

Review

Driving to Safety: CRISPR-Based Genetic Approaches to Reducing Antibiotic Resistance

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Bacterial resistance to antibiotics has reached critical levels, skyrocketing in hospitals and the environment and posing a major threat to global public health. The complex and challenging problem of reducing antibiotic resistance (AR) requires a network of both societal and science-based solutions to preserve the most lifesaving pharmaceutical intervention known to medicine. In addition to developing new classes of antibiotics, it is essential to safeguard the clinical efficacy of existing drugs. In this review, we examine the potential application of novel CRISPR-based genetic approaches to reducing AR in both environmental and clinical settings and prolonging the utility of vital antibiotics.

The Antibiotic Resistance Crisis

Since their introduction, antibiotics have reduced human mortality rates from infectious diseases by 80% [1]. Unfortunately, antibiotic resistance (AR) among leading bacterial pathogens is currently estimated to cost >700 000 lives annually [2], nearly equal to the mortality attributed to all the world's most deadly mosquito-borne diseases combined [3]. Widespread overprescription of antibiotics and their misuse in animal husbandry have increased the prevalence of AR in medical facilities [4] and in the environment [5–7]. Evidence indicates that environmental sources of AR are transmitted via bacterial intermediates to human populations and contribute significantly to the current health crisis of antibiotic treatment failures in resistant infectionsⁱⁱ [6,8,9].

As troubling as the current situation is, health experts predict that AR threats could markedly worsen in the coming decadesⁱⁱⁱ [10], leading to some 10 million AR disease deaths per year by 2050 if left unchecked [2]. This ballooning crisis can only be addressed by synergistic efforts to develop strict new antibiotic stewardship guidelines by the medical establishment [11]; legislation to prohibit inappropriate agricultural practices, such as adding antibiotics in animal feed to enhance livestock growth; and robust partnerships spanning academia, industry, philanthropies, and government agencies to develop new natural or synthetic antibiotics [12], innovative immunotherapies [13], or novel antibacterial [14] and anti-AR compounds [15,16] to extend the longevity of existing antibiotics.

CRISPR-Based Strategies to Combat AR

The discovery of a bacterial immunity system referred to as **CRISPR (clustered regularly interspaced short palindromic repeats; see Glossary)** has given rise to a revolution in precision genetic engineering in both prokaryotic and eukaryotic organisms [17,18]. Among this ever-expanding array of immune recognition and protective mechanisms, type II CRISPR systems, the best studied and most widely applied to practical ends, include both protein (e.g., Cas9) and RNA [e.g., endogenous cRNAs and trans-activating CRISPR RNAs (tracrRNAs), and synthetic **guide RNAs** that fuse the cRNAs and tracrRNAs into a single transcript, referred to hereafter as gRNAs], which form ribonucleotide–protein complexes that cut DNA bases at sites complementary to a 20–base pair target recognition sequence in the gRNA (Figure 1A).

Highlights

Synthetic CRISPR systems have been developed to combat antibiotic resistance (AR).

Phage and conjugative horizontal gene transfer vehicles can disseminate CRISPR anti-AR platforms throughout bacterial populations.

Anti-AR CRISPR systems may reduce AR prevalence in experimental infection models.

Self-amplifying proactive genetic systems increase anti-AR efficiency approximately 100-fold.

Guide RNA–directed transposons should allow insertion of anti-AR CRISPR platforms into multiple defined genomic or episomal target sites.

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In prokaryotes, CRISPR has been used for efficient genome editing (combined with the λ Red homology-directed DNA repair cassette), to kill specific bacterial strains (Cas9 cuts strain-specific genomic sequences in bacteria, unable to repair double-strand DNA breaks) [19–21], or to serve in anti-AR systems to deplete plasmid-encoded AR genes (by cleaving the AR targets and destroying the plasmid) [19–23] (Figure 1B). These latter platforms for eliminating high-copy number plasmids typically reduce the prevalence of encoded AR by two to three logs [19,20,22].

Horizontal Gene Transfer Systems for Disseminating Anti-AR CRISPR Systems

Two **horizontal gene transfer (HGT)** systems for disseminating CRISPR-based anti-AR components have been developed. The first makes use of either phagemids, which are packaged with helper phages [19–21], or temperate phages inserted as prophages into the bacterial genome [23]. These constructs displayed modest efficacy in reducing systemic bacterial load [20], eliminating bacteria from exposed surfaces [21], and topical treatment of bacterial skin infections in a mouse model [19,23]. Embedding the CRISPR machinery in a phage-like pathogenicity island has also shown promise in blocking the development of subcutaneous or systemic *Staphylococcus aureus* infections by killing the bacteria or rendering them avirulent [24].

The second dispersal approach makes use of **conjugal transfer** plasmids using type IV secretory systems (TSS4) to disseminate CRISPR components to either Gram-negative Enterobacteriaceae [25] or Gram-positive *Enterococcus* spp. [26,27]. The Gram-negative targeting system, developed first in *Escherichia coli* and carrying essential conjugal transfer genes (including a TSS4 cassette) *in cis* (i.e., on the plasmid), was transferred with great efficiency both within its own species and to *Salmonella enterica*, where it targeted specific genomic loci with efficient bactericidal action. The Gram-positive targeting system, although effective in reversing AR under laboratory culture conditions, provided only minimal protection *in vivo* in a murine intestinal infection model, potentially reflecting difficulties in gaining access to isolated tissue or intracellular foci of pathogenic bacteria. A plasmid-based broad host range RP4 conjugal system, dubbed MAGIC (metagenomic alteration of gut microbiome by *in situ* conjugation), also demonstrated robust conjugation to both Gram-positive and Gram-negative bacteria among the mammalian gut microbiota [28]. Promiscuous classes of **integrative and conjugative elements (ICEs)** that are inserted into the bacterial chromosome provide another potential avenue for mediating efficient HGT into diverse recipient species [29,30].

