NEW TECHNOLOGIES: METHODS AND APPLICATIONS

CRISPR-based genomic tools for the manipulation of genetically intractable microorganisms

Rebecca S. Shapiro, Alejandro Chavez and James J. Collins

Abstract | Genetic manipulation of microorganisms has been crucial in understanding their biology, yet for many microbial species, robust tools for comprehensive genetic analysis were lacking until the advent of CRISPR–Cas-based gene editing techniques. In this Progress article, we discuss advances in CRISPR-based techniques for the genetic analysis of genetically intractable microorganisms, with an emphasis on mycobacteria, fungi and parasites. We discuss how CRISPR-based analyses in these organisms have enabled the discovery of novel gene functions, the investigation of genetic interaction networks and the identification of virulence factors.

Microbial species represent the most abundant and diverse organisms on Earth, with critical roles in environmental homeostasis, industrial manufacturing, agriculture, and human health and disease. Understanding the complex biology of microorganisms has largely been dependent on our ability to genetically manipulate them. Genetic analysis of microorganisms has a long history of pioneering and innovative experiments, including the formative discovery of transformation in *Streptococcus pneumoniae*[1] and the subsequent identification of DNA as the carrier of genetic information[2]. The earliest accounts of genetic engineering involved the generation of transgenic lineages of *Escherichia coli* through transformation of a recombinant plasmid that encoded an antibiotic resistance gene[3]. Since then, genetic manipulation of microorganisms has been pivotal for the development of biotechnological tools and the study of microorganisms themselves.

Recent advances in molecular techniques have improved our ability to perform genetic manipulation in diverse microorganisms. Currently, modern technology platforms exist for functional genomic analysis and systems-level forward and reverse genetics in many microbial species, particularly model organisms. Such platforms include genome-wide genetic deletion libraries in *E. coli*[4], whole-genome single-deletion[5] and double-deletion[6] libraries in the model yeast *Saccharomyces cerevisiae* and transposon sequencing for systems-level genetic analysis of several bacterial species[7], including *Salmonella enterica* subsp. *enterica* serovar Typhimurium[8] and *Pseudomonas aeruginosa*[9]. These tools have enabled large-scale genetic analyses to assess gene function and identify genetic interactions.

Despite advances in technologies for systems-level functional genomic analysis in many microbial species, other microorganisms have remained difficult to engineer genetically, which has hindered our potential to unlock their secrets. New advances in genomic manipulation — particularly CRISPR–Cas-based tools — have revolutionized our ability to perform targeted genetic manipulations in diverse organisms and have been instrumental in enabling us to alter the genomes of even the most notoriously intractable microbial species. CRISPR is a group of DNA sequences in bacterial and archaeal genomes that have an important role in immunity in these organisms. In type II CRISPR systems, short DNA sequences that originate from invading foreign DNA, such as viruses or plasmids (termed spacers), are inserted into the CRISPR locus, transcribed and processed into small CRISPR RNAs (crRNAs). A crRNA and a *trans*-acting RNA (tracrRNA) target Cas endonucleases to protospacer sequences based on complementarity to the crRNA sequence and the presence of a protospacer adjacent motif (PAM) site. Cas proteins mediate double-strand breaks (DSBs) at the target protospacer locus.

