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Sensitizing pathogens to antibiotics using the CRISPR-Cas system

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ABSTRACT

The extensive use of antibiotics over the last century has resulted in a significant artificial selection pressure for antibiotic-resistant pathogens to evolve. Various strategies to fight these pathogens have been introduced including new antibiotics, naturally-derived enzymes/peptides that specifically target pathogens and bacteriophages that lyse these pathogens. A new tool has recently been introduced in the fight against drug-resistant pathogens–the prokaryotic defense mechanism–clustered regularly interspaced short palindromic repeats-CRISPR associated (CRISPR-Cas) system. The CRISPR-Cas system acts as a nuclease that can be guided to cleave any target DNA, allowing sophisticated, yet feasible, manipulations of pathogens. Here, we review pioneering studies that use the CRISPR-Cas system to specifically edit bacterial populations, eliminate their resistance genes and combine these two strategies in order to produce an artificial selection pressure for antibiotic-sensitive pathogens. We suggest that intelligent design of this system, along with efficient delivery tools into pathogens, may significantly reduce the threat of antibiotic-resistant pathogens.

Text

It is more than a century since the first effective compound for the treatment of syphilis, arsphenamine, was synthesized in Paul Erlich's laboratory (Ehrlich, 1910; Gelpi et al., 2015). Arsphenamine, the first antimicrobial compound, was replaced in the following three decades by more advanced antibiotics that were characterized by easier storage, easier administration to the patient and relatively minor side effects (Aminov, 2010). The introduction of penicillin as an efficient antibiotic revolutionized the treatment of bacterial infections; indeed, rather than being life-threatening diseases, bacterial infections became minor nuisances that were easily cured. The following decades saw the introduction of over 150 different antibiotics and their derivatives, which in turn introduced a new and significant artificial selection pressure for bacteria to become antibiotic-resistant. The widespread use of antibiotics has driven bacterial populations to become antibiotic-resistant through genetic changes and additions. Thus, the bacterial populations that infected people a century ago were different from those that infect people today.

In a 2014 report, the World Health Organization (WHO) recognized that bacterial resistance to antibiotics is a serious threat to public health http://www.who.int/mediacentre/news/releases/2014/amr-report/en/. Among the key findings, the 2014 WHO report noted that all regions of the world now have *Klebsiella pneumonia*, a major hospital-acquired infection that is resistant to carbapenems, which are a group of antibiotics of last resort; carbapenem resistance is observed in half of the infected patients in some countries. Similar findings were observed for a major antibiotic family, the fluoroquinolones, which have now lost their complete efficacy that was observed in the 1980's such that they are now effective in only half of patients in some countries. Likewise, cephalosporins, a group of antibiotics that was once effective against gonorrhea, now fails to be effective in many Western countries such as Austria, Australia, France, Japan and the UK. In general, the WHO report indicated that antibiotic resistance prolongs sickness, increases the risk of death and increases healthcare costs significantly. The Centers for Disease Control and Prevention (CDC) in the USA reported that annually over two million people become infected with antibiotic-resistant pathogens, and that over 23,000 people die as a direct result of these infections in the USA alone (https://www.cdc.gov/drugresistance/).

Interestingly, a relatively small selection of pathogens is responsible for most antibiotic-resistant infections, as highlighted by The Infectious Diseases Society of America (Boucher et al., 2009). This group of pathogens, acronymically labeled as the ESKAPE pathogens, is comprised of ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa* and ***E****nterobacter* spp. These pathogens are known to escape antibiotic treatment by having

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or acquiring resistance, and they account for the majority of antibiotic-resistant infections (Boucher et al., 2009). Since only a small selection of pathogens is responsible for the majority of antibiotic-resistant infections, it is possible to prioritize new therapeutic solutions for these pathogens, and thereafter handle the remaining pathogens.

Although the current pace of development of new antibiotics may be insufficient to combat the rise in antibiotic resistance, there is minimal motivation to develop new antibiotics (Verhoef and Morris, 2015). The low level of motivation is largely due to the high cost of research and development of new drugs compared to the price charged per treatment. As opposed to therapeutics for chronic pathological states, antibiotics are used for a relatively brief treatment period, resulting in a rather low per-treatment profit. The low economic motivation, along with the inherent difficulties in finding new therapeutic families in this heavily researched field, has resulted in slow development of new antibiotics.

Research on the effectiveness of bacteriophage (phage) therapy has been renewed as a result of the ever-growing problem of antibiotic-resistant pathogens, for which current therapies are still inadequate. The evolutionary ability of lytic phages to propagate optimally in bacteria as a means of killing them makes the lytic phage a strong candidate as a treatment for bacterial infection. This potential for therapy has resulted in the testing of lytic phages against a wide range of pathogens such as *Staphylococcus aureus* (Matsuzaki et al., 2003) and *Pseudomonas aeruginosa* (Watanabe et al., 2007).

It is possible that future treatments for infections of mucosal and external tissues, such as the skin and ears, will be based on phage therapy. While several phase I and II clinical trials have been completed with success for the use of phage therapy to treat chronic bacterial ear infections in humans or burn wound-associated infections (Wright et al., 2009), many challenges remain if phage therapy is to replace the use of antibiotics to treat infection of internal tissues.

