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Using Genome-Editing Technologies to Mitigate Antimicrobial Resistance

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Abstract (50 words)

• Please provide a short teaser to set the scene and introduce the main take home-message of the article.
• The limit of 50 words is strict.
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Length (1500 words, 15 references, 2 extra elements)

One of the most urgent public health concerns in the United States is the arms race between the rapid emergence of antimicrobial resistant (AMR) bacteria and the development of novel bactericidal agents for combating AMR infections. According to a 2013 report by the Centers for Disease Control and Prevention, over 2 million people become infected with antibiotic resistant bacteria and at least 23,000 of those individuals die annually as a result of the infection[1]. Overuse of antibiotics has resulted in rapid bacterial adaptation and evolution to develop resistances to the most common FDA-approved antibiotic treatments.

While small molecule antibiotics are the most commonly used, the development, screening and testing of these types of antibiotics is costly and time and labor-intensive. As of March 2017, there are an estimated 41 new antibiotics in clinical trials in the United States[2]. The success rate of new antibiotics in human patients, however, is historically low, with only 20% of the tested products being approved as a treatment option. These limitations have prompted the need for building an arsenal of unique bactericidal agents with precise targeting capabilities. Researchers are tackling these issues through the development of novel nucleic-acid and peptide-based antimicrobials, bacteriocins, bacteriophage therapy, antibodies and anti-virulence compounds[3].

Here, we suggest that a simple, easily adaptable and more effective method for developing antibiotics is to instead exploit defense systems that Nature has already evolved. The methods listed above use a bottom-up approach and rational design, which requires exhaustive screening, with the final product being a single new antimicrobial agent. However, the goal in the development of antimicrobials is to generate safe, effective and programmable treatments that can be rapidly modified and adapted to 1) specifically target other bacterial pathogens[3] or 2) modify the treatment to target the same species differently to stay ahead of developing resistances. Furthermore, there is a need for selective delivery of antibiotics to reduce off-target effects, both systemically and within complex populations of bacteria (i.e. the gut microbiome).

One such natural defense mechanism that meets these criteria is the Type II CRISPR/Cas bacterial adaptive immune system. With the wave of research and interest in CRISPR/Cas systems currently at the scientific forefront, the novel use of CRISPR/Cas as antimicrobials will be the focus here.

Repurposing Bacterial Adaptive Immune Systems as Antimicrobial Agents

In the last few years, much attention has been directed towards bacterial evolution of the CRISPR/Cas9 adaptive immune system. This system evolved to counter invasion by foreign genetic material, including mobile genetic elements and bacteriophage. In short, CRISPR/Cas9 is an effective method to modify genomes. The system consists of an RNA-guided endonuclease, Cas9, which induces a double stranded DNA break. Cas9 is guided to any genomic loci of interest by a single strand RNA that can be designed to contain complimentary base pairs for the purpose of directing the enzyme. Induction of a double-stranded DNA break rapidly activates non-homologous end-joining, which results in the insertion or deletion (indel) of a base pair, ultimately introducing an early stop codon and disrupting gene expression. While many bacteria natively use CRISPR/Cas9 (or other CRISPR/Cas systems) as a defense mechanism against bacteriophages and mobile genetic elements, the system can instead be repurposed to attack bacteria, ultimately resulting in CRISPR/Cas9 being used as antimicrobial agent.

Small molecule antibiotics are able to readily pass the bacterial membrane layer. However, given the uniqueness of using a protein/RNA complex as an antibacterial (which is considerably larger), significant challenges arise with how to effectively deliver CRISPR/Cas9 antibacterials to the site of infection. Furthermore, the delivery challenge becomes two-fold when the pathogenic bacteria are intracellular microbes; the antibiotic must be 1) released into the bacteria residing within the cell but also 2) selectively delivered to the infected cells. While this challenge enhances the complexity of delivery, adding two layers of specificity for delivery will also mitigate off-target effects.

How then can a large protein/RNA complex be delivered to Gram negative and/or Gram positive bacteria? Several groups have cleverly taken advantage of the species-level specificity of bacteriophage for delivery. Bacteriophage are naturally occurring bacterial predators and are able to proficiently inject DNA into bacteria. Using bacteriophage specific to the species of interest, CRISPR/Cas9 can be encapsulated into bacteriophage capsids (i.e. protein coats) by genetically encoding the machinery onto a phagemid (a plasmid designed to be packaged into bacteriophage capsids). One of the first examples of this was displayed in 2014[4]. Citorik *et al.* first transformed *E. coli* with 1) plasmid born CRISPR/Cas9 that targeted antibiotic resistance in addition to 2) a chromosomal copy of target antibiotic resistance genes. *E. coli* transformed with CRISPR/Cas9 targeting antibiotic resistance genes resulted in nearly a thousand-fold decrease in transformation efficiency in presence of selection agents. These promising *in vitro* results prompted the researchers to then use bacteriophage to package vectors encoding for CRISPR/Cas9 targeting the same antibiotic resistance genes. Upon addition of CRISPR/Cas9-packaged bacteriophage, rapid killing of target bacterial cells was observed with the maximal bactericidal effect occurring as quickly as 2-4 hours.

