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

Citation: Shapiro, Rebecca S. et al. "CRISPR-Based Genomic Tools for the Manipulation of Genetically Intractable Microorganisms." Nature Reviews Microbiology 16, 6 (March 2018): 333–339 © 2018 Macmillan Publishers Limited, part of Springer Nature

As Published: https://doi.org/10.1038/s41579-018-0002-7

Publisher: Nature Publishing Group

Persistent URL: http://hdl.handle.net/1721.1/119469

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

CRISPR technologies: a toolkit for making genetically intractable microbes tractable

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Abstract
Genetic manipulation of microbial species has been critical in dissecting their biology — yet many microbial species lacked robust tools for comprehensive genetic analysis until the advent of CRISPR-based techniques. Here, we review CRISPR-related advances that have enabled genome engineering and genetic analysis of difficult-to-manipulate microbial organisms, with an emphasis on mycobacteria, fungi, and parasites. We discuss how CRISPR-based analyses in these organisms have uncovered novel gene functions, dissected genetic interaction networks, and identified virulence factors.

Introduction

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. Unravelling the complex biology of these microorganisms has largely been dependent on our ability to manipulate them genetically. Genetic analysis of microbial organisms 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 critical carrier of genetic information[2]. The earliest accounts of genetic engineering involved the generation of transgenic *Escherichia coli* lineages through transformation of a recombinant plasmid encoding an antibiotic-resistance gene[3]. Since then, genetic manipulation of microbial organisms has been critical for the development of new biotechnologies and the study of microbial organisms themselves.

Recent advances in molecular techniques have improved our ability to perform genetic manipulation in diverse microbial organisms. Modern technology platforms currently 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*[8] and *Pseudomonas aeruginosa*[9]. These biotechnologies 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 relatively 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 a diversity of organisms and been instrumental in enabling us to alter the genomes of even the most notoriously intractable microbial species. CRISPR, or Clustered Regularly Interspaced Short Palindromic Repeats, are a group of DNA sequences in prokaryotes that play an important role in bacterial and archaeal immunity, and have been co-opted for genome editing across the tree of life[10,11]. In type II CRISPR systems, “spacer” sequences from CRISPR loci (derived from viral, plasmid or other foreign DNA which have been inserted into the CRISPR locus) are transcribed into RNA, and processed into small CRISPR RNAs (crRNAs), which then pair with CRISPR-associated (Cas) proteins and a trans-activating crRNA (tracrRNA). Together, this complex is targeted to “protospacer” sequences based on complementarity to the crRNA sequence and the presence of a protospacer adjacent motif (PAM) site. Cas proteins are endonucleases that cause double-strand breaks (DSBs) at the target protospacer locus.

While this system is used by prokaryotes to detect and cleave invading foreign DNA, it can also be exploited as a biotechnology tool for precise genome editing at a targeted locus. This was first demonstrated using the Cas9 protein from *Streptococcus pyogenes* and a modified chimeric single-guide RNA (sgRNA), which fused together 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]. While sgRNA targeting is relatively flexible, the requirement of a PAM sequence can limit the availability of genomic target sites. Furthermore, organisms with extreme skew in GC or AT richness will influence whether or not a particular PAM is likely to be present at a high frequency. Once a DSB is generated, the manner in which it is repaired is responsible for the observed genome editing outcomes: breaks can be repaired by non-homologous end joining (NHEJ) that can result in insertions or deletions (indels) at the target locus, by alternative NHEJ pathways such as microhomology-mediated end joining that 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 allows for precise mutations or alterations at the target locus. A property of Cas9 targeting in many microbial species is that the generated DSBs tends to be poorly repaired by NHEJ mechanisms, despite the presence of such machinery within the genome of the targeted organism[12]. In the case where a homologous donor is simultaneously provided, Cas9 is able to negatively select against any unmodified cells when targeted to a genomic locus[13]. In contrast, if Cas9 is directed towards a 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) 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].

Here we review advances in CRISPR-based techniques for rigorous genetic analysis of otherwise difficult-to-work-with microbial organisms. We describe challenges and limitations associated with using traditional methods for genetic manipulation, and highlight how CRISPR-based technologies are helping to overcome these biological and technological hurdles. We focus on recent developments in CRISPR-based techniques for the analysis of genetically unwieldy microbial organisms, including mycobacteria, microbial fungi, and eukaryotic parasites, and explain how CRISPR-based work in these organisms has been instrumental for generating genetic mutants and performing functional genomic analysis, dissecting genetic interaction networks, and conducting complex genome engineering. We close by discussing how ongoing technological advances in CRISPR-based platforms will undoubtedly yield exciting new capabilities for the study of the microbial world.

Generating microbial mutants with CRISPR

A critical component of functional genomic analysis is the ability to generate genetic mutations or deletions, or otherwise knock-down gene function so as to assess resultant phenotypes. This reverse genetic analysis strategy has been instrumental in dissecting genetic perturbations and understanding gene function in many microbes. However, similar analysis in

genetically unwieldy microbial organisms has lagged behind, due to limitations associated with generating genetic perturbations in such organisms.

