Programmable sensitivity

By Stephen Parmley, Senior Writer

Since the GAIN Act was passed in 2012 to stimulate new antibiotic development, there has been more interest in the field but no game changers have emerged, and antibiotic resistance is still a major problem. Two independent teams believe they could have a far-reaching solution in the gene editing technology CRISPR, which allows them to tailor therapies to specific resistance mutations.^{1,2}

The most promising application of the findings is the potential to resensitize resistant bacteria to standard antibiotics, but the researchers will have

to develop better delivery methods to fully capitalize on the system's possibilities.

The teams, led by Luciano Marraffini at **The Rockefeller University** and Timothy Lu at the **Massachusetts Institute of Technology (MIT)**, both employed the CRISPR (clustered, regularly interspaced short palindromic repeats)-Cas9 system to produce programmable therapies that target antibiotic resistance genes directly.

Marraffini is an assistant professor in the laboratory of bacteriology at Rockefeller University. Lu is an associate professor of biological engineering and electrical engineering and computer science at MIT. He is also cofounder of **Sample6**, a company using engineered phages to develop rapid point-of-care microbial diagnostics for food safety.

"Instead of the way that antibiotics currently work—which is targeting proteins most of the time—we asked: can we actually develop a sequence-specific antimicrobial that really targets things based on genetic information?" said Lu.

Although the Generating Antibiotic Incentives Now (GAIN) Act created a new impetus for innovation in antibiotic development, most

Figure 1. CRISPR's double-edged knife.

Treatment of antibiotic-resistant bacteria with a clustered, regularly interspaced short palindromic repeats (CRISPR)-Cas9 agent yields different outcomes depending on whether acquired resistance genes are present on the bacterial chromosome or on an episome.

[a(1)] Antibiotic-sensitive bacteria can acquire resistance genes (R) from other bacteria that become chromosomally integrated and cause the bacteria to become resistant to the antibiotic.

[b(1)] Antibiotic-resistant bacteria can be selectively killed with a CRISPR-Cas9 agent that targets the chromosomally integrated resistance gene. Delivery of a gene cassette encoding a small *trans*-activating CRISPR RNA (tracrRNA), Cas9 and a single guide RNA (sgRNA) (orange boxes) into the cell results in expression and assembly of the CRISPR-Cas9 agent (yellow oval). This agent cleaves the targeted resistance gene, leading to chromosomal degradation and bacterial cell death.

[a(2)] Antibiotic-sensitive bacteria also can acquire episomes encoding resistance genes from other bacteria (R) to become antibiotic resistant.

[b(2)] These antibiotic-resistant bacteria can be selectively killed with a CRISPR-Cas9 agent that specifically targets a resistance gene in the episome. Delivery of the appropriate gene cassette results in expression and assembly of a CRISPR-Cas9 agent that cleaves the episomal resistance gene, leading to episomal degradation and restoring antibiotic sensitivity to the bacterium.

efforts on the research front have remained old school by targeting pathways or proteins unique to bacteria. Those approaches yield broad-spectrum antibiotics that carry the liability of suppressing beneficial microorganisms as well as pathogenic ones.

In addition, most compounds in development target Gram-positive bacteria, and Gram-negative infections remain severely under-addressed. In both types of organisms, genetic resistance to antibiotics can arise through mutations in the bacteria's chromosomal DNA or by acquisition of foreign episomes that contain resistance genes. Episomes are replicating extrachromosomal DNA elements that either remain as intact entities inside the bacterium or integrate into the bacterial chromosome.

The CRISPR system involves using Cas9 nuclease to create double-stranded breaks in DNA and a sequence-specific guide RNA to direct Cas9 to the target sequence. By using CRISPR to target the DNA rather than the resistance pathway, the two groups have developed techniques to attack resistance acquired through chromosomal mutations or episome acquisition that can be applied to Gram-positive or Gram-negative bacteria.

Where a resistance mutation is present on the chromosomal DNA, CRISPR-Cas9 cleaves the DNA at the site of the resistance gene, leading to chromosomal degradation and bacterial cell death. For bacteria that have acquired an episome containing a resistance gene, CRISPR-Cas9 can eliminate the episomal DNA and thus resensitize the organism to antibiotics (see Figure 1, "CRISPR's double-edged knife").

Overcoming resistance

Both groups started by testing whether CRISPR-Cas9 could kill bacteria containing resistance genes in their chromosomes.

Lu's group created a CRISPR-Cas9 that targets a carbenicillin resistance gene of *Escherichia coli*—a Gram-negative strain. The researchers used a bacteriophage system to deliver the agent to *in vitro* cultures of *E. coli* containing a chromosomal carbenicillin resistance gene. The compound reduced bacterial viability 1,000-fold in the cultures and produced its peak bactericidal effect in 2–4 hours. Carbenicillin is a generic antibiotic in the carboxypenicillin group that is active against Gram-negative bacteria.