Just one month later, Bikard *et al*. published their findings in which they also used CRISPR/Cas9 encoded onto phagemids and packaged into bacteriophage to target antibiotic resistance and virulent strains of *S. aureus*[5]. They observed similar results and found species and target specific

killing of antibiotic resistance *S. aureus* using bacteriophage-packaged CRISPR/Cas9 targets. However, they also looked at an *in vivo* mouse skin colonization infection model. Following bacterial colonization of mouse skin, the infected areas were topically treated with bacteriophage-encapsulated CRISPR/Cas9 antimicrobials, which resulted in a significant decrease in target bacterial colonization.

Other groups have explored using CRISPR/Cas9 to selectively remove individual species within complex bacterial populations[6], engineering bacteriophage scaffolds to change species specificity of targeting[7] and exploring different genetic strategies to resensitize bacteria to antibiotics[8].

Combined, these findings strongly support repurposing CRISPR/Cas9 machinery to attack, rather than defend bacteria for the treatment of antibiotic resistance bacteria or newly emerging strains of bacteria. CRISPR/Cas9 has proven to be highly programmable and adaptable simply by altering the targeting RNA sequence. Proof of principle studies using mouse skin colonization models demonstrate the ability to topically treat infections using bacteriophage genetically encapsulating CRISPR/Cas9. While the delivery mechanism of CRISPR/Cas9 antibacterials to bacteria can be solved by identifying species specific bacteriophage or engineering bacteriophage scaffolds, this only addresses externally- and topically-treatable infections, such as MRSA (or methicillin-resistant *Staphylococcus aureus*). Additional strategies are needed to address systemic infections or tissue/organ specific infections.

With the advent of any new treatment, how to effectively, selectively and efficiently deliver the treatment is the greatest challenge for drug therapy development and implementation. By tuning effectiveness, selectivity and efficiency of drug delivery, off-target effects can be largely mitigated and lower doses can be used, reducing treatment cost. Reviews have detailed the different techniques that have been tested to deliver CRISPR/Cas9 therapies, which include adeno-associated viral vectors, cell-penetrating peptides, lipid based encapsulation and direct injection[9]. Further, much attention has been devoted to understanding the different efficacies of delivering 1) CRISPR/Cas9 in the form of expression DNA encoding the Cas9 protein and expression of the RNA, 2) mRNA encoding for Cas9 coupled with the transcribed RNA or 3) the delivery of Cas9 protein coupled with transcribed RNA. While these all have varying degrees of efficacy in different contexts, when exploring how to deliver bacteriophage encoding CRISPR/Cas9 targets to intracellular infections, the delivery becomes more challenging. These challenges arise due to the inherent structural diversity of bacteriophages; both bacteriophage size and structure vary wildly across different species. CRISPR/Cas9 offers a reasonable strategy for developing easily programmable antibacterial agents; however, an easily adaptable and robust technology must be developed for the delivery of highly diverse bacteriophage structures for intracellular infections.

Due to the structural diversity of bacteriophages, using a strategy such as mesoporous nanoparticles with defined binding chemistries as well as specific adsorptive pore-size is not an effective strategy for delivering many different bacteriophages. Current biotechnology techniques need to be explored for the encapsulation of diverse structures. Carnes *et al.* used an evaporation-induced self-assembly process to directly encapsulate bacteria into silica and lipid-

based nanostructures to study bacterial quorum sensing[10]. This type of strategy is ideal for direct encapsulation of non-symmetrical cargoes, including bacteriophages. By using complex matrices for encapsulation, including doping in silica to the matrices, allow for nanoparticle functionalization. Matrix compositions can be tuned for delayed cargo release and different functionalization capacities. The silica can be functionalized with lipid or polymer layers to mask the nanoparticle as a biological component. By altering lipid and/or polymer composition, functionalization of the membrane layer can be achieved by attaching targeting peptides to direct the particles to the cell type of interest in addition to self-recognition peptides to evade immune clearance. Using strategies such as these offer solutions to the two-fold delivery problem for treating intracellular bacterial infections (e.g. *Burkholderia pseudamallei*).

Encoding proven CRISPR/Cas9 antibacterial machinery into bacteriophages for bacterial delivery and subsequently using encapsulation techniques as described above to modify selective cellular delivery for the purpose of intracellular infections is currently achievable. The next, most significant feat is to tackle non-laboratory strains of bacteria. The question we must now ask ourselves is how can we modify our proof-of-principle laboratory strain studies to emerging biological threats?

Acknowledgements

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