HGT elements can also be used to disseminate non-CRISPR-based anti-AR factors. For example, using an RP4 *OriT* cis-acting conjugal configuration, a split intein toxin–antitoxin system was efficiently transmitted from *E. coli* to *Vibrio cholerae* [31]. In this well-crafted system, expression of an intein toxin was placed under control of the endogenous ToxR, a transcriptional activator of its own regulon [32]. The STX pathogenicity island, an ICE carrying ToxR, is associated with AR resistance and regulates cholera toxin expression in the pandemic *V. cholerae* O139 strain. Using ToxR regulated intein toxin expression, the authors selectively targeted the AR *V. cholerae* O139 while sparing the non-AR strain *V. cholerae* O1, providing a proof of concept for achieving strain-specific targeting of anti-AR genetic tools.

Gene Drive Systems in Eukaryotes

Gene drives developed in diploid eukaryotes greatly bias their transmission to offspring beyond the expected 50% rate of Mendelian inheritance. **Super-Mendelian elements** can increase transmission of entire chromosomes, as typified by meiotic drives [33–40] or balanced **chromosomal translocations** [41]. Alternatively, driving systems can simply copy the element itself, as is typical for so-called **selfish genes** such as homing endonuclease genes [42,43] or transposons [44–47], the latter type of element being well represented in prokaryotes.

Glossary

Active genetics: copying of a genetic element from one chromosome to its homolog in response to a double-strand DNA break being generated in the homolog at the same genomic site at which the active genetic element is inserted. Copying, which results from directional gene conversion, is typically mediated in the germline by the synthesis-dependent strand annealing (or D-loop) branch of the homology-directed repair pathway.

Allelic drive: a genetic system biasing the inheritance of a particular allelic variant, typically altering only one or a few base pairs. CRISPR-based allelic drive systems can be either of two types: copy cutting or copy grafting.

CRISPR (clusters of regularly interspaced short palindromic repeats): a bacterial immunity system from which the synthetic CRISPR/Cas9 genome editing system was derived by Jennifer Doudna and colleagues.

Chromosomal translocations: rearrangements of the genome resulting from chromosomal segments breaking off from their original chromosomal locations and being rejoined to other chromosomal sites on the same or different chromosomes. In the case of reciprocal translocations, which can be viable and have normal fitness, breaks on two different chromosomal arms result in portions of one chromosome arm being switched for another to create two new chromosomes with the same gene complement present in the original strain.

Conjugal transfer: genetic entities that can be transmitted actively between bacteria. Examples include F-factors mediating transfer of plasmids or portions of the genome (high-frequency recombination or ‘HFR’ strains), which is a bacterial form of sexual reproduction. Full autonomous conjugal elements typically carry cis-acting *OriT* sequences required for their transfer to other cells as well as type IV secretory systems that encode the transfer machinery, including pili through which the DNA is transferred. Many plasmids carry only *OriT* sequences and can only be transferred passively to recipient cells.

Guide RNAs (gRNAs): expressed RNAs that bind to Cas9 and direct its cleavage of specific DNA targets. Endogenous bacterially expressed CRISPR gRNAs consist of separate crRNAs and tracrRNAs that bind to Cas9

Recently, highly efficient synthetic CRISPR-based gene drive systems have been developed in yeast [48] and insects [49–55], each relying on a simple concept: a cassette encoding Cas9, a gRNA, and optional cargo (such as antimalarial effector molecules in mosquitoes) are inserted precisely at the genomic site targeted for Cas9/gRNA-mediated cleavage. Expression of the Cas9 and gRNA in germline cells then leads to cleavage of the homolog chromosome, and **homologous DNA repair** copies the element into the DNA break, resulting in nearly all offspring inheriting the element (Figure 1C).

The bipartite nature of the Cas9/gRNA system also makes it possible to insert only the gRNA component (with or without cargo) while providing Cas9 from a separate chromosomal location. Such ‘split-drive systems’ efficiently propagate in insect populations [56–58,141], and a similar prototype split drive in mammals also shows promise [59]. Additional gRNAs can be included in the same cassette to bias inheritance of a favored allelic variant at a different chromosomal location (**allelic drive**) [60] (Figure 1D) or to alter secondary gene targets [61]. It is also possible to deploy dual gRNA drives to delete and replace target sequences [57,62].

Bacterial Drive Systems

It might seem odd to consider developing CRISPR-related drive systems in nondiploid bacteria, since prokaryotes do not require sexual reproduction, a property that is generally considered as defining for gene drives. Although pathogenic or clonal organisms cannot readily acquire a drive element from others of their kind, bacteria often contain a variety of autonomously replicating genetic entities, including large circular chromosomes, plasmids of varying copy number, and an array of transposable elements and phage-related elements. Thus, one could imagine systems capable of copying genetic cassettes between such entities. Also, bacteria frequently share DNA within their own species or between species by various means of HGT [4,63]. These include naked DNA **transformation** [64], conjugation of genomic or plasmid sequences [65–67], **phage transduction** [68], and even via vesicular transfer of nucleic acids [69]. In this context, recent advances suggest that self-amplifying drive-like CRISPR systems could contribute to efforts to modify or suppress AR bacterial populations.

A bacterial split gene drive system targeting an AR determinant has recently been developed, referred to as ‘proactive genetics’ (pro-AG) [70]. The core pro-AG component is a gRNA expression cassette targeting a high-copy number AR gene (β -lactamase) that is flanked by homology sequences to the β -lactamase cleavage site. This homology-flanked gRNA was carried on a low-copy number plasmid that also harbored an arabinose-inducible λ Red DNA repair cassette [70] (Figure 2). A Cas9 transgene encoded on a separate low-copy number plasmid was inducible by anhydrotetracycline (aTc). Following induction of Cas9 (by aTc) and the λ Red DNA repair cassette (by arabinose), the gRNA cassette was copied into the β -lactamase target, disrupting its activity (Figure 2C,D) but not damaging the plasmid, in contrast to the destructive effect of the control CRISPR system (Figure 2A,B). The pro-AG system was shown to be self-amplifying on the basis of levels of the gRNA being limiting (Figure 2E). As the gRNA copied from its low-copy number plasmid of origin to its high-copy number plasmid target, its levels increased, leading to positive feedback amplification of the copying process. This self-amplification of the pro-AG system achieved an ultimate 5-log fold reduction in AR prevalence, which was over 100 times more efficient than observed with the control CRISPR system or with an editing configuration that did not amplify the gRNA cassette (Figure 2E).