A common biological limitation amongst 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 microbes[24–27]. For targeted gene disruption, CRISPR-based editing can bypass the need to use traditional homologous recombination-based approaches, since CRISPR-Cas-induced DSBs can be repaired via NHEJ, in a manner that is mutagenic to the target locus and independent of homologous recombination (Figure 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 make targeted mutations in the genome, by enabling users to simply “knock down” gene expression for their target of interest (Figure 1b). This approach has proven itself particularly useful in cases when one wants to study a gene that is essential for cell growth[32,33]. These CRISPR-based techniques have all been successfully implemented to expand the genetic toolkit available for genetic perturbation and analysis in otherwise unwieldy microbial organisms.

Genomic perturbations using CRISPR technologies have greatly facilitated functional genomic analysis in *Mycobacterium tuberculosis* — a notoriously difficult-to-manipulate pathogen and the causative agent of tuberculosis. CRISPRi techniques have bypassed the need for homologous recombination and allowed precise knock-down expression of *M. tuberculosis* genes[34–36]. The ability to genetically regulate genes to confirm their essentiality and study their function has important applications, but has been difficult in *Mycobacteria* with traditional methods, where much of the genome remains uncharacterized. By optimizing a novel CRISPRi platform in *Mycobacteria*, the necessity of several putative essential genes, including those involved in folate metabolism was readily determined[37] (Figure 1b). Given the scalable nature of this platform, future high-throughput CRISPRi 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[29,38–40] 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[38]. This system was used to simultaneously target two key fungal efflux pumps — *CDR1* and *CDR2* — for mutation 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 *C. glabrata*[29] — another important and increasingly prevalent human fungal pathogen. By generating CRISPR-mediated mutations, two previously uncharacterized factors were identified as key virulence genes and important regulators of the pathogen's ability to cause infection in a model host[29]. Together with recent work in newly emerging fungal pathogens[40], these tools are proving instrumental for genetic perturbation and functional characterization of fungal microorganisms.

As with microbial fungi, clinically important eukaryotic parasites, including *Trypanosoma cruzi*, *Toxoplasma gondii*, *Leishmania donovani*, *Cryptosporidium parvum,* and the malarial parasite *Plasmodium falciparum*, have historically had limited genetic tools available for the facile generation of genetic mutants[25,26,41]; yet new applications of CRISPR technology, are enabling efficient genetic disruptions in these parasites[24,42–46]. An impressive use of CRISPR technology, was applied in the apicomplexan parasite *T. gondii*[42] — a ubiquitous parasite that can cause devastating congenital disease or fetal death if transmitted from mother to fetus. A CRISPR-Cas9 platform was used to construct a genome-wide loss-of-function library in the parasite — the first of its kind in *T. gondii* or any apicomplexan. This pooled genetic mutant library was used in a host-parasite interaction screen, to identify critical genes required to invade and infect human cells (Figure 1a). Amongst the genes identified as playing a role in host infection, ICAP12/CLAMP was found to be an essential invasion factor, and further shown as an essential gene in a different apicomplexan, *P. falciparum*[42], demonstrating the potential of CRISPR-based screening for deciphering parasite genetics and identifying key virulence factors.

CRISPR-enabled microbial 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 activities for previously well-characterized genes. Assessing these epistatic relationships between genes relies on the generation of double genetic mutants and the comparison of their resultant phenotype to that of the parental single mutants. These techniques have been exploited in model microbial species including *S. cerevisiae*[6] and *E. coli*[47], and have yielded a thorough understanding of complex, systems-level genetic interactions in these organisms.

Due to the requirement for combinatorial genetic perturbations of multiple loci, genetic interaction analysis has historically been limited in non-model microbial organisms. For diploid microorganisms (such as certain microbial fungi[48]) or polyploid microorganisms (including bacterial and archaeal species[49,50]), 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[51,52] and microbial parasites[53,54] also hinders the generation of multi-locus mutant strains. CRISPR-based technologies can overcome many of these limitations since Cas9 editing targets all homologous genomic loci simultaneously and allows for simultaneous multi-gene targeting (via the use of multiple sgRNAs). By facilitating the generation of multiple genetic mutants, CRISPR techniques are

improving our ability to perform versatile genetic interaction analysis in non-model microbial species as well.

In *Mycobacteria*, CRISPRi-mediated gene silencing of multiple genetic loci has been used to identify genetic interactions within the folate biosynthesis pathways[37] — an important pathway for antibiotic drug targeting. Using a tunable CRISPRi system, hypomorphic sgRNAs were utilized to obtain simultaneous partial knockdowns of several target genes involved in folate biosynthesis. This identified synthetic lethal interactions between different folate biosynthesis genes — where individual knockdown of each gene had a mild growth defect, but the combined knockdown of two factors had a synergistic antimicrobial effect and was lethal to the mycobacteria[37]. These synergistic interactions were confirmed by integrating CRISPR-mediated genetic interaction analysis with chemical-genetic assays using folate-targeting antibiotic drugs. Together, these CRISPR-based methods establish a powerful platform for identifying and developing effective combination therapies for treating mycobacterial infections.

CRISPR-based multi-gene perturbations have also facilitated complex genetic interaction analysis for large gene families in eukaryotic microorganisms, including *C. albicans*[55] 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- and double-gene deletion mutants in the diploid fungal pathogen, to facilitate genetic interaction analysis. This work targeted gene families of adhesin and efflux factors for combinatorial deletion, 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[55] (Figure 1c). This genetic interaction analysis further identified synergistic genetic interactions that render the fungal pathogen less able to form biofilm or more susceptible to antifungal drugs (e.g. *CDR1* and *CDR2*, or *CDR2* and *TPO3* deletions), proving the utility of such analysis in deciphering complex genetic regulation of important fungal virulence phenotypes.

