The Minutes of The CRISPR Conference

CRISPR APPLICATION GUIDELINES

Author: iGEM Tianjing 2021

Date: Sep. 12, 2021

Version: 1.1

CRISPR was discovered and invented in the early 1990s. Nowadays, CRISPR-related technologies and applications have developed rapidly, becoming the most popular gene-editing tool in human biology, agriculture and microbiology. We all know that the CRISPR gene-editing system won the Nobel Prize in chemistry in 2020.

CRISPR technology is also one of the powerful and commonly used tools for our research in the field of synthetic biology. When using CRISPR, teams more or less always encounter some problems. Therefore, our team Tianjin planned to hold a CRISPR conference, collect the problems from each team, and discuss solutions together.

As the organizer of the conference, Tianjin, invited iGEM teams from ZJUT, OUC, BUCT, NNU, NWU, NEFU, XJTLU, CPU, TJU, LZU and Tongji universities. And we successfully held an online CRISPR discussion conference on September 12, 2021.

CONTENTS

ATTENDING TEAM

Team：**Tianjin**
Project Name：Creation of the CREATEs
Contact：E-mail: 2429736514@qq.com

Team：**BUCT**
Project Name：Delivering gaba and 5Htp through fatty acid lowering (DeliGHtFAL)
Contact：Tel / WeChat: 18718806286

Team：**OUC-China**
Project Name：ALLPASs（Amplifying, Low-leakage Platform for Antibiotic Sensors）
Contact：E-mail: OUCiGEM@163.com Tel: 18939794377

Team：**NNU-China**
Project Name：Constructing the strain library of E. coli for improving the antimicrobial peptides production
Contact：Tel: 16651698356

Team：**NWU_China_A**
Project Name：An adventure of ancient snails
Contact：E-mail: 2991088806@qq.com

Team：**ZJUT-China**
Project Name：CRISPR/Cas9-based Cell-Free Biosensors For RNA Biomarker
Contact：E-mail: 262758046@qq.com Tel: 13615715986

Team：**XJTLU_China**
Project Name：Dr. Phage
Contact：E-mail: igem@xjtlu.edu.cn Tel: 18765438960

Team：**NEFU_China**
Project Name：Visible Diagnosis Platform by G-quadruplex(VDPG)
Contact：QQ：1341851925 Tel/ WeChat: 18801203060

Team：**CPU_China**
Project Name：PEDe.
Contact：Email: zhenglinyu2000@163.com

Team：**LZU_China**
Project Name：Targeted inhibition of *SARS-CoV-2* infection based on CRISPR-Cas13 system
Contact：Email: habe19@lzu.edu.cn

Team：**Tongji_Software**
Project Name：Phage Microbiome Assist Phagotherapy, Phage-MAP
Contact：E-mail: tj_software2021@163.com

CHAPTER 1

INTRODUCTION OF CRISPR/CAS

CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated Proteins) is an acquired immune defense system widely available in prokaryotes to resist the invasion of foreign genetic material. CRISPR/Cas is the third generation of gene editing techniques following zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENS) [1].

1.1 the Structure of CRISPR/Cas9

CRISPR/Cas9 from Streptococcus pyogenes is the most studied and widely used. It's comprised of three components: the Cas9 protein, crRNA and the trans-activating CRISPR RNA (tracrRNA) gene. In 2014, Doudna reported on how CRISPR/Cas9 works and how double-stranded DNA is cleaved[2]. They optimized CRISPR/Cas9 by fusing separate crRNA and tracrRNA to form a single guide RNA (sgRNA). SgRNA can specifically bind the target sequence, and then guide Cas9 to perform site-specific cleavage of the DNA double strand. Cas9 can recognize PAM sequences of target DNA, then unbinds the double strand and pairs sgRNA with the target sequence, resulting in DNA double strand broken at the first three bases of PAM sequence. When DNA double-strand breaks, the cell activates automatic repair.

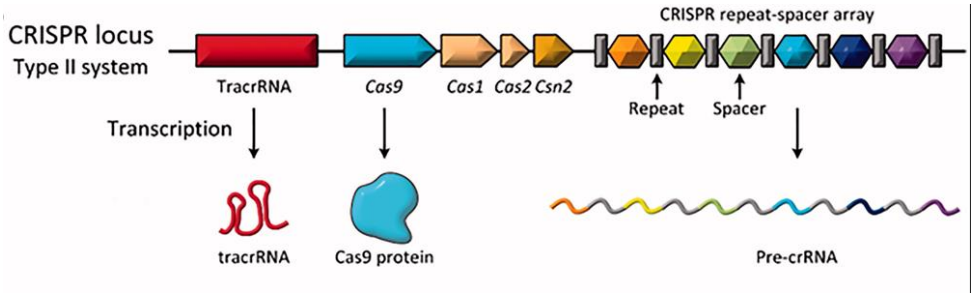

Figure 1.1 genomic CRISPR/Cas9 locus[3]

The genetic sequences of CRISPR are mainly composed of the leading region, multiple highly repetitive sequences and spacer sequences[3]. The leading region is usually 300-500bp in length and is rich in A and T bases. The leading region can act as a promoter to initiate transcription of CRISPR sequences, but it has no open reading frame and can't encode proteins. The downstream of the leading region is repeat sequences, generally composed of 23 to 50bp, with an average length of about 31bp. The repeated sequences contained palindromes, and the RNA transcribed from them could form a structurally stable stem loop that mediated the formation of CRISPR and Cas protein complex. Spacer sequences consist of 17 to 84bp bases with an average length of about 36bp. In the same CRISPR site, there are basically no identical or relatively similar spacer sequences.

1.2 CRISPR/Cas9 System Working Principle

When a phage invades a cell that contains the CRISPR system, the CRISPR-related protein complex quickly binds to it. The foreign DNA was cleaved into small fragments with the length of 17-48bp by Cas1 and Cas2 proteins. Under the action of related Cas proteins, the small fragments with PAM sequences will be integrated into the area between the leading region and the first repeat sequence, forming a new region. The formation of the new region indicates that the invading phage information has been stored in the CRISPR locus.

CRISPR was transcribed into pre-crRNA and tracrRNA under the regulation of the leading region when the same foreign DNA invaded. With the help of tracrRNA, pre-CrRNA was cleaved into mature crRNA by RNaseIII. Then, crRNA, tracrRNA and Cas9 protein will form a complex that can recognize and bind to DNA fragments complementary to crRNA. Cas9 protein acts as an endonuclease, causing DNA double-strand breaks.

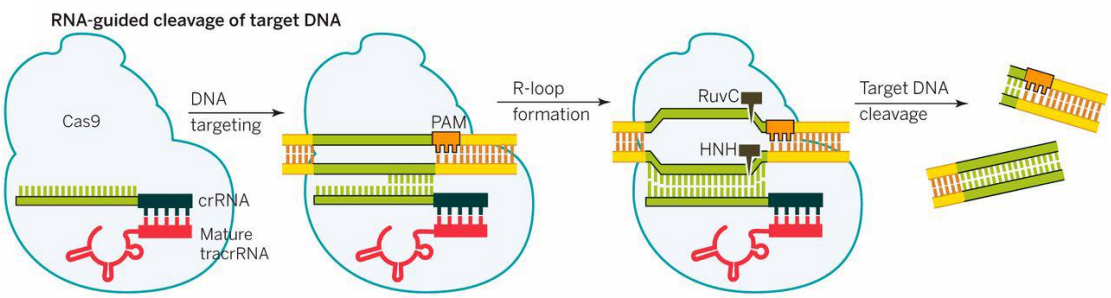

Figure 1.2 RNA- guide cleavage of DNA[2]

CHAPTER 2

COMMON PROBLEM SOLUTIONS

2.1 the restriction of PAM sequence

CRISPR/Cas9 genome editing relies on sgRNA-target DNA base pairing and a short downstream PAM sequence to recognize target DNA. The strict protospacer adjacent motif (PAM) requirement hinders applications of the CRISPR/Cas9 system since it restricts the targetable sites in the genomes. In theory, the more complex the PAM sequence is, the fewer target sites can be accessed by the Cas9 protein.

