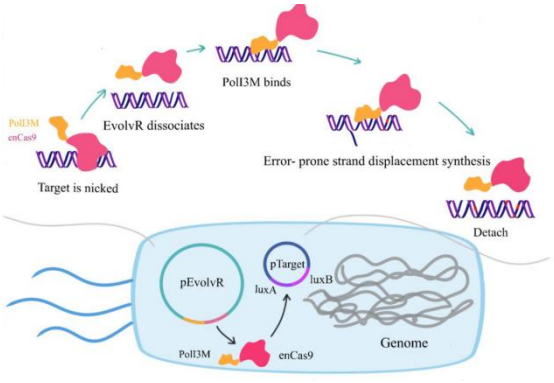


Fig-1a. brief description of EvolvR

EvolvR is a two-plasmid system, plasmid pEvolvR involve enCas9-PolI3M-TBD and gRNA, plasmid pTarget carries the target gene. When gRNA targets the specific site, gRNA-mediated nickase (enCas9) cleave single strand and detach. Then low fidelity DNA polymerase I (PolI3M), binds to the nick and resynthesizes downstream strand from 5' to 3', introducing base mutations.

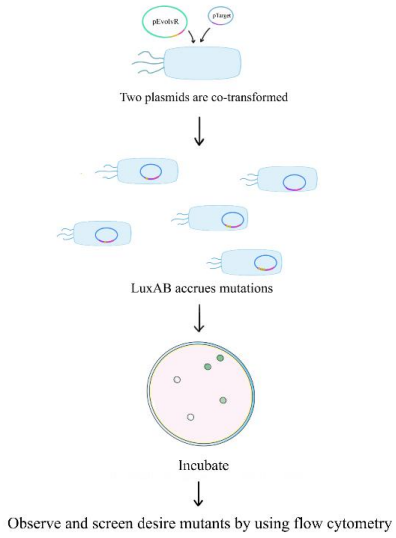


Fig-1b . flow chart of evolving of mutants

The flow chart of producing and screening the luciferase *luxA* mutants by EvolvR system. Flow cytometry is used to test and screen the wavelength of luciferase mutants.

Result

1. Species identification and structural prediction

We constructed phylogenetic tree of 16S rRNA gene to identified a cyan light-emitting luminescent

bacteria strain, *Vibrio FG-1*, which was isolated from the Yellow sea. Close phylogenetic relationship between *Allivibrio finisterrensis* and *Vibrio FG-1* was reflected by MEGAX analysis (Figure 2a). Phylogenetic tree construction of *luxA* has shown that the *luxA* of *Vibrio FG-1* is closely related to *luxA* of *Aliivibrio Salmonicida* and *Aliivibrio Fischeri* (Figure 2b).

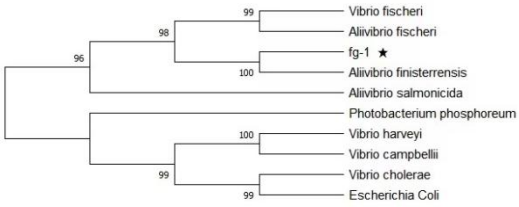


Fig-2a.FG-1's Phylogenetic tree of 16s

Phylogenetic tree was constructed based on 16S rRNA genes. Vibrio FG-1 belonged to Allivibrio group with a confidence level of 96% and has a high phylogenetic relationship with Aliivibrio finisterrensis.

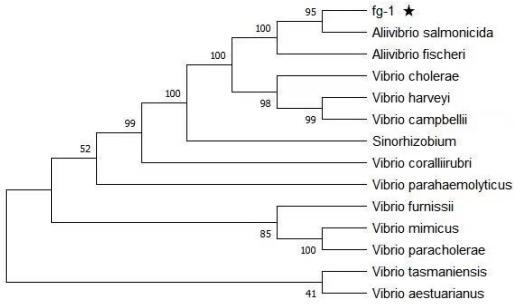


Fig-2b.lux protein's Phylogenetic tree of FG-1

Phylogenetic tree construction of *luxA* has shown that Vibrio FG-1 is closely related to *Aliivibrio Salmonicida* and *Aliivibrio Fischeri*, with a confidence level of 95%.