Genetic interaction analysis using CRISPR techniques has not been extensively employed 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[56]. This technology platform was used to identify interactions between RNA binding proteins, including critical interactions that are required for organismal health and lifespan[56]. Applying similar techniques to other nematode species that are parasitic to humans holds great promise for dissecting the complex biology and virulence factors utilized by these organisms.

Microbial genome engineering with CRISPR

Bacterial and fungal microorganisms play 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 critical interest. Genome-editing technologies, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-Cas systems, have moved to the forefront of genome engineering due to their relative efficiency, precision, and versatility[57]. Amongst these technologies, CRISPR-based techniques permit a simpler design process, and more affordable and faster execution than engineered nuclease platforms, making them highly favourable engineering tools for industrially important microorganisms with limited availability of genetic tools.

For bacteria used in industrial manufacturing including many *Clostridium*, *Streptomyces*, and *Corynebacterium* species, 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 industrial-exploited *Clostriudium* species, newer CRISPR-based techniques have resolved these issues and facilitated precise and scarless editing of ethanol production genes and other relevant pathways with important applications for optimized biofuel production[58–60]. 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 to improve the efficiency of genome editing in *Streptomyces*[61] and to perform strategic genetic knock-ins to activate silent biosynthetic gene clusters and increase metabolic output[62] (Figure 1d).

Similar advances have been made with CRISPR as a means to improve genetic engineering in industrially relevant filamentous fungi including *Aspergillus*, *Penicillium*, and *Trichoderma* species[31,52,63], along with other fungal yeast species critical for bioproduction such as *Pichia pastoris* and *Yarrowia lipolytica*[64,65]. For *Myceliophthora thermophila* — an important thermophilic biomass-degrading fungus that produces industrially important thermostable enzymes — CRISPR-Cas9 editing was used to engineer four loci simultaneously to 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, suggesting these tools will greatly accelerate the engineering of diverse fungal organisms with important implications for industrial biotechnology and the production of critical enzymes and chemicals.

Future prospects

Our ability to genetically manipulate and study a diversity of microbial organisms has been enhanced by contemporary CRISPR-based technologies, which improve upon many previous genetic techniques. For instance, while RNAi has proved to be a crucial technology for understanding genetic function through inhibition of gene expression[66], the canonical RNAi machinery is absent or non-functional in bacteria[67–69], certain protozoan parasites[70,71], and other microorganisms, limiting its use amongst these species. Transposon sequencing (Tn-seq) has also been used as a powerful genetic tool that combines transposon insertional mutagenesis with massively parallel sequencing of transposon insertion sites for functional

genomic analysis[7,72], but has mainly been limited to bacterial species, and has more limited applications to diploid or polyploid microbial organisms. Recently, genome editing technologies, including zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have moved to the forefront of genetic engineering due to their relative efficiency, precision, and versatility[57]. Indeed, these engineered nuclease systems have been used for genetic manipulation of microbial species including *S. cerevisiae*[73] and *P. falciparum*[74], however these techniques also tend to be costly, laborious, and time-intensive, and for these reasons have not been widely adopted in the field of microbial genetics and bioengineering.

Compared to 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 relatively simple and cost effective for use across diverse microorganisms. Additionally, variable delivery methods for CRISPR-Cas systems into microbial cells, including the use of plasmids, bacterial conjugation[75], bacteriophage[14,15,76], or ribonucleoprotein particles (RNPs) of Cas proteins with sgRNAs — which circumvents the need for species-specific CRISPR plasmids[40,77] — has facilitated this technology in a diversity of microorganisms. While this review has focused on expressing heterologous type II CRISPR platforms in microorganisms, other CRISPR machinery, including type I and type III CRISPR, and endogenous CRISPR systems represent additional mechanisms for genome editing in bacterial and archaeal species[78,79]. While using endogenous CRISPR systems is not a widely deployed strategy for genome engineering (as it is predicated on knowing the crRNA sequence, PAM requirements and sgRNA design preferences), harnessing an microorganism's native CRISPR system may be expected to improve overall targeting efficiency, and may therefore be advantageous.

While 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. While CRISPRi can overcome the need for NHEJ or HDR, such platforms will repress an entire genetic operon, as opposed to targeting individual genes, and must be optimized to achieve robust genetic repression. Additionally, all CRISPR-Cas systems must be efficiently delivered into microbial cells, and their utility 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, these technologies are not immune to issues surrounding specificity and off-target mutations[80], and anti-CRISPR systems in some host backgrounds may counteract of the efficiency of CRISPR editing[81]. 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 microbial organisms. For example, CRISPR

base editing platforms[82,83], including CRISPR-STOP[84], 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)[85] could be utilized in less tractable microbial organisms, and employed to optimize cellular output and identify drug targets, as in model microbial organisms[86,87] (Figure 2a). Additionally, given the critical role of epigenetic modifications in mediating bacterial antibiotic stress survival[88], fungal phenotypic plasticity[89], and host-parasite interactions[90], CRISPR-based epigenetic modifications[91] is a promising technology for the study of microbial organisms. Moreover, technology platforms such as Perturb-seq, which pairs CRISPR-mediated genetic perturbations with droplet-based single-cell RNA-seq[92], could facilitate systems-level dissection of gene function and genetic regulation in many microorganisms.