Solutions: For a long time, researchers have been working to optimize Cas9 to extend its compatibility with different PAM sequences, with the hope that one day, Cas9 will be free of PAM and have genome-wide editing capability. Take SpCas9 as an example: Keith Joung's lab obtained spCAS9-VRQR variants of NGA and SPCAS9-VRER variants of NGCG as early as 2015 through sequential error-prone PCR [4]. In 2018, David Liu constructed xCas9 3.7 variants that recognize NGG, NG, GAA and GAT using his unique directional evolution technique PACE [5]. In the same year, Nureki laboratory constructed a more active variant of SPCAS9-NG, whose recognized PAM sequence was extended to NG [6]. In 2020, David Liu further developed a series of PAM sequences identified by SpCas9 mutants using PACE technology and extended them to NRNH (R is A/G, H is A/C/T). [7] In the same year, Benjamin P. Kleinstiver's laboratory made a strong upgrade of SpCas9 protein, and the transformed SpCas9 variant SpRY was almost completely free of PAM. PAM sequences are NRN and NYN (Y is C/T) (NRN> NYN).[8]

2.2 off-target

The CRISPR-Cas9 system relies on specific binding of sgRNA recognition sequences to targeted sequences. Due to the complexity of the genome, sgRNA may match with other untargeted sequences, which may also activate Cas9 activity, resulting in off-target and potential safety hazards.

Solutions:

1) VIVO： Researchers from Astrazeneca and Harvard described “verification of in vivo off-targets” (VIVO), a highly sensitive strategy that can robustly identify the genome-wide off-target effects of CRISPR–Cas nucleases in vivo. VIVO consists of two steps.

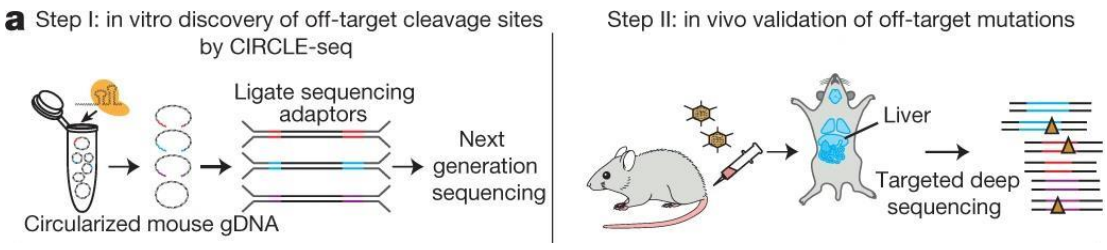

Figure 2.1 Schematic illustrating the two-step VIVO method.

In step I, CIRCLE-seq identifies off-target sites cleaved in vitro. In step II, the sites identified in step I are assessed in vivo for indel mutations by targeted amplicon sequencing performed.

In an initial in vitro “discovery” step, a superset of potential off-target cleavage sites for a nuclease is identified using circularization for in vitro reporting of cleavage effects by sequencing (CIRCLE-seq). This method is highly sensitive, avoids potential confounding effects associated with cell-based assays8 and can successfully identify supersets of sites that include bona fide off-targets in cultured human cells8[9].

In a second in vivo “confirmation” step, sites identified by CIRCLE-seq are examined for indel mutations in target tissues that have been treated with the nuclease.

2) DISCOVER-Seq: After the introduction of DSBs by Cas9 cutting the genome in the cell, the cell will repair it through the damage repair pathway, during which DNA repair proteins bind to the DSBs site caused by Cas9.Researchers found that MRE11 binding peaked before the appearance of insertions and deletions (indels) and was readily detected at a known guide RNA (gRNA) off-target.

DISCOVER-Seq is a universal approach for unbiased detection of genome-editing off-targets in the whole genome, and the operation steps are simplified. The accuracy is significantly better than that of VIVO system, so it may have a greater possibility in clinical application. [10]

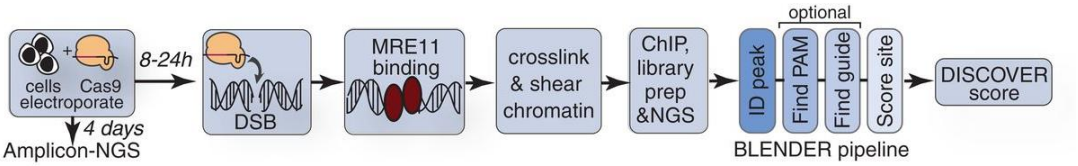

Figure 2.2 DISCOVER-Seq workflow[7]

2.3 Mosacism

In order to obtain genetically modified embryos or mice by CRISPR/Cas9 system, Cas9 mRNA and sgRNA are usually introduced into zygotes by microinjection [11] or electroporation [12]. However, most mutants generated with this method are genetically mosaic, composed of several types of cells carrying different mutations, which complicates phenotype analysis in founder embryos or mice. To simplify the analysis and to elucidate the roles of genes involved in developmental processes, a method for producing non-mosaic mutants is needed.

Solutions: Some researchers established a method for generating non-mosaic mouse mutant embryos. They introduced Cas9 protein and sgRNA into in vitro fertilized (IVF) zygotes by electroporation, which enabled the genome editing to occur before the first replication of the mouse genome. [13] As a result, all of the cells in the mutant carried the same set of mutations. This method solves the problem of mosaicism/allele complexity in founder mutant embryos or mice generated by the CRIPSR/Cas9 system.

CHAPTER 3
TEAM PROJECT

3.1 Tianjin

(1) Project name

Creation of the CREATE

(2) Project introduction

It’s generally considered that cells have chromosomes to carry genetic information, which plays an important role in the growth and proliferation of organisms. What if cells lose their chromosomes? Are they still alive? We composed chromosome released eukaryote which is active, transitory and environment-friendly(CREATE).

(3) Question

Cas9 protein multi-target editing efficiency is low.

3.2 BUCT

(1) Project name

Delivering gaba and 5Htp through fatty acid lowering (DeliGHtFAL)

(2) Project introduction

Humans have been plagued by obesity and anxiety for a long period of time. Studies have shown that obesity can aggravate anxiety while depression and anxiety also fuel obesity, namely the "depression-obesity circle". In order to break this circle and alleviate people's "body shame" and anxiety, we used E. coli Nissle 1917 as a chassis with enhanced capacity of β-oxidation of fatty acid thereby increasing fatty acids consumption from diets to facilitate weight loss. Meanwhile, GABA and 5-HTP with anxiety-relieving effects were synthesized from the catabolic products of fatty acids and glycerol in our engineered bacteria. To sum up, this project aims to ameliorate obesity and anxiety simultaneously by converting fatty acids, which causes "body shame", to GABA and 5-HTP, ultimately breaking the "depression-obesity circle".

(3) The CRISPR-related part of the experiment

Knocking down fadR with CRISPR technology to boost the beta-oxidation of fatty acids.

To render E. coli to absorb more fatty acids, we increase the expression of FadL (long-chain fatty acid transporter protein) on the surface and FadD (acyl-CoA synthetase). Because the FadR protein repressed the expression of about 15 genes concerning with beta oxidation, so we have to knock down the fadR gene.