We designed primers and amplified *luxA* and *luxB* genes of luciferase. In the predicted structure of the α and β subunit of the luciferase we obtained, similarities to the same subunits of *Vibrio harveyi* were observed. Under this circumstance, we hypothesized that two subunits will combine with each other and form an asymmetrical structure (Figure 3a, b). The Asp113, RHis44, RLys112 and RHis45 at the α subunit we predicted (Figure 3e), which will electrostatic stability interact with each other after binding to FMN [8], indicates that this α subunit will also bind to FMN. In this luciferase, the FMN binding site at α subunit, Ala75, mutated to Gly (Figure 3c), but considering that this situation also exists in some bacterial luciferase [9], we could not confirm the influence of this substitute of amino acid. In addition, mutations in Cys106 at α subunit may lead to the instability of intermediate 4a,5-dihydro-4a-hydroperoxy flavin [10] and further reduce the enzyme activity. Also, the substitutions Y151D on the β subunit (Figure 3d) may also lead to reductions in luciferase activity and total quantum yield [8]. Besides, A series of mutations occurred in amino acid residues on mobile loop， which did not report in previous studies. Considering that mobile loop is the boundary between the active center and the outside world [10], the mutation of contact site probably result in the decrease of enzyme activity. Furthermore, A series of mutations occurred on the mobile loop of α subunit, including an F272H, which has not been reported before. Given that the mobile loop worked as a boundary between the active center and the solvent [8], the mutation of this contact site may lead to the decrease of enzyme activity and deserves more attention.

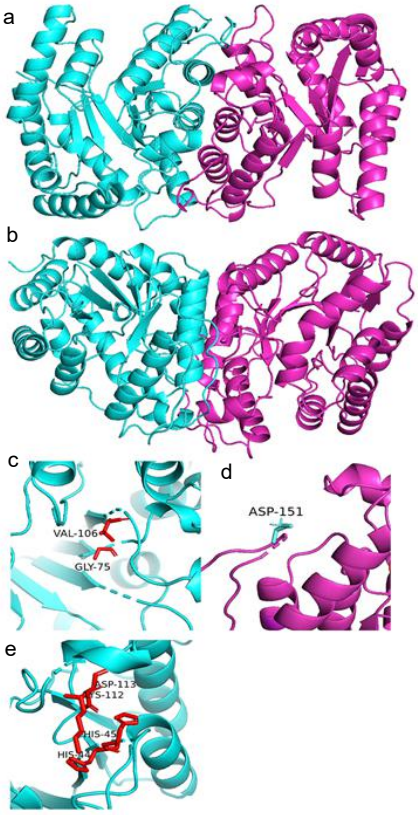


Fig-3. The structure of the α and β subunit of the luciferase

a, b. The asymmetrical dimer formed by α and β subunit. c, d. Substitutions of some active site at α and β subunit that may lead to decrease in enzyme activity. Red structure is FMN. e. The Asp113, RHis44, RLys112 and RHis45 at the α subunit interact with each other after binding to FMN. The structure predicted phyre2 and label by PyMol

2. Construction of EvolvR system

We attempt to express luciferase in *Escherichia coli DH5α* and directed evolve luciferase by using enCas9-Pol13M-TBD meidated EvolvR system. We construct and transform pTarget plasmid with luciferase genes *luxA* and *luxB*, as well as pEvolvR plasmid with gRNA mediated EvolvR system into *Escherichia coli*. By observing the phenotype, testing wavelength of luciferase mutants, screening and amplifying, luciferase mutants with desired characteristics will be finally obtained [1].

2.1 Construction of pTarget

We amplified luxA and luxB genes from *Vibrio FG-1* by PCR, and added J23119 promoter to them. DNA fragments *luxAB*, *ampR* and *p15a* ori were assembled by seamless cloning method. Three fragments with equal molar ratio and Seamless cloning MasterMix was added to incubate according to the instructions of seamless cloning kit. About 20bp homologous sequence between each fragment permit the construction of plasmid. Single colony will be screened on plate containing ampicillin, further colony PCR will be used to verified.