This system is used by bacteria and archaea to detect and cleave invading foreign DNA, but it can also be exploited as a biotechnology tool for precise genome editing at a targeted locus[10,11]. This was first demonstrated using the Cas9 protein from *Streptococcus pyogenes* and a modified chimeric single-guide RNA (sgRNA), which links the crRNA and tracrRNA. By manipulating the sequence of this sgRNA, Cas9 could be programmed to target specific DNA sequences for cleavage, generating a DSB[10]. Although sgRNA-mediated targeting is relatively flexible, the requirement of a PAM sequence can limit genomic target sites. Furthermore, an extreme skew in GC or AT richness will influence whether a particular PAM is likely to be present at a high frequency. The pathway used to repair the induced DSB determines the type of genome editing: breaks can be repaired by non-homologous end joining (NHEJ), which can result in insertions or deletions (indels) at the target locus; by alternative NHEJ pathways (such as microhomology-mediated end joining), which can result in genetic mutations, deletions and translocations; or by homology-directed repair (HDR) if a donor DNA template with homology to the target locus is supplied. The latter strategy enables precise mutations or alterations at the target locus. A property of Cas9-mediated targeting in many microbial species is that the generated DSB tends to be poorly repaired by NHEJ mechanisms despite the presence of the repair machinery within the genome of the targeted organism[12]. When a homologous DNA donor is simultaneously provided, Cas9 negatively selects against any unmodified cells when targeted to a genomic locus[13]. By contrast, if Cas9 is directed towards an episomal plasmid, the element will be lost from the resulting population of Cas9-expressing cells[14,15]. Newer variants of this technology such as CRISPR

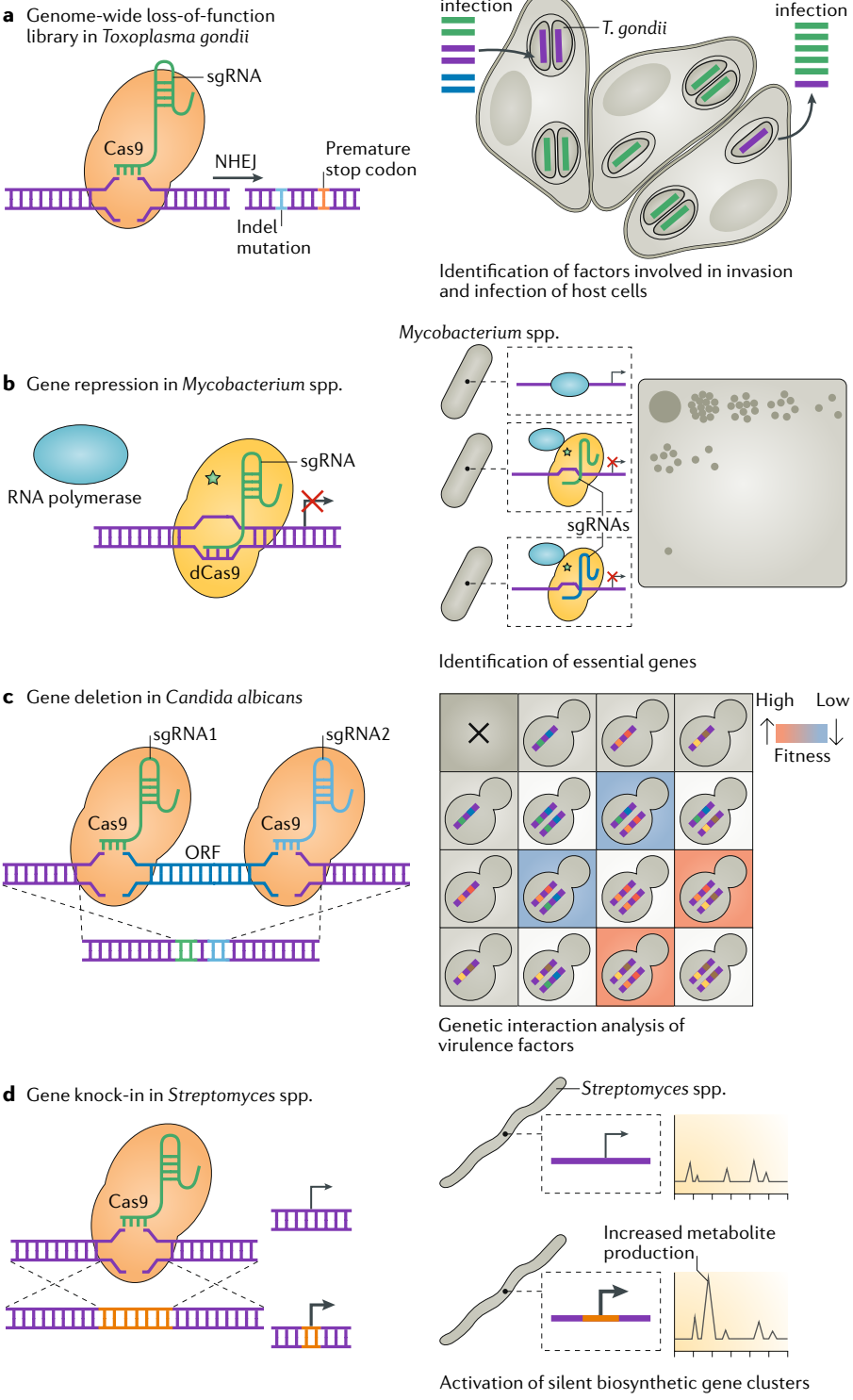
interference (CRISPRi) exploit a nuclease-dead version of the Cas9 enzyme (dCas9) that is targeted to specific genomic loci by sgRNAs to achieve steric hindrance of RNA polymerase, thus blocking transcription initiation or elongation[16–18]. Together, these groundbreaking technologies have been used to alter the sequence and modulate the expression of genes in a remarkably wide variety of species[11].