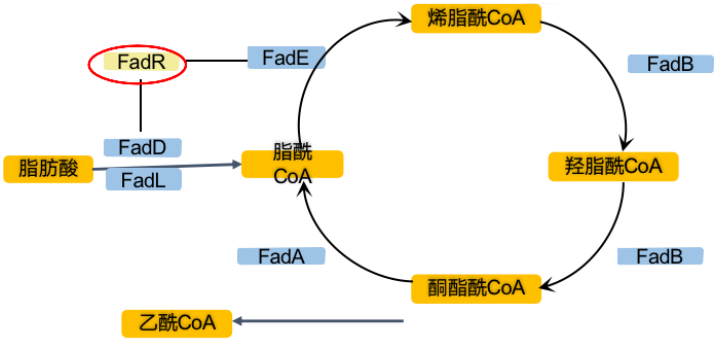

Figure 3.1 E. coli acid beta oxidation pathway

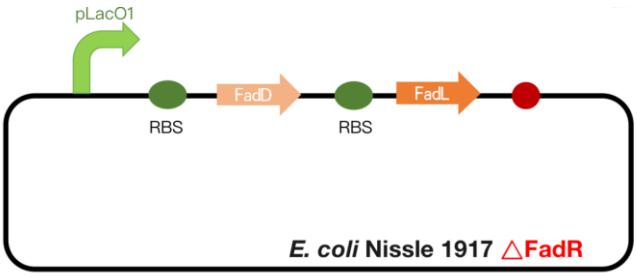

Figure 3-2 plasmid

(4) Question

At present, our team has been successfully using CRISPR technology to knock out fadR gene.we hope to obtain the improvement of experimental design and system from the conference, so as to provide technical support for the experimental part .

How to eliminate the resistance efficiently?

3.3 OUC-China

(1) Project name

ALLPASs（Amplifying, Low-leakage Platform for Antibiotic Sensors）

(2) Project introduction

Due to the large-scale abuse of antibiotics, the environmental pollution and food residues caused by antibiotics have posed a great threat to the ecological environment and human health. Therefore, this project aims to design a series of whole cell biosensors (WCBs) to detect three kinds of common antibiotics.. To break through the common limitations of WCBs, a fluorescent RNA aptamer is used as the output signal to increase the response speed, and it is hoped that the signal-noise ratio and dynamic range of the sensor can be improved by the NIMPLY logic gate gene circuit composed of CRISPRi and strand replacement reactions.

(3) Use of CRISPR in the project

CRISPR is not the main research content of this project, but it is also critical as a module in our genetic circuit. In the whole genetic circuit, an RNA fluorescent aptamer (3WJdB) serves as the output signal, but a complementary antisense RNA (KB2) is constitutively expressed to repress the leakage of the output signal. In this project, CRISPRi was used to remove the repression when the input inducible signal was present. Specifically, dCas9 protein is constitutively expressed, while sgRNA is induced by the input signal (i.e., the antibiotic under test) to guide dCas9 to bind to the upstream binding site of antisense RNA and ultimately to repress the downstream transcription.

(4) Question

Since the plasmids have not arrived yet and some plasmid construction problems, we have not carried out the CRISPR module experiments, so we cannot ask very specific questions. But here are some of the questions that might be troubling in the future.

i. How fast does the Cas9 or dCas9 protein take effect?

It has been pointed out in some studies that the time required for gRNA binding to Cas protein and guiding it to the target is not short. Therefore, will it affect the reaction time of the gene circuit, and is it suitable for the design of rapid detection? Is there any way to speed it up?

ii. As Cas9 protein is relatively large, how does it burden cells?

iii. What is the actual repression effect of dCas9 protein on the target in prokaryotic system?

iv. What are the advantages of using CRISPR to regulate gene circuits compared to traditional methods such as repressor or Riboswitch?

3.4 NNU-China

(1) **Project name**

Constructing the strain library of E. coli for improving the antimicrobial peptides production.

(2) **Project introduction**

With the increasing exacerbation of antibiotics' overuse, more and more antibiotic-resistant bacterial strains have emerged. Antimicrobial peptides (AMPs) are considered one of the promising alternatives to antibiotics. Escherichia coli is the first microorganism to be extensively used for the production of recombinant proteins. However, the expression of AMPs genes is problematic due to the toxicity of the proteins to the expression host. In order to improve the production of various AMPs, the RBS library of T7 RNA polymerase based on the E. coli BL21 (DE3) will be constructed using CRISPR gene editing tools. For target AMPs, the most suitable expression host could be screened for library in only three days, thus realizing "private antimicrobial peptide customization". Based on this, our project covers two aspects: the construction of library and the application of library. We aim to protect the human health and environment by reducing the abuse of antibiotics.

(3) **Question**

At first, we used CRISPR single gene editing editor to change the RBS sequence to achieve the purpose of database construction, but the effect was not very good. Later, we used crispr-cas9 double plasmid to build the database, and the situation was improved.

3.5 NWU_China_A

(1) Project name

An adventure of ancient snails

(2) **Project introduction**

Tyrian purple, also known as,6,6-dibromoindigo, is a kind of natural pigment with a long history of production and application, which was always been preferred by the royal. ln addition to its value in history and culture, as a kind of novel biocompatible semiconductor it also receive much attention. However, due to natural yield little, difficulty in chemosynthesis and the potential pollution, its high yield production hasn't been realized. Therefore, we plan to implement a two-cell reaction by spatiotemporally separating the two consecutive procedures of the production, so that the enzyme in tyrian purple metabolic pathway could achieve its highest efficiency. Moreover, we can choose some gene expression regulatory parts to improve the high-yield and high-purity dual cell system.

(3) **Question**

i. We are in the early stage of the experiment, no one in this team has relevant experience, everything is still groping, can you provide some small tips to avoid some detours?

ii. To obtain efficient gene editing, what aspects should be paid attention to in sgRNA design?

3.6 ZJUT-China

(1) Project name

CRISPR/Cas9-based Cell-Free Biosensors For RNA Biomarker

(2) **Project introduction**

According to literature, various RNA molecules in urine or blood have proven to serve as biomarkers for the diagnosis and prognosis of various diseases. Based on that, we are developing a cell-free RNA biosensor which comprises a cell-free system, engineered DNA transcription templates and RNA-responsive CRISPR/Cas9 System. We hope our project will provide patients with a simple, safe, low-cost diagnostic method.

(3) Question

i. How to quantitatively judge the cleavage activity of gRNA in vitro?
ii. The gRNA obtained by IVT is easily degraded after purification. Is there a good way to improve it?
iii. How to perform an EMSA experiment for two RNAs?
iv. Cas9 gene is long and it is difficult to construct plasmids
v. The off-target rate is high, and it takes time to screen gRNA with high cleavage activity, and we don’t know how to quantitative comparison of cleavage activity

3.7 XJTLU_China

(1) **Project name**

Dr. Phage

(2) **Project introduction**

In a brief summary, our project is to use edited phages containing specific protein-coding genes to infect and lyse bacteria and release the transfected exogenous protein “luxR”, which can then activate downstream cell-free gene circuit and generate visual signal. At this point we use CRISPR-Cas9 system and the method of homologous recombination to edit the target phages. The purpose of phages in our project is to specifically identify bacteria, while the edited phage will not be assembled as the gene of related key protein is cleaved. The downstream circuit outputs binary signals indicating the bacteria concentration meets the national standard or not. It means if the concentration of the target bacteria is higher than the standard, the system will indicate visual signal, otherwise the signal will not be displayed.

(3) **Question**

Screening methods for effective crRNA for phage genes.

Most screened CrRNAs cannot cleave phage genes.

3.8 NEFU_China

(1)Project name

Visible Diagnosis Platform by G-quadruplex(VDPG)

(2) **Project introduction**

We found that in the spread of large-scale epidemics such as COVID-19, traditional PCR nucleic acid testing may not be the best solution for some developing countries, and it has high technical and qualification requirements. Therefore, we proposed VDPG: Visible Diagnosis Platform by G-quadruplex. It can get rid of the limitations of laboratory conditions, and can achieve rapid nucleic acid detection at home or even in the field. We use Recombinase Polymerase Amplification (RPA) to selectively amplify the sequence to be tested, and then use the nCas9-Phi29 fusion protein for specific recognition and cutting. The product of the fusion protein is ssDNA, which is the primer that initiates Rolling Circle Amplification (RCA). The product of RCA is repetitive G-rich ssDNA. This ssDNA can form a large number of G-quadruplexe to catalyze the color reaction. Through the color reaction, we can visualize the nucleic acid detection results. Our design can improve the ability of some underdeveloped countries and regions to deal with large-scale epidemics.