As microbiome and metagenomic analysis becomes 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 (Figure 2a). CRISPR techniques will also serve as powerful tools for analyzing the genetic interface between microbial pathogens and their hosts. CRISPR-based editing techniques are well-developed in mammalian cell lines[93,94], as well as in organisms that can serve as model species for animal-pathogen interactions (*Caenorhabditis elegans*[95], zebrafish[96]) or plant-pathogen interactions (*Arabidopsis*[97]). Thus, combining CRISPR-based genetic perturbations in host species with those in microbial pathogens, could provide a mechanism to systematically analyze host and pathogen genetic factors that are critical for the host-pathogen interaction (Figure 2b). These and other applications of CRISPR techniques will accelerate the study of diverse microbial organisms, with important implications for understanding microbial biology, improving production of critical biomolecules, and identifying key virulence factors and targets for antimicrobial therapeutics.

Figure legends

Figure 1. Applications of different CRISPR technologies across diverse microbial organisms. a. CRISPR-based mutation via NHEJ has been used for genetic disruption in the parasite microbe *T. gondii*, and exploited for the generation of genome-wide mutation libraries to screen for factors involved in parasite infection of mammalian cells. **b.** CRISPRi-based genetic depletion has been used in *Mycobacterium* species to identify essential genes involved in bacterial metabolism. Different color sgRNAs indicate targeting of different genomic loci for genetic repression. **c.** A modified CRISPR-based gene drive has been used in *C. albicans* for the generation of single- and double-gene deletions, and genetic interaction analysis of virulence regulators. **d.** A CRISPR-based genetic knock-in strategy has been exploited for genetic engineering to activate silent biosynthetic gene clusters in *Streptomyces* species.

Figure 2. Future applications of CRISPR techniques for microbial analysis. a. CRISPRa-based gene activation may be exploited in a diversity of microbial organisms in order to over-express genes, and help identify targets of antimicrobial therapeutics. **b.** CRISPR engineering can be used in both microbial as well as model host organisms (such as the *C. elegans*) to determine the genetic basis of host-microbe interactions.

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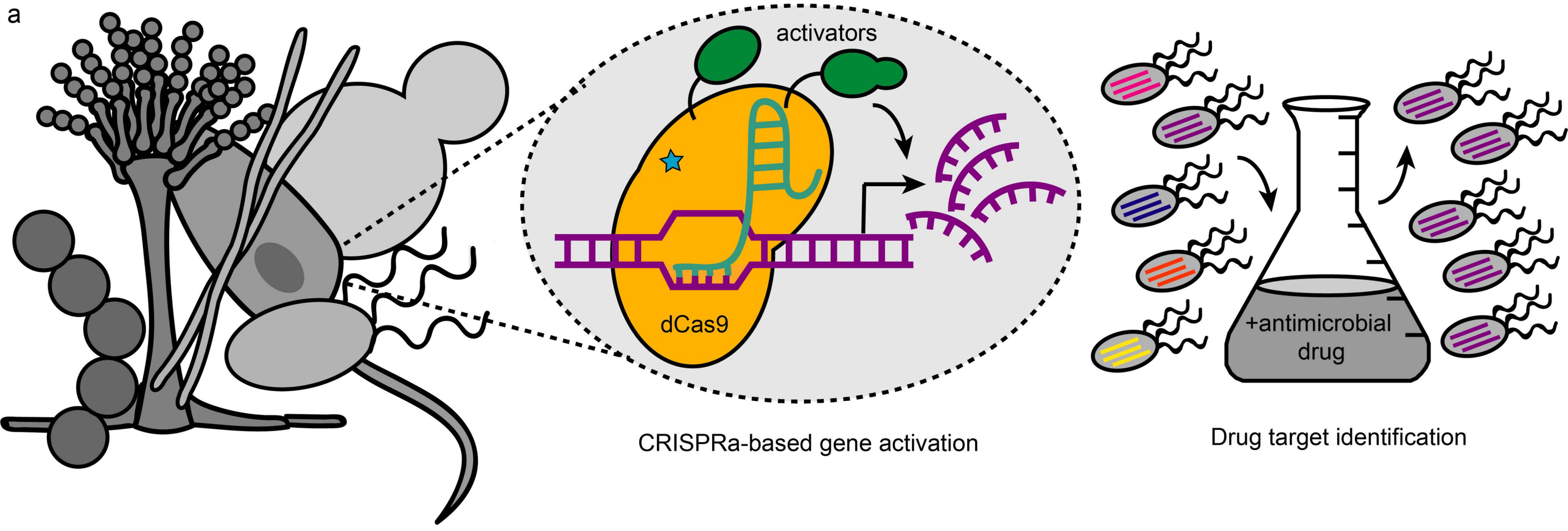
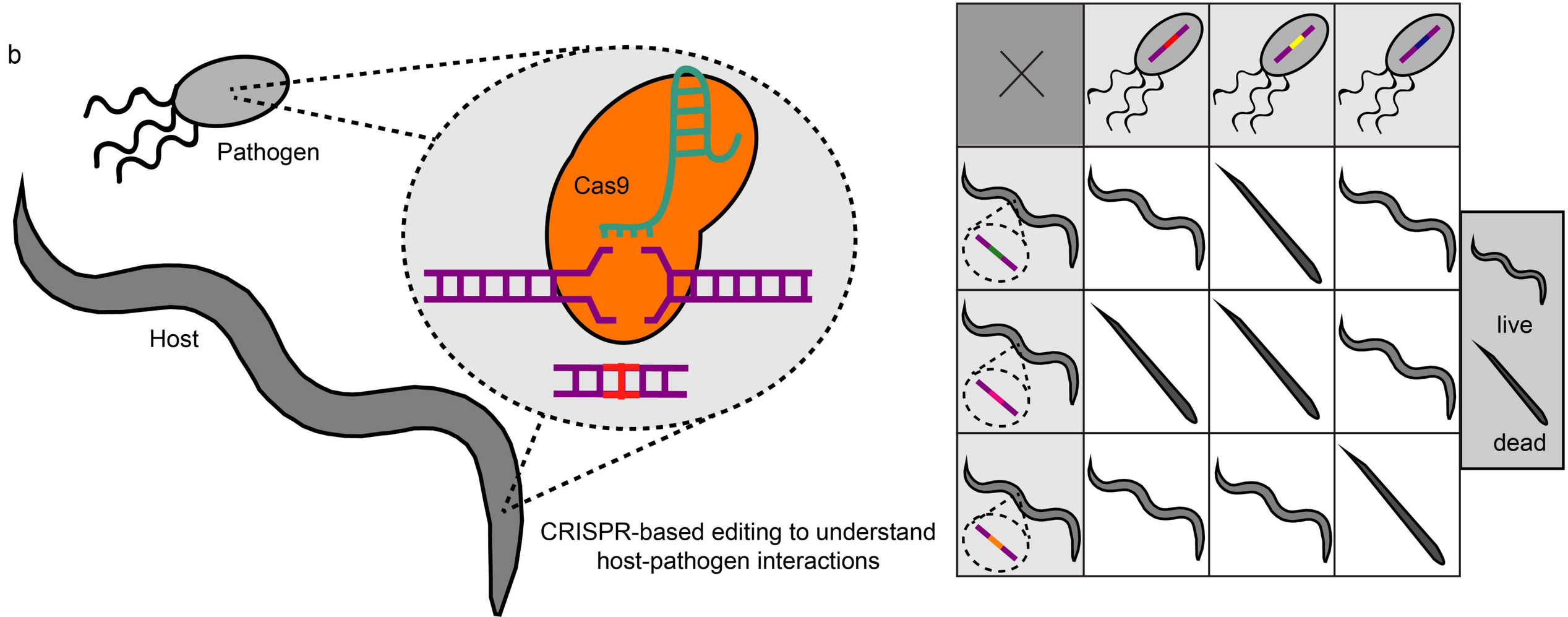
a



