CRISPR-Cas9 mediated phage therapy provides a sequence-specific alternative to antibiotics

Josephina Hendrix\textsuperscript{1,2,3}, Daren Kraft\textsuperscript{1,2}, Leighla Tayefeh\textsuperscript{3,4}, Kirill Novik\textsuperscript{1,3}, Rishabh Yadav\textsuperscript{5}, Kendra Shattuck\textsuperscript{1}, Joshua Ivie\textsuperscript{1}

Advisors: Dr. Robin Dowell, Tim Read, Samantha O’Hara


Introduction

The emergence of pan-resistant strains of bacteria poses an imminent risk to public health. To combat the evolution of antibiotic resistance, we have demonstrated the potential for CRISPR-Cas9 mediated phage therapy as an antibacterial agent. *Escherichia coli* (kan\textsuperscript{+}) were infected by non-replicating CRISPR-Cas9 phage resulting in decreased survival from the targeting phage-variant versus the non-targeting control. CRISPR-Cas9 mediated phage therapy presents the opportunity to target bacterial species with a high degree of sequence specificity.

More specifically, type II CRISPR-Cas9 plasmids (BBa_K1218011) were modified to target a sequence existing within the neomycin phosphotransferase gene, which confers kanamycin resistance, yet does not appear elsewhere in the *E. coli* genome. The targeting and unmodified BBa_K1218011 were transformed into *E. coli* (kan\textsuperscript{+}). Results showed greatly reduced transformation efficiency with the targeting plasmid. The packaging signal from M13 phage was then cloned into BBa_K1218011 to form BBa_K1445001. The targeting sequence, previously mentioned, was cloned into BBa_K1445001 to create a targeting variant. Helper phagemids in *E. coli* synthesize phage capsids to package either BBa_K1445001 or the targeting variant phagemid, producing non-replicating CRISPR-Cas9 phage. *E. coli* (kan\textsuperscript{+}, F\textsuperscript{−}) were infected by the produced phage. Our infection results demonstrate the potential for CRISPR-Cas9 mediated phage therapy as a powerful and sequence specific antibacterial agent.

Introduction to CRISPR-Cas9

The components
- Cas9 is an endonuclease
- CRISPR codes for a guide RNA (gRNA) whose specificity is determined by its spacer sequence

Cas9 targeting
1. Cas9 and guide RNA form complex
2. Complex scans the DNA for Protospacer Adjacent Motifs (PAM site)
3. Cas9 binds the PAM and unravels adjacent DNA
4. gRNA anneals to complementary sequences
5. Cas9 cleaves DNA, causing cell death

BioBrick Parts

BBa_K1445000: The M13 phage packaging signal. This part is necessary and sufficient for plasmid packaging into M13 phage.

BBa_K1445001: M13 packaging signal and entire CRISPR-Cas9 machinery on one plasmid.

BBa_K1445002: This BioBrick backbone contains the M13 phage packaging signal and allows BioBrick parts to be incorporated into M13 phage.

Methods

Design spacer sequence
1. Identify a PAM site and its complementary spacer
2. Verify that the spacer is unique to the pathogen using our software

Construction of phagemids
3. Add packaging signal to CRISPR-Cas9 plasmid
4. Incorporate identified spacer sequence

Phage production
5. Transform CRISPR-Cas9 and helper phagemid
6. CRISPR-Cas9 phagemid replicates while helper phagemid expresses phage coat proteins
7. CRISPR-Cas9 Phagemid is packaged into phage

Infection
8. Phage deliver CRISPR-Cas9 phagemid to target cells
9. Cell expresses Cas9 and gRNA
10. CRISPR-Cas9 complex binds target sequence
11. Cas9 cleaves genomic DNA

Results

Phagemid delivery
M13 phage can deliver plasmids encoding the packaging signal (A) but not plasmids without a packaging signal (B).

CRISPR-Cas9 killing through transformation
*E. coli* (kan\textsuperscript{+}) were transformed with a CRISPR-Cas9 plasmid having a genome targeting (A) or non-targeting (B) gRNA. The difference in transformation efficiency demonstrates the specific killing ability of targeted CRISPR-Cas9.

CRISPR-Cas9 killing through phage delivery
The CRISPR-Cas9 phagemid can be delivered by M13 phage to infect *E. coli* (kan\textsuperscript{+}, F\textsuperscript{−}). Decreased cell growth is observed in the population infected with targeting (A) gRNA compared to the non-targeting (B) variant, demonstrating specificity of CRISPR-Cas9 phage.

Future Directions

Increase proficiency of phage packaging: Helper phagemids are packaged at low frequency, creating replicating phage and contaminating the product. Insertion of phage structural genes into the genome of a phage producing cell will eliminate helper phagemid packaging, thus purifying produced phage.

Accounting for mutation in target organism: Mutations in the target sequence can protect a bacterium from CRISPR-Cas9 targeting. Addition of multiple CRISPR arrays into CRISPR-Cas9 phagemid, each spacer targeting different sequences within a pathogen, will enable CRISPR-Cas9 mediated cell death in pathogen mutants.

Prevent proliferation of antibiotic resistance: Antibiotic resistance can be spread through plasmid transfer between species. Elimination of antibiotic resistance as a selection marker will improve the safety of CRISPR-Cas9 mediated phage therapy. By creating trpC auxotrophic bacteria and inserting the rescue trpC gene into CRISPR-Cas9 phagemids we can ensure selective retention of phagemid without proliferating antibiotic resistance.

Human Perspective

We supported the efforts of the STEAM summer camp by teaching adolescents about antibiotic resistance. Students engaged in a lab experiment to identify the mock pathogen samples.

We worked with the CSU team to validate our phage packaging signal part (BBa_K1445000). During this time we discussed common problems and solutions experienced by both teams.

With limited time to reach our project goals, we needed all the motivation (coffee and late nights) we could get. Regardless of previous experience, each of us gained a unique, hands on experience and learned to problem solve. More importantly, we pulled together from various backgrounds to completed a project we could call our own.

Acknowledgments

References

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