(3) **Question**

i. The self-expressed and purified Cas9 protein is not efficient when cleavage dsDNA in vitro.
ii. nCas9 cause single-strand break (SSB), Phi29 polymerase initiates the strand displacement from the cut, how to sensitively detect the displaced ssDNA?
iii. Binding Verification of nCas9 and dsDNA (EMSA)

3.9 CPU_China

(1)Project name

PEDe.

(2) **Project introduction**

Examplified by polyethylene, undegradable plastics have caused severe environmental crisis worldwide. Manganese peroxidase (MnP) uses hydrogen peroxide (H2O2) to produce high-redox-potential Mn3+, possessing a PE-degrading potential. Our project takes MnP as our key PE-degrading enzyme, employs aryl alcohol oxidase (AAO) to provide H2O2 for MnP, and utilizes hydrophobin-1 (HFB1) to increase substrate accessibility, meanwhile applying SpyCatcher-SpyTag system with CRISPR/dCas9 system to anchor MnP, AAO and HFB1 onto one double-stranded DNA scaffold according to certain spatial order, distance and proportion. The final complex works as a molecular machine that can adhere to and degrade PE in a green and swift way.

(3) **Question**

In our design, CRISPR/dCas9 system is applied in combination with SpyCatcher-SpyTag system to assenble our molecular machine. The C-terminal of dCas9 is fused with SpyCatcher, which enables dCas9 to identify and combine with protein components modified with SpyTag by forming an Isopeptide bond between SpyCatcher and SpyTag. Three kinds of complexes are built through this way. Mediated by 3 kinds of sgRNA, dCas9-SpyCatcher-SpyTag-protein conplexes will anchor to the specific sequences on dsDNA, eventually forming a complete system in vitro.

The problem we face now is how to complete the characterization of the dCas9-functional protein-dsDNA complex in vitro. Previously, we decided to use the AKTA purification system to separate proteins of different molecular weights in the assembled system, and perform systematic characterization based on the absorption peak detection results and electrophoretic mobility shift assay (EMSA). However, in the follow-up experiments, we found that the content of unwanted impurities in the obtained crude protein products was too high, thus preventing our targeted functional protein to be purified with the affinity chromatography column (Ni-NTA). Also, the molecular weight of the complex was too large for it to be separated from other proteins with EMSA, the differentiation was found to be poor. Therefore, we sincerely hope to be able to get everyone's help in the characterization of the big system.

3.10LZU_China

(1) Project name

Targeted inhibition of *SARS-CoV-2* infection based on CRISPR-Cas13 system

(2) **Project introduction**

Currently, the world is stillfaced with the COVID-19 epidemic caused by novel coronavirus which urgently requires flexible and targeted protection measures. Novelcoronavirus belongs to the family of forward RNA viruses. The spike proteins on its surface bind to the human cell surface receptors called ACE2 and then enter the cell through endocytosis. Reducing the expression of ACE2 does not cause significant structural abnormalities. Therefore, recognizing and inhibiting the expres- sionlevel of ACE2 is considered to be an effective treatment for COVID-19. CRISPR-Cas13d is an RNA-guided ribonuclease which targets ssRNA .The RNA-targeted ribonuclease activity of Cas13d is independent of the specific adjacent sequences, which meets the requirements of rapid devel opment of gRNA.

SARS-CoV-2 mainly infects the human respiratory and digestive systems. It causes disease through direct cytotoxic effects and inducing inflammatory response. We select human embryonic kidney cells (HEK293T), lung cells (HLF-a), colorectal cells (CCD-18Co) and gastric cells (GES-1)to establish hACE2 stable transfection lines respectively and lentivirus with novel coronavirus spike protein is used as pseudovirus.Through bioinformatics screening of sgRNA pool for ACE2 conserved sequence, the lentivirus /CRISPR-Cas13d system was constructed. Specific crRNA-mediated Casl3d system is used to target and knock down the mRNA level of ACE2 in four stable celllines,which leads to a decrease in the expression of the receptor protein ACE2 on the cell surface.Thus, entry of the pseudovirus into cells was inhibited. We will use vitro and cell experiments toverifythe effect of CRISPR-Cas13d system designed by us.

(3) **Question**

We plan to use a lentiviral vector to express RfxCas13d and sgRNAs. It’s designed to target the mRNA of hACE2 in HEK293T/hACE2 stable cell line. Our problem is to choose 30nt Cas13d direct repeats or 36 nt Cas13d direct repeats when express multiple pre-sgRNAs in a lentiviral vector.

3.11 Tongji_Software

(1) Project name

Phage Microbiome Assist Phagotherapy，Phage-MAP

(2) **Project introduction**

i. **Background**

Antibiotics are considered as a sharp tool to kill bacteria and treat related/relevant diseases. However, the extensive use of antibiotics may lead to the emergence of superbugs that are highly resistant to antibiotics. Once being infected with superbug it may be in a hopeless predicament. It is expected that more and more people will die of superbug in the next few decades.

Figure 3-3

Interestingly, there is a natural bacterial killer, Phage, which specifically infects and destroys pathogenic bacteria, killing only one or one class of bacteria, so they will not damage the normal flora of human body and produce toxic and side effects. Thus, phage therapy has been developed and become a new weapon of targeted anti-bacterial infection.

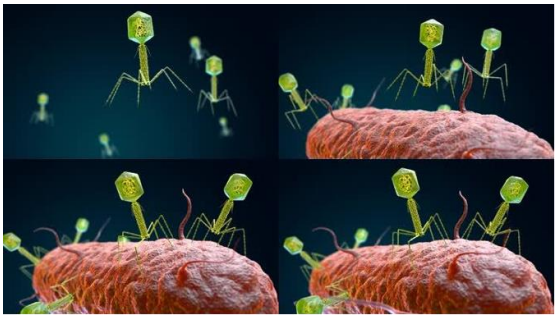

Figure 3-4 Phages are infecting bacteria

But how can we choose phage to intervene in treatment? Due to the high specificity of bacteriophages, it is usually necessary to screen a large number of phages to find bacteriophages against specific strains.

ii. Project purpose

The project of Tongji_Software, Phage-MAP, is using computer methods to solve this problem. The team members plan to use the CRISPR immune system of bacteria to find the same Spacer sequence in the phages and the bacteria, so as to establish a map of the interaction network between the two.The following figure shows the role of spacer sequence in CRISPR system.

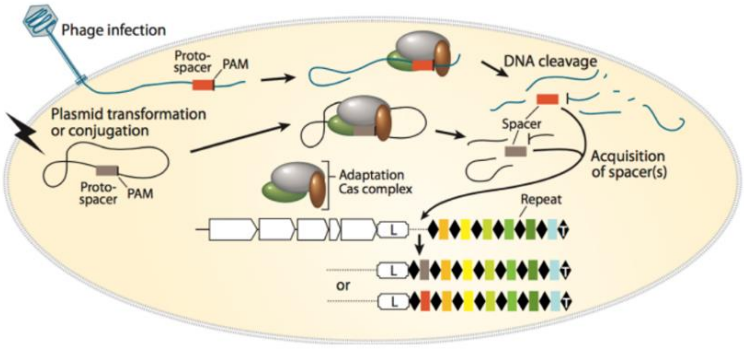

Figure 3-5 The role of spacer sequence in CRISPR system when bacterial resist phage invasion

Project flow

In order to achieve this goal, they did three parts of work: 1. Establish a database, 2. Create an efficient search tool, 3. Establish a visual network map. First, when establishing the database, the team considered 26,773 sequences of 10,819 species of bacteria and 14,571 sequences of 12041 species of phage. Through the BLAST alignment algorithm, 22220 positive alignment records were obtained. Secondly, they provide a search function that supports multiple keywords. In order to speed up the search, team members put the above data into a database with a well-organized structure. Finally, to improve the user experience, the team established a website and implemented an interactive visual network graph function on the website.