CRISPRa-based gene activation

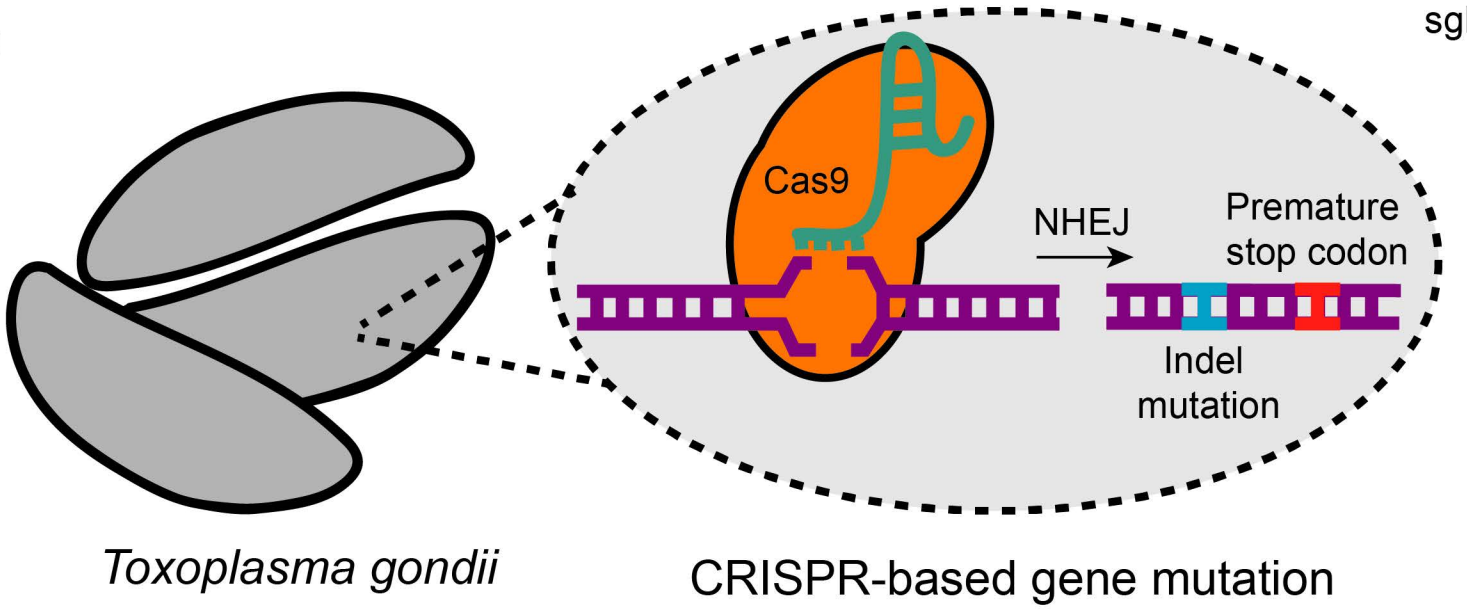
Drug target identification

b



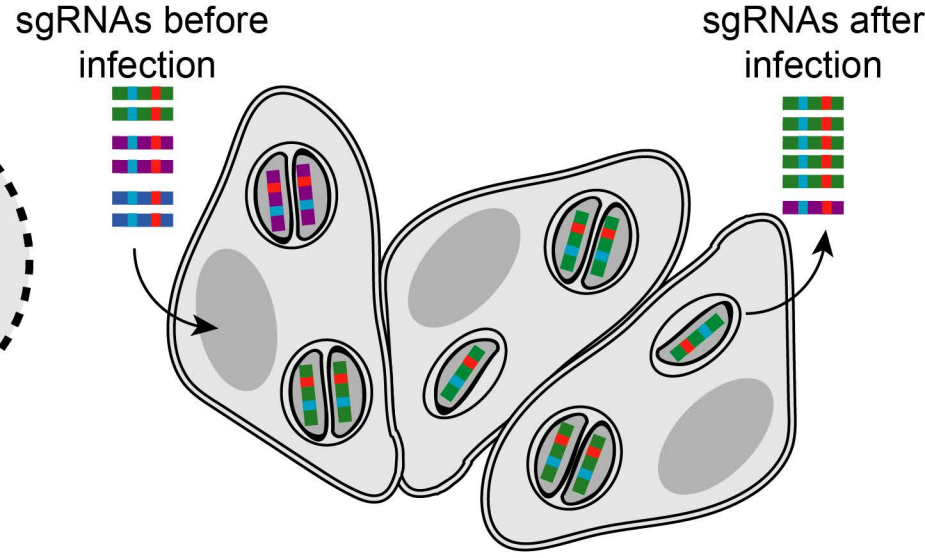
CRISPR-based editing to understand host-pathogen interactions