2.2 Design of gRNA

We selected some gRNA candidates to target luxA gene for directed mutation. Our gRNA design is mainly based on the following key points:

a. Expression of two gRNAs that nick separate strands at genomic loci separated within 100 bp was lethal, whereas nicking the same strand at this 100-bp distance was not lethal.

b. In this project, we used enCas9, a mutant of Cas9 (H840A). So 20bp-NGG-spCas9 was used for Protospacer Adjacent Motif (PAM) searching when the online software CRISPROR was used to select and design gRNA [11, 12].

c. Avoid GC levels that are too high (above 80%) or too low (20%)

d. Select gRNA candidates with low off-target rate

e. Due to the uncertainty of which sites will determine the change of luminescence wavelength of luciferase, we analyze the conserved domains of α unit of luciferase by MEME (Figure 4). Referring the structure and conserved domain prediction to choose the targeting site.

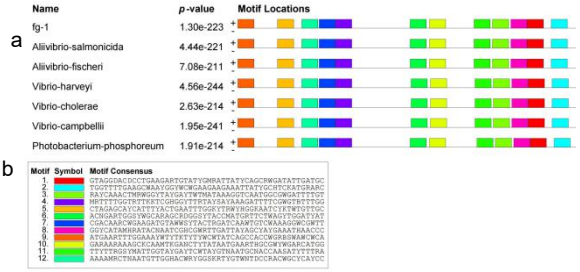


Fig-4.The conserved domain of the amino acid sequence of luxA protein

a. the comparison of the conserved domain of the amino acid sequence of *luxA* protein of Vibrio fg-1 with other vibrio luxA. Rectangles with the same color represent the same motif. b. specific amino acid sequences of motif represented by each color rectangle.

According to above several rules, we design the following five gRNA candidates (figure 5a). Due to uncertainty of the specific sites that potentially determine the change of luminescence wavelength of luciferase, we design some gRNA targeting the key domain, gRNA candidates 1,2,3, and others targeting the conserved region, gRNA candidates 4 and 5 (figure 5b).

a

sgRNA1	site: 102	GGGGTTGCCTCTGAAGAGCT
sgRNA2	site: 118	AGCTTGGATTTGATACCTAC
sgRNA3	site: 555	ACCGAATGGCTAGCAAAACA
sgRNA4	site: 702	ATATGCTCTGTTGACGATGT
sgRNA5	site: 901	GCAATGAAATAAACCCCGTA

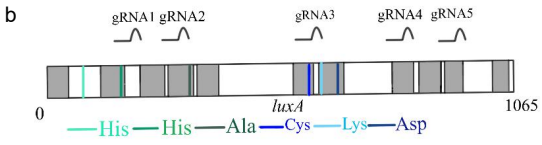


Fig-5 .gRNA candidates targeting sites

a. five gRNAs candidates sequences b. The gray bands in the figure represent the conserved regions(motifs) of *luxA* gene. The colored lines mark the corresponding active sites of luciferase.

2.3 Construction of pEvolvR

We inserted promoter J23119 and gRNA in front of the gRNA scaffold on the pEvolvR plasmids by recombination. Five pEvolvR plasmids were separately constructed for the five gRNA candidates. After pEvolvR was introduced, the expression of enCas9-Pol13M-TBD was induced by dehydrating tetracycline.

3. Fluctuation analysis assay

To sensitively quantify the mutation rate, so as to verify that the mutagenesis is created by EvolvR system instead of the basic mutation rates, we designed a Fluctuation analysis to verify.