If the pro-AG system were to be combined with a method for dissemination between bacteria, such as conjugal transfer plasmids [25,27], ICE [30], or phage [19–21,23] delivery systems (Figure 3A, Key Figure), such engineered platforms could potentially modify bacterial populations

independently. Synthetic gRNAs fuse the cRNAs and tracrRNAs into a single transcript, referred to as either ‘sgRNAs’ or more simply ‘gRNAs,’ the latter being the term used in this review.

Homologous DNA repair: DNA repair pathway initiated following induction of double-strand DNA breaks in which the break is repaired by copying sequences (or a template) from an identical sister chromosome (typically following DNA replication in somatic cells) or from the homolog chromosome (typically during meiosis, although sometimes also in somatic cells).

Horizontal gene transfer (HGT): gene transfer between individual organisms. In bacteria, HGT can be mediated by naked DNA (transformation), conjugal transfer, transduction (by phage or phage-related entities including gene transfer agents), or by vesicular fusion.

Integrative and conjugative elements (ICEs): integrative and conjugal elements are segments of DNA flanked by inverted repeat sequences that can be carried in the genome, plasmids, or phages and can excise and circulate via the terminal repeats to form transient or stable replicating autonomous plasmids.

MAGIC (metagenomic alteration of gut microbiome by *in situ* conjugation): using this method, replicative or integrative transfer vectors are delivered from an engineered donor strain into amenable recipients in a complex microbiome.

Phage-inducible chromosomal islands (PICIs): a family of pathogenicity islands that are readily spread between bacteria that carry cis-acting sequences allowing them to be packaged into infective particles by phage-dependent mechanisms.

Phage transduction: transfer, by incorporation into a phage genome, of bacterial host sequences to cells infected, and typically lysogenized, by the transducing phage.

Selfish genes: genes that are transmitted from parents to progeny in a biased fashion. See ‘super-Mendelian elements’ entry below. Typically, selfish genes are naturally occurring entities.

Transformation: uptake of naked extracellular DNA sequences into bacterial cells and insertion of these sequences into the genome or episomal elements.

Super-Mendelian elements: genetic entities in diploid eukaryotic organisms, such as gene drives, selfish genes, or

by reducing AR prevalence and/or temporally suppress or alter the proportions of competing bacterial populations.

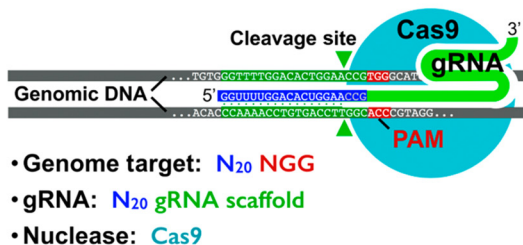
other active genetic elements, that are inherited more often than by chance alone (i.e., by >50% of their offspring).

An advantage of conjugative systems is the wide host range that they can achieve [28,30,31]. Such broad dissemination could be of great benefit in reducing AR across a range of bacterial species in complex populations, such as those residing in the gut microbiome or in environmental contexts, including water treatment plants and aquaculture facilities [5–7]. Deployment of such systems may also be envisioned for prophylactic applications, such as probiotics for high-risk patient subgroups, or as sentinel bacterial strains carrying arrays of gRNAs directed against potential AR determinants designed to impede influx of new AR factors into defined agricultural environments.

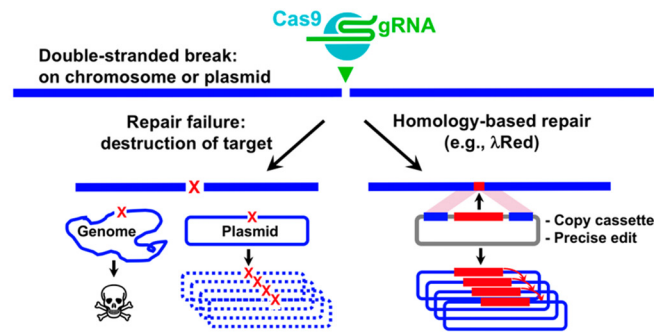
gRNA-Directed Transposons

Another potentially powerful drive-enabling tool derives from the discovery of RNA-guided transposon systems [71–75]. These mobile elements lack typical transposase functions and instead

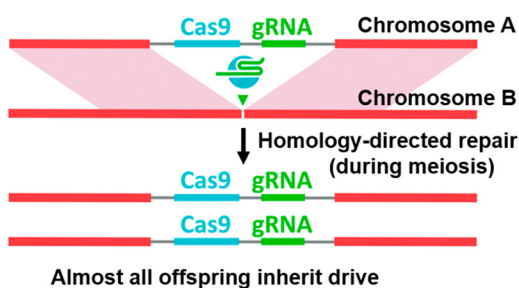
(A) Bi-partite CRISPR system



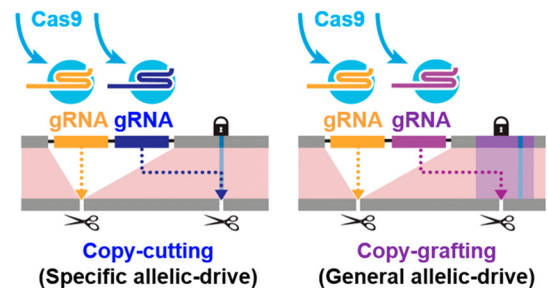
(B) Prokaryotic CRISPR outcomes



(C) Eukaryotic gene-drive

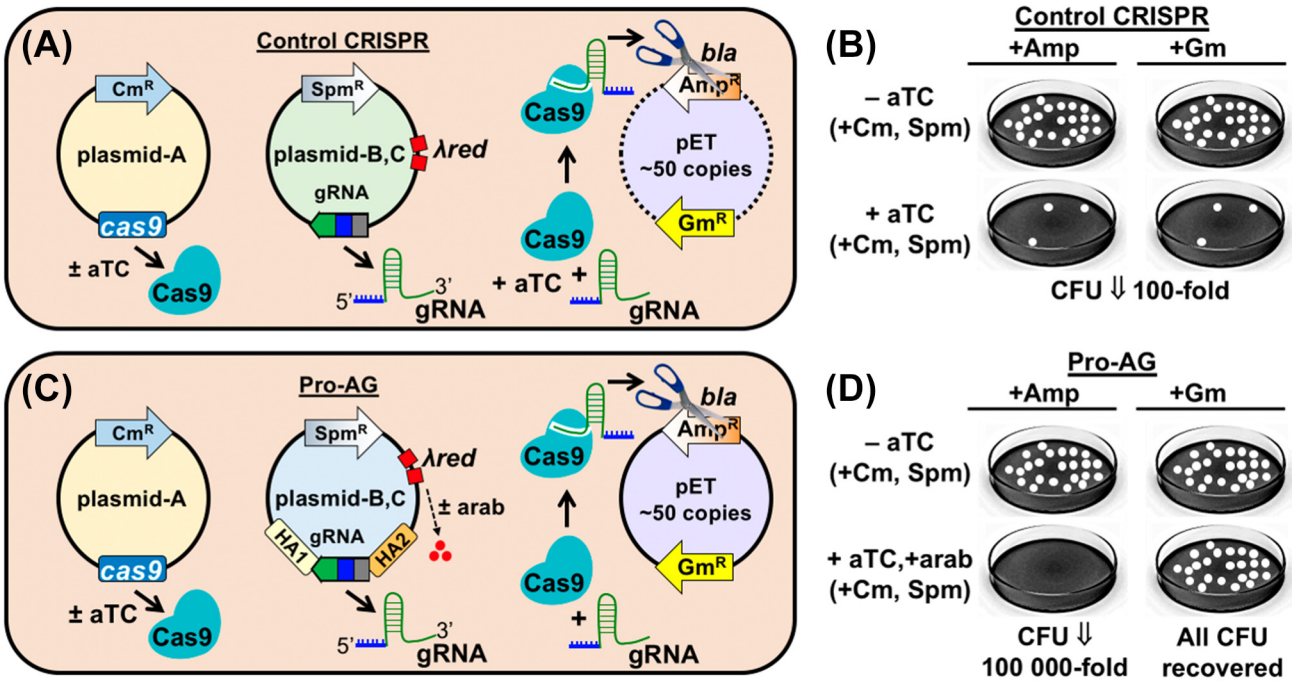


(D) Allelic-drive

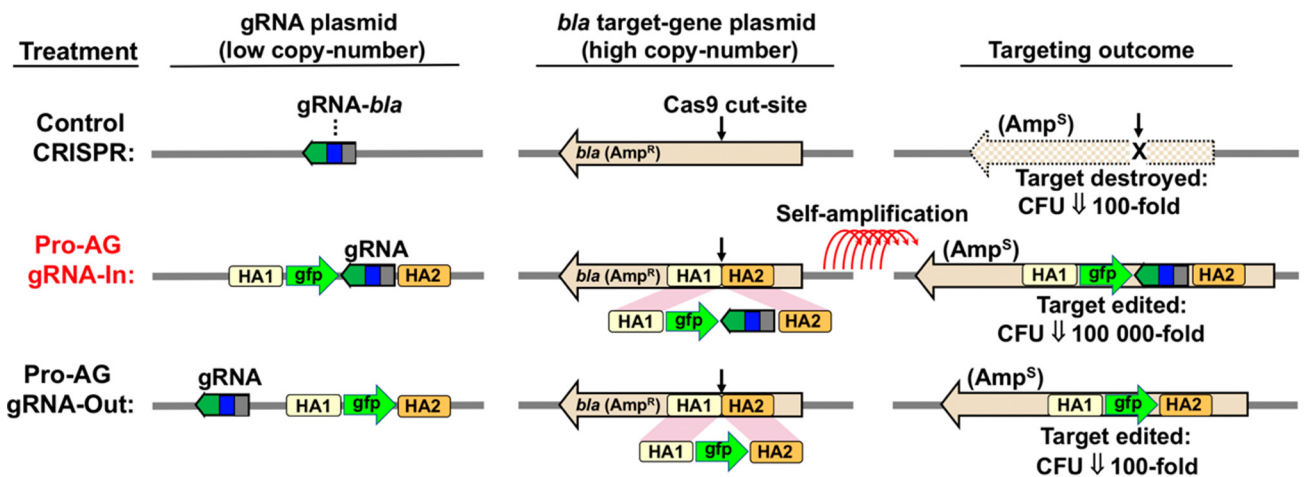


Trends In Genetics

Figure 1. Eukaryotic Gene-Drive Systems. (A) The bipartite synthetic CRISPR (clustered regularly interspaced short palindromic repeats) system: a guide RNA (gRNA; green/blue) binds Cas9 (cyan), directing it to bind and cleave DNA at complementary sites 20 nucleotides in length (dark green). The PAM site (NGG, red), required for *Streptococcus pyogenes* Cas9 (SpCas9) binding to genomic targets, is absent in the gRNA, which binds to the template strand, displacing the nontemplate strand. The two strands are cleaved by different Cas9 catalytic centers that each generate single-strand breaks. (B) In most prokaryotic cells, double-strand DNA breaks are not efficiently repaired without a repair template and dedicated homology-based repair enzymes such as those encoded by the λ Red cassette. Such DNA breaks in genomic targets lead to cell death or destruction of plasmid templates (left outcomes). If a homologous DNA template is provided together with λ Red repair proteins, gene cassettes (red box) or preferred allelic variants (see panel D) can be copied into the DNA break via a self-amplifying positive feedback loop. (C) A Cas9+gRNA cassette inserted in one chromosome of a diploid eukaryotic organism directs cleavage of its homolog during meiosis and is copied into the DNA break by homology-directed DNA repair, and nearly all progeny inherit the ‘gene-drive’ cassette. (D) Copy-cutting versus copy-grafting allelic drive strategies. A split-drive element carries a gRNA to copy itself (yellow) and a second gRNA (blue or purple) to propagate a preferred allelic variant (lock and copying indicated by dark blue vertical line). In copy cutting, the allele-driving gRNA selectively cuts the unfavored allele (left panel, blue gRNA and broken arrow), a constraint met approximately 50% of the time. In the more generally applicable copy grafting, a noncleavable site is engineered within approximately 25 bp of the favored allele, resulting in all alleles other than the engineered protected allele being cut by the driving gRNA (right panel, purple gRNA and broken arrow).



(E) Pro-AG is self-amplifying due to limiting gRNA levels



Trends in Genetics

Figure 2. Pro-Active Genetics (Pro-AG) Systems in *Escherichia coli*. (A) Anti-AR CRISPR system components: low copy-number plasmid-A (Cm^R), expressing Cas9 upon induction with anhydrotetracycline (aTC) and plasmid-B (Spm^R), constitutively expressing a gRNA (gray promoter box). High-copy-number plasmid pET (Amp^R, Gm^R) carries the antibiotic resistance (AR) target, *beta lactamase* (*bla* = Amp^R), for guide RNA (gRNA)-directed Cas9 cleavage. (B) Control CRISPR colony-forming units (CFU) recovered on either Amp or Gm plates (+Cas9) are reduced ~100-fold. (C) Proactive genetic (pro-AG) system components: plasmid pET and Plasmid-A as in (A). Plasmid-B,C carries the gRNA flanked by *bla* homology arms (HA1, HA2) and a *lred* DNA repair cassette (red boxes), inducible by arabinose (*arab*, red dots). (D) Induction of pro-AG system (+aTC, +*arab*) reduced CFU recovered by ~100 000-fold on Amp plates. Edited colonies could be quantitatively recovered on Gm plates, however. (E) Pro-AG is based on a positive feedback cycle of gRNA amplification. Top row: Control CRISPR configuration [depicted in (A)]. Middle row: Pro-AG gRNA-In configuration: a gene cassette <gRNA + *gfp* cargo gene> flanked by homology arms (HA1, HA2) inserts into the *bla* target gene thereby disrupting its

(Figure legend continued at the bottom of the next page.)

include nuclease-deficient Cas-related Cascade proteins that do not cut DNA but rather direct the complex in a gRNA-dependent fashion to specific target sites. Once bound in a sequence-specific fashion to their target DNA sequence, Cascade proteins recruit an excised paired-end transposon complex to insert the element at a fixed distance from the complex in either orientation. Since these RNA-directed transposons can be programmed to insert themselves at multiple different target sites, scenarios can be imagined in which they could be designed to copy back and forth between the genome and conjugal transfer plasmids or phage constructs to generate bacterial analogs of gene drive systems (Figure 3A). If combined with other CRISPR tools, including self-amplifying pro-AG systems, next-generation configurations of self-propagating elements might be developed to reduce AR prevalence in environmental and clinical settings.

Countering Evolution of Resistance to Anti-AR CRISPR Systems

Although anti-AR CRISPR systems can be highly effective, mutations in their basic components (e.g., gRNA cassettes or Cas9 transgenes) can lead to selection of ‘escapers’ that become recalcitrant to AR reversion [20,70,76]. Several complementary strategies can be envisioned to counter such inevitable evolutionary outcomes (Figure 3B). One tactic that has shown promise is to render bacteria simultaneously antibiotic sensitive and refractory to infection by a lytic phage (Figure 3B, cooperative systems). In this dual-acting system, cells are first infected with a temperate phage that inserts into the genome as a lysogen and expresses CRISPR components directed against both a high-copy number AR plasmid target and a receptor for the lytic phage. Next, the bacteria are infected with the lytic phage, leaving only colonies of AR-sensitive lysogens [21]. Another approach could be to incorporate redundant anti-AR systems into HGT platforms, reducing the likelihood that mutations in single CRISPR components would lead to loss of anti-AR targeting (Figure 3B, redundant systems). Alternatively, conjugal and phage delivery systems could be combined by placing *att* phage landing sites in the conjugal element. These approaches may be supplemented with phagemids [19–21] carrying minimal anti-AR systems or CRISPR-bearing prophages inserted into an ICE carried by the host chromosome [30,77], either of which might offer alternative HGT routes. Having dual modes of carriage should also help break down barriers to accessing different microenvironments. Similarly, benefits might be gained by incorporating gRNA-directed transposons [71–73] into conjugal transfer platforms to facilitate shuttling of constructs between shared genomic and plasmid integration sites (Figure 3A).

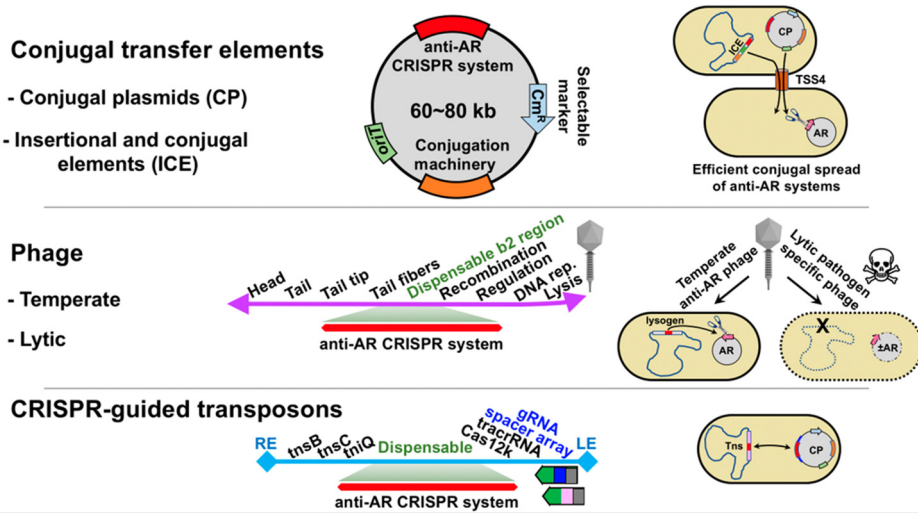
It will also be important to anticipate resistance mechanisms arising in nature, where a dizzying variety of mobile genetic elements and host mechanisms could reduce the efficacy of anti-AR systems. For example, acquisition of anti-CRISPR factors [78], phages that interfere with pilus-dependent TSS4-mediated conjugation [79], or phage-neutralizing activities of **phage-inducible chromosomal islands (PICIs)** [80] could conspire to attenuate anti-AR CRISPR platforms. A potentially fruitful approach to overcome such mechanisms would be to evolve prototype conjugal and phage systems over many serial generations to expand their host range and performance, as recently accomplished in phage bacterial evolution experiments [81–83]. Integrating other orthogonal CRISPR-including different class II Cas9 systems (SaCa9 [84] or Cas12a [85]) or Cas3-based systems [86] to delete large segments of DNA offers additional avenues to pursue. Other important challenges to practical implementation of HGT-mediated anti-AR CRISPR platforms will be gaining adequate penetrance into the target populations (Box 1) and pursuing open and transparent paths to acquiring regulatory approval for deployment of these systems in clinical or environmental settings (Box 2).

function. The pro-AG element copies itself through a self-amplifying mechanism (red arrow array) reducing Amp^R CFU ~100 000-fold. All targeted plasmids analyzed carried accurately edited insertions of the <gRNA, gfp> cassette. Bottom row: gRNA-Out configuration: places the targeting gRNA outside of the homology arm flanked <gfp> cassette. Since the gRNA is not coamplified with the <gfp> cassette, Amp^R CFU are only reduced ~100–1000-fold ≈ the control CRISPR configuration. All targeted plasmids analyzed carried precisely edited <gRNA, gfp> cassette insertions.

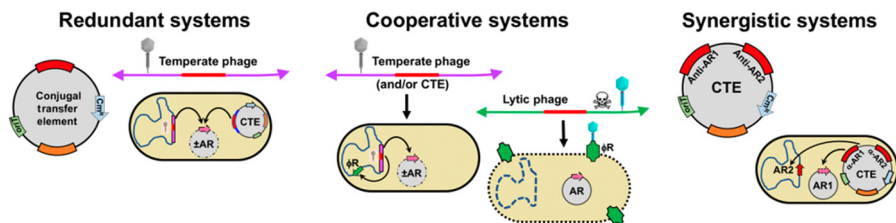
Key Figure

Deploying Anti-Antibiotic Resistance (Anti-AR) CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Platforms

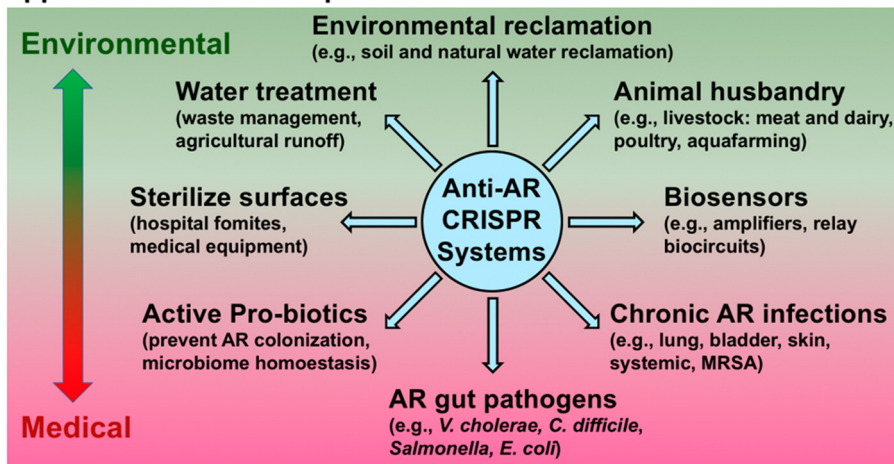
(A) Mobilizing anti-AR CRISPR systems with HGT platforms



(B) Combination anti-AR platforms



(C) Applications of anti-AR platforms



Looking Forward

An important future objective will be integrating anti-AR CRISPR platforms with other synthetic systems, which should enhance their efficiency and extend their protective effects to scrub AR from both clinical and environmental settings (Figure 3C). Examples of such add-on technologies include quorum sensing [87,88], components that mediate plasmid replicon and segregation functions [89–91], or toxin–antitoxin systems [92] to stabilize retention of the editing platform. Also, deeper forms of protection might be provided by targeting a range of collateral AR functions, such as antibiotic decoys (e.g., methicillin-resistant *S. aureus* *mecA*) [93,94] or extrusion pumps/transporters [95]. These objectives could be achieved by using RNA-targeting Cas13a anti-AR systems [96] or programmable dCas9 systems to target inactivation of host virulence factors [97–100]. Developing bacterial counterparts of eukaryotic allelic drive strategies [60] also holds potential for re-establishing the normal activity of altered targets (Figure 3B, synergistic systems). For example, one might seek to replace alleles of essential chromosomal genes that have evolved to reduce fitness costs of carrying AR plasmids [101] with less plasmid-friendly wild-type alleles of these genes.

Pro-AG cassettes could also carry dosage-sensitive *cis*-acting elements to develop tunable amplifier biocircuits and logic-gated relay circuits. These configurations could drive sequential pro-AG catalyzed events to create conditionally ordered editing outcomes, thereby enriching the currently expanding toolbox of genetic biocircuits to refine anti-AR accessory systems and linking these technologies to the burgeoning field of synthetic biology [102]. Also, incorporating newly characterized retron systems [103–107] in which bacterial reverse transcriptases synthesize multicopy single-strand DNA (ssDNA) from noncoding RNA templates could provide amplified ssDNA homology templates for directing gene-editing events [104,107]. Additionally, the recently demonstrated antiphage activities of retrons [103,105] could be exploited as deployed by Yosef and colleagues [21] to render anti-AR systems carrying such elements immune to productive infection by several different phages, thereby increasing the fraction of bacteria in populations carrying anti-AR systems.

Another important challenge will be to demonstrate efficacy of anti-AR CRISPR platforms in the context of *in vivo* infection models where complex interactions with anti-AR donor and intended AR recipient bacteria are examined in broader microbial communities. For example, in *Drosophila*, which serves as an excellent model to study basic cellular functions underlying disease [108] or host–pathogen interactions [109–111], full conjugal systems have been developed [27,28,30] for

Figure 3. (A) Mobilizing anti-antibiotic resistance (anti-AR) CRISPR systems on horizontal gene transport (HGT) platforms. Conjugal transfer elements: conjugal plasmids (CP; middle panel) and integrative and conjugal elements (ICE) carry both anti-AR CRISPR components and conjugal transfer machinery (e.g., *cis*-acting *oriT* and TSS4 systems; brown cylinder in right panel). Phage: temperate phage (depicted) carrying anti-AR CRISPR cassettes (red box) insert into the host genome as lysogens protecting against AR-bearing plasmids. Lytic phage kill targeted pathogenic bacteria that may or may not also carry AR-plasmids. CRISPR-guided transposons: Programmable transposons (Tsn) encode a Cascade complex directing cassette insertion into specific target sites directed by guide RNA (gRNA) spacer arrays and anti-AR CRISPR systems (red box). Tsn with two gRNAs directing insertion into both chromosomal and plasmid sites (e.g., on a conjugal transfer plasmid) disseminate anti-AR systems throughout bacterial target populations. (B) Combination anti-AR platforms. Redundant systems: anti-AR cassette carried by conjugal plasmids or temperate phage reduce AR frequency in target cells; Cooperative systems: anti-AR systems carried by temperate phage (depicted) or conjugal transfer elements (CTEs) that also eliminates a lytic phage receptor combined with a lytic phage that kills non-lysogenic bacteria expressing the phage receptor. Synergistic systems: an HGT CRISPR platform performing two independent edits such as inserting a gRNA-bearing cassette into a high copy number target (AR1) and also performing an allelic edit to neutralize other AR determinants (AR2) such as an overactive efflux pump. (C) Applications of anti-AR systems span medical (red) to environmental (green) uses including combatting intestinal and chronic AR infections. Abbreviation: MRSA, methicillin-resistant *S. aureus*.

Box 1. Disseminating Anti-AR Systems Through Relevant Bacterial Populations

In addition to bacteria evolving resistance to anti-AR CRISPR platforms (see main text), two major challenges could limit dissemination of these systems throughout bacterial populations. First, bacteria are well equipped to resist mechanisms of HGT by deployment of restriction modification systems or one of the myriad of naturally occurring CRISPR immunity cassettes. With regard to the latter, many studies have shown that naturally occurring CRISPR systems can limit HGT [123–125] in some cases, convergently acquiring repeats targeting genes required for plasmid transfer such as TSS4-encoded nickase genes [125]. However, on larger evolutionary timescales, evidence suggests that CRISPR systems may minimally impact HGT, since the positive effects of acquiring new genetic information eventually balance out short-term costs [126]. Similarly, CRISPR has been proposed to have balancing or even net positive effects on HGT mediated by phage transduction [127] or even naked DNA transformation in the highly competent bacterium *Acinetobacter baylyi* [128]. Thus, it is unclear whether resident CRISPR systems in native bacterial populations will greatly attenuate HGT in practice. Anti-AR platforms could also include broad-spectrum anti-CRISPR that promote HGT [129] or deployment of orthogonal CRISPR systems that actively target those present in the recipient species.

The second great challenge for practical deployment of anti-AR CRISPR systems is ensuring pervasive spread of the anti-AR system throughout the native target bacterial species, which often differ in a variety of respects from laboratory strains. Thus, even robust conjugal transfer systems with broad host range, such as MAGIC [28] or Mobile-CRISPRi [99] or promiscuous ICE elements [29,30], might be hampered in the context of natural bacterial targets that may carry a diversity of unanticipated factors rendering them less competent as conjugal recipients. There may also be important target species that are not easily cultured or manipulated in the lab and that may not serve as efficient recipients of conjugal elements delivered from more tractable donor species engineered to carry the anti-AR constructs. Likewise, it is likely to prove challenging in clinical settings to gain access to sequestered bacterial populations that are either encapsulated extracellularly within tissues or hidden within intracellular compartments. Developing new genetic systems that could permit donor anti-AR bacteria from gaining access to native target bacteria or to the same internal host refuges occupied by pathogenic target strains may help mitigate these challenges.

Pseudomonas aeruginosa, *V. cholerae*, and Gram-positive bacteria (e.g., enterococci). In these *Drosophila in vivo* studies, biofilm formation in the foregut reduced *Pseudomonas* dispersal into the body cavity [112,113], while conversely, for *V. cholerae*, biofilms in the hindgut promoted colonization of this noninvasive pathogen [114]. Conjugation between *E. coli* and *V. cholerae* is efficient [31] and occurs between *E. coli* and *Pseudomonas* using specialized strains [115–117], which may allow delivery systems to be tested experimentally in the *Drosophila* model. Insights provided from these high-resolution genetic studies in *Drosophila* could then be validated in and extended to mammalian infection systems.

Box 2. Regulatory Challenges

In addition to the practical challenges outlined in Box 1, safety concerns and public acceptance of the technology will be significant hurdles to overcome in navigating an open and transparent regulatory path. For example, in the sister field of gene drives, these issues have been vigorously debated in scientific journals [130–133] and the public media and have been the subject of in-depth review by the National Academy of Sciences [134]. The parallels of gaining public acceptance and approval for gene drives and anti-AR systems disseminated through the environment by HGT are obvious in that they share the core controversial feature of spreading transgenes through native populations [135,136]. Similar levels of scrutiny regarding safety will be forthcoming in gaining approval for self-propagating anti-AR systems in clinical settings. Indeed, even nonspreading CRISPR technologies are likely to face significant regulatory hurdles due to their novelty [137,138]. In healthcare settings, the first use of these systems to gain regulatory approval may reside in decontaminating surfaces, since the complex issues of patient safety would not apply [138].

Counterbalancing the significant barrier to gaining approval for implementing anti-AR systems, particularly those incorporating drive-like HGT systems, is the great need for new solutions to the AR problem. Again, there is a persuasive parallel with gene drives, namely the pervasive and potentially unsurmountable problem of escalating pathogen resistance: AR in the case of bacteria [2] and insecticide resistance in mosquitoes [139] as well as antimalarial drug resistance in malarial parasites in the case of controlling mosquito-borne diseases [140]. As conventional control measures fail, the importance of considering novel solutions becomes paramount – a strong argument for developing state-of-the-art genetic interventions to be used in combination with existing tools. Moving forward, it will be important to emphasize the potential benefits offered by new genetic technologies while being open and honestly attentive to considering the potential risks associated with these potent yet unproven weapons in our continuing fight with microbes.

In mammalian infection systems, several of the studies cited previously have tested efficacy of anti-AR CRISPR systems for clearing bacterial infections in the intestine [26,27] or epidermis [19,23]. The infant rabbit ileum is also suitable for studying *V. cholerae* infections [118] and for assessing the efficacy of anti-AR platforms [31]. Such studies could also shed light on the role that gRNA-directed transposons such as Tn6677 [71] play in the pathogenesis of *V. cholerae* infections.

Persistent urinary tract infections (UTIs) by uropathogenic *E. coli* (UPEC) provide another excellent model for chronic infections. UTIs affect >50% of women and cost >\$2.4 billion annually in the USA [119,120], and they are the leading condition for antibiotic prescription in primary care [121]. Well-established UPEC infection models could be used to assess the benefits of targeting known virulence factors such as *fimH*, which encodes the FimH pilus protein. Type 1 pili encoded by *fimH* have been shown to be necessary and sufficient for invasion of HTB-9 human bladder epithelial cells and establishment of intracellular bacterial communities [122], an important factor in chronic or recurrent UTI. Eliminating such host virulence factors or altering other host target genes contributing to AR phenotypes, using **active genetics** CRISPR systems that function analogously to allelic drives developed in insects [60], could also extend the range and depth of next-generation genetic interventions.

Another impactful arena for second-generation CRISPR systems may lie in scrubbing AR from environmental settings, including industrial scale animal husbandry and agriculture (Figure 3C). Since such contamination contributes significantly to transfer of AR determinants to humans [6,8,9], reducing environmental AR prevalence should help break this sustaining cycle.

Concluding Remarks

CRISPR-based technologies have demonstrated considerable promise as weapons in the recurring and escalating battle against multidrug-resistant bacterial infections. These highly specific platforms can be used to eliminate AR bacteria or scrub AR factors from infectious pathogens or environmental AR reservoirs. Incorporating self-amplifying pro-AG strategies, including precise allelic editing, should enhance the efficacy and extend the range of potential applications in developing next-generation anti-AR strategies. Using these novel genetic advances to develop more sophisticated logic-gated and amplifier circuits should also expand the toolbox for combatting AR and restoring healthy homeostatic balance to microbial communities (see Outstanding Questions).

Acknowledgments

These studies were supported by National Institutes of Health grant R01 GM117321, a Paul G. Allen Frontiers Group Distinguished Investigator Award to E.B., and a gift from the Tata Trusts in India to Tata Institute for Genetics and Society, University of California, San Diego.

Declaration of Interests

E.B. has equity interests in two companies: Synbal Inc. and Agragene, Inc. These companies may potentially benefit from the research results. E.B. also serves on Synbal Inc.'s Board of Directors and Scientific Advisory Board and on Agragene Inc.'s Scientific Advisory Board. The terms of these arrangements have been reviewed and approved by the University of California, San Diego, in accordance with its conflict-of-interest policies. V.N. participated in a clinical advisory board meeting for SNIPR Biome.

Resources

ⁱ <https://apps.who.int/iris/handle/10665/259205>

ⁱⁱ www.who.int/glass/resources/publications/early-implementation-report-2017-2018/en/

ⁱⁱⁱ <https://www.cdc.gov/drugresistance/biggest-threats.html#:~:text=2019%20AR%20Threats%20Report,-CDC's%20Antibiotic%20Resistance&text=According%20to%20the%20report%2C%20more,at%20least%2012%2C800%20people%20died>

Outstanding Questions

Can CRISPR anti-AR platforms be deployed to scrub AR determinants from pathogens causing persistent infections?

Will it be possible to disseminate CRISPR anti-AR platforms in the environment with HGT systems to reduce the pool of AR determinants transferable to clinically relevant bacterial pathogens?

Can combinatorial platforms be developed to neutralize multiple AR mechanisms such as modification enzymes, uptake and efflux pumps, and drug decoys simultaneously?

Might solid-state digital amplifier and conditional logic biocircuits be designed for synthetic biology applications (sensor and next-generation anti-AR systems)?

How could evolution of bacterial resistance mechanisms to anti-CRISPR systems (e.g., plasmid and genomic AR resistance mutations, anti-CRISPR factors, phage or conjugal interference factors) be remedied by developing redundant or integrated anti-AR platforms?

How could sequestered bacterial populations be accessed in the environment or in tissues/organs and would enhanced and optimized efficiency of anti-CRISPR platforms, and/or parallel delivery systems help address these challenges?

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