a



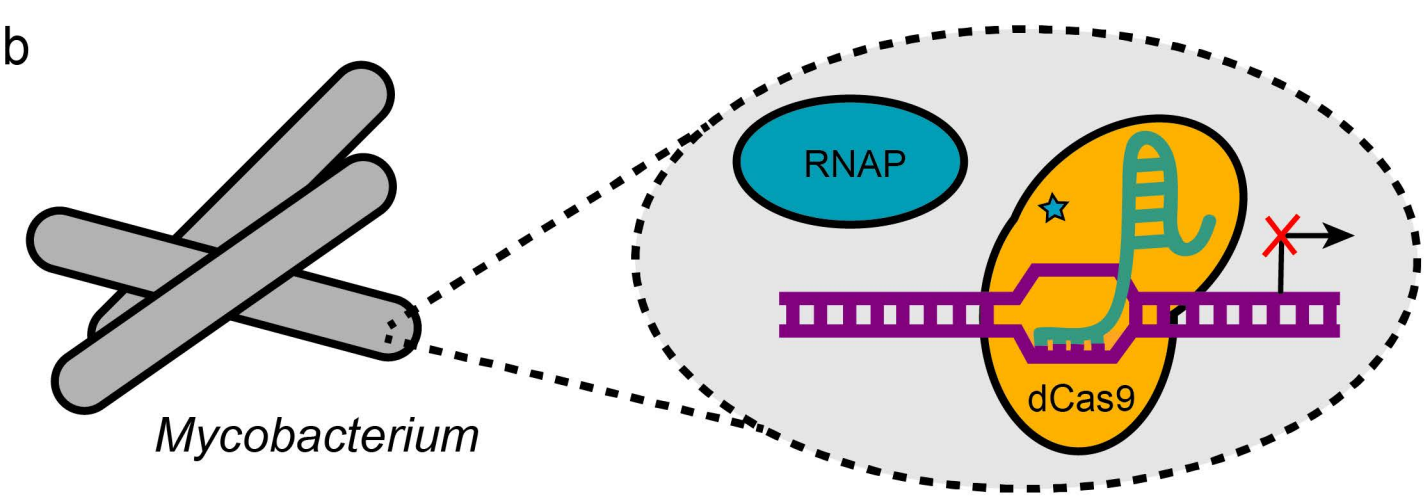
Toxoplasma gondii

CRISPR-based gene mutation



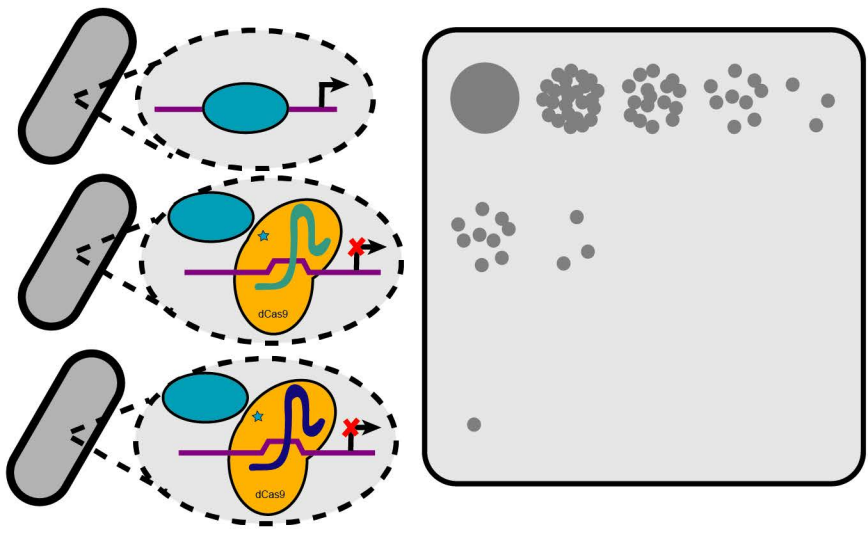
Identification of parasite infection factors