Figure 3-6 This is a simple example

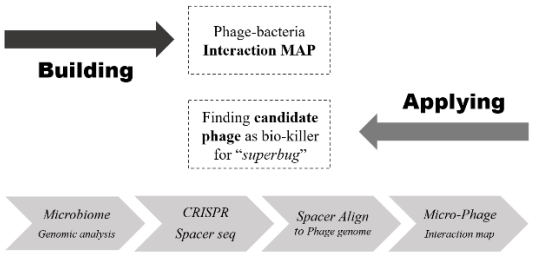

Figure 3-7 The flow for building and using databases

The team members of Tongji_Software hope that with the help of Phage-MAP, doctors can quickly find the corresponding phage for patients infected with superbugs, and bring hope to patients.

(3) **Question**

i. Only using spacer comparison, the detection rate is low;

ii. It is difficult to find the validation set;

iii. At present, the most difficult thing is that some phages are lysogenic. We want to screen them, but there is no very effective tool and database in this regard.

CHAPTER 4
QUESTION & ANSWER

4.1 Tianjin

(1) Question

Cas9 protein multi-target editing efficiency is low.

(2) Answer

Reason of low editing efficiency:

i. Poor Cas9 cleavage activity and easy off-target may be caused by the design of sgRNA. Optimized sgRNA may change this situation. There are some data base could provide reference[14].

ii. It may be related to the DNA repair mechanism: try to transfer an oligonucleotide sequence that is not homologous to the yeast gene[15].

iii. The problem caused by genome structure and chromatin state is more complicated to be solve[16].

iv. The constructed plasmid has low expression efficiency and can be optimized from the vector, promoter, RBS, and codon preference.

4.2 BUCT

(1) Question

At present, our team has been successfully using CRISPR technology to knock out fadR gene.we hope to obtain the improvement of experimental design and system from the conference, so as to provide technical support for the experimental part .

How to eliminate the resistance efficiently?

(2) Answer

Cultivate the bacteria in Non-resistant medium. The Pcas plasmid will be eliminated after several generations.

4.3 OUC-China

(1) Question

Since the plasmids have not arrived yet and some plasmid construction problems, we have not carried out the CRISPR module experiments, so we cannot ask very specific questions. But here are some of the questions that might be troubling in the future.

i. How fast does the Cas9 or dCas9 protein take effect?

It has been pointed out in some studies that the time required for gRNA binding to Cas protein and guiding it to the target is not short. Therefore, will it affect the reaction time of the gene circuit, and is it suitable for the design of rapid detection? Is there any way to speed it up?

ii. As Cas9 protein is relatively large, how does it burden cells?

iii. What is the actual repression effect of dCas9 protein on the target in prokaryotic system?

iv. What are the advantages of using CRISPR to regulate gene circuits compared to traditional methods such as repressor or Riboswitch?

(2) Answer

i. Cas9: RNA complex cutting DNA rate

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4106473/

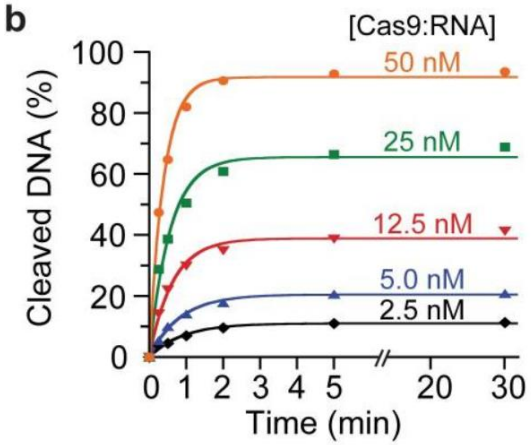

Figure 4.1 RNA complex cutting DNA rate

a. Changing the existing mode of transport is certainly not realistic, so one of the feasible ways is to change the concentration of the substrate, speed up, and, in general, more easily reach a certain amount of encounter and then reaction. Consideration may be given to enhancing the expression of the relevant genes. For example, increase the concentration of guided RNA or protein.

b. The rate of degradation of the original antonymic RNA may be more important than inhibiting the expression of antonymic RNA

c. Specifically, tests are also needed to see the results.

ii. a. Cas protein 158KDa, green fluorescent protein 28KDa. The difference between the two protein molecular weights is not large. The OUC team wants to use Cas protein to regulate a specific line. Green fluorescence can detect the amount required should be relatively large. So don't worry too much about the burden of engineering cells.

b. The later design of manual detection devices, cell environment can be set.

iii. a. Compared to the characteristics of CRISPR in mammals, which requires the fusion of chromic glycoplasmic syringes with dcas9, the CRISPR system alone can significantly inhibit gene expression in bacteria.

b. If you want to describe specifically, you may be able to measure it with parameters such as dissocation constants.

c. If you want exact data, you may still have to experiment.

iv. a. Editability can be achieved by regulating RNA sequences to regulate gene expression. Unlike riboswitch, which requires a variety of substrates to regulate, CRISPR simply changes the RNA sequence. But if you target a target, the advantage is not obvious.

b. The response speed is faster than the inhibitor protein, which is expressed in a fixed way. Synthesis guides RNA when needed, without having to resynthesis to deter proteins coming out.

4.4 NNU-China

At first, we used CRISPR single gene editing editor to change the RBS sequence to achieve the purpose of database construction, but the effect was not very good. Later, we used crispr-cas9 double plasmid to build the database, and the situation was improved.

4.5 NWU_China_A

(1) Question

i. We are in the early stage of the experiment, no one in this team has relevant experience, everything is still groping, can you provide some small tips to avoid some detours?

ii. To obtain efficient gene editing, what aspects should be paid attention to in sgRNA design?

(2) Answer

Efficient gene editing-sgRNA design principles

sgRNA contains the target sequence (crRNA sequence) and Cas9 nuclease recruitment sequence (tracrRNA).

The crRNA sequence is a 20-nucleotide sequence homologous to the target gene and guides Cas9 nuclease activity.

crRNA sequence contains seed sequence and non-seed sequence. The seed sequence determines the specificity of Cas9, while the non-seed sequence affects the conformational changes of Cas9 and participates in the targeted cleavage of DNA by CRISPR/Cas9, so both are essential.

How can we design a good sgRNA? Both cutting efficiency and specificity must be considered.

i. Cutting efficiency-the location of the target site

a. Avoid targeting the N-terminal or C-terminal of the gene:

Avoid targeting the N-terminus of genes to reduce the use of alternative ATG downstream of the start codon. Avoid targeting the C-terminus of genes to maximize the chance of creating non-functional alleles. (Because mutations in fragments near the end of the protein are unlikely to interfere with gene expression)

b. Avoid targeting 5' and 3' UTRs (untranslated) regions

c. Some gene-specific modes, such as steric hindrance, can also reduce the efficiency of gene editing.

ii. Specificity

a. Truncated gRNAs (trugRNAs)

The crRNA sequence is less than 20 nucleotides, which can reduce off-target site mutations up to 5000 times or more without sacrificing the efficiency of target genome editing. Truncated gRNAs with 17 or 18 nucleotides are called "trugRNA". 17 nucleotides is the minimum length for effective gene editing. According to experience, the use of 18-19bp trugRN is the best, and some 17bp trugRNA may not work. (The possible reason is: the 20bp gRNA has more affinity for its target site than required, so the 18-19bp trugRNA will also work, and the trugRNA may make the tru-RGN/DNA complex more sensitive to mismatches.) trugRNA can effectively introduce targeted indel or HDR-mediated genome editing events into human cells.

b. Specific tru-gRNA target site

tru-RGN (RNA-guided nucleases; RNA-guided nucleases) usually induce low-level mutations at potential off-target sites with 1 or 2 mismatches; in contrast, standard RGNs have as many as 4 or 5 Multiple off-target sites with multiple mismatches caused high levels of mutations. Therefore, a reasonable strategy to reduce off-target effects may be to select specific tru-gRNA target sites on the genome, and have the smallest number of potential off-target sites containing 1 or 2 mismatches.