In this Progress article, we review advances in CRISPR–Cas-based techniques for rigorous genetic analysis of genetically intractable microorganisms. We describe challenges and limitations associated with the use of traditional methods for genetic manipulation in these organisms and highlight how CRISPR-based technologies can overcome these biological and technological hurdles. We focus on recent developments in CRISPR-based techniques using type II CRISPR platforms for the analysis of mycobacteria, microbial fungi and eukaryotic parasites and explain how CRISPR-based work in these organisms has been instrumental in generating genetic mutants and performing functional genomic analysis, dissecting genetic interaction networks and conducting complex genome engineering. Finally, we discuss how ongoing technological advances in CRISPR-based platforms will undoubtedly yield exciting new research avenues.

Generating mutant microorganisms

A crucial component of functional genomic analysis is the ability to generate genetic mutations or deletions or otherwise knock down gene function to assess the resultant phenotypes. This reverse genetic analysis strategy has been instrumental in dissecting genetic perturbations and understanding gene function in many microorganisms. However, similar analysis in genetically intractable microorganisms has lagged behind owing to limitations associated with genetic manipulation in such organisms.

A common biological limitation among intractable microorganisms is inefficient homologous recombination, which is often needed for classic genetic manipulation techniques. Low rates of homologous recombination and the requirement for very long stretches of homologous sequence for effective recombination have hindered the generation of genetic mutants in many mycobacterial[19,20], fungal[21–23] and parasitic microorganisms[24–27]. For targeted gene disruption, CRISPR-based editing can bypass the need to use traditional homologous recombination-based approaches, as CRISPR–Cas-induced DSBs can be repaired via NHEJ in a manner that is mutagenic to the target locus and independent of homologous recombination (FIG. 1a). To introduce a precise genetic alteration, CRISPR–Cas-based editing systems can improve the efficiency of homologous recombination-based genome editing through the generation of a genomic DSB and stimulating homologous recombination at the locus of interest[24,28–31]. Additionally, CRISPRi can bypass the need to induce targeted mutations in the genome, as the target gene can be knocked down (FIG. 1b). This approach is particularly useful to study a gene that is essential for cell growth[16,32]. These CRISPR-based techniques