b



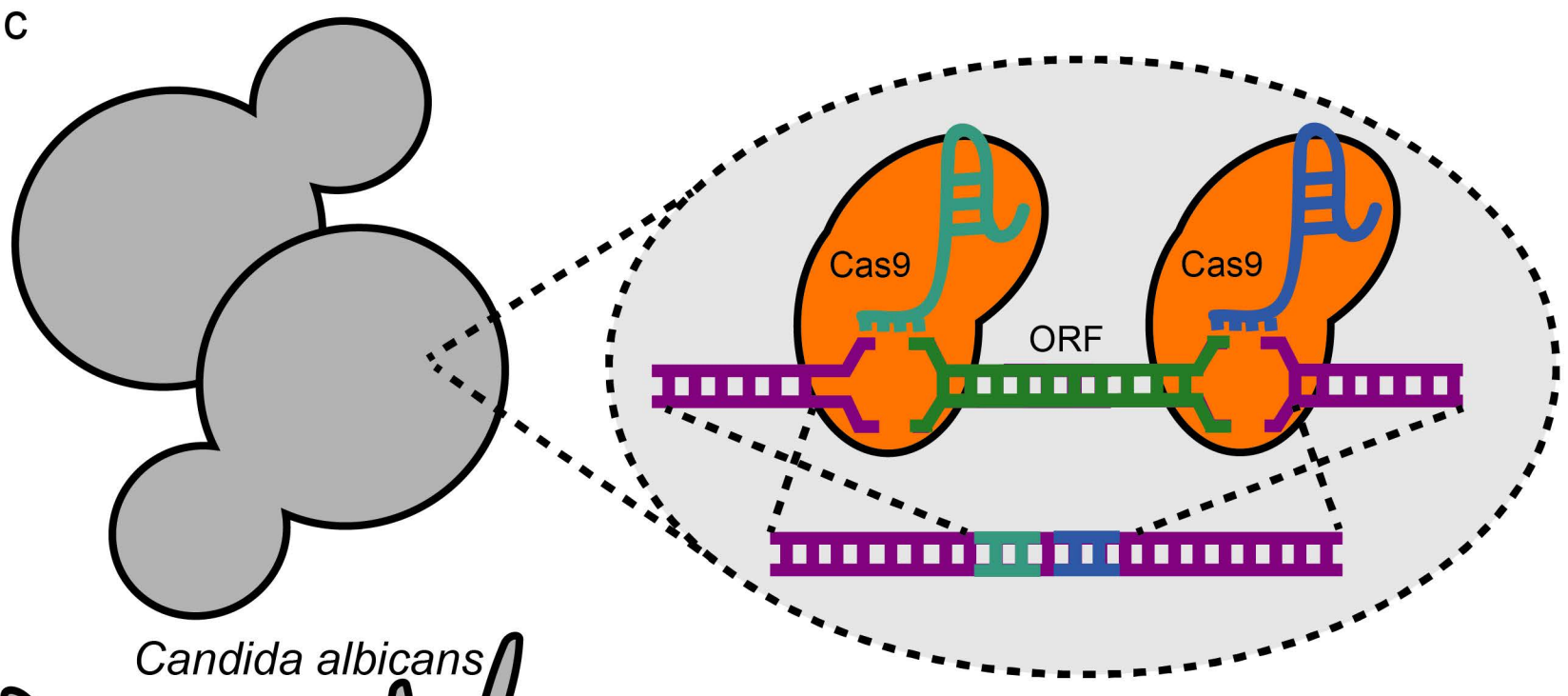
Mycobacterium

CRISPRi-based gene repression



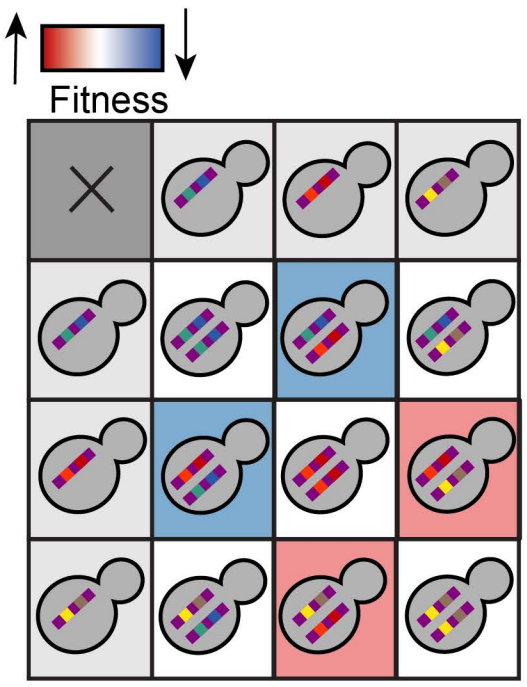
Essential gene identification

c



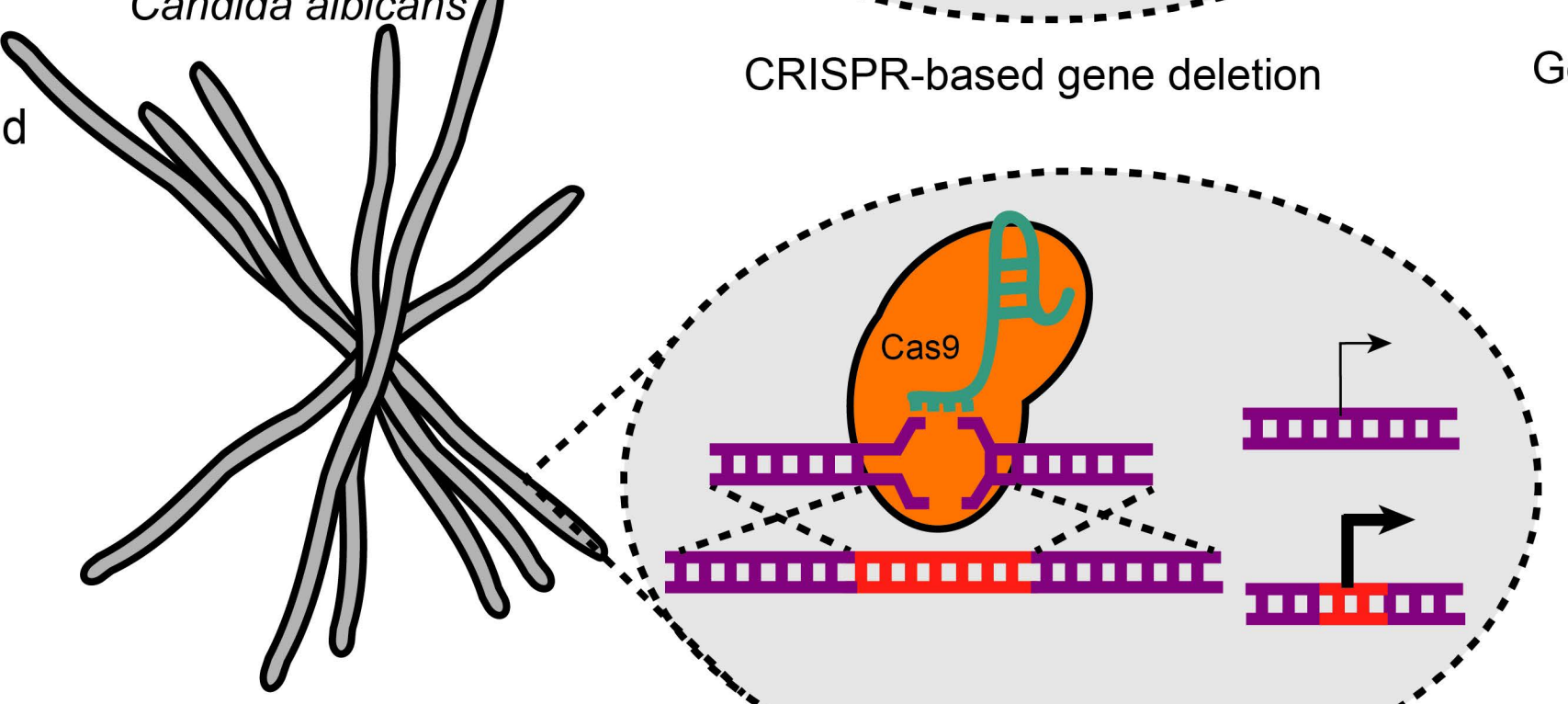
Candida albicans