Merril et al. (1996) demonstrated that the majority of lambda phages injected into mice is sequestered by the liver and the spleen within minutes of entering the bloodstream. While they succeeded in selecting mutant phages that remained unsequestered by the liver or the spleen, the issue of sequestration remains a barrier in the development of other therapeutic phages. There are a number of additional drawbacks in the use of phages against bacterial infections (Loc-Carrillo and Abedon, 2011). Firstly, the effectiveness of the frequent use of phages is hindered by the observed and significant antibody-neutralizing response. Secondly, most phages are unable to diffuse into the entirety of the infected tissue as a result of their large sizes; phages are generally physically larger than antibiotics, so that this is a greater issue for phages than it is for antibiotics. Thirdly, since individual phages have a narrow host specificity, they are effective against a similarly narrow range of pathogens; this can also be viewed as an advantage since narrowness implies specificity, which in turn results in maximum preservation of healthy natural microflora. Fourthly, bacteria evolve to become phage-resistant in the same way that they evolve to become antibiotic-resistant. Phage-resistance may develop by a number of ways including insertion of phage-specific DNA into the clustered regularly interspaced short palindromic repeats (CRISPR) arrays (Barrangou et al., 2007), modification of the bacterial metabolic pathways and modification of the phage receptor on the bacterial membrane (Qimron et al., 2006). A discussion of the advantages and disadvantages of phage therapy can be found in a review by Lu and Koeris (2011).

It is likely that antibiotic resistance will be addressed by new and innovative phage-based approaches in the coming years. Phages are already in use as approved disinfectants in the food industry. For example, the United States Food and Drug Administration (US FDA) has approved a mixture of six naturally occurring lytic phages for the elimination of Listeria in food products (Abuladze et al., 2008; Lang, 2006).

Several recent studies proposed combined treatments using phages as delivery vehicles as well as CRISPR-Cas for controlling antibiotic-resistant pathogens. CRISPR-Cas has been used for specific editing of numerous genomes including cells obtained from humans (Cong et al., 2013; Mali et al., 2013), rodents (Li et al., 2013; Wang et al., 2013), fish (Hwang et al., 2013), flies (Gratz et al., 2013), worms (Friedland et al., 2013), plants (Feng et al., 2013; Shan et al., 2013), yeast (DiCarlo et al., 2013), bacteria (Jiang et al., 2013) and bacteriophages (Kiro et al., 2014; Martel and Moineau, 2014). It is also being used for other applications, as shown in Fig. 1. The system, which is comprised of the CRISPR and CRISPR-associated (Cas) proteins, has been identified as an adaptive immune system (Barrangou et al., 2007). This intriguing system is found in ~87% of archaeal genomes and in ~50% of bacterial genomes (Makarova et al., 2015), and it is analogous to the mammalian immune system (Abedon, 2012; Goren et al., 2012). The genetic loci of all systems include a CRISPR array that is comprised of short repeated sequences, known as 'repeats', which flank similarly sized sequences, known as 'spacers'. The spacers are acquired from DNA sequences known as 'protospacers'. RNA transcribed from the CRISPR array (crRNA) is processed by Cas proteins into RNA-based spacers that are flanked by partial repeats. These RNA spacers direct Cas proteins to specifically target and cleave nucleic acids that encode matching protospacers. Thus, the system can be programmed to specifically target any DNA based on the information provided in the CRISPR array. CRISPR-Cas has been exploited recently to target bacterial populations that carry specific genes and, in particular, genes that encode antibiotic resistance (Bikard et al., 2014; Citorik et al., 2014; Gomaa et al., 2014; Hale et al., 2012; Yosef et al., 2015).

Gomaa et al. (2014) used the CRISPR-Cas system to target specific sequences in the genomes of different strains and subtypes of bacteria. This targeting resulted in bacterial death. Mixing different strains of bacteria and then introducing spacers that were specific against one strain resulted in significant elimination of the targeted strain from the mixed population while the other strain remained viable. The study had two key weaknesses. Firstly, it did not use a potent method for delivering the CRISPR-Cas system into bacteria. Secondly, rather than targeting resistance genes on extra-chromosomal elements, chromosomal genes that were unique to part of the mixed population were targeted. Targeting the chromosome results in bacterial killing, which consequently selects for non-targeted mutants, whereas targeting extra-chromosomal elements does not result in such mutants as long as antibiotics are absent. Nevertheless, the study did demonstrate experimentally, for the first time, that the CRISPR-Cas system is able to discriminate between different strains in a manner that is superior to phages, antibiotics, or other selective agents. This impressive discrimination is possible because even minor sequence differences suffice for the CRISPR-Cas system to discriminate between strains.

Experiments to target resistance genes using the CRISPR-Cas system were reported soon after the system was first characterized (Hale et al., 2012). However, only in 2014 did two papers simultaneously describe using CRISPRs to target specific antibiotic resistant bacteria (Bikard et al., 2014; Citorik et al., 2014). Citorik et al. (2014) used two different tools to deliver the CRISPR-Cas system into bacteria for targeting antibiotic resistance genes. Their first delivery method was a conjugative plasmid carried by an *E. coli* strain; when the desired host came into contact with the *E. coli*, the plasmid was able to conjugate into the host. However, conjugation was inefficient due to improper selection for hosts acquiring the plasmid, prompting the use of another method, *i.e.* M13-based phagemids. M13-based phagemids are plasmids that encode specific genes, in