◀ Fig. 1 | **Applications of different CRISPR technologies in diverse microorganisms. a** | CRISPR-based mutation via non-homologous end joining (NHEJ) has been used for genetic disruption in the parasite *Toxoplasma gondii*[40]. In this system, Cas9 is targeted to genomic loci by a small guide RNA (sgRNA), where it creates a double-strand break (DSB). The DSB is repaired via NHEJ in a manner that is mutagenic to the cell, causing an insertion or deletion (indel) mutation that can cause a premature stop codon. This platform was exploited for the generation of genome-wide mutation libraries to screen for factors involved in parasite infection of mammalian cells. Genome-wide libraries of sgRNAs (depicted as green, purple or blue bars to depict different sgRNAs targeting different genes) were transformed into *T. gondii*, which were then used to infect mammalian host cells. The relative abundance of sgRNAs represented before and after host cell infection helps identify factors involved in invasion and infection of host cells. **b** | CRISPR interference (CRISPRi)-based genetic depletion has been used in *Mycobacterium* spp. to identify essential genes involved in bacterial metabolism[34]. CRISPRi relies upon a nuclease-dead version of Cas9 (dCas9, depicted with a star), which uses an sgRNA to target genomic loci and block transcription from this site. Different coloured sgRNAs indicate targeting of different genomic loci for genetic repression (two sgRNAs (green and blue) are shown here as an example). **c** | A modified CRISPR-based gene drive has been used in *Candida albicans* for the generation of single-gene and double-gene deletions and for genetic interaction analysis of virulence regulators[52]. The gene drive uses Cas9 and two sgRNAs (depicted in blue and green) to target a gene for DSBs at the 5′ and 3′ ends of the gene. The DSBs are repaired by homologous recombination using the gene drive itself (which contains the sgRNAs flanked by regions of homology upstream and downstream of the gene of interest), leading to complete deletion of the gene. *C. albicans* haploids were transformed with unique gene drives targeting different genes for deletion and were then mated in a pairwise manner to generate double genetic deletion mutants, which were assessed for fitness and defects in virulence and antifungal drug resistance. **d** | A CRISPR-based genetic knock-in strategy has been exploited for genetic engineering to activate silent biosynthetic gene clusters in *Streptomyces* spp.[59]. Using a CRISPR–Cas9 system, strong heterologous promoters were inserted to drive high levels of expression from otherwise transcriptionally silent biosynthetic gene clusters. Activation of gene clusters and metabolite production was measured by high-performance liquid chromatography (HPLC) analysis.

have all been successfully implemented to expand the genetic toolkit available for genetic perturbation and analysis in otherwise intractable microorganisms.

Genomic perturbations using CRISPR technologies have greatly facilitated functional genomic analysis in *Mycobacterium tuberculosis*, the causative agent of tuberculosis that has been notoriously difficult to manipulate and whose genome remained uncharacterized for a long time. CRISPRi techniques have enabled transcriptional repression of specific genes in *M. tuberculosis*[20,33,34]. By optimizing a novel CRISPRi platform in *Mycobacteria*, several putative essential genes, including genes involved in folate metabolism (an important antibiotic target), were readily identified[34] (FIG. 1b). Given the scalable nature of this platform, future high-throughput CRISPRi-mediated repression studies could identify essential genes and potential synergistic genetic interactions on a larger scale, with important implications for antibiotic target discovery.

Generating genetic mutations and gene deletions using CRISPR has also been instrumental in the functional characterization of microbial fungal species, including industrially important filamentous fungi[30,31] and clinically relevant pathogenic yeasts[29,35–37] and moulds[28]. Pioneering work in the opportunistic human fungal pathogen *Candida albicans* used a Cas9 system to disrupt gene function and generate both conditional loss-of-function mutations and inducible promoter replacements in essential genes[35]. This system was used to simultaneously target two key fungal efflux pumps — *CDR1* and *CDR2* — in a hyper-drug-resistant *C. albicans* clinical isolate, rendering it sensitive to antifungal drugs and indicating an important application of this system in studying genetic mechanisms of antifungal drug resistance. Additionally, CRISPR-Cas9 has been used for generating loss-of-function mutants in *Candida glabrata*[29], which is another important and increasingly prevalent human fungal pathogen. By generating CRISPR-mediated mutations, two previously uncharacterized factors were identified as key virulence factors that affect the ability of the pathogen to infect a model host[29]. Together with recent work in newly emerging fungal pathogens[37], these tools are proving instrumental for genetic perturbation and functional characterization of fungal microorganisms.