(3) share

i. Preparation

- Transform DH5α with pCas and pTarget.
- Pick a single colony for sequencing.
- Check out the sequencing result and extract plasmids.

ii. Transform

- Transform the BL21(DE3) competent cells with pCas. Inoculated the recovered seed cultures into LB media with 50μg/L kanamycin and incubate at 30℃.
- Pick a single colony of transformant on the plate. Prepare competent cell by following the protocol of TaKaRa competent cell preparation Kit. Add arabinose to a final concentration of 10 mM in the culture when its OD600 reach 0.1, which will induce the expression of λ-Red to mediate homologous recombination.
- Centrifugate the culture to collect the cell when the OD600 reach 0.6. Mix 50 μl of cell with 100ng pTarget, to conduct the electroporation.
- Recover the cell in 1 ml LB media for 1 hour at 30 ℃ after electroporation. Spread the cell onto LB agar containing kanamycin (50 mg/liter) and spectinomycin (50 mg/liter) and incubated overnight at 30°C.

iii. Validation

- Perform PCR with transformant as template. Use untreated strain as control to compare the length of PCR product and sequence for validation.

iv. Plasmid curing

- Inoculate the validated cell into LB media containing kanamycin (50 mg/L) and IPTG (0.5mM), spread the cell onto LB agar containing kanamycin after 8-16 hours incubation. Use LB agar containing ampicillin as control (plasmid is successfully cured if the control group doesn't have any visible colony).
- Incubate the cell at 43℃ (or 37℃) to cure pCas.

4.6 ZJUT-China

(1) Question

i. How to quantitatively judge the cleavage activity of gRNA in vitro?

ii. The gRNA obtained by IVT is easily degraded after purification. Is there a good way to improve it?

iii. How to perform an EMSA experiment for two RNAs?

iv. Cas9 gene is long and it is difficult to construct plasmids

v. The off-target rate is high, and it takes time to screen gRNA with high cleavage activity, and we don't know how to quantitative comparison of cleavage activity

(2) Answer

i. LZU-China: ImageJ can be used for grayscale analysis.

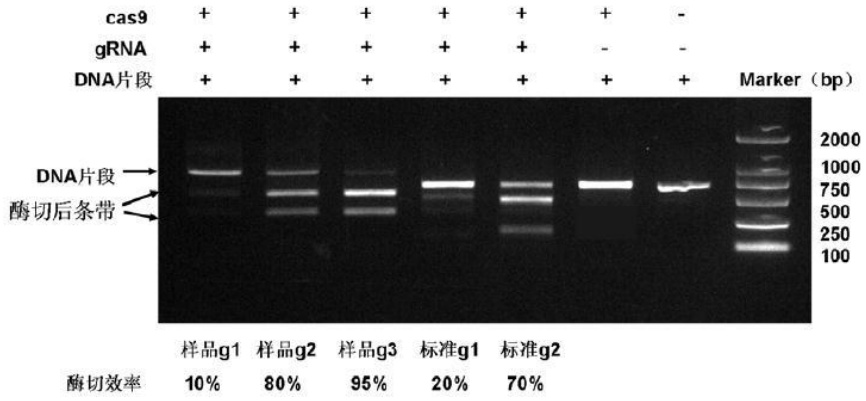

Figure 4.2 ImageJ can be used for grayscale analysis

ii. LZU-China:You can use transgene's purification kit for RNA purification. At the same time, it is recommended to add 1 microliter of RNase inhibitor during the IVT reaction. Also, it is significant to wipe the ultra-clean table with detergent before using it, and tips and ep tube are treated with nuclease-free treatment. During sample loading, take 2 microliters of purified RNA diluted with DEPC water to 10 microliters with 10x DNA loading buffer, then run the gel at 120V for about 10 minutes.

iii. CPU-China: The team has done the binding between RNA and protein. First, Cas9 protein and sgRNA were mixed on ice to incubate for 30 minutes, and then 6% EMSA gel was run to set up three groups of cas9 protein, pure RNA, and protein RNA complex. Simultaneously, running two gels, one is stained with ethidium bromide and the other is stained with Coomassie Brilliant Blue. Finally, by comparing the positions of protein and RNA on the two gels, determine whether there is binding.

iv. LZU-China:After communicating with the ZJUT team,we learned that they are planning to use a cell-free system to express Cas9.The ZJUT team has difficulty in constructing the plasmid without

arac and λ-Red.It's a pity that we don't have enough experience in molecular cloning and constructing plasmid,but we have had similar problem.When we were constructing two lentiviral vectors longer than 10kb, a lentiviral vector used to establish a stable cell line of HEK293T was inserted into mRFP through one-step cloning. It took a month and was not constructed.Finally we entrusted GENEWIZ with plasmid construction. We suggest that if the future teams have difficulty in plasmid construction,they should purchase the original plasmid from Addgene or entrust a company to construction.

v. LZU-China: The ZJUT team plans to use a cell-free system. The DNA fragments are added during the reaction, and it does not involve off-target problems but only the cleavage activity. We use Cas13d and don't have much practical experience with Cas9. We communicated with ZJUT students and learned that highly active gRNAs are generally predicted by software. If it is predicted that there are more off-targets, manual design might be better. In addition to improving the specificity of sgRNA, multiple sgRNAs should be designed to target the target sequence at the same time.

We found some methods for manually designing Cas9 sgRNA from a website, and we can also refer to GenScript's CRISPR headbook.

For quantitative comparison of cleavage activity, after discussion, we think that the cleavage activity of Cas9 can be semi-quantitatively analyzed by gray-scale analysis of gel electrophoresis with software such as ImageJ.

4.7 XJTLU_China

(1) Question

i. Screening methods for effective crRNA for phage genes.

ii. Most screened CrRNAs cannot cleave phage genes.

(2)Answer

i. The crRNA selection method provided in the literature was adopted;

a. R code screening,

b. Geneious software screening;

Both methods have a phage-specific DNA database to test for effectiveness.

ii. CrRNAs with low GC content in the double strand were selected, and crRnas that might affect the structural proteins of phages were selected.

4.8 NEFU_China

(1) Question

i. The self-expressed and purified Cas9 protein is not efficient when cleavage dsDNA in vitro.

ii. nCas9 cause single-strand break (SSB), Phi29 polymerase initiates the strand displacement from the cut, how to sensitively detect the displaced ssDNA?

iii. Binding Verification of nCas9 and dsDNA (EMSA)

(2)Answer

i. a. Design multiple groups of sgRNA, which can be assisted by various websites.

The CG content of the seed sequence is controlled at 40%-60%, screen out the best cutting efficiency from multiple groups of sgRNA.

(https://crispy. secondarymetabolites.org/#/input)

b. Pay attention to RNase free enviornment during the purification process of Cas9 protein, which may affect the binding efficiency of Cas9 and sgRNA.

c. Refer to the commercial kit to optimize the cutting buffer.

ii. a. Molecular probes can be designed to label ssDNA, but dsDNA may cause a certain background signal.

b. Use ssDNA specific dyes (such as oligreen) for quantitative analysis, which has a very high detection sensitivity.

iii. a.Nucleic acid removal treatment is carried out before nCas9 protein is combined with dsDNA. Nuclease can be added when Escherichia coli is lysed; ion-exchange chromatography(IEC) is used to remove nucleic acid. The Q column has a strong adsorption effect on nucleic acid and requires a high salt concentration. Only the nucleic acid can be eluted, and the nucleic acid in the protein solution can be easily removed.

b. Try to make the length of dsDNA as small as possible. In this way, the mobility of the protein and dsDNA complex will change more.

c. Use about 6% non-denaturing polyacrylamide gel, if the gel concentration is too high, the complex is difficult to migrate and difficult to analyze.