In an *in vivo* model of systemic infection in wax moth larvae infected with a virulent strain of *E. coli*, the CRISPR-based therapy—directed against a key virulence factor gene—increased survival fourfold compared with a control bacteriophage.

Marraffini's team set out to program CRISPR to create an antibacterial that would selectively kill virulent bacteria but not affect the healthy host microbiota. The researchers designed a CRISPR-Cas9 agent to target a chromosomally integrated methicillin resistance gene in methicillin-resistant *Staphylococcus aureus* (MRSA) and used a bacteriophage delivery system to test it in a mixed culture containing equal parts MRSA and methicillin-sensitive *S. aureus*.

The percentage of methicillin-sensitive bacteria increased from 50% before treatment to 99.6% after treatment, which suggested that

predominantly resistant organisms had been killed. A nonspecific control bacteriophage caused no significant change in the ratio.

In a mouse model of Gram-positive bacterial skin infection, a CRISPR-Cas9 bacteriophage targeting a kanamycin resistance gene decreased by fivefold the proportion of resistant bacteria in a mixed population of bacterial colonies on the skin.

Next, both studies used the technology to resensitize resistant bacteria to specific antibiotics.

The Rockefeller group created a Cas9-CRISPR bacteriophage that targeted an episomal tetracycline resistance gene. After treatment with bacteriophage, 99.99% of the cells became sensitive to tetracycline.

The MIT group used its carbenicillin resistance gene–targeted Cas9 bacteriophage to treat cultures of *E. coli* containing an episomal carbenicillin resistance gene. In this case, too, treatment with bacteriophage resulted in 99.9% of the cells gaining sensitivity to carbenicillin.

Results of both studies were published in *Nature Biotechnology*.

Delivery issues

Although the CRISPR approach was effective in selectively killing or resensitizing resistant bacteria, the bacteriophage-based delivery system provides both advantages and drawbacks for using the technology to develop antibacterials.

Both labs showed that their CRISPR-based antimicrobials killed pathogenic bacteria *in vitro* and *in vivo*, but the reductions were modest and were most likely limited by the number of cells successfully infected with the Cas9 bacteriophage.

The two studies showed at least a three-log reduction in pathogenic bacteria *in vitro*. However, the Rockefeller group saw

only about one-log reduction *in vivo*, and the MIT group saw only a twofold protective effect *in vivo*. Typically, antibiotics reduce the counts of sensitive bacteria *in vivo* by greater than four logs and fully protect infected animals.

According to Marraffini, a modest effect might be enough. "We only need to help the immune system when it is overloaded, and studies have shown that in some cases reducing the bacterial pathogen count by only one log will be sufficient and the immune system clears the rest," he said.

Marraffini added that although the number of phage-susceptible bacterial strains amenable to this delivery system is relatively small, the CRISPR-based approach provides a selectivity advantage over other bacteriophage delivery systems because the latter kill pathogenic and beneficial bacteria indiscriminately.

Lu told *SciBX* that his team is developing a better delivery system by using rational engineering and high throughput screening to modify the bacteriophage and by exploring alternative delivery methods as well.

The use of the phage system to deliver CRISPR-Cas9 might also face difficulties in the clinic because clinicians are reluctant to give bacteriophage-based therapies orally or parenterally owing to the potential safety risk from contaminating endotoxins.

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**—Timothy Lu,
Massachusetts Institute of Technology**

James Collins—an early developer of bacteriophage systems that sensitize cells to antibiotics—said that one option might be to start with other routes of administration. “You could use the phage in a topical application in conjunction with existing antibiotics,” he said. “Or you could envision using the phage in an aerosolized fashion to go after lung infections.” Collins is a professor of biomedical engineering at **Boston University**.

He added that although the two studies provide powerful proof of concept for the approach, key questions still have to be addressed, such as what level of penetrance into virulent bacterial populations can be achieved and how fast resistance could arise.

“Bacteria evolve quickly against any strategy we appear to be coming up with, so the issue is how quickly?” Collins said. “Bugs are quite good at evolving against bacteriophage and evolving ways to pump out antibiotics. What would be the signs to pick up that resistance is developing and needs to be controlled?”

Indeed, a key point raised at a hearing last month about antibiotic resistance held by the U.S. House of Representatives’ Energy and Commerce Committee’s Subcommittee on Health was the need for diagnostic technologies that can keep pace with the emergence of antibiotic-resistant bacteria.

Rockefeller University and MIT have both filed patent applications covering their respective *Nature Biotechnology* studies, but neither Marraffini nor Lu disclosed the licensing status.

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