As with microbial fungi, limited genetic tools were available for clinically important eukaryotic parasites, including *Trypanosoma cruzi*, *Toxoplasma gondii*, *Leishmania donovani*, *Cryptosporidium parvum* and the malarial parasite *Plasmodium falciparum*[25,26,38], but new applications of CRISPR-based technology are enabling efficient genetic disruptions in these parasites[24,39–43]. The first CRISPR–Cas9 screen in an apicomplexan resulted in the generation of a genome-wide, loss-of-function library in *T. gondii* (a ubiquitous parasite that can cause devastating congenital disease or fetal death if transmitted from mother to fetus)[39]. This study identified critical genes required to invade and infect human cells (FIG. 1a), including *ICAP12* (renamed claudin-like apicomplexan microneme protein (*CLAMP*)), which was shown to be an essential invasion factor and further revealed to be an essential gene in *P. falciparum*[39]. Those findings demonstrate the potential of CRISPR-based screening for deciphering parasite genetics and identifying key virulence factors.

Genetic interaction analysis

Genetic interaction analysis is a powerful tool for assessing the functional relationship between genes, performing pathway analysis, uncovering the function of uncharacterized genes and identifying new functions for previously well-characterized genes. Assessing epistatic relationships between genes relies on the generation of genetic double mutants and the comparison of their resultant phenotype with that of the parental single mutants. These techniques have been exploited in model microbial species, including *S. cerevisiae*[6] and *E. coli*[44], and have yielded a thorough understanding of complex, systems-level genetic interactions in these organisms.

Owing to the requirement for combinatorial genetic perturbations of multiple loci, genetic interaction analysis has historically been limited in non-model microorganisms. For diploid microorganisms (such as certain microbial fungi[45]) or polyploid microorganisms (including bacterial and archaeal species[46,47]), genetic interaction analysis is hindered by the need to repeatedly delete or mutate multiple copies of a genetic locus. Lack of functional selectable markers for genetic analysis in many filamentous fungi[48,49] and microbial parasites[50,51] also hinders the generation of multilocus mutant strains. CRISPR-based technologies can overcome many of these limitations as Cas9-mediated editing targets all homologous genomic loci simultaneously and enables the simultaneous targeting of multiple genes (through the use of multiple sgRNAs), thus enabling versatile genetic interaction analysis in non-model microbial species.

In *Mycobacteria*, CRISPRi-mediated gene silencing of multiple genetic loci has been used to identify genetic interactions within the folate biosynthesis pathways[34]. Using hypomorphic sgRNAs, the authors were able to simultaneously induce partial knockdown of several target genes involved

in folate biosynthesis. The results uncovered synthetic lethal interactions between different genes in the folate biosynthesis pathway: individual knockdown of each gene resulted in a mild growth defect, whereas the combined partial knockdown of two genes was lethal[34]. Moreover, partial knockdown of individual genes sensitized mycobacteria to antibiotics that inhibit different steps in the folate biosynthesis pathway. Together, these CRISPR-based methods establish a powerful platform for identifying and developing effective combination therapies for treating mycobacterial infections.

CRISPR-based multigene perturbations have also facilitated complex genetic interaction analysis for large gene families in eukaryotic microorganisms, including *C. albicans*[52] and *T. cruzi*[24]. Recent work in *C. albicans* demonstrated the use of a CRISPR-Cas9-based gene drive platform for the rapid generation of single-gene and double-gene deletion mutants in the diploid fungal pathogen to facilitate genetic interaction analysis. This work targeted both gene families of adhesin and efflux factors and showcased the power of this technology to identify the complex genetic network topology underlying key fungal virulence traits, including biofilm formation and antifungal drug resistance[52] (FIG. 1c). This genetic interaction analysis further identified synergistic genetic interactions that render the fungal pathogen less able to form a biofilm or more susceptible to antifungal drugs (for example, *CDR1* and *CDR2* or *CDR2* and *TPO3* deletions), highlighting the use of such analysis in deciphering complex genetic regulation of important fungal virulence phenotypes.