In order to optimize the culture scale, different glucose concentrations were applied to control the number of cell divisions when the culture reached saturation. Based on the colony number of gentamicin-sulfate resistant LB plate, the plate has no colony when the dilution ratio is 10^{-6}. At this time, OD600 = 0.05 corresponds to colony number is 5×10^6 CFU / ml (N_0). After diluting to 10^6 CFU / 30ml, the plate is added to the LB liquid medium with different glucose concentration and coated on the complete LB medium. According to the experimental steps in Methods, the glucose concentration 0.1%, 0.05%, 0.01% and 0.005%, respectively refer to the ratios of unmutated plates to total plates 44.7%, 30.6%, 89% and 64.4%. P_0 method (Poisson distribution) requires that the percentage of culture medium (p_0) without mutants should be between 10% and 80%, so 0.01% LB liquid culture medium with glucose concentration was selected. To calculate the viability and the mutation rate of bacteria in formula (Figure 7), we calculate the number of bacteria that have mutated (***a***), the total number of bacteria after culture (N_f) and the actual number of viable bacteria (***b***) [13].

$$\textbf{viability} = \frac{b-a}{N_f-a}$$

$$p_0 = \frac{N_f-c}{N_f-a}$$

$$u = \frac{-ln(p_0)}{\text{vability}\times N_f}$$

Fig-6. **The formula of fluctuation test**

The colony number (***a***) is 1.3 CFU per plate. After incubation for one day in the 96-well plate, OD600 = 0.0699, meaning total number of bacteria $N_f = 2\times10^5$ CFU. The actual number of viable bacteria coated in the complete culture medium(***b***) is 322.5 CFU per plate, and the ***viability*** of bacterial was calculated (***viability*** $= \frac{b-a}{N_f-a} = 0.0016$). The number of mutated cells (***c*** =28.64 CFU) was obtained when they were coated in selective medium. Therefore, zero-level events - $p_0 = \frac{N_f-c}{N_f-a} = 0.99986$.

The mutation rate - $u = \frac{-ln(p_0)}{\text{vability}\times N_f} = 4.38\times10\text{-}7$.

The original mutation rate is higher than our expectation, and the analysis results are inaccurate due to fewer samples and fewer repeated experiments. Due to the restriction of time, we have not test the basic mutation rate of E.coli with EvolvR system.

4. Multi-gRNAs EvolvR system

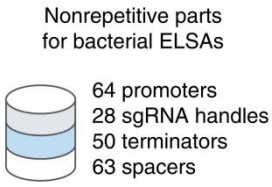
In industrial production, the desired product or phenotype often requires the co-regulations of multiple genes, such as regulating the metabolic pathway, studying complex gene regulatory networks or polygenic diseases. Multiple gRNAs-guided EvolvR system is of great application value. If multiple gRNAs guided EvolvR system is designed to target a series of genes and simultaneously direct evolve them, it has the potential to complete the co-directed evolution of an entire metabolic pathway or family of genes in a short period of time. If multiple gRNA guided EvolvR system used to target the same gene, it will

increase the overall length of the mutation window, and could even customize the length of the mutation window by selecting the locus and number of gRNAs.

EvolvR system has been proved to simultaneously diversify several genomic locus by expressing gRNAs [1]. However, only five gRNAs system was tested in the research, EvolvR system with more gRNAs need further verification.

However, some studies have indicated that if multiple gRNAs were expressed at the same time in CRISPR/Cas9 system, those repeated DNA sequences increase difficulty in plasmid assembly and decrease genetic stability, especially in organisms with frequent homologous recombination [14, 15]. The problem also need to be solved in EvolvR system, which is adapted from CRISPR/Cas9 system. Alexander C et al. designed and screened a series of non-repeating sequence toolbox to help build multiple gRNA Arrays targeting different loci [7]. The toolkits consist of a sequence non-repeating series of promoters and spacers, and a series of non-repeating gRNA scaffolds designed through RNA structure prediction and Monte Carlo optimization, as well as experimental testing. In our design of the gRNA, we attempt to use nonrepetitive extra-long gRNA arrays (ELSA) from the literature to construct a series of gRNA arrays targeting different loci of luciferase genes for EvolvR system(figure 7c) [7].

a



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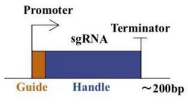

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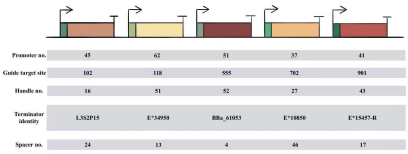

Fig-7. Introduction of Nonrepetitive parts of ELSA to develop the EvolvR system

a. the non-repetitive extra-long sgRNA arrays literature, including promoters, gRNA handles, spacers and terminators. Different combination of these parts can help avoid repetition. b. the circuit of expressing gRNA. c. using some of parts candidates in ELSA library to construct the circuit of co-expressing our 5 gRNA candidates.