4.9 CPU_China

(1) Question

In our design, CRISPR/dCas9 system is applied in combination with SpyCatcher-SpyTag system to assenble our molecular machine. The C-terminal of dCas9 is fused with SpyCatcher, which enables dCas9 to identify and combine with protein components modified with SpyTag by forming an Isopeptide bond between SpyCatcher and SpyTag. Three kinds of complexes are built through this way. Mediated by 3 kinds of sgRNA, dCas9-SpyCatcher-SpyTag-protein conplexes will anchor to the specific sequences on dsDNA, eventually forming a complete system in vitro.

The problem we face now is how to complete the characterization of the dCas9-functional protein-dsDNA complex in vitro. Previously, we decided to use the AKTA purification system to separate proteins of different molecular weights in the assembled system, and perform systematic characterization based on the absorption peak detection results and electrophoretic mobility shift assay (EMSA). However, in the follow-up experiments, we found that the content of unwanted impurities in the obtained crude protein products was too high, thus preventing our targeted functional protein to be purified with the affinity chromatography column (Ni-NTA). Also, the molecular weight of the complex was too large for it to be separated from other proteins with EMSA, the differentiation was found to be poor. Therefore, we sincerely hope to be able to get everyone's help in the characterization of the big system.

(2)Answer

In terms of purification, you can consider replacing the label to perform a Pull-Down experiment for separation, and the labeled and interacting proteins are precipitated together and then characterized

4.10 LZU_China

(1) Question

We plan to use a lentiviral vector to express RfxCas13d and sgRNAs. It's designed to target the mRNA of hACE2 in HEK293T/hACE2 stable cell line. Our problem is to choose 30nt Cas13d direct repeats or 36 nt Cas13d direct repeats when express multiple pre-sgRNAs in a lentiviral vector.

(2)Answer

The length of Direct repeat should not be too long, it is recommended to be controlled within 30nt. Different laboratories may use different lengths of Direct repeat to obtain different results, and you can try different length aspect ratios.

4.11 Tongji_Software

(1) Question

i. Only using spacer comparison, the detection rate is low;

ii. It is difficult to find the validation set;

iii. At present, the most difficult thing is that some phages are lysogenic. We want to screen them, but there is no very effective tool and database in this regard.

(2)Answer

i. Bacterial resistance to phages involves a variety of immune systems, and CRISPR / CAS system plays limited roles. Therefore, it is not comprehensive to predict the interaction between bacteria and phages only based on spacer sequence. In order to make the prediction more comprehensive and reliable, more parameters should be added. Among them, the first and second generation gene editing techniques, namely zinc finger proteins (ZFN) and talens are important immune mechanisms of bacteria.

Secondly, phage-bacteria recognition experiences nonspecific recognition and specific recognition in chronological order. The former is based on random collision, while the latter depends on the recognition mechanism between phage' surface protein and bacterial cell receptor, and theses proteins correspond to a specific DNA sequences, which may become another comparison parameter.

Moreover, for lysogenic phage, its integration site on the bacterial genome is relatively conservative. As such, lysogenic integration site may be another candidate parameter.

A new question: the bacteria with spacer sequence represents that they can be infected by the corresponding phage, but it also proves that they have a certain resistance to such phage. Therefore, how to evaluate whether the phage is suitable as a candidate for phage therapy? We think a rational edition of the phage candidate might be needed to have better performance.

ii. Practical verification is needed to test the accuracy of database. One of the solutions is to cooperate with the teams with phage related experimental certificate and let the team assist the verification. Or it may work to cooperate with the laboratories exploring phage therapy. Ask them to use the database to select phage candidates to see whether the success rate is improved compared with the randomly selected candidates. However, if you want to verify the accuracy and universality of the database more comprehensively, the amount of data generated by cooperation with several laboratories is obviously not enough. Therefore, it is necessary to widely search the existing experimental data or the established database of phage infection of E. coli based on reality.

iii. Database collection is not our area of expertise, but we have also selected some databases or websites for reference.

a. Actinobacteriophage Database at PhagesDB（https://phagesdb.org/phages/）

The Actinobacteriophage Database at PhagesDB.org is a website that collects and shares data, pictures, protocols, and analysis tools associated with the discovery, sequencing, and characterization of **mycobacteriophage**—viruses that infect the *Mycobacteria* and also other bacterial hosts in the phylum Actinobacteria.

b. CRISPRCasdb (https://crisprcas.i2bc.paris-saclay.fr)

This is a database that can simultaneously query and identify CRISPR sequences and Cas genes online, providing valuable data resources and analytical tools for CRISPR-CAS system diversity research. It includes 16,990 complete prokaryotic genomes (16,650 bacteria from 2,973 species and 340 archaea from 300 species). The simultaneous identification of CRISPR sequences and Cas genes and the type and subtype of the system were realized using the CRISPRCasFinder program. And this website built in a variety of query screening and BLAST tools, very convenient.

c. CRISPRminer(http://www.microbiome-bigdata.com/CRISPRminer/)

It's a knowledge base and web server to comprehensively collect and investigate the knowledge of CRISPR-Cas systems and generate instructive annotations, including CRISPR arrays and Cas protein annotation, CRISPR-Cas system classification, self-targeting events detection, microbe–phage interaction inference, and anti-CRISPR annotation.

d. International Nucleotide Sequence Database Collaboration (INSDC).

ISDC is a long-standing foundational initiative that operates between DDBJ, EMBL-EBI and NCBI. INSDC covers the spectrum of data raw reads, through alignments and assemblies to functional annotation, enriched with contextual information relating to samples and experimental configurations. It may be possible to obtain genetic information beyond NCBI alone.

e. Streamlining CRISPR spacer-based bacterial host predictions to decipher the viral dark matter

In this study[21], the authors created a database of >11 million spacers and a program to execute host predictions on large viral datasets. Addtionally, they evaluated the performance using 9484 phages with known hosts and obtained a recall of 49% and a precision of 69%.

f. the Phage Receptor Database (PhReD)

This is a database of known and newly identified phage receptors[22]. Specific receptors on bacterial surfaces are closely related to phage adsorption, and understanding the mechanisms and influencing factors is also crucial for studying host-phage interactions

References:

[1] Thomas Gaj,Charles A. Gersbach,Carlos F. Barbas. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering[J]. Trends in Biotechnology,2013,31(7):

[2] Jennifer A. Doudna,Emmanuelle Charpentier. The new frontier of genome engineering with CRISPR-Cas9[J]. Science,2014,346(6213)

[3] Lino Christopher A,Harper Jason C,Carney James P,Timlin Jerilyn A. Delivering CRISPR: a review of the challenges and approaches.[J]. Drug delivery,2018,25(1)

[4] Kleinstiver Benjamin P,Prew Michelle S,Tsai Shengdar Q,Topkar Ved V,Nguyen Nhu T,Zheng Zongli,Gonzales Andrew P W,Li Zhuyun,Peterson Randall T,Yeh Jing-Ruey Joanna,Aryee Martin J,Joung J Keith. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. [J]. Nature, 2015, 523(7561).

[5] Hu Johnny H,Miller Shannon M,Geurts Maarten H,Tang Weixin,Chen Liwei,Sun Ning,Zeina Christina M,Gao Xue,Rees Holly A,Lin Zhi,Liu David R. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity.[J]. Nature, 2018, 556(7699).