Genetic interaction analysis using CRISPR techniques has not been extensively used in microbial parasites, but recent work suggests the exciting potential for CRISPR-based analysis in parasitic worms. A CRISPR-Cas9-based synthetic genetic interaction (CRISPR-SGI) approach was developed in the model nematode worm *Caenorhabditis elegans* to overcome existing technical limitations and permit the systematic generation of double-gene mutants for genetic interaction analysis[53]. This technology platform was used to identify interactions between RNA-binding proteins, including critical interactions that are required for organismal health and lifespan[53]. Applying similar techniques to other nematode species that are parasitic to humans holds great promise for dissecting their complex biology and virulence factors.

Genome engineering

Bacterial and fungal microorganisms have a crucial role in the industrial manufacturing of biofuels, pharmaceutical agents and other biomolecules and metabolites; thus, the ability to precisely alter their genomes to optimize a desired output is of great interest. Genome-editing technologies, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR–Cas systems, are widely used genome-engineering tools owing to their efficiency, precision and versatility[54]. Among these technologies, CRISPR-based techniques permit a simpler design process and more affordable and faster execution than engineered nuclease platforms, making them highly favourable tools to genetically engineer industrially important microorganisms for which genetic tools are not readily available.

For bacteria used in industrial manufacturing, including many *Clostridium* and *Streptomyces* spp., traditional genome-editing techniques relied on inefficient homologous recombination and resulted in markers or other genetic 'scars' in the genome, which are undesirable for industrial applications. For *Clostridium* spp., newer CRISPR–Cas-based techniques have resolved these issues and facilitated precise and 'scarless' editing of genes involved in ethanol production and other relevant pathways with important applications for optimized biofuel production[55–57]. CRISPR-Cas9 has also been used for targeted genome engineering in *Streptomyces* bacteria, which are prolific producers of bioactive natural products, including antibiotics and anticancer agents. Recent advances have exploited CRISPR–Cas to improve the efficiency of genome editing in *Streptomyces* spp.[58] and to perform strategic genetic knock-ins to activate silent biosynthetic gene clusters and increase metabolic output[59] (FIG. 1d).

CRISPR-based techniques have also improved our ability to genetically engineer industrially relevant filamentous fungi, including *Aspergillus* spp., *Penicillium chrysogenum* and *Trichoderma reesei*[31,49,60], as well as other fungal yeast species that are important for bioproduction, such as *Pichia pastoris* and *Yarrowia lipolytica*[61,62]. For *Myceliophthora thermophila* — an important thermophilic biomass-degrading fungus that produces industrially important thermostable enzymes — CRISPR-Cas9-mediated editing was used to engineer four loci to simultaneously boost cellulase production and overcome the difficulties associated with multiple gene editing in this species[30]. This work also highlighted that the CRISPR editing platform was readily adaptable for flexible use in other thermophilic fungi, which suggests that these tools will greatly accelerate the engineering of diverse fungal organisms with important implications for industrial biotechnology and the production of enzymes and chemicals.

Future prospects

Our ability to genetically manipulate and study diverse microorganisms has been improved by contemporary CRISPR-based technologies, which improve many previous genetic techniques. For instance, although RNAi has proved to be a crucial technology for understanding genetic function through inhibition of gene expression[63], the canonical RNAi machinery is absent or non-functional in bacteria[64–66], certain protozoan parasites[67,68] and other microorganisms, which limits its use in these species. Transposon sequencing has also been used as a powerful genetic tool that combines transposon insertional mutagenesis with sequencing of transposon insertion sites for functional genomic analysis[7,69], but it has mainly been limited to bacterial species and has more limited applications to diploid or polyploid microorganisms. Engineered nuclease systems such as ZFNs and TALENs have been used for genetic manipulation of microbial species, including *S. cerevisiae*[70] and *P. falciparum*[71]; however, these techniques also tend to be costly, laborious and time-intensive.