Discussion

Vibrio luciferase has been used as a tracer or reporter gene in cytology or genetic engineering studies, marking the movement of cellular structures and gene expression products. However, the performance of wild-type vibrio luciferase could not preferably fulfill the complex cytological or genetic engineering studies, for low luminescence intensity and fluorescence at restricted wavelength. Mutation and optimization of luciferase is necessary and meaningful. Luciferase with high luminescent intensity will become an excellent reporter, easy to detect without excitation light, while “colorful” luciferase toolkit, which can emit light at different wavelengths, will better suit different experimental demands of researchers. The universality and hypotoxicity of luciferase Lux permit its application in prokaryote and eukaryotes, including yeast and mammalian cells. Thus, we believe that the optimized luciferase system is worth expecting, and set up the project.

In our study, the luciferase *luxAB* from *Vibrio FG-1* is mutated by hypermutation EvolvR system, expecting that the mutants with higher luminescence intensity or various luminescent wavelength can be screened and reserved. Unfortunately, we have not completed our project. The screening and part of verifying experiments

are still in process. We envision that luciferase mutants with ideal characteristic will be evolved, providing optimized reporter system for future cytological, genetic, and synthetic biology studies.

In addition, for further improvement of luminescence system, association with bioluminescence resonance energy transfer system (BRET) is well worth be taken into consideration. In BRET system, luciferase (as a donor) transfers its energy to fluorescent protein (as an acceptor), which efficiency of BRET relies on the overlap degree between the donor emission spectrum and acceptor absorbance spectrum [17]. Matching luciferase mutants to befitting fluorescent protein will enable auto-bioluminescent report system emit longer wavelength light.

Meanwhile, according to current researches and comments, EvolvR system is a continuous directed evolution machine worth considering. Though other methods, like error-prone PCR, are well understood and developed, the advantages of EvolvR system indicate its great potentiality. Therefore, on one hand, we attempt to apply this system to directed evolve luciferase. Also, the feasibility of EvolvR system will be verified, and extra experiment testing data will share to the public.

On the other hand, EvolvR system, a comparatively new directed evolution system, has a lot of room for improvement and optimization. We focus on the potential problem of multiple gRNAs construction. We propose the idea of using ELSA (Extra-long sgRNA Arrays) to help build multiple gRNAs-guided EvolvR system. Regretfully, this is still an idea without experimental testification. We are going to carry forward our project to finish the proof of idea. We envision that a stable optimized multiple gRNAs-guided EvolvR system will be create in the future, providing a new strategy for simultaneous evolution of multiple genes like metabolic pathways, as well as extending diversifying window by using several gRNAs.

Materials and Methods

1. Seamless cloning MasterMix constructs plasmid

1.1. DNA manipulation.

PCR was performed with 2×Taq PCR MasterMix (Solarbio, Beijing, China). Plasmid DNA was isolated with the TIANprep Mini Plasmid Kit (TIANGEN, Bejing, China). DNA fragments were purifified from agarose gels by using the Universal DNA Purufucation Kit (TIANGEN, Bejing, China) or the Monarch DNA Gel Extraction Kit (NEB). DNA sequencing and primer synthesis were carried out by Sangon Biotech (Shanghai, China).

1.2 Plasmid construction.

a. The plasmids and primers used in this study are listed in the supplemental material.
b. To construct pTarget-luxAB, the coding regions of luxAB, p15A, and AmpR are amplifified from the genome of Fg-1, a plasmid from 2021OUC-China iGEM and Cloned UpB_4A3m with three different primer pairs luxAB-F/luxAB-R, p15A-R/p15A-F, AmpR-R/AmpR-F, respectively. The J23119 promoter is added to PCR products of luxAB by primersluxAB-F and J23119R, resulting in J&luxAB. Oligonucleotides are designed to contribute flanking homologous regions to adjacent DNA fragments of 20-25bp in length, resulting in p15A-homo and AmpR-Homo.
c. A new pEvolvR plasmid expressing enCas9-PolI3M-TBD with gRNA targeting luxAB is constructed. Three fragments pEA, pEB and pEC with flanking homologous regions to adjacent DNA fragments of 20-25bp are amplifified from pEvolvR-enCas9-PolI3M-TBD with primer pairs pEA-R/pEA-F, pEB-R/pEB-F, pEC-R/pEC-F, respectively.