[6] Hiroshi Nishimasu,Xi Shi,Soh Ishiguro,Linyi Gao,Seiichi Hirano,Sae Okazaki,Taichi Noda,Omar O. Abudayyeh,Jonathan S. Gootenberg,Hideto Mori,Seiya Oura,Benjamin Holmes,Mamoru Tanaka,Motoaki Seki,Hisato Hirano,Hiroyuki Aburatani,Ryuichiro Ishitani,Masahito Ikawa,Nozomu Yachie,Feng Zhang,Osamu Nureki. Engineered CRISPR-Cas9 nuclease with expanded targeting space [J].Science, 2018, 361(6408): compatibility and high DNA specificity. [J]. Nature, 2018, 556(7699).

[7] Miller Shannon M,Wang Tina,Randolph Peyton B,Arbab Mandana,Shen Max W,Huang Tony P,Matuszek Zaneta,Newby Gregory A,Rees Holly A,Liu David R. Continuous evolution of SpCas9 variants compatible with non-G PAMs.[J]. Nature biotechnology, 2020, 38(4).

[8] Walton Russell T,Christie Kathleen A,Whittaker Madelynn N,Kleinstiver Benjamin P. Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants.[J]. Science (New York, N.Y.), 2020, 368(6488).

[9] Akcakaya Pinar,Bobbin Maggie L,Guo Jimmy A,Malagon-Lopez Jose,Clement Kendell,Garcia Sara P,Fellows Mick D,Porritt Michelle J,Firth Mike A,Carreras Alba,Baccega Tania,Seeliger Frank,Bjursell Mikael,Tsai Shengdar Q,Nguyen Nhu T,Nitsch Roberto,Mayr Lorenz M,Pinello Luca,Bohlooly-Y Mohammad,Aryee Martin J,Maresca Marcello,Joung J Keith. In vivo CRISPR editing with no detectable genome-wide off-target mutations.[J]. Nature,2018,561(7723).

[10] Wienert Beeke,Wyman Stacia K,Richardson Christopher D,Yeh Charles D,Akcakaya Pinar,Porritt Michelle J,Morlock Michaela,Vu Jonathan T,Kazane Katelynn R,Watry Hannah L,Judge Luke M,Conklin Bruce R,Maresca Marcello,Corn Jacob E. Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq.[J]. Science (New York, N.Y.),2019,364(6437).

[11] Kalebic N, Taverna E, Tavano S, et al. CRISPR/Cas9-induced disruption of gene expression in mouse embryonic brain and single neural stem cells in vivo［J］. EMBO Rep, 2016, 3 ：338-348.

[12] Hara S, et al. Microinjection-based generation of mutant mice with a double mutation and a 0. 5 Mb deletion in their genome by the CRISPR/Cas9 system［J］. J Reprod Dev, 2016, 62（5）：531-536.

[13] Hashimoto M, Yamashita Y, Takemoto T. Electroporation of Cas9 protein/sgRNA into early pronuclear zygotes generates non-mosaic mutants in the mouse［J］. Dev Biol, 2016, 418（1）：1-9.

[14] CRISPR/Cas9 Methodology for the Generation of Knockout Deletions in Caenorhabditis elegans. G3 (Bethesda). 2019 Jan 9;9(1):135-144.

[15] Non-homologous DNA increases gene disruption efficiency by altering DNA repair outcomes. Nature Communications, 2016; 7: 12463

[16] Computational Tools and Resources Supporting CRISPR-Cas Experiments. Cells. 2020 May 22;9(5):1288.

[17] Zhang XH, Tee LY, Wang XG, et al. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. Mol Ther Nucleic Acids. 2015 Nov 17;4:e264. doi: 10.1038/mtna.2015.37.

[18] Fu Y, Sander JD, Reyon D, et al. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nat Biotechnol. 2014 Mar;32(3):279-84. doi: 10.1038/nbt.2808. Epub 2014 Jan 26.

[19] Doench JG, Hartenian E, Graham DB, et al. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. Nat Biotechnol. 2014 Dec;32(12):1262-7. doi: 10.1038/nbt.3026. Epub 2014 Sep 3.

[20] Ran FA1, Hsu PD, Lin CY, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell. 2013 Sep 12;154(6):1380-9. doi: 10.1016/j.cell.2013.08.021. Epub 2013 Aug 29.

[21] Moïra B Dion, Pier-Luc Plante, Edwige Zufferey, Shiraz A Shah, Jacques Corbeil, Sylvain Moineau, Streamlining CRISPR spacer-based bacterial host predictions to decipher the viral dark matter, Nucleic Acids Research, Volume 49, Issue 6, 6 April 2021, Pages 3127–3138, https://doi.org/10.1093/nar/gkab133.

[22] *FEMS Microbiology Letters*, Volume 363, Issue 4, February, 2016, fnw002, https://doi.org/10.1093/femsle/fnw002

Appendix 1: Agenda for the meeting

the CRISPR conference,		
preparation		
Time	Schedule	The staff
9.5-9.6	Contact teams to collect specific questions.	Huang Yu Huang Yuxin Xu Yingjia
9.7	Data summary	
9.8	Count the team questions and draw lots to decide the questions corresponding to each team.	
9.11	Draw lots to determine the order of speech.	
9.12	Online meeting	
9.12 Meeting Process		
Time	Schedule	The staff
9:00-9:30	Opening Speech	Huang Yu Chang Jingyu Dong Yufeng Yang Yuchen Xu Yingjia
9:30-9:45	Team 1 shares	
9:50-9:55	discussion	
9:55-10:10	Team 2 shares	
10:10-10:15	discussion	
10:15-10:30	Team 3 shares	
10:30-10:35	discussion	
10:35-10:45	Intermission and free discussion	
10:45-11:00	Team 4 shares	
11:00-11:05	discussion	
11:05-11:20	Team 5 shares	
11:20-11:25	discussion	
11:25-11:55	Experience sharing	
11:25-14:05	Lunchtime	
14:10-14:25	Team 6 shares	
14:25-14:30	discussion	
14:30-14:45	Team 7 shares	
14:50-14:55	discussion	
14:55-15:10	Team 8 shares	
15:10-15:15	discussion	
15:15-15:30	Team 9 shares	

15:30-15:35	discussion	
15:35-15:45	Intermission and free discussion	
15:45-16:00	Team 10 shares	
16:00-16:05	discussion	
16:05-16:20	Team 11 shares	
16:20-16:25	discussion	
16:25-16:35	The closing ceremony	

Material sorting after the meeting

Time	Schedule	The staff
9.12-9.13	Each team sorted out the information and sent it to the staff, who sorted it out and collected it.	Wu Xu Sun Dawei
9.13-9.15	Make the first draft of the CRISPR Application Guide.	Dong Yufeng
9.15-9.17	Revise and finalize the Guide.	

Appendix 2: Links to teams' web pages

Team：Tianjin

Website link: https://2021.igem.org/Team:Tianjin

Team：BUCT

Website link: https://2021.igem.org/Team:BUCT

Team：NNU-China

Website link: https://2021.igem.org/Team:NNU-China

Team：NWU_China_A

Website link: https://2021.igem.org/Team:NWU-CHINA-A

Team：ZJUT-China

Website link: https://2021.igem.org/Team:ZJUT-China

https://video.igem.org/w/qgfpuHjrEcw1JgiY8o75y8

Team：XJTLU_China

Website link: https://2021.igem.org/Team:XJTLU-CHIN

XJTLU-CHINA: Dr. Phage (2021) - Project Promotion [English] - iGEM Video Universe

Team：NEFU_China

Website link: https://video.igem.org/w/4WNTQJUHEP6a1gXkYBsZ4s

Team：CPU_China

Website link: Team:CPU CHINA - 2021.igem.org

Team：LZU_China

Website link: https://2021.igem.org/Team:LZU-CHINA

Team：Tongji_Software

Website link: http://47.100.225.207/