CRISPR-based gene deletion



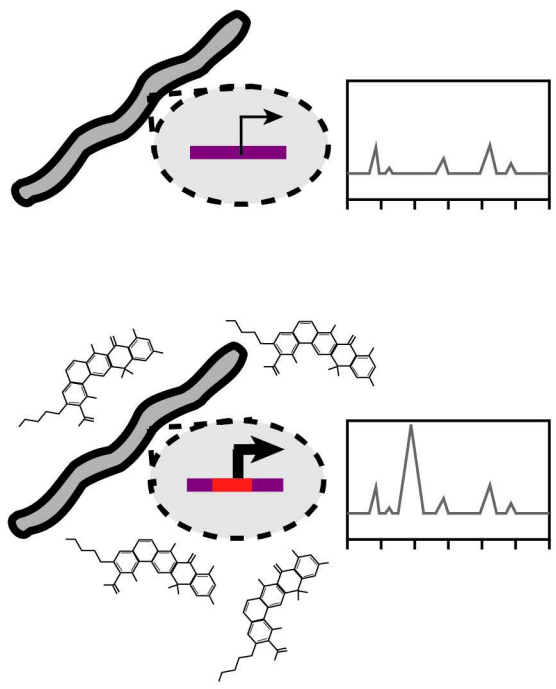
Genetic interaction analysis of
virulence factors

d



Streptomyces

CRISPR-based gene knock-in



Genetic engineering to activate
silent biosynthetic genes