Compared with other methods, CRISPR offers many unique advantages for microbial genetic manipulation as it is more universal, can be efficiently used at a large scale, is multiplexable and is relatively simple and cost-effective. Additionally, variable delivery methods for CRISPR–Cas systems into microbial cells, including the use of plasmids, bacterial conjugation[72], bacteriophages[14,15,73] or ribonucleoprotein particles (RNPs; comprising Cas proteins in complex with sgRNAs), that circumvent the need for species-specific CRISPR plasmids[37,74] have facilitated the use of this technology in a diversity of microorganisms. Although this Progress article focuses on expressing heterologous type II CRISPR platforms in microorganisms, other CRISPR machinery (including type I and type III CRISPR systems) and endogenous CRISPR systems represent additional mechanisms for genome editing in bacterial and archaeal species[75,76]. Although using endogenous CRISPR systems is not a widely adopted strategy for genome engineering (as it is

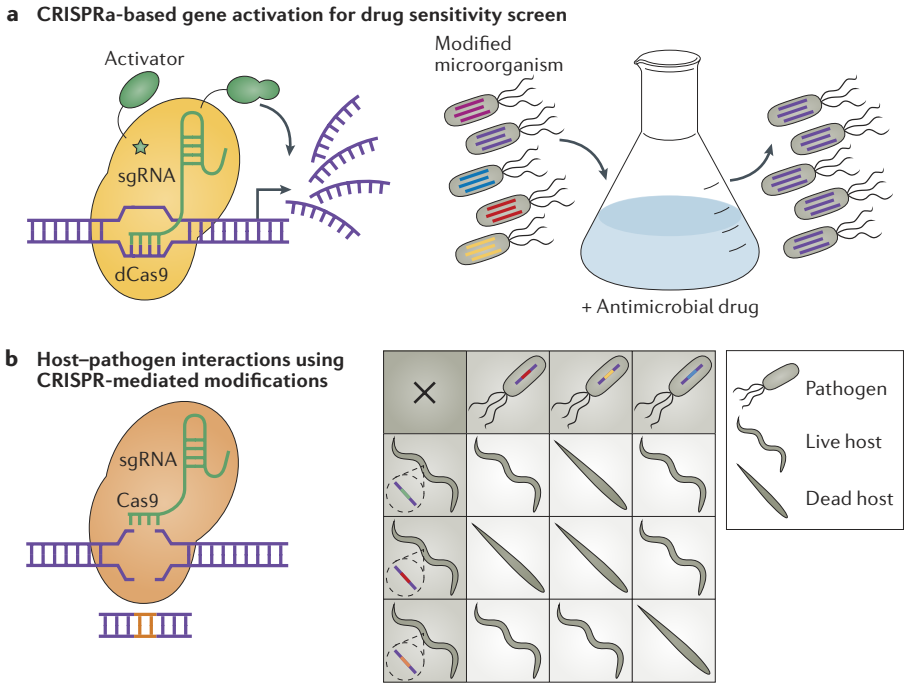


Fig. 2 | **Future applications of CRISPR–Cas-based gene editing techniques. a** | CRISPR-based gene activation (CRISPRa) may be exploited in less tractable microorganisms to overexpress genes and identify targets of antimicrobial therapeutics. A nuclease-dead Cas9 enzyme (dCas9, indicated with a star) is fused with strong transcriptional activation domains (such as VP64 for eukaryotes or σ-factors for bacterial species) to drive expression from promoters based on targeting from a small guide RNA (sgRNA). Pooled libraries of CRISPRa mutant microorganisms can be subjected to antimicrobial drugs, and the relative abundance of mutant strains that survive in the presence of the antimicrobial drugs can be assessed. Overexpressed genes that facilitate survival following growth in the presence of the antimicrobial drug are possible candidates as novel drug targets. **b** | CRISPR engineering can be used in both microbial and model host organisms (such as *Caenorhabditis elegans*) to determine the genetic basis of host–microorganism interactions. In this scenario, CRISPR–Cas-based platforms could be used in both microbial strains and simple model host organisms such as *C. elegans*. Combining genetic mutant microorganisms and genetic mutant hosts could increase our understanding of key factors involved in host–pathogen interactions.

predicated on knowing the crRNA sequence, PAM requirements and sgRNA design preferences), harnessing the native CRISPR system of a microorganism is expected to improve overall targeting efficiency and may therefore be advantageous.

