CRISPR-Based Approaches for Gene Regulation in Non-Model Bacteria

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CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) have become ubiquitous approaches to control gene expression in bacteria due to their simple design and effectiveness. By regulating transcription of a target gene(s), CRISPRi/a can dynamically engineer cellular metabolism, implement transcriptional regulation circuitry, or elucidate genotype-phenotype relationships from smaller targeted libraries up to whole genome-wide libraries. While CRISPRi/a has been primarily established in the model bacteria Escherichia coli and Bacillus subtilis, a growing numbering of studies have demonstrated the extension of these tools to other species of bacteria (here broadly referred to as non-model bacteria). In this mini-review, we discuss the challenges that contribute to the slower creation of CRISPRi/a tools in diverse, non-model bacteria and summarize the current state of these approaches across bacterial phyla. We find that despite the potential difficulties in establishing novel CRISPRi/a in non-model microbes, over 190 recent examples across eight bacterial phyla have been reported in the literature. Most studies have focused on tool development or used these CRISPRi/a approaches to interrogate gene function, with fewer examples applying CRISPRi/a gene regulation for metabolic engineering or high-throughput screens and selections. To date, most CRISPRi/a reports have been developed for common strains of non-model bacterial species, suggesting barriers remain to establish these genetic tools in undomesticated bacteria. More efficient and generalizable methods will help realize the immense potential of programmable CRISPR-based transcriptional control in diverse bacteria.

Keywords: bacterial gene regulation, CRISPR interference (CRISPRi), CRISPR activation (CRISPRa), transcriptional interference, transcriptional activation, non-model bacteria, genome-wide library

INTRODUCTION

Since the development of CRISPR interference (CRISPRi) (Qi et al., 2013) and CRISPR activation (CRISPRa) (Bikard et al., 2013) in 2013, they have become efficient and prevalent tools for transcriptional regulation in bacteria. CRISPR-Cas originates as a form of prokaryotic immunity, with systems comprising one or more CRISPR-associated (Cas) proteins and a short guide RNA (gRNA) that complex together to target and cleave foreign DNA or RNA molecules, such as viruses (Nussenzweig and Marraffini, 2020). The gRNA leads the complex to target sequence via complementarity between the protospacer sequence of the gRNA and the target site on the DNA/RNA molecule. Various mechanisms exist to prevent cleavage of chromosomal DNA, which most often involves a protospacer adjacent motif (PAM) or equivalent next to the target site that is not present in the CRISPR arrays on the chromosome (Jackson et al., 2017).
Researchers developed CRISPRi technology by deactivating the nuclease activity of select Cas enzymes to create mutant dCas proteins that bind, but do not cleave, the DNA target (Qi et al., 2013). Most CRISPRi systems repress a gene’s expression through steric inhibition of RNA polymerase binding or extension (Qi et al., 2013), although some repress gene expression through RNA cleavage (Zhang K. et al., 2020; Rahman et al., 2021). Gene repression over 100-fold has been reported for several diverse CRISPRi tools and can approach near knockout levels of gene expression (Qi et al., 2013; Miao et al., 2019). Targeting a different sequence is easily achieved by changing the short protospacer sequence on the gRNA to bind a location within the promoter, untranslated region, or coding sequence of the target gene based on simple design rules (Qi et al., 2013; Zetsche et al., 2015; Zhang et al., 2017). Additionally, multiplexed gene repression can be achieved by simply expressing multiple gRNA within a cell (Qi et al., 2013; Zhang et al., 2017).

Shortly after the development of CRISPRi, researchers developed CRISPRa for bacterial transcriptional activation by combining the dCas protein with a transcriptional activator that recruits transcription machinery to the target gene’s promoter to increase gene expression (Bikard et al., 2013). The specific mechanism for transcriptional activation depends on the activator, which can be incorporated by directly fusing a transcriptional activator domain to the dCas protein (Bikard et al., 2013; Ho et al., 2020; Schilling et al., 2020), incorporating RNA scaffolds into the gRNA sequence to recruit activator domains to the dCas complex (Dong et al., 2018; Liu Y. et al., 2019; Fontana et al., 2020a), or using non-covalent protein-protein interaction domains to complex the transcriptional activator and dCas protein (Villegas Kcam et al., 2021; Villegas Kcam et al., 2022).

Unlike CRISPRi, however, CRISPRa has complex design rules that often strongly depend on the CRISPRa technology (i.e., type of activation domain and approach to couple the activation domain and dCas complex) as well as several other factors (Liu Y. et al., 2019; Fontana et al., 2020a; Ho et al., 2020; Villegas Kcam et al., 2021). These other considerations include the basal expression of the target gene and the location of the binding site for the CRISPRa complex, where activation is typically achieved in a narrow range upstream of the target gene’s promoter and the activation strength fluctuates sharply as the nucleotide position shifts. Combined with the PAM requirement for DNA binding, these requirements greatly restrict the available DNA target sites for effective gene activation, especially for endogenous genes. Due to these relatively stringent design rules for gene activation and often low (<10-fold) activation levels compared to CRISPRi repression (Bikard et al., 2013; Liu W. et al., 2019; Fontana et al., 2020a; Villegas Kcam et al., 2021), CRISPRa development has been slower in bacteria than in eukaryotes (Kampmann, 2018; Fontana et al., 2020b). Despite the current limitations of CRISPRa, however, the simplicity and inherent properties of CRISPRi/a gene regulation can provide strong transcriptional control of multiple genes simultaneously, making these approaches often easier and faster than traditional methods and allowing for dynamic transcriptional control.

CRISPR systems are classified into a variety of classes, types, subtypes, and variants, each with unique genes and properties (Koonin et al., 2017; Makarova et al., 2020). Many systems have been engineered to create effective CRISPR/a tools. The first and most common tool is derived from the Type II Cas9 system, which comprises a single deactivated Cas9 (dCas9) protein and two small RNAs that create the gRNA (Qi et al., 2013). These two RNAs can be combined into a synthetic single guide RNA (sgRNA) for easier synthesis, but each sgRNA requires an independent promoter for expression (Jiang et al., 2015). Although many different dCas9 variants exist, the Streptococcus pyogenes dCas9 (SpCas9) system is the most common due to its short PAM sequence and strong transcription regulation abilities. Recently, tools derived from the Type V Cas12a (formerly Cpfl) system have been developed, which uses a single deactivated Cas12a (dCas12a) protein and one gRNA (Zetsche et al., 2015; Kim et al., 2017; Zhang et al., 2017; Miao et al., 2019). Unlike dCas9, dCas12a can process its gRNA from CRISPR arrays, providing easier multiplexed regulation (Fonfara et al., 2016; Zhang et al., 2017). Additionally, several studies have suggested that dCas12a variants are less toxic than dCas9 variants across different bacterial phyla (Liu W. et al., 2019; Knoot et al., 2020; Kuo et al., 2020), making them an attractive alternative to dCas9. The most common dCas12a variants used in bacteria are derived from Francisella tularensis subsp. novicida (Fn dCas12a) and Acidaminococcus sp. BV3L6 (As dCas12a). Several Type I CRISPRi/a tools have been designed, but due to the large number of genes in these systems, most tools are implemented by reprogramming the host species’ endogenous CRISPR system for gene repression (Luo et al., 2015; Xu et al., 2021; Villegas Kcam et al., 2022). Only a handful of CRISPRi tools from other systems have been reported for transcriptional regulation in bacteria, likely due to the novelty of the system (Rahman et al., 2021) or high cellular toxicity observed upon expression (Zhang K. et al., 2020).

Despite the unique traits and relevance of a vast diversity of bacteria, CRISPRi/a tools have been primarily developed in the model bacteria Escherichia coli and Bacillus subtilis. Yet, non-model bacteria (a broad definition of non-model, excluding E. coli and B. subtilis, is used here) offer great promise in research and industry spanning a wide range of medical, environmental, and biomanufacturing applications. For example, Streptomyces, Sorangium, and Photorhabdus spp. naturally produce bioactive secondary metabolites, such as antibiotics, and contain silent biosynthetic gene clusters with unknown and potentially useful products (Ye et al., 2019; Tian et al., 2020; Ke et al., 2021). Additionally, Rhodococcus and Corynebacterium spp. can produce valuable chemicals from cheap and simple feedstock and are tolerant to harsh conditions, making them ideal cell factories (Cleto et al., 2016; De Lorenzo et al., 2018). However, several conditions must be reached to successfully establish efficient CRISPRi/a tools in a non-model bacterium. In this mini-
review, we detail these criteria, emphasizing the importance of characterized genetic parts to tightly control the expression of CRISPRi/a systems to limit potential toxicity while providing sufficient expression for effective transcriptional control. We demonstrate that despite the potential difficulties in creating these tools in non-model bacteria, they have been established across eight different bacterial phyla and have been used for a variety of applications, including high-throughput genome-wide selections. Finally, we highlight the current challenges to developing CRISPRi/a tools in non-model bacteria and novel species, which suggest directions for future progress.

**REQUESTS AND CHALLENGES TO ESTABLISH CRISPRi/A IN NON-MODEL BACTERIA**

Several criteria must be met to successfully establish an effective CRISPRi/a tool in a non-model species or strain. First, the conditions for culturing, maintaining, and genetically manipulating the strain (often referred to as strain "domestication") must be determined. For a phylogenetically similar strain to a previously established model bacteria, such as many Bacillus species (Zhan et al., 2020) and Enterobacteriaceae (Ho et al., 2020), suitable culture conditions may be similar to those previously determined. For novel or fastidious species, however, trial and error and patience may be required to determine appropriate culture conditions for growth and genetic manipulation, such as the obligate intracellular pathogen Chlamydia trachomatis (Ouellette, 2018).

Additionally, introducing foreign DNA is often challenging for a non-model bacterium, as many are genetically recalcitrant, especially pathogens (Fernandes et al., 2021b) and novel strains (Zhao et al., 2020; Jin et al., 2022), and establishing a sufficient genetic transformation method can require significant effort. Additionally, care must be taken when introducing synthetic DNA to circumvent the bacterial host’s native immunity that may degrade foreign DNA, including restriction-modification and CRISPR systems (Marraffini and Sontheimer, 2008; Jin et al., 2022), such as by mimicking the recipient strain’s methylation patterns (Monk et al., 2015; Zhao et al., 2020). More discussion on the isolation and domestication of non-model bacteria can be found in other reviews (Vartoukian et al., 2010; Lewis et al., 2021; Riley and Guss, 2021).

Next, reliable genetic parts for the non-model bacterium are required to be able to express and tightly control the CRISPRi/a tool, including promoters, ribosome binding sites, terminators, and expression or integration vectors. For many non-model bacteria, especially novel species, these genetic part libraries are unavailable, and so, the necessary genetic parts must be created and characterized. In some cases, established genetic parts may be transferable from a model bacterium to a related species, such as promoters between Gram-positive bacteria (Liew et al., 2010). However, genetic parts often do not function equivalently between bacterial species or even strains (Tong et al., 2015; Leonard et al., 2018). Each CRISPRi/a component should be expressed using unique genetic parts to prevent repeated DNA sequences.

Since dCas protein expression can elicit cytotoxicity, high strength promoters used for overexpression may not be optimal. If existing genetic parts are insufficient for a new bacterial species, identifying genetic regulatory elements from the endogenous genome provides an alternative to synthetic DNA design strategies (Fernandes et al., 2019). Libraries of genetic parts and inducible promoters are excellent tools to tune the expression of CRISPRi/a systems, and several studies have established such toolboxes in non-model bacteria to facilitate the development of genetic tools such as CRISPRi/a (Mimee et al., 2015; Leonard et al., 2018; Shin et al., 2019; Teh et al., 2019; Liow et al., 2020). These libraries and tunable parts are especially important to control the expression of the CRISPRi/a tool to minimize potential cellular toxicity and to precisely control transcriptional regulation (Qu et al., 2019; Bosch et al., 2021; Shabestary et al., 2021).

In the design of a synthetic CRISPRi/a system for a bacterium, consideration should be given to prevent interference with endogenous CRISPR systems and/or anti-CRISPR genes harbored on the strain’s genome. If the foreign and native CRISPR-Cas types are too similar, the introduction of the synthetic gRNA may induce cleavage of the host bacterium’s genome (via the catalytically active endogenous Cas enzyme) and can cause cell death in a DNA repair-deficient strain or undesired mutations if the strain has appropriate DNA repair pathways. This can be avoided by choosing a CRISPRi/a tool that does not share significant homology to any endogenous CRISPR-Cas.

Native CRISPR-Cas systems can be predicted from the sequenced genome or proteome using computer software (Couvain et al., 2018; Chai et al., 2019), aiding in CRISPRi/a tool selection for novel strains. Alternatively, the native system can be engineered to create a CRISPRi/a tool via genetic manipulation, such as the deletion of the native cas2/3 or cas3 gene responsible for cleavage in Type I-F systems (Zheng et al., 2020; Qin et al., 2021; Xu et al., 2021) or mutating the native cas9 sequence for Type II systems (Shields et al., 2020; Dammann et al., 2021). Anti-CRISPR proteins, which inhibit CRISPR systems through a variety of mechanisms (Pawluk et al., 2018), may require deletion or disruption before a heterologous CRISPRi/a tool can be expressed (Xu et al., 2021). Online tools and databases are available to predict and describe anti-CRISPR proteins from protein sequences to help select an appropriate CRISPRi/a system (Wang et al., 2020; Wang et al., 2021).

Finally, the CRISPRi/a components should be expressed at a level that provides adequate transcriptional repression or activation for the given application without significant cellular toxicity. Many studies have reported CRISPRi toxicity for diverse bacteria, while little is known about CRISPRa toxicity due to limited reports in the literature. These observed forms of toxicity include changes in cell morphology (Cho et al., 2018; Ouellette et al., 2021) and slower growth or complete growth inhibition (Rock et al., 2017; Yu et al., 2018; Wurihan et al., 2019; Zhang K. et al., 2020; Brito et al., 2020). To prevent toxicity, one can use a less toxic CRISPRi/a system for the host species (Rock et al., 2017;
# TABLE 1 | CRISPRi/a studies in non-model bacteria and their key characteristics.

| Bacterium | Application | CRISPRi/a | CRISPR System(s)* | GW<sup>b</sup> | References<sup>c</sup> and type of study |
|-----------|-------------|-----------|-------------------|--------------|--------------------------------------|
| **Actinomycetota** | | | | | |
| Burkibacillus, Burkholderia cepacia | Probiotic | CRISPRi | As dCas12a | N | TD: Jin et al. (2022) |
| Corynebacterium glutamicum | Bioproduction | CRISPRi | Fn dCas12a | N | TD: Liu et al. (2019a); Li et al. (2020b); ME: Liu et al. (2019a); Li et al. (2020b); Huang et al. (2021) |
| M. smegmatis, M. tuberculosis | Pathogen | CRISPRi | Fn dCas12a | N | TD: Fleck and Grundner, (2021) |
| M. smegmatis, M. tuberculosis, M. bovis | Pathogen | CRISPRi | NdCas9 | N | TD: Choudhary et al. (2015); Singh et al. (2016); Xiao et al. (2019); Agarwal, (2020); Nadolinikaaia et al. (2021); MGF: Thakur et al. (2016); Singh et al. (2017); Choudhary et al. (2019); Dutta et al. (2019); Agarwal, (2020); Lunge et al. (2020); Faulkner et al. (2021); Gan et al. (2021); Gibson et al. (2021) |
| M. smegmatis, M. tuberculosis | Pathogen | CRISPRi | Sth1 dCas9 | N | TD: Rock et al. (2017); Cheung et al. (2021); Judd et al. (2021); Baranowski et al. (2018); Landetta et al. (2019); Mai et al. (2019); McNeil and Cook, (2019); McNeil et al. (2020); McNeil et al. (2022); Randall et al. (2020); Brzostek et al. (2021); Quinones-Garcia et al. (2021); Savkova et al. (2021) |
| M. tuberculosis | Pathogen | CRISPRi | Native Type III-A | Y | TD: Rahman et al. (2021); SS: Rahman et al. (2021) |
| Rhodococcus opacus | Bioproduction | CRISPRi | Sth1 dCas9 | N | TD: DeLorenzo et al., 2018, 2021; ME: DeLorenzo et al. (2018) |
| Saccharopolyspora erythraea | Bioproduction, bioresearch | CRISPRi | Sp dCas9 | N | ME: Liu et al. (2021b) |
| **Streptomyces** | | | | | |
| S. venezuelae | Bioproduction, bioresearch | CRISPRa | Sp dCas9-aNTD | N | TD: Ameruoso et al. (2021) |
| S. coelicolor | Bioproduction, bioresearch | CRISPRi | Fn dCas12a | N | TD: Li et al. (2018); MGF: Yan et al. (2022); ME: Liu et al. (2021c) |
| S. coelicolor, S. venezuelae, S. rapamycinicus, S. spp. | Bioproduction, bioresearch | CRISPRi | Sp dCas9 | N | TD: Tong et al. (2015); Tong et al. (2020); Zhao et al. (2018); Tian et al. (2020); Ameruoso et al. (2021); Wang et al. (2021b); ME: Tian et al. (2020); MGF: Ultee et al. (2020); Zhang et al. (2020b); Zhang et al. (2021); TRN: Tian et al. (2020) |
| **Bacteroidetes** | | | | | |
| Bacteroides thetaiotaomicron | Probiotic | CRISPRi | Sp dCas9 | N | TD: Mimo et al. (2015); TRN: Mimo et al. (2015); Takekari et al. (2020) |
| Bacteroides, Parabacteroides, Prevotella spp. | Probiotic | CRISPRi | As dCas12a | N | TD: Jin et al. (2022) |
| **Chlamydiae** | | | | | |
| Chlamydia trachomatis | Pathogen | CRISPRi | As dCas12a | N | TD: Ouellette et al. (2021) |
| **Cyanobacteria** | | | | | |
| Anabaena sp. PCC 7120 | Bioproduction, bioresearch | CRISPRi | Sp dCas9 | N | TD: Higo et al. (2018); Higo and Ehira, (2019); ME: Higo et al. (2018); Higo and Ehira, (2019); MGF: Higo et al. (2019) |

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TABLE 1 | Continued CRISPRi/a studies in non-model bacteria and their key characteristics.

| Bacterium | Application | CRISPRi/a | CRