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Bacterial CRISPR screens for gene function

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Abstract

In this review we describe the application of CRISPR tools for functional genomics screens in bacteria, with a focus on the use of interference (CRISPRi) approaches. We review recent developments in CRISPRi titration, which has enabled essential gene functional screens, and genome-scale pooled CRISPRi screens. We summarize progress toward enabling CRISPRi screens in non-model and pathogenic bacteria, including the development of new dCas9 variants. Taking into account the current state of the field, we provide a forward-looking analysis of CRISPRi strategies for determining gene function in bacteria.

Introduction

The exponential increase in bacterial genome assemblies and the growing importance of studying the full diversity of bacterial life have led to an increased focus on functional genomic approaches. By coupling genome-scale genetic perturbations with high-throughput phenotypic assays, functional genomics systematically defines gene-phenotype relationships, allowing functional inferences for genes of unknown function. Several high-throughput methods exist for perturbing gene function, including transposon-based approaches such as Tn-seq and TraDIS, knockout collections, and CRISPR approaches. These methods have unique strengths and weaknesses and are often deployed in a complementary fashion. However, advances in our understanding of CRISPR, decreases in DNA synthesis costs, and new CRISPR modalities have led to broad adoption of CRISPR for functional genomics studies across the bacterial domain.

There are many families of CRISPR systems, but most bacterial applications use the Type II-A system from *Streptococcus pyogenes* in which a nuclease effector protein ($Cas9_{Spy}$, [1]) is targeted to a specific DNA sequence by a complementary RNA (either a crRNA:tracrRNA complex [1] or a fused single guide RNA [2]). The Cas9-sgRNA complex binds complementary DNA in a two-step process, first recognizing a short DNA sequence (NGG)

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called the protospacer adjacent motif (PAM), and then progressively unwinding the DNA and forming a DNA:sgRNA R-loop [3]. Once bound, Cas9 cuts DNA in a stereotypical position, enabling DNA editing [4,5]. In bacteria, more attention has been focused on using a catalytically deactivated Cas9 (dCas9) to downregulate transcription (CRISPR interference - CRISPRi [2,6]). CRISPRi blocks RNA polymerase (RNAP) elongation (Figure 1A, potentially perturbing the expression of downstream and upstream genes in the targeted operon ([7–10], reviewed in [11]). dCas9 has also been used to direct transcriptional activators to specific genomic sites (e.g. CRISPR activation - CRISPRa [6,12,13]).

CRISPR methodologies offer three major advantages. First, because CRISPR activity depends on the expression of both the sgRNA and dCas9 components, it is inducible and titratable [2,14]. This enables the creation of libraries targeting essential genes and the measurement of gene dosage effects, neither of which cannot be achieved through transposon mutagenesis or traditional knockout approaches. Second, because CRISPR sgRNA spacers contain only 20bp of unique sequence, genome-scale sgRNA libraries can be quickly synthesized, cloned, and quantified through deep sequencing using the sequence of the sgRNA spacer as the bar-code. Alternatively, small, targeted libraries can be constructed to target a specific process. Third, due to their modular nature, CRISPR systems can be engineered (e.g. different promoters, dCas9 variants, delivery systems) to function in diverse microbial species enabling similar approaches in different organisms. Limitations of CRISPRi approaches include operon level knockdown and potential dCas9 toxicity. Additionally, when inducible systems are employed, there is a temporal delay between dCas9 production and target depletion *via* cell division. In this review, we discuss recent developments in CRISPR technology that have buttressed these strengths and furthered the application of CRISPR systems for characterizing gene function and highlight technological and conceptual advances in functional genomics afforded by CRISPR approaches.

Titration of CRISPRi activity

The ability to control CRISPR activity has been a central motivation driving its adoption. In contrast to transposon or knockout approaches, which inactivate gene function during strain construction, most bacterial CRISPRi approaches tightly control the expression of dCas9 and/or sgRNAs to modulate knockdown, usually using inducible promoters [2,7,8,10,14–16] (Figure 1B). By constructing CRISPRi libraries in permissive conditions (dCas9/sgRNA not expressed) and performing experiments in non-permissive conditions (dCas9 and sgRNA expressed), CRISPRi enables the exploration of essential and conditionally-essential genes. All CRISPRi screens in bacteria performed to date are collated in Table 1.

Bacillus subtilis [7] and *Streptococcus pneumoniae* [15] studies were the first to take advantage of this inducibility by building arrayed libraries of CRISPRi strains targeting all essential genes (identified using orthogonal genetic approaches) and probing the chemical sensitivities and cellular morphology of these strains. By identifying phenotypes shared by genes of known and unknown function, these screens revealed the roles of previously uncharacterized genes in iron-sulfur cluster biogenesis [7], peptidoglycan synthesis [15] and teichoic acid synthesis [15]. The utility of the *B. subtilis* CRISPRi library for determining the targets of drugs was also demonstrated both in the initial study [7] and in subsequent

trials [17]. More recently, the Burne lab [18] targeted the essential genes of *Streptococcus mutans,* a pathogen associated with dental caries, using dCas9 from *S. mutans* itself. By characterizing the growth and morphology of their library, they identified the function of a poorly annotated essential gene and dissected a novel virulence determinant. Likewise, the Warner lab [19] applied CRISPRi (using dCas9 from *Streptococcus thermophilus* - $dCas9_{Sth}$ [16]) to the essential genes of *Mycobacterium smegmatis,* a close relative of the human pathogen *M. tuberculosis*. Using high-throughput microscopy, they determined the function of an uncharacterized essential gene.

Titrating CRISPR activity *via* inducible promoters allowed the probing of essential gene function, however testing multiple knockdown levels has remained difficult. In contrast, programming CRISPRi activity level into sgRNA sequences allows quantification of the fitness impact of multiple knockdown levels in a single pooled experiment. Early studies leveraged the decreased activity of sgRNAs designed to pair with the template (non-coding) strand [7,8,10,20], (likely because those complexes are more easily surpassed by RNAP [21]) to perform initial analyses of gene-specific susceptibility to knockdown. Control of sgRNA activity by varying the extent of complementarity between sgRNAs and their targets has also been demonstrated [22]. More recently, the Gross lab demonstrated precise control of sgRNA activity by introducing single, targeted mismatches into the sgRNA (Figure 1B, mismatch-CRISPRi [23,24]), likely by affecting DNA binding kinetics [25]. Mismatch-CRISPRi functions across species, allowing the first cross-species comparison of essential gene expression-fitness relationships. The Bikard lab [26] recently identified additional sequence determinants of sgRNA activity. Because sgRNA sequences are constrained by the genome of interest, this method does not allow precise engineering of sgRNA efficacy but does enable the design of high-activity sgRNA libraries.

A different type of gene perturbation is achieved with CRISPRa, which uses dCas9 to position a transcriptional activator adjacent to a gene and activate its expression [6,12,13]. Despite its promise, this technique remains of limited utility for high-throughput approaches due to variable efficacy and strict targeting requirements. Recent work from the Wang lab has identified and engineered a dCas9-linked activation with relaxed spacing requirements, potentially relaxing targeting requirements [27]. While no CRISPRa genetic screens have been reported in bacteria, several [28,29] have been performed in eukaryotic systems, where differential responses to knockdown and activation allow stringent identification of genes with specific phenotypes. This is an exciting prospect for bacterial functional genomics, especially because CRISPRi/CRISPRa experiments can be performed simultaneously [12,13].

Construction of sgRNA libraries

The relative simplicity of constructing and assaying libraries is a major advantage of CRISPRi. Because sgRNA spacers targeting each gene are cloned into a single chromosomal or plasmid site, construction of arrayed and pooled CRISPR libraries is simplified compared to gene deletion libraries. Compared to transposon-based approaches with similar genomic coverage, CRISPRi libraries are more compact, which facilitates construction, handling, and sequencing. This size allows their use in situations in which Tn-seq would be bottlenecked

(e.g., pathogenesis experiments). The decreasing cost of DNA synthesis has made it feasible to synthesize large sgRNA libraries, leveraging CRISPRi for larger scale assays (Figure 2).

A major limitation of large libraries is the cloning efficiency required to maintain good representation of sgRNAs from oligo-chip to flask. Previously, this hurdle was overcome using highly efficient natural competence to introduce sgRNAs into a genomic locus (*B. subtilis* [24]) or by cloning sgRNAs into plasmids that can be efficiently transformed (e.g. by electroporation) [8–10,24]. Recently reported chromosomal "landing pads" [30] allow high efficiency cloning of sgRNAs directly into the genome of *E. coli* and other species. Integrating plasmids may allow similar functionality in other species [31–33]. Chromosomally integrated sgRNAs allow finer control of sgRNA expression and more accurate quantification of strain abundance through sequencing than plasmid-based systems. The efficiency gains from working with large, complex libraries come from assaying the collection as a pool, in many cases by quantifying the relative abundance of each strain before and after a process of selection or enrichment (e.g. during normal growth conditions to calculate relative growth rate) (Figure 2). Because substantial sequencing depth is required for accurate quantitation (for discussion, see [9,24]), these screens have benefited from the continuing reduction in sequencing costs.

The Bikard, Xing, and Warner labs performed the first sets of large library functional genomic experiments in *E. coli* [8–10] and *M. smegmatis* [34]. Using libraries of ~60,000, ~90,000, and ~12,000 elements (respectively) targeting almost all genes, these studies demonstrated the robustness of pooled screens and their utility for essential gene identification. Demonstrating the utility of these libraries to address specific biological questions, one study from the Bikard lab [10] also identified genes, including essential genes, whose knockdown reduced infection by 3 bacteriophages. A recent study expanded this approach to additional phages [35]. This highlights the ability of pooled CRISPRi approaches to determine essential gene phenotypes besides growth and paves the way for pooled CRISPRi-based chemical genomic screens, analogous to previously reported transposon studies [36]. These studies also revealed toxicity caused by high dCas9 expression and the presence of specific sgRNA seed sequences [8], similar to reports in *E. coli* [37] and other organisms [16,38]. These effects should be considered and addressed by evaluating large numbers of non-targeting sgRNAs to control for any non-specific phenotypes. The Church lab applied CRISPRi to *Vibrio natriegens* [20]. Using a library of ~14,000 sgRNAs targeting all genes, they identified its essential genes and refined metabolic gene annotations. More recently, the Arkin lab built a library of ~33,000 sgRNAs targeting all genes and almost all putative sRNAs in *E. coli* and screened it at various intervals post-induction [39]. These time-resolved measurements of fitness revealed gene function-specific transient responses and novel gene phenotypes. Pooled CRISPRi approaches have also been successfully applied to optimize bacteria for industrial uses [40].

The Tavazoie lab performed a large CRISPRi screen using a different approach. Rather than commercial synthesis of sgRNAs, they used the natural adaptation machinery of the *S. pyogenes* CRISPR-Cas system to produce sgRNAs targeting *E. coli.* These crRNA systems appear to function analogously to sgRNAs, but further work is required to characterize their utility [41]. Finally, recent proof-of-principle work from the Elf lab [42] demonstrated a

novel method, DuMPLING, which allows cellular morphology to be assayed in a pooled setting. Scaling this concept to entire genomes and even communities is an exciting prospect.

Application of CRISPRi to diverse organisms

A final key advantage of CRISPRi is its applicability to diverse organisms, including many that lack genetic tools. The recognition of the importance of specific bacteria in the microbiome and environment has driven the development of techniques for working with non-model organisms, including CRISPRi approaches. In the last few years, CRISPRi has been demonstrated in many bacteria, including human associated bacteria (Figure 3). In some species, such as *Bacteroidetes* [43], *Staphylococcus aureus* [44], *Borrelia burgdorferi* [45], *Burkholderia* [32], *Clostridioides difficile* [46], *Vibrio cholerae* [47], *Lactobacillus plantarum* [48], *Enterococcus faecalis* [49], *Zymomonas mobilis* [50], *Lactococcus lactis* [51], *Myxococcus xanthus* [52], *Streptomyces* [53] and *Corynebacterium glutamicum* [54], it is possible to use the canonical *S. pyogenes* dCas9 ($dCas9_{Spy}$). In other species, specialized dCas9 proteins must be used. For example, $dCas9_{Sth}$ is used in *Mycobacterium* species [16], while either $dCas9_{Sth}$ or *S. pasteurianus* dCas9 ($dCas9_{Spa}$) can be used in *Caulobacter crescentus* [55]. Moreover, although $dCas9_{Spy}$ functions in *Pseudomonas* species, $dCas9_{Spa}$ was shown to be more effective in some strains [33,38,56]. A recently developed system, Mobile-CRISPRi, enables modular engineering of CRISPRi components and delivery systems, with demonstrated efficacy in ESKAPE pathogens [31]. Importantly, several studies have demonstrated that CRISPRi is maintained and functions when microbiome strains or pathogens are in the host [18,38,43], suggesting that pooled CRISPRi screens can be performed *in vivo* to identify genes involved in commensalism and pathogenesis. Indeed, this approach has already been applied to *S. pneumoniae* to successfully identify *in vivo* essential genes for targeted drug development [57].

An example of the kinds of insights that can be gained by applying CRISPRi to multiple bacterial strains can be found in the recent work from the Bikard lab [58], in which a library of 11,629 sgRNAs targeting conserved *E. coli* genes was introduced into 18 ecologically distinct strains. Both the fitness effects of gene knockdown and the subset of essential genes varied across strains and conditions, highlighting previously unappreciated differences within a single species. Performing CRISPRi screens in pathogenic bacteria will identify novel points of vulnerability in these species, such as the rhamnose-glucose polysaccharide synthesis pathway identified in *S. mutans* [18], informing antibiotic development. Cross species comparisons will enable deeper evolutionary comparisons of essential gene functions [24].

Future directions

When we last reviewed CRISPR methods in bacteria [59], no high-throughput CRISPRi screens had been performed. This has been rectified in the intervening years with a veritable explosion of high-throughput, high-dimensional CRISPRi screens of essential genes and whole genomes. These studies have revealed not only novel gene functions, but also novel connections between pathways.

Despite the inherent modularity and portability of CRISPRi approaches, almost all high-throughput screens were performed in model systems. Considering the breadth of biologically and industrially relevant organisms in which CRISPRi has been demonstrated (reviewed above), and the expanding toolkit of sgRNA design software, cloning, and sequencing approaches, CRISPRi screens in diverse bacteria (e.g. *S. mutans* [18], *M. smegmatis* [19]) may become increasingly common. In contrast to studies in well-characterized bacteria, CRISPRi screens in non-model organisms may generate numerous novel functional annotations, which will undoubtedly propagate to related species. Importantly, comparative functional genomics analyses across strains/species will allow us to study the functional conservation of genes. For example, comparison of *E. coli* and *B. subtilis* essential gene expression fitness curves has already revealed shared constraints on essential gene expression [24].

Just as CRISPR based screens have expanded from model systems to non-model organisms, CRISPR based techniques are expanding into fully fledged screens. In particular, continued engineering of dCas9 variants [12,13,60] with relaxed PAM requirements and the discovery of new transcriptional activators [27] may enable the first CRISPRa screens in bacteria. Such screens will reveal new biology. Advances in sequencing and synthesis may also drive new CRISPRi methodologies. In particular, the decreasing price and increasing quality of paired-end sequencing strategies allows the sequencing of two proximally encoded sgRNAs. Since CRISPRi can effectively target numerous genes simultaneously, it allows the quantification of fitness in strains depleted for two genes. Double knockout analysis in the yeast *Sacchromyces cerevisiae* furthered the understanding of its genetics by uncovering redundancy and revealing its network of genetic interactions [61]. Likewise, applying this technique to bacteria will expand our understanding of their gene networks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Bacterial CRISPR approaches are used for gene editing, repression, and activation.
- CRISPRi, which blocks transcription of targeted genes, is the main bacterial modality.
- Chemical genomics and titratable CRISPRi enable studies of essential gene functions.
- Pooled, genome-wide CRISPRi screens identify novel gene functions.
- CRISPRi has been demonstrated in many non-model bacteria.

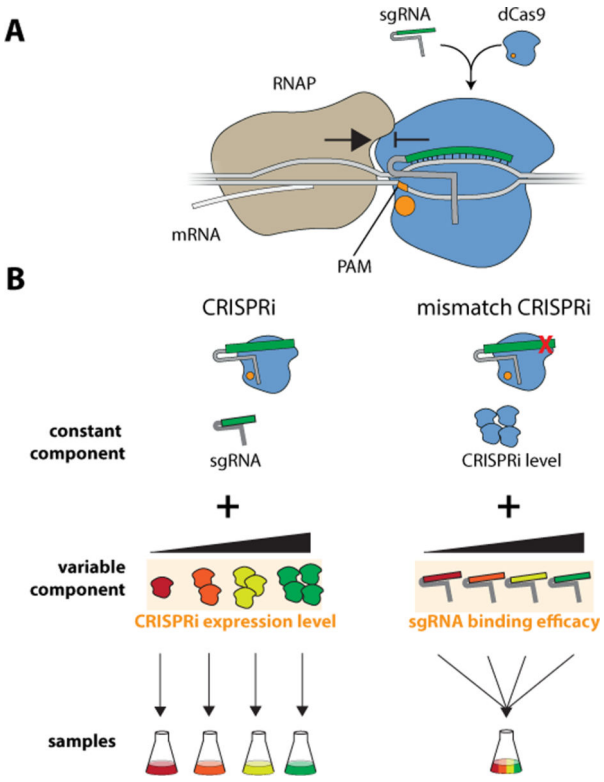

Figure 1.

A) Schematic of CRISPRi mechanism. A catalytically inactivated Cas9 (dCas9) recognizes a PAM site and unwinds the DNA allowing the base-pairing region (green) of its sgRNA to bind the complementary DNA sequence in a target gene. dCas9 acts as a roadblock to elongating RNA polymerase (RNAP) and thus represses the expression of the targeted gene. B) Adjusting repression strength in CRISPRi and mismatch CRISPRi. In regular CRISPRi (left), repression is controlled by adjusting the expression level of CRISPRi components, and each level of repression necessitates a separate experiment. In mismatch CRISPRi (right), the same effect is achieved by adjusting the binding strength of sgRNAs through the introduction of mismatches. This allows many interference levels to be queried in a single pooled sample.

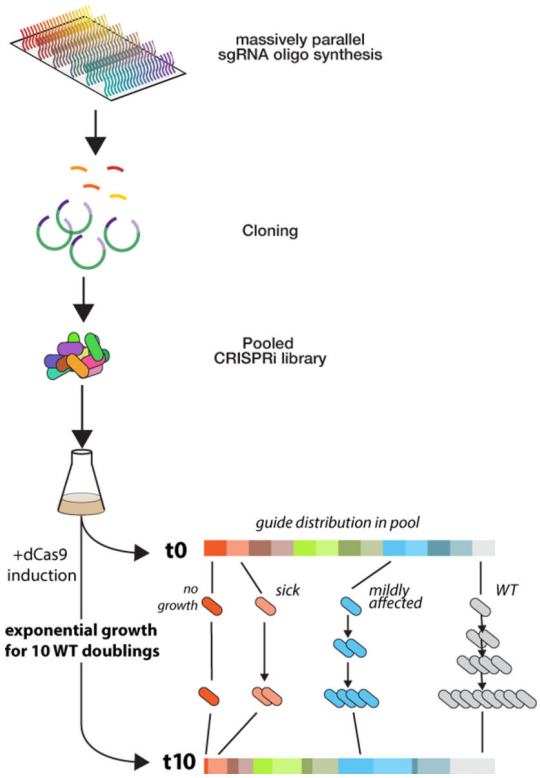

Figure 2.

Flow-chart of a pooled CRISPRi experiment. DNA oligos coding for individual sgRNAs are synthesized in bulk on a DNA chip, and cloned into the target organism as a pool. To determine the effect of downregulating a target gene through an sgRNA, the relative abundance of an sgRNA is determined via deep sequencing before and after several generations of growth. De-enrichment of an sgRNA sequence indicates a fitness defect in cells containing that sgRNA.

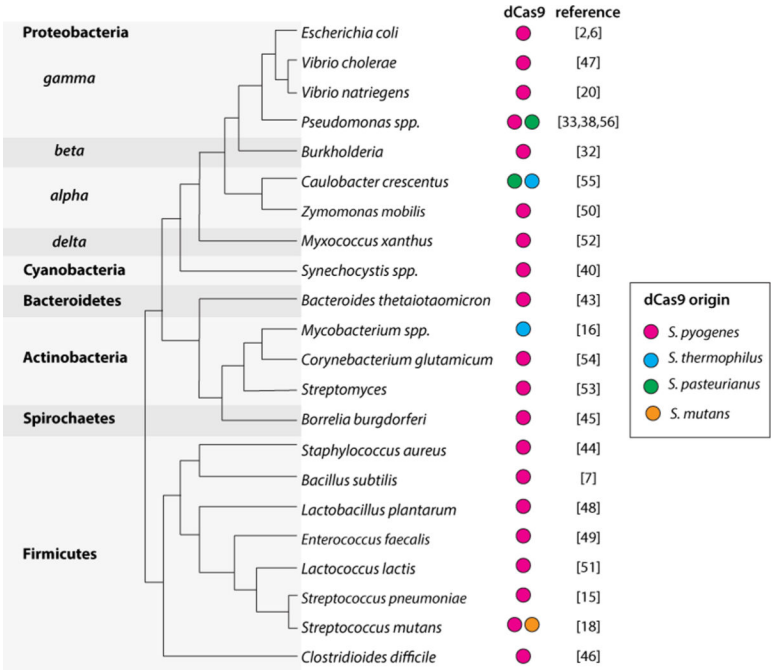

Figure 3.

Bacteria in which CRISPRi has been successfully established, and origin of the dCas9 systems used.

Table 1 –

All CRISPRi screens in bacteria to date.

Organism	Screen	sgRNAs	Genes	Reference
B. subtilis	Arrayed chemical genomics	289	Essential	[7]
	Pooled growth/mismatch	33,585	Essential	[24]
S. pneumoniae	Arrayed growth/microscopy	384	Essential	[15]
	Pooled in vivo growth	1,499	All	[57]
E. coli	Pooled growth	92,919	All	[8]
	Pooled growth	~60,000	All	[9]
	Pooled growth/phage	92,919	All	[10]
	Pooled Microscopy/Growth	235	Cell-cycle	[42]
	Pooled growth	~33,000	All	[39]
	CRISPR adaptation/growth	462,382	All	[41]
	Pooled growth in multiple strains	11,629	Most	[58]
	Pooled growth/phage	~33,000	All	[35]
	Pooled growth/mismatch	36,291	Essential	[24]
M. smegmatis	Pooled growth	11,467	2,385	[34]
	Arrayed growth/microscopy	272	Essential	[19]
V. natriegens	Pooled growth	13,567	All	[20]
S. mutans	Arrayed growth/microscopy	259	Essential	[18]
Synechocystis sp.	Pooled growth	10,498	All	[40]