Although these new CRISPR platforms have endowed us with the capacity to efficiently alter microbial genomes for a myriad of purposes, there are still certain hurdles and limitations to implementing such techniques. CRISPR-based manipulation relies on NHEJ or HDR, and although recombination rates are improved through CRISPR-mediated DSBs, this can still be a limitation, as low HDR efficiency will limit CRISPR-based editing capabilities. Although CRISPRi can overcome the need for NHEJ or HDR, such platforms will repress an entire genetic operon instead of targeting individual genes and must be optimized to achieve robust gene repression. Additionally, all CRISPR–Cas systems must be efficiently delivered into microbial cells, and their use is limited to organisms with effective transformation tools. Even in microorganisms that can be readily transformed and for which there are efficient CRISPR-based tools, specificity and off-target mutations pose additional challenges[77], and anti-CRISPR systems present in some hosts may counteract the efficiency of CRISPR-mediated editing[78]. Moreover, for some organisms, there are still technical and cost-related limitations of scaling up these techniques for systems-level functional genomic analysis.

Despite these limitations, CRISPR technologies are rapidly evolving, and newer techniques hold immense promise for the study of microorganisms. For example, CRISPR-mediated base-editing platforms[79,80], including CRISPR-STOP[81], can be used to generate precise single nucleotide conversions and introduce stop codons to silence target genes, which could be exploited to study microbial gene function. CRISPR-based gene activation or overexpression (CRISPRa)[82] could be used in less tractable microorganisms to optimize cellular output and identify drug targets, as in model microorganisms[83,84] (FIG. 2a). CRISPR-based epigenetic modification[85] is a promising technology for the study of survival of bacteria during antibiotic stress[86], fungal phenotypic plasticity[87] and host-parasite interactions[88]. Moreover, technology platforms such as Perturb-seq, which pairs CRISPR-mediated genetic perturbations with droplet-based, single-cell RNA sequencing (RNA-seq)[89], could facilitate systems-level dissection of gene function and genetic regulation in many microorganisms.

As microbiome and metagenomic analyses become increasingly pervasive, new microbial species are being identified at unprecedented rates, and CRISPR-based technologies are likely to enhance and expand our ability to establish genetic analysis tools in many of these previously uncharacterized microorganisms (FIG. 2a). CRISPR techniques will also be powerful tools for analysing the genetic interface between microbial pathogens and their hosts. CRISPR-based editing techniques are well developed in mammalian cell lines[90,91] as well as in model organisms for animal–pathogen interactions (*C. elegans*[92] or zebrafish[93]) or plant–pathogen interactions (*Arabidopsis thaliana*[94]). Thus, combining CRISPR-based genetic modifications in host species with genetic modifications in microbial pathogens could provide a mechanism to systematically analyse host and pathogen genetic factors that are involved in the interaction (FIG. 2b). These and other applications of CRISPR techniques will accelerate the study of diverse microorganisms, with important implications for understanding microbial biology, improving production of critical biomolecules and identifying key virulence factors and targets for antimicrobial therapeutics.

*Rebecca S. Shapiro[1,2,3], Alejandro Chavez[4] and James J. Collins[1,2,5]**

[1]Department of Biological Engineering, Institute for Medical Engineering and Science, Synthetic Biology Center, MIT, Cambridge, MA, USA.

[2]Broad Institute of MIT and Harvard, Cambridge, MA, USA.

[3]Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada.

[4]Department of Pathology and Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY, USA.

[5]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA, USA.

**e-mail: jimjc@mit.edu*

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