Double-stranded gRNA is gained after DNA annealing, and it is modified by the promoter with the primer pair——. Similarly, then homologous regions are added to produce J&gRNA-Homo.

d. Two plasmids were both constructed using the Seamless cloning Master Mix provided by Sangon Biotech (Shanghi, China). The plasmids pTarget-luxAB and pEvolvR were cotransformed into E. coli strainDH5α, following the specification steps provided.

2. Structural forecasting

The nucleotide sequences of gene *luxA* and *luxb* of *vibrio* Fg-1 were translated into amino acid sequences from the initiation codon. The structures of both *luxa* and *luxb* protein were predicted by phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) through homology modeling method. Futher analysis of protein structure including the alignment and sites labeling were accomplished by PyMol.

3. Fluctuation Test

3.1. Optimize the scale of training

- Start LB cultures of each E. coli strain and grow cultures overnight at 37°C with orbital shaking at 170 rpm.
- Prepare selective media.
- Dilute the bacteria until the culturedoes not produce colonies on the resistance plate.
- Determine the cell density of the overnight culture as precisely as possible，and the total number of cells at this time is N0.
- Depending on the measured bacterial density, the overnight culture was diluted to 30 ml with a complete medium with different glucose concentrations (0.1%, 0.05%, 0.01%, 0.005%) so that each 30 ml solution contained a total of 100,000 cells.
- Measure the cell density of the four conditions to determine dilution.
- Divided into two 96-well plates (48holes per condition), each with 30ul.
- Incubate two plates (37℃) at the right temperature and do not shake.
- Obtain cell density for ten of the 48holes under each condition to ensure that cell counts vary with glucose concentration.
- The remaining holes add 70ul distilled water to the 30ul culture and mix well before applying the plate to prevent the bottom residue. The coated plate remains dry.
- Calculate the number of colonies of zero-level events and find an appropriate proportion of zero-level events (unmutated plates/total plates between 10% and 80%)

3.2. Fluctuation test

- Overnight culture the bacteria liquid.
- Break up the bacteria liquid and measure cell density.
- Dilute the bacteria into a fully cultured solution at the selected glucose concentration.

3.3. Apply a separate strain

- Confirm the dilution multiple, then remove a certain volume of the bacteria fluid from the diluted bacteria to coat the common medium, and then count the colonies (which can be used as the initial number of cells in each hole).
- Add the diluted bacteria to 4 96-wellplates.
- Two of the 96-well plates were cultured, and the other two were coated in the same selected medium, where only mutated bacteria could grow, and then count the colonies (the number of bacteria that have mutated- “a”)
- After the culture of the two 96-wellplates, ten of the holes were extracted to measure the cultured bacterial density, and the total number of cultured bacteria (Nf) was calculated. Then the ten holes of bacteria liquid coated into a complete medium, calculate the number of colonies, at this time the number of colonies is the actual number

of active bacteria (b), so as to obtain the survival rate of bacteria viability.

- Spread all remaining bacteria into the same selected medium and count (number of mutant cells- “c”).

3.4. Data analysis:

Calculatemutation rates:

$$u = \frac{-ln(p_0)}{\text{vability} \times N_f}$$

$$p_0 = \frac{N_f - c}{N_f - a}$$

$$viability = \frac{b-a}{N_f - a}$$

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Author Attribution

W.C. conceived the project designs, contributed to the execution of experiments, analyzed the data and wrote the manuscript; J.W., R.Z., contributed to plasmid pTarget construction and assay execution for fluctuation analysis; X.Z.,X.S.,Z.Z, contributed to plasmid pEvolvR construction; J.G. contributed to luciferase structure prediction. The manuscript was read, edited and approved by all authors.

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