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CRISPR-Cas9 technology: applications in genome engineering, development of sequence-specific antimicrobials, and future prospects

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The development of CRISPR-Cas9 technology has revolutionized our ability to edit DNA and to modulate expression levels of genes of interest, thus providing powerful tools to accelerate the precise engineering of a wide range of organisms. In addition, the CRISPR-Cas system can be harnessed to design "precision" antimicrobials that target bacterial pathogens in a DNA sequence-specific manner. This capability will enable killing of drug-resistant microbes by selectively targeting genes involved in antibiotic resistance, biofilm formation and virulence. Here, we review the origins and mechanistic basis of CRISPR-Cas systems, discuss how this technology can be leveraged to provide a range of applications in both eukaryotic and prokaryotic systems, and finish by outlining limitations and future prospects.

Insight, innovation, integration

The development of CRISPR-Cas9 technology has revolutionized our ability to edit DNA, thus opening the door to the targeted engineering of a wide range of organisms. In addition, the CRISPR-Cas system can be harnessed to design "precision" antimicrobials that eradicate bacterial pathogens by targeting specific DNA sequences. Here, we review the origins and mechanistic basis of CRISPR-Cas systems, discuss how this technology can be leveraged to provide a range of applications in both eukaryotic and prokaryotic systems, and end by enumerating existing limitations and future prospects.

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Introduction

The clustered regularly interspersed short palindromic repeats (CRISPR)-Cas system is widespread in the microbial world.[1] Indeed, it is found in 40% of all bacterial species and 90% of archaea.[1,2] However, insights into its biological significance as a genetic adaptive immune system against invading DNA elements such as infectious viruses and plasmids have only recently emerged. In addition, type II CRISPR-Cas systems have been engineered to allow straightforward RNA-guided genome editing in numerous eukaryotic and prokaryotic organisms.[1–3] This system has further been repurposed to substantially improve our ability to regulate gene expression.[4–10] One of the keys to the system is the unprecedented ability of the Cas9 nuclease to be guided by programmable small RNAs to a specific double-stranded (ds)DNA sequence, thus allowing this protein to be used to make specific target changes to the genome and other cellular DNA. CRISPR-Cas technology has already been harnessed for a wide range of applications both in mammalian and microbial cells, including the generation of mutants for genetics studies, dissection of gene pathways, execution of high-throughput genetic screens, or providing targeted approaches for narrow-spectrum treatment of antibiotic-resistant infections. Here, we review the bacterial origins of CRISPR-Cas, describe several applications derived from this technology, and discuss the hurdles that need to be tackled in coming years for this tool to become even more useful.

Origins of CRISPR-Cas systems

CRISPRs and Cas genes were first described in the genome of the bacterium *Escherichia coli* in 1987.[11] However, it was not until the year 2000 that more progress was made in the field, when Mojica *et al.* identified CRISPR systems in other bacteria and archaea.[12] Another key observation was made in 2005 when several research groups noticed that many spacer sequences within CRISPRs were actually derived from exogenous DNA elements such as plasmids and bacteriophages.[13–15] These discoveries led to the proposal

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Table 1 Glossary

CRISPR	Clustered regularly interspaced short palindromic repeat: a DNA locus of short conserved repeat sequences flanking sequences that are often derived from exogenous DNA such as phages or plasmids.
CRISPR RNA	crRNA: a small RNA molecule generated by transcription and processing of the CRISPR array. crRNA is composed of conserved repeat fragments and a variable spacer sequence, which matches the complementary sequence in the invading DNA.
cas	CRISPR-associated genes located near the CRISPR array that are necessary to interfere with invading nucleic acid.
Cas9	CRISPR-associated protein containing two nuclease domains, which is guided by small RNAs to cleave target DNA.
dCas9	Nuclease-deficient Cas9 used to regulate transcription levels.
HNH	Endonuclease domain whose name derives from its characteristic histidine (H) and asparagine (N) amino acid residues.
RuvC	Endonuclease domain named after a protein involved in DNA repair in *E. coli*.
Protospacer	A sequence in the target DNA in either the host genome or a foreign genetic element (*e.g.*, plasmid), which matches a CRISPR spacer sequence in the bacterial CRISPR array.
PAM	Protospacer adjacent motif. A conserved sequence that is located near the protospacer in the target DNA and is required for DNA cleavage by CRISPR-Cas9.
Spacer	Short sequences located between repeat sequences that correspond to target genomes.
tracrRNA	Trans-activating CRISPR RNA, which hybridizes with a repeat fragment of crRNAs and directs Cas9 activity.
DSB	Double-strand break of DNA.
NHEJ	Non-homologous end joining. Ligase-dependent, error-prone DSB repair pathway that often leads to insertions or deletions (indels).
HDR	Homology-directed repair. Error-free DNA repair mechanism that seals the broken DNA molecule by using a donor homologous template DNA sequence.
Indel	Insertion and/or deletion.
Cas9 D10A nickase	Mutated Cas9 created by a point mutation [by changing the 10th residue Asp to Ala (D10A)] in the RuvC nuclease domain. This nCas9 can only nick (single-strand break) but not cleave the PAM site in the DNA strand complementary to the gRNA.
Cas9 H840A nickase	Cas9 nickase created by a H840A point mutation in the HNH nuclease domain. Cleaves the DNA strand that is non-complementary to the gRNA.
sgRNA	Single guide RNA. Engineered chimera that links crRNA and tracrRNA and therefore combines the functions of both molecules.
TALEN	Transcription activator-like effector nuclease. Nuclease resulting from fusing transcription activator-like effector DNA binding domains to a nuclease domain.
ZFN	Zinc finger nuclease generated by fusing DNA-recognition zinc finger motifs to a nuclease domain of the restriction enzyme *FokI*.
Seed	The first 8–12 bp of a protospacer immediately near the PAM. Involved in hybridization between the crRNA and its target DNA (*i.e.*, successful targeting).

that CRISPR-Cas may act as an adaptive defense system in bacteria.[16] Shortly after, in 2007, this hypothesis was confirmed experimentally in a study that established that CRISPR-Cas systems from *Streptococcus thermophilus* indeed operated as an adaptive immune system against lytic phages.[17] In 2008, mature CRISPR RNAs (crRNAs) were shown to act as guides when present in a complex with Cas proteins, and this complex was demonstrated to interfere with phage proliferation in *E. coli*[18] and *Staphylococcus epidermidis*.[19] These studies laid the foundation for the use of CRISPR-Cas as a DNA-targeting tool (Table 1).

This review provides an overview of the origins and biology of CRISPR-Cas, and highlights its use as a tool for genome engineering both in eukaryotic and prokaryotic organisms, and its potential as a technology to generate selective, sequence-specific antimicrobials. Although the majority of scientific disciplines note the promise of CRISPR-Cas as a groundbreaking new technology for genome engineering, it is important to note that the scientific community has expressed concern about the ethical implications of gene editing in humans, particularly when thinking about modifying the germline.[20]

CRISPR-Cas: components and biological mechanism

To date, six types of CRISPR-Cas systems termed I–VI have been identified based on the different molecular mechanisms for nucleic acid recognition and cleavage used by each system.[21]

Here, we focus on the biology of the type II CRISPR-Cas system (Fig. 1) due to its widespread use in genome engineering. This system consists of CRISPR (cr) RNAs that guide Cas (CRISPR-associated) proteins to specific foreign DNA sequences. A short sequence motif known as the protospacer adjacent motif (PAM), which is adjacent to the crRNA-targeted sequence on the invading genome, plays a key role in the stages of adaptation and interference, and in spacer acquisition[22] (Fig. 1). The PAM may differ from organism to organism, but it is NGG in the commonly studied bacterium *Streptococcus pyogenes*. Examples of PAMs in other bacterial species include: NNNNGATT in *Neisseria meningitidis*, or NNAGAA in *Streptococcus thermophilus*.[1]

The mechanism of CRISPR-Cas-mediated immunity consists of three different stages (Fig. 1):[23] (i) adaptation phase, where exposure to foreign DNA causes Cas proteins to promote the insertion of a short nucleotide sequence from the invader's genome as a new spacer into the bacterial host CRISPR array; (ii) expression phase, in which the repeat-spacer array is transcribed as a long precursor CRISPR RNA (pre-crRNA) that is then processed by trans-activating crRNA (tracrRNA) in the type II system (or by Cas nucleases in other CRISPR systems) into short individual crRNAs composed of a spacer portion and flanking repeats; (iii) interference phase, in which the small crRNAs associate with Cas proteins to form a ribonucleoprotein complex. crRNAs are then used as guides to direct the crRNA-Cas complex to its target site/s present in the invader's genome, which are complementary to the crRNA spacer sequence.

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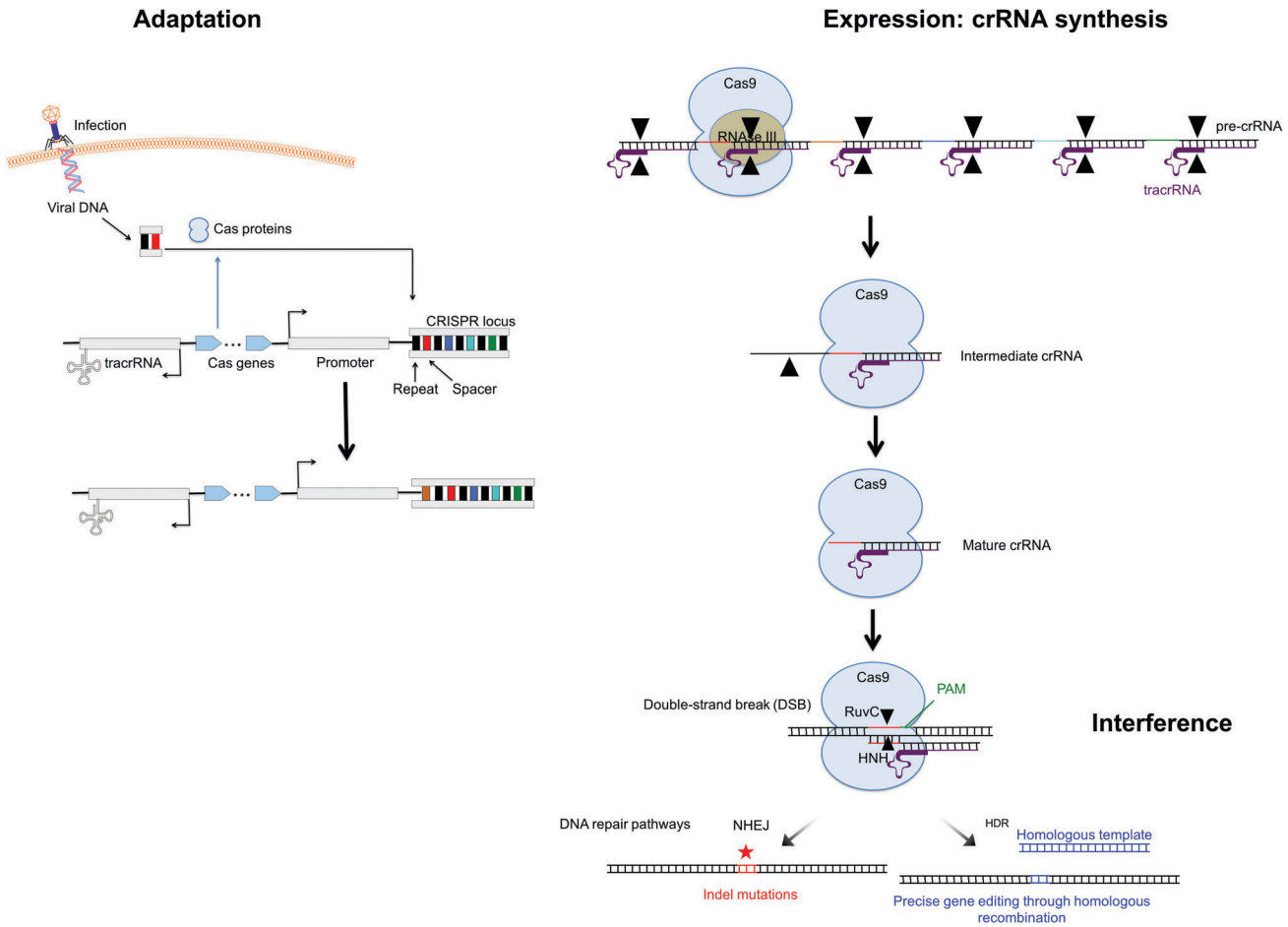


Fig. 1 Biology of type II CRISPR-Cas system. Type II CRISPR-Cas immunity consists of three distinct phases. Phase I or adaptation phase: injected DNA material from invading bacteriophages or plasmids establishes a memory of infection in the cell. The bacterial CRISPR array acquires a short sequence from the infecting DNA element. This spacer sequence is integrated into the first repeat of the bacterial CRISPR array by Cas proteins and is accompanied by the duplication of this repeat sequence. Phase II or expression phase: the spacer sequence is transcribed and the resulting pre-crRNA is further processed into a short crRNA. Phase II or interference phase: the crRNA guides Cas9 for specific DNA targeting.

The sequences of crRNAs that determine DNA targeting by CRISPR result from the acquisition of foreign nucleotides into the CRISPR array during previous exposures of the bacterium to a phage or plasmid. This process constitutes a bacterial adaptive immune system, as it enables precise recording of past encounters with invading DNA elements.

Genome engineering

Genome editing using Cas9: basics and mechanism of action

As mentioned above, the genome engineering functionality of the type II different CRISPR systems relies on the association of CRISPRs with Cas proteins. This is exemplified in the type II CRISPR-Cas system of the bacterium *S. pyogenes*, which consists of a single Cas protein (*i.e.*, the nuclease Cas9), and two noncoding RNAs referred to as crRNA and trans-activating crRNA (tracrRNA) that guide the tracrRNA:crRNA:Cas9 complex to their target site in the invading DNA for subsequent recognition and cleavage of dsDNA[24,25] (Fig. 2). Therefore, the selection of proper protospacers is critical because these RNA molecules determine specificity as they harbor target recognition sequences. For example, the protospacer of the widely used *S. pyogenes* Cas9 contains a 20 nucleotide base-pairing region followed by the PAM consisting of nucleotides NGG (*i.e.*, N_{20}NGG).

The simplicity and efficiency of this system has made Cas9 the nuclease of choice for a range of genome engineering applications. The system was further improved and simplified by engineering the dual tracrRNA:crRNA into a chimeric, single guide RNA (sgRNA) that retains its ability to target Cas9 to specific dsDNA sequences in the genome (Fig. 2). Indeed, the majority of the tracrRNA portion of sgRNA is able to bind to Cas9 by means of its double-stranded structure at the 3′ end of the sgRNA, and has been shown to target DNA by Watson–Crick base pairing through the first 20 nucleotides located at the 5′ end of the sgRNA.[24] Cas9 is capable of mediating genome editing by cleaving DNA through double-strand DNA break (DSB) formation at a specific site determined by the 20-nucleotide target recognition sequence present within the crRNA[24,26] (Fig. 1 and 2) and predicated on the target dsDNA sequence

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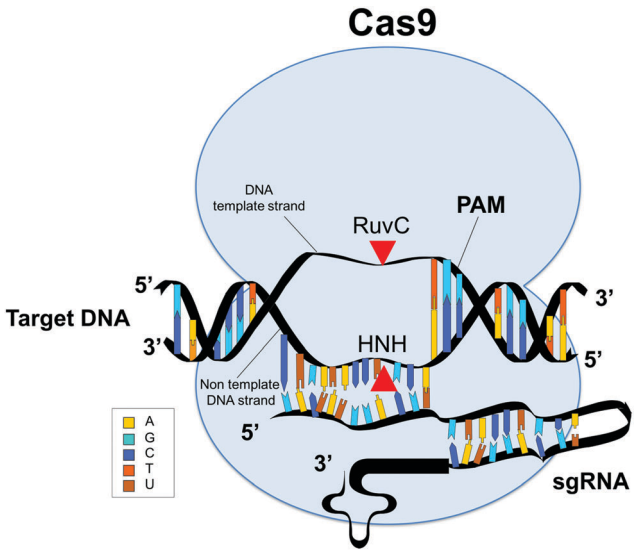


Fig. 2 Genome engineering applications of CRISPR-Cas. The system consists of two components: a single guide RNA (sgRNA) and a CRISPR-associated endonuclease called Cas9. The sgRNA is a small RNA whose sequence (typically ~20 nucleotides) allows binding to Cas9 and specifies the targeting sequence that can be re-programmed to target any gene of interest. The sgRNA consists of a 20-nucleotide base pairing region, a 42-nucleotide dCas9 binding handle, and a 40-nucleotide *S. pyogenes* transcription terminator.

being followed by a PAM. In eukaryotic and some prokaryotic systems, once DSBs are formed, they are re-ligated by cellular DNA repair systems that will be described in the next section. The engineering of sgRNAs simplified gene editing and democratized its use across disciplines.

Double-strand DNA break repair pathways: nonhomologous end joining (NHEJ) and homology-directed repair (HDR)

Once the sgRNA:Cas9 complex is bound to its target DNA, the Cas9 nuclease domains HNH and RuvC each cleave one of the DNA strands in a location 3 bases upstream of the PAM, leaving a blunt end DNA DSB. When a DSB occurs in eukaryotic cells, it is repaired primarily through either the error-prone non-homologous end joining (NHEJ) pathway or by homology-directed repair (HDR) (Fig. 1). On the other hand, many bacterial species, including *Escherichia coli*, lack a NHEJ pathway and thus rely on the cellular homologous recombination system in conjunction with chromosome or plasmid-borne templates to repair DSBs.[27] However, exceptions to this rule have been found in *Bacillus subtilis*, *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*, which have been shown to undergo NHEJ through the Ku/LigD pathway.[27] Interestingly, recent work has challenged the notion that CRISPR-based genome targeting is necessarily lethal to bacteria, as *E. coli* was shown to survive genome editing by Cas9.[28]

During repair, NHEJ usually re-ligates the lesion in a process that involves the generation of undefined, short insertion or deletion (indels) of several base pairs in a position near the cutting site.[29] This often alters the open reading frame of the target gene, for example by causing a frame shift, and therefore leads to the creation of premature stop codons and thus gene knockouts.[29–32] On the other hand, HDR uses as a template for homologous recombination an exogenous DNA fragment that contains homologous flanking arms. This pathway has been leveraged to efficiently introduce specific sequences into the cutting site, given that an appropriate donor DNA template is provided. Indeed, HDR can be used for precise replacement of DNA sequences with defined modifications such as insertions, deletions[32,33] and nucleotide substitutions.[34–36] Interestingly, the efficiency of CRISPR-Cas9-mediated HDR can be modulated by small molecules.[37] For example, molecules L755507 and Brefeldin A have been shown to enhance HDR efficiency by 3-fold compared to the untreated control in mouse embryonic pluripotent stem cells, while others (*e.g.*, the thymidine analogs AZT and TFT) have been shown to reduce it.[37] These or other yet to be discovered small molecule enhancers could be used to improve CRISPR-mediated HDR efficiency.

In many bacterial genomes, Cas9-mediated DSB formation leads to cell death due to the absence of a functional NHEJ pathway. This has been harnessed to facilitate bacterial genome editing procedures, as Cas9 can be directed to a specific sequence in the genome. CRISPR-Cas9 thus eliminates wild-type clones (*i.e.*, that contain the target gene) and can be used to select for recombinants that are designed to lack either the target sequence or the PAM.[38,39]

Genome engineering tools: ZFNs, TALENs and CRISPR-Cas9

Several sequence-specific nucleases were used before the discovery of CRISPR-Cas, including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). These tools were developed based on the original observation that, in mammalian cells, DSBs triggered up to 1000-fold increased recombination events in the cleaved region.[40] This led to the notion that nucleases such as ZFNs and TALENs could theoretically be programmed to target any gene of interest.

Zinc fingers (ZFs) and transcription activator-like effectors (TALEs) were originally discovered in nature. For instance, TALEs are secreted by microorganisms *via* their type III secretion system when they infect plants. They are characterized by possessing a relatively large DNA recognition sequence (*i.e.*, target site) that consists of 12–40 base pairs.[41] The ZFNs and TALENs used for genome modifications are engineered nucleases in which a ZF or TALE DNA-binding domain is linked to nuclease domains, for example those derived from the restriction enzyme *FokI*. The ability of ZFNs to create DNA modifications through DSBs depends on a pair of DNA-binding zinc fingers comprised of ~30 amino acid modules that interact with nucleotide triplets, and the restriction endonuclease *FokI*, which is responsible for catalytic cleavage of DNA. Each ZFN typically recognizes 9–18 nucleotides. Because the nucleases to which they are attached only function as dimers, pairs of ZFNs are required to target any specific locus; one that recognizes the sequence upstream and the other that recognizes the sequence downstream of the site to be modified. The main limitation of this system is the difficulty and high cost associated with the modular assembly and development of custom proteins for the recognition

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of specific target DNA sequences, in addition to the ZF modules not being 100% orthogonal.[42]

TALENs, on the other hand, require DNA-binding proteins called transcription activator-like effectors that are combined with a DNA cleavage domain such as *FokI*. TALEs contain multiple 33–35 amino acid repeat domains, each recognizing a single base pair, and their specificity is determined by two hypervariable amino acids that are known as the repeat-variable di-residues. TALENs offer some advantages over ZFNs, including the tendency to have a higher editing success rate. For example, in human cells TALENs achieved an editing rate of >99% *cf.* ~24% for ZFNs and an average off-target rate of ~20% (*cf.* <10% for ZFNs).[43,44] On the other hand, ZFNs are typically smaller than TALENs, which makes it easier to deliver and express them in cells. Indeed, a typical cDNA encoding a ZFN is approximately 1 kb, whereas for a cDNA encoding a TALEN is usually around 3 kb. Additionally, ZFN engineering does not involve assembling as many highly repetitive elements as for TALENs. However, both technologies present limitations because they rely on protein engineering procedures to change specificity, which has prevented ZFNs and TALENs from being widely adopted for genome engineering applications across different fields.

The discovery of the gene-editing abilities of the Cas9/sgRNA complex paved the way for the use of Cas9 as a genome-engineering tool in numerous organisms as it offered several unprecedented advantages over ZFNs and TALENs, including ease of use, simplicity, low cost, programmability and multiplexing. The CRISPR system is programmable, as the nuclease Cas9 can be guided to any target gene by simply changing the guide sequence of the sgRNA, as long as there is a PAM flanking the target DNA sequence. This system can also be multiplexed to target more than one gene simultaneously by introducing multiple sgRNAs into the cell.[45] This, at least in theory, should enable the design and construction of genome-wide array libraries containing sgRNAs, each of which targets a specific gene in a given genome. Among other examples, our recent technology for scalable barcoded combinatorial gene assembly, CombiGEM, created a library of 23 409 barcoded dual guide-RNA (gRNA) combinations, followed by a high-throughput pooled screen. This CombiGEM-CRISPR approach identified gene pairs that inhibited ovarian cancer cell growth when they were targeted for knockouts, along with drug pairs that targeted these same gene pairs and achieved synergistic activity when used together.[46] In addition to all these advantages, the specificity of CRISPR-Cas technology towards its target sequence in human cells is comparable to that of ZFNs and TALENs,[42–44] with high efficiency rates (~90%) and average off target effect rates of ~20%.

As described previously (Fig. 1), three components need to be expressed in foreign hosts for the type II CRISPR-Cas system to be fully functional: the Cas9 protein, tracrRNA, and customized crRNA (or a chimeric sgRNA in place of the tracrRNA:crRNA). For instance, transfer of the type II CRISPR-Cas system by genomic DNA transformation into *S. pneumoniae* led to targeted genome editing.[36] A CRISPR-Cas system consisting of two plasmids was also described to edit the genome of *E. coli*: one plasmid encoded tracrRNA and Cas9, and the other expressed the crRNA array.[36] Editing of multiple genes in a single step has also been achieved in the genomes of the bacterium *E. coli*[36] and humans[32,34,47,48] by using customized target recognition sequences composed of 20-nucleotides in a crRNA array. Thus, CRISPR-Cas systems can be used for multiplex editing of both prokaryotic and eukaryotic genomes simply by programming the crRNA array (or gRNA when using a sgRNA).

Regulating the expression levels of Cas9 through inducible promoters has been shown to be important, particularly to (i) reduce the likelihood of potential off-target effects and (ii) minimize the occurrence of suppressor mutations that inactivate either Cas9 or sgRNA. These suppressor mutations may result in so-called ''escaper'' colonies that are indistinguishable from expected recombinants.[36] Another consideration to take into account when using Cas9 from bacteria with low GC content such as *S. pyogenes* (35% GC) is that it may need to be codon-optimized for proper functioning in organisms with high GC content such as *Streptomyces*.[49]

Despite the extensive use of CRISPR-Cas as a genome-editing tool in eukaryotic organisms, this technique has not been as widely adopted by the microbial sciences community. However, we predict this will change in coming years and CRISPR-Cas will become widespread in microbiology laboratories around the world for some of the following applications: to generate deletion mutants, perform complex genetic studies and high-throughput genetic screens, target pathogenic bacteria, microbiome and biofilm research, study previously intractable bacterial species by providing a genome engineering strategy, *etc.*

Genome editing using Cas9 nickases and Cas9 derivatives

The Cas9 nuclease can be further modified into a nickase, which cleaves a single strand of DNA at the target site (*i.e.*, creates a nick) as opposed to cutting both DNA strands and forming a DSB, which is lethal in bacteria.[50,51] Nickases can be generated either by introducing a D10A mutation in the RuvC domain, or a H840A mutation in the HNH domain of Cas9. The HNH nuclease domain cleaves the DNA strand that binds to crRNA, and the RuvC-like domain cuts the opposite strand, thus creating DSBs at specific sites.[51,52] Since Cas9 nickases are unable to form DSBs, they do not induce the NHEJ repair pathway. This is particularly useful in prokaryotes, where wild-type Cas9-induced DSBs are lethal due to minimal expression (or lack) of NHEJ. Cas9 nickases help circumvent this lethality by generating non-lethal single-nicks, which trigger homologous recombination and allow one-step editing at specific target genomic loci. This strategy was used for high efficiency editing (>95%) in *Clostridium cellulolyticum*, and to deliver foreign genes into the genome in a single step, without the need for a selective marker.[53]

However, nicked DNA can be repaired *via* HDR, which is typically associated with a lower incidence of off-target effects, as mentioned above. Indeed, it has been shown that Cas9 nickases stimulate HDR at levels similar to wild-type Cas9[50] and the approach of combining nickases and HDR has successfully edited genomes of mammalian cells.[32]

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Cas9 nickases can also be leveraged to introduce double nicks, by using them in combination with a pair of sgRNAs targeted to adjacent sites on opposite DNA strands. The system operates as follows: each sgRNA recruits a Cas9 D10A nickase that is programmed to target and create two individual single-stranded breaks a short distance apart from each other. This approach retains on-target efficiency while allowing the generation of DSBs and NHEJ-induced mutations with a 50–1500-fold reduction in off-target effects.[50] In addition, paired-nicking allows HDR at levels comparable to those mediated by wild-type Cas9, and at higher rates than single Cas9 nickase-mediated HDR. Despite its promise, several considerations have to be taken into account for optimal functionality of this technology, including: (i) the design of properly offset gRNAs that are essential for efficient DSB formation, and (ii) the use of paired nickases that are offset by <20 bases to ensure efficiency.

Another strategy that has been explored in an attempt to reduce off-target effects is the use of truncated sgRNAs composed of 17 or 18 base pairs that can be generated by truncating the 5′ end of the sgRNA sequence to reduce the target complementarity to <20 nucleotides in length.[54] These truncated sgRNAs retained on-target activity while decreasing off-target effects by around 5000-fold. This approach reduced the likelihood of mutations caused by RNA-guided nucleases at sites that differ by as many as 5 nucleotides from the target. An additional strategy to further reduce off-target mutations involves the combination of two truncated sgRNAs with paired Cas9 nickases.[51]

A variant of Cas9 has been engineered by combining dCas9 with the nuclease domains of *FokI*, an endonuclease mentioned earlier that is often used in ZFN and TALEN technologies. The dCas9-*FokI* dimers (known as fCas9) are capable of editing genes in mice,[55] and have been shown to increase sequence specificity/on-target activity by >140-fold when compared with wild-type Cas9 in human cells.[56] Moreover, this Cas9 derivative generally induces less off-target effects when compared to the paired Cas9 nickase strategy.[57] This approach is mainly limited by the requirement to have two defined PAM sites, which lowers the frequency of available targets in a given genome.

CRISPRi and CRISPRa: programmable regulation of transcription using dCas9

Different approaches have been used in the past to regulate gene expression, including RNAi[58] and expressing the gene of interest under the control of inducible promoters. In addition to its ability to edit DNA, CRISPR technology has been repurposed for use in transcriptional regulation both in mammalian and bacterial cells (Fig. 3 and 4). This was achieved by mutating the two nuclease domains of Cas9 (HNH- and RuvC), which resulted in an engineered "dead" variant of Cas9 (dCas9) that is catalytically inactive. dCas9 is unable to cleave DNA but can be used to activate (CRISPRa) or inhibit (CRISPRi) gene expression when used in conjunction with a designed sgRNA that is complementary to the desired DNA target sequence.[6–8,59,60] The main advantage of

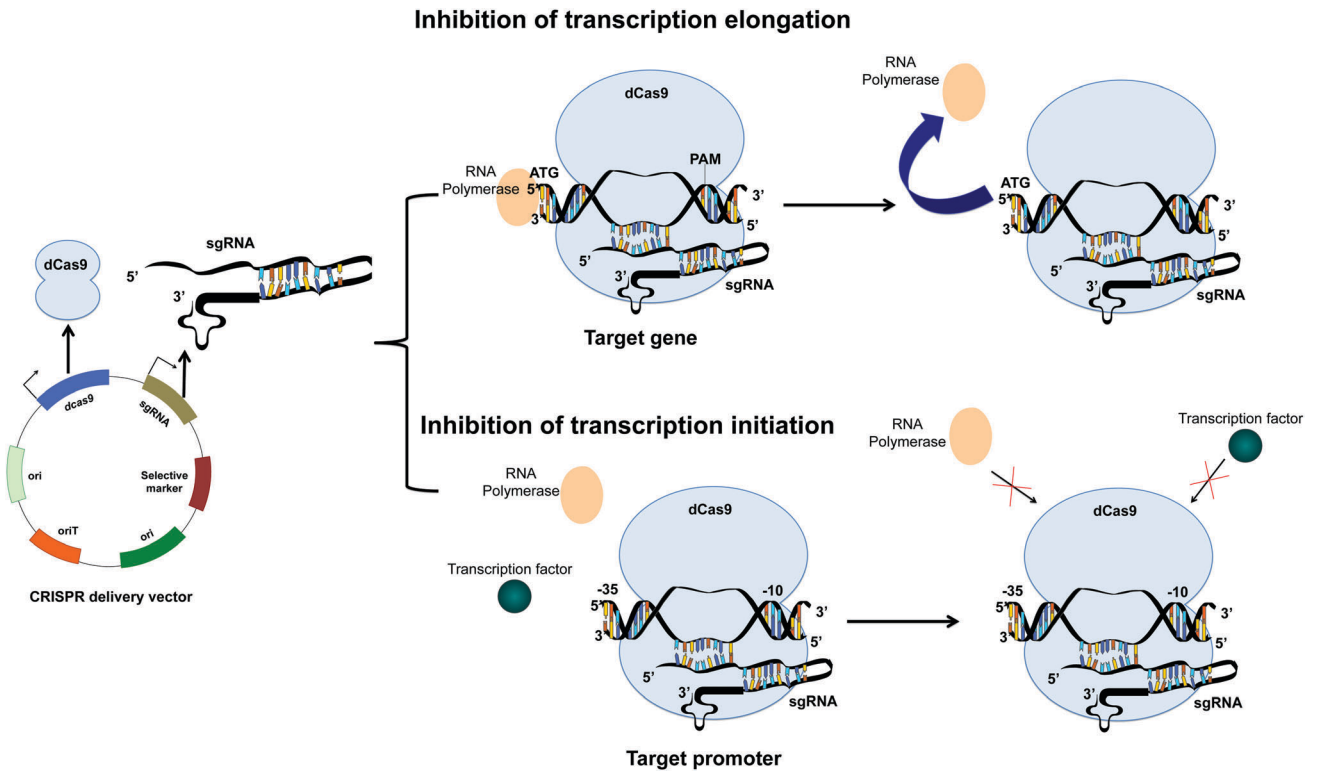


Fig. 3 CRISPR-dCas9 for genome interference. CRISPRi enables transcriptional repression of any gene of interest by either inhibiting transcription elongation or transcription initiation.

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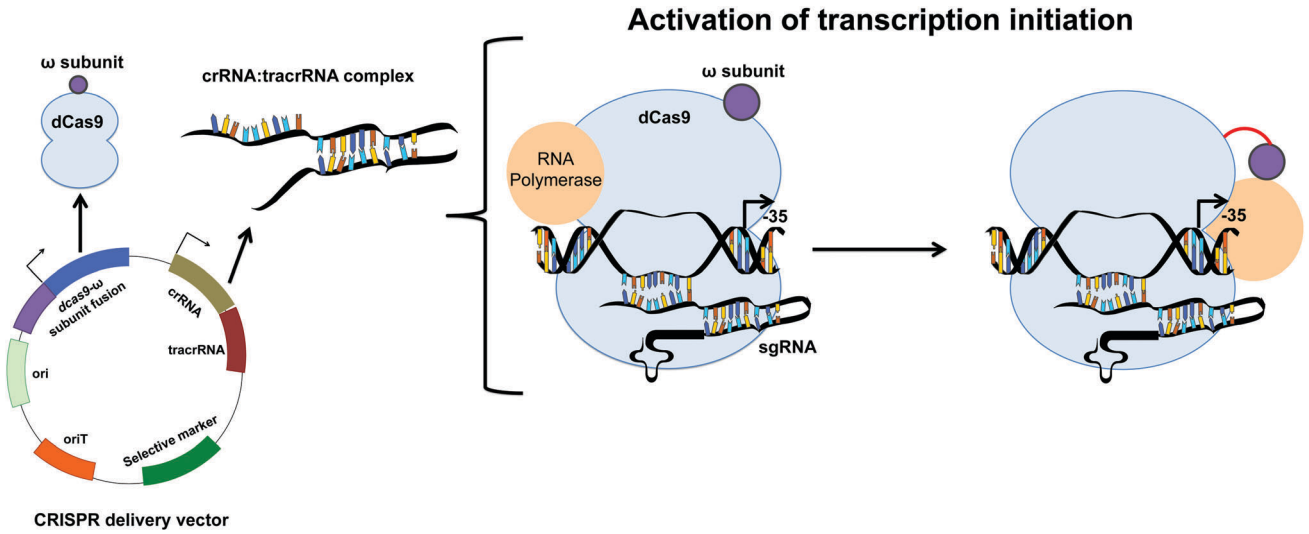


Fig. 4 CRISPR-dCas9 for genome activation in bacteria. CRISPRa can be used to enhance gene expression by fusing dCas9–sgRNA complexes with transcriptional activator domains that recruit RNA polymerase to the promoter region of the target gene.

dCas9 over wild-type Cas9-based genome editing is that it does not require cellular repair pathways such as NHEJ, which makes it a suitable technology for difficult-to-engineer microbes.

CRISPRi causes gene repression through the ability of the dCas9–sgRNA complex to bind DNA and sterically hinder transcription of the targeted DNA (Fig. 3), which is different than its preceding RNAi technology that acts at the mRNA level. CRISPRi was first demonstrated in the bacterium *E. coli*, where dCas9 was shown to operate as a potent programmable transcriptional repressor. Mechanistically, the dCas9–sgRNA complex is capable of repressing transcription in two ways: (i) by occluding RNA polymerase binding to promoter DNA, or (ii) by sterically hindering transcription elongation (Fig. 3). Indeed, RNA-guided dCas9 binding of either promoter or open reading frame regions decreases transcription by two orders of magnitude in *E. coli*.[6,7] When repressing transcription by blocking the elongation step also in *E. coli*, repression efficiency has been shown to increase when the gRNA anneals to the non-template DNA strand.

CRISPRi presents additional important advantages compared to traditional techniques used for regulating gene expression such as RNAi, which does not work in bacteria. First, it is easy to redirect the system against new gene targets by simply inserting a specific 20 nucleotide base-pairing region into the sgRNA. Moreover, CRISPRi is scalable as thousands of sgRNAs can be constructed using pooled cloning strategies with oligonucleotide libraries. Another advantage of the CRISPRi system is its inducibility by means of inducible promoters, which enables manipulation of essential genes[60,61] by partial repression of gene expression. Also, CRISPRi can be multiplexed to repress the expression of several genes simultaneously by using multiple sgRNAs that can be encoded in the same plasmid.[6,7,59,60] One issue with CRISPRi is that it can affect genes downstream of the target gene if this is part of an operon, as dCas9 operates by blocking elongating RNA polymerase.

Recent efforts have successfully adapted CRISPRi to a number of microorganisms. For example, the CRISPRi system has been implemented in *Mycobacterium tuberculosis*.[62] Recent work has also achieved transfer of CRISPRi constructs into *E. coli* through conjugation.[63] Moreover, CRISPRi systems have been successfully integrated into the chromosomes of bacteria that have traditionally been difficult to genetically manipulate, such as the common gut commensal bacterium *Bacteroides thetaiotaomicron*[64] and *Actinomycetales*.[65,66] Chromosomal integration eliminated the need for selective markers associated with plasmid-based CRISPR, and allowed implementation of the system in long-term experiments such as those involving animal models. These integrated strains are valuable as they could be used in prolonged studies that were previously very difficult to perform, largely due to the need of selection markers. For example, they could be used in work directed at investigating living bacterial communities that require several days to fully develop, such as microbial biofilms.

dCas9 can also be used to enhance gene expression (Fig. 4). This technology has been termed CRISPR activation (CRISPRa) and is capable of stimulating transcription of target genes by using a dCas9–sgRNA complex that is fused with activator domains (*e.g.*, VP64 in eukaryotes) that recruit RNA polymerase to promoter DNA (Fig. 4). Bikard *et al.*[6] demonstrated this in *E. coli* by fusing dCas9 to the ω subunit of RNA polymerase and expressing the resulting dCas9-ω in an *E. coli* mutant lacking the gene encoding for ω. In this mutant, and in the presence of an appropriate sgRNA, dCas9-ω takes the place of ω in the RNAP complex where it can mediate the recruitment of RNAP to a position upstream of the promoter, therefore activating transcription. More recently, multiple activators have been either fused to dCas9 or recruited by dCas9 or the sgRNA to achieve increased levels of gene expression control.[67] CRISPRa has been shown to potentiate transcription in *E. coli* by around one order of magnitude mainly from weak promoters, and

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activation is dependent on the position of the guide RNA. In contrast to the efficiency of gene repression by CRISPRi, dCas9-mediated gene transcription activation has been shown to be substantially less efficient in both prokaryotic and eukaryotic cells[68–70] and therefore will need to be optimized in the future.

Further, expression of both dCas9 and sgRNA can be placed under the control of inducible promoters,[61] which allows titratable and reversible gene repression by CRISPRi. It is also possible to combine multiple activators or repressors to achieve synergistic interactions between the different components to either enhance or down-regulate gene expression.

Recently our group described the activation and repression of natural and artificial eukaryotic promoters. In this study the CRISPR-Cas system of *Streptococcus pyogenes* were programmed through the engineering of guide RNAs with base-pairing complementarity to target DNA sites. We demonstrated that the activity of CRISPR-based transcription factors (crisprTFs) can be tuned by directing multiple crisprTFs to different positions in natural promoters and by arraying multiple crisprTF-binding sites in the context of synthetic promoters in yeast and human cells.[70]

The need for new antibiotics: CRISPR-Cas9 sequence-specific antimicrobials

Antibiotic resistance represents one of the biggest challenges of our time as drug-resistant bacteria continue emerging, which has coincided with the lack of new antibiotics either being developed or approved for human use. Indeed, it is estimated that antibiotic-resistant infections will kill 10 million people a year by 2050 if no new antimicrobial approaches are developed, costing the global economy $100 trillion.[71]

Several new alternative approaches have been developed in recent years to combat antibiotic resistance, including the use of bioengineered and biologically inspired synthetic peptides,[72,73] engineered bacteriophages,[74] and others. CRISPR technology represents a unique opportunity to combat antibiotic resistance through its ability to selectively target any gene of interest in a particular pathogen (Fig. 5). Indeed, CRISPR-Cas has been recently harnessed to design antimicrobials that work by targeting specific DNA sequences. These sequence-specific antibiotics operate by using the RNA-guided nuclease Cas9 to exert a toxic effect in cells through chromosomal cleavage.[75–77] For instance, potential target genes could include those involved in antibiotic resistance, biofilm development, formation of persisters, virulence, *etc.* In contrast with conventional antibiotics that are broad-spectrum in terms of their activity, these CRISPR-Cas antimicrobials can be programmed with a sgRNA matching a specific target gene of choice. Therefore, these antimicrobials could be capable of selectively killing the bacteria that harbor this gene while leaving all other neighboring bacteria unaffected, even in complex bacterial populations such as biofilms or the human microbiota.[76]

Recent work has shown that this approach can be used for selective killing of bacterial populations.[75–78] These studies showed efficient delivery of RNA-guided nucleases by using phagemids, which are plasmids that contain signals for bacteriophage packaging. These phagemids were engineered to contain genes that encode for both *cas9* and gRNAs. For example, Citorik *et al.*[75] showed efficient delivery of RNA-guided nuclease Cas9 into *E. coli* by using phagemids with packaging signals for the

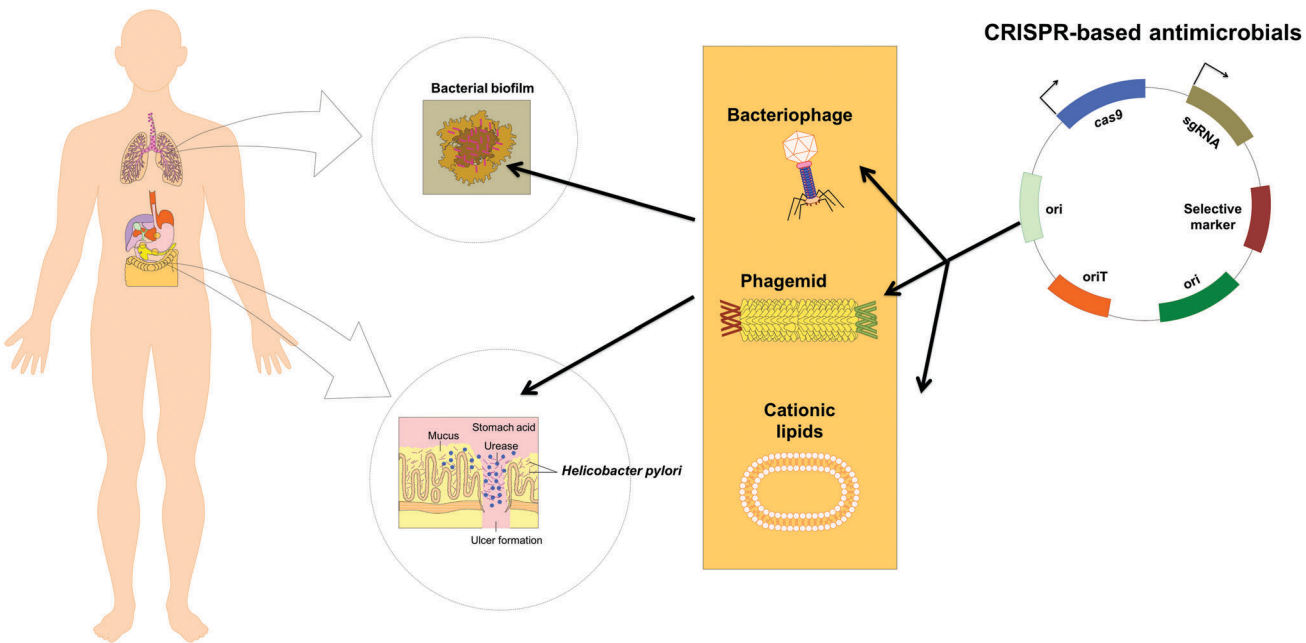


Fig. 5 CRISPR-Cas-based antimicrobials as therapeutics and for microbiome engineering. The future of antibiotic therapeutics may involve CRISPR-Cas antimicrobials that are efficiently delivered into different sites of infection in the host (*e.g.*, lungs, stomach) to help eradicate drug-resistant infections such as those caused by bacterial biofilms. These systems can also be used for selective removal of target species from the microbiome.

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filamentous phage M13. This strategy led to selective killing of pathogenic *E. coli* strains that expressed specific target genes including those that encode for antibiotic resistance. Importantly, phagemid-mediated delivery of Cas9 antimicrobials led to increased survival in a *Galleria mellonella* (*i.e.*, wax moth) model of *E. coli* EHEC 0157:H7 intestinal infection. Another study[76] used a similar approach but focused on the Gram-positive bacterium *S. aureus* and its temperate phage ΦNM1. The authors delivered phagemids to the skin of infected mice in a *S. aureus* skin colonization model, which led to substantial and selective Cas9-mediated killing of antibiotic-resistant bacteria. Some "escaper" colonies were able to evade the treatment; however these bacteria were not genetically resistant mutants, therefore indicating that under the conditions tested, resistance was not readily selected for. Indeed, these colonies either had not received the Cas9 plasmid or had received a defective plasmid. More recently, temperate and lytic phages were shown to efficiently deliver CRISPR-Cas to selectively sensitize and kill antibiotic-resistant bacteria.[78]

In conclusion, these seminal studies provided proof-of-concept demonstrations that Cas9-carrying phagemids could be used to selectively target and eliminate antibiotic-resistant bacteria. Another benefit of this approach is that the Cas9 plasmid can be used to immunize its host against undesirable genes (*e.g.*, antibiotic resistance or virulence genes), therefore preventing future acquisition of such genes. As a result, this therapy could potentially be used to limit the spread of antibiotic resistance.

These studies change the way we traditionally think about antibiotic discovery, as they suggest that "personalized" nuclease-guided antimicrobials can be generated to target any gene of interest in a given bacterial genome (Fig. 5).

Future research using CRISPR-Cas antimicrobials will likely focus on targeting biofilms (Fig. 5), which are multicellular communities of bacteria that are estimated to cause at least 65% of all persistent infections in humans.[79–82] The inner layers of biofilms are inhabited by persister cells that are dormant variants of regular cells that are highly tolerant to many stresses including antibiotics.[80] Currently, there are no available effective drugs that selectively target biofilms or persisters.[83] Several gene circuits and global regulators are known to be involved in biofilm development and the formation of persister cells, and thus represent attractive therapeutic targets. However, the genetics of biofilm and persister cell formation are not yet well understood. CRISPR-Cas technology will be useful to genetically dissect these processes. Another key challenge in the near future will be to program CRISPR-Cas-based antibiotics against pathogenesis-related genes and to efficiently deliver these constructs into biofilms for their subsequent eradication (Fig. 5). A key potential of CRISPR-Cas therapeutics is their specificity, which allows them to exclusively target pathogenic bacteria, while leaving non-pathogenic bacterial populations such as the human microbiota unaffected. Therefore, in the future it may be necessary to have a detailed profile of the composition of a patient's microbiota to avoid unwanted targeting of beneficial commensal populations.

Limitations: off-target effects and the delivery problem

As mentioned throughout the text, a fundamental requirement of any genetic engineering technology is keeping to a minimum the frequency of secondary mutations, often referred to as off-target effects. In the case of genome editing mediated by ZFNs, TALENs, or Cas9, this is directly related to the target specificity of these nucleases, since the recognition and cleavage of off-target sites will lead to the introduction of undesired indels by the NHEJ repair system. To avoid off-target effects, it is imperative to properly design sgRNAs that direct Cas9 to their exact target in the genome. To ensure on-target activity and proper functioning of Cas9, studies using the type II CRISPR-Cas system and characterization of Cas9[23,24,84,85] have demonstrated the requirement of a perfect match to the seed sequence (*i.e.*, 8–12 bp near the PAM involved in hybridization between the crRNA and its target DNA). One of the main goals in the near future, particularly for larger genomes, will be to determine the specific effects of off-target effects in the genome under study, and ideally prevent their incidence. Progress has been made along these lines, as machine learning computational tools have been developed to design optimized sgRNAs that drastically reduce off-target effects of CRISPR-Cas9.[86] In addition, approaches such as fusing dCas9 to *FokI* have resulted in decreased off-target effects.[87–89]

Apart from the generation of off-target effects, another limitation to translating CRISPR-Cas into a suitable technology for human medicine is the lack of efficient delivery methods.[90,91] Several approaches have been recently proposed for efficient delivery of CRISPR-Cas constructs into living cells. For example, the recombinant sgRNA:Cas9 complex has been directly delivered into cultured human cells, mice, and zebrafish by means of electroporation or microinjection.[1]

Electroporation has been proposed as a method to deliver CRISPR-Cas constructs into mouse cells.[92] A plasmid encoding for CRISPR-Cas9 can be introduced into target cells by exposing those cells to electrical pulses. A new method that involves membrane deformation has also been shown to efficiently deliver CRISPR-Cas constructs into hard-to-transfect human immune and stem cells.[93]

As mentioned above, another physical method consists of microinjecting the plasmids directly into the target cells. This technique provides an alternative strategy to deliver CRISPR-Cas9 into cells with high efficiency and target specificity.[94] Further, this method has been shown to be compatible with CRISPR multiplexing as it has been used to generate multi-gene knockout rabbits.[94] However, disadvantages associated with this technique include a high level of manual skill required to prevent cell damage, and the fact that it is low-throughput as only a limited number of eukaryotic cells can be microinjected at once. In addition, microinjection of Cas9 mRNA transcribed *in vitro* has been used to generate genetically modified zebrafish, mice, pigs and monkeys.

Alternatively, the CRISPR-Cas9 system can be introduced by ribonucleoprotein delivery.[95] This approach is advantageous in

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that protein delivery does not require codon optimization or selection of promoters, which have to be carefully designed and represent a limitation in plasmid-mediated delivery. In addition, protein delivery has the important advantage of resulting in fewer off-target effects because ribonucleoproteins are degraded rapidly in the cells shortly after their delivery and therefore exhibit less toxicity. Ribonucleoprotein delivery also reduces the effective concentration and stability of the Cas9–sgRNA complex inside cells.[95] Recently, ribonucleoprotein delivery has been achieved *via* electroporation in a procedure that involved recombinant Cas9 in a complex with sgRNA that had been previously transcribed *in vitro*. Electroporation was then used to transfect the fused ribonucleoprotein (Cas9:sgRNA) into target human cells. Importantly, ribonucleoprotein delivery has been shown to impose less stress on human embryonic stem cells, resulting in a 2-fold greater number of colonies formed post-plasmid transfection.[92]

Another method that has been recently explored for CRISPR-Cas delivery into human cells is cationic lipid-mediated transfection.[96] This approach consists on fusing a natively cationic Cas9 nuclease with polyanionic gRNA to make the gRNA efficiently interact with cationic lipids. When cationic lipid particles are added, they form a lipid bilayer around the gRNA–Cas9 complex, which protects the complex as it enters the cell. Cationic lipid-mediated delivery has been shown to increase uptake efficiency of the gRNA–Cas9 complex into cells. Lipid-mediated transfection resulted in up to 80% genome modification and 10-times greater genetic modification specificity *cf.* transfection of plasmids. This method also showed reduced off-target effects due to rapid degradation of directly delivered proteins and RNA into the cells. Different strategies using bioreducible lipid nanoparticles[97] and self-assembled DNA nanoclews[98] have also been shown to lead to increased delivery of CRISPR-Cas9.

On the other hand, bacterial CRISPR-Cas editing is also plasmid-based and it is introduced to recipient bacteria *via* direct transformation or conjugation methods.[1,54,57,62] Similarly to human cells, to introduce plasmids that encode sgRNA and Cas9 into target bacterial cells, researchers rely on traditional methods such as electroporation,[90,92] chemical transfection, or newer alternatives such as phagemids.[75,76]

Phagemids are a very interesting delivery option as they have been used to efficiently deliver into bacterial cells RNA-guided nucleases targeting specific DNA sequences. However, this delivery route is limited by the fact that phagemids have the same narrow host range as the bacteriophage they are derived from. This substantially limits the number of bacterial species that can receive the CRISPR-Cas plasmid, as only those expressing the appropriate phage receptor are susceptible to phagemids.

Overall, substantial progress has been made in recent years in the area of delivery methods for CRISPR-Cas and other nucleic acid-based therapeutics. However, these methods also have their limitations as they are not high-throughput, and their efficiency can still be improved. Optimized or completely new delivery methods will be needed to make CRISPR-Cas technology suitable for human applications.

Conclusions

CRISPR-Cas9-based tools have proven to be widely applicable in the biomedical sciences and biotechnology due to their simplicity and efficiency. However, several aspects around the technology still need further improvement, including: rational design of sgRNAs, reduction of off-target effects, and optimization of existing delivery methods. Despite these current limitations, CRISPR-Cas applications have already transformed eukaryotic genome engineering and are increasingly being used for microbial synthetic biology applications. For instance, it has thus far allowed for facile generation of deletion mutants in a number of bacterial species.[23,24] This was previously unthinkable with existing methods, as the process was laborious and often unpredictable, particularly in bacterial species with limited available molecular cloning tools. In addition, high-throughput genetic screens can now be performed relatively quickly, and can help study human disease, and track previously intractable organisms such as those that compose our microbiota, for which there were no genetic tools until recent years.

Another application is the use of CRISPR-Cas as antibiotics that can target any gene of interest and can be applied to any bacterium of choice, given that the delivery problem is solved. This capability opens the door to next-generation antimicrobials of exceptional selectivity toward their target or targets (through multiplexing), and to study and dissect genetic pathways in bacteria. Another area of focus will be the discovery of novel nucleases with improved physical and functional properties compared to those of Cas9. Progress has been made with the recent identification of a new nuclease called Cpf-1 that belongs to type II CRISPR systems.[99] Cpf-1 offers several advantages over Cas9 including its smaller size that improves transfection efficiency into cells, and apparently also higher precision of DNA insertions as it works by making staggered cuts to DNA that leave a 5′ overhang as opposed to Cas9 that generates blunt ends.

The unprecedented power of CRISPR carries numerous ethical concerns that need to be addressed when considering applying this technology to humans. This is specially the case when thinking about modifying the germline in humans using CRISPR-Cas, which is an intense area of debate[20] and will continue to be so as we move forward.

In conclusion, since its discovery, CRISPR-Cas technology has changed our ability to edit and regulate prokaryotic and eukaryotic genomes. We believe CRISPR-Cas has the potential to constitute the basis for therapies to treat a range of human diseases, including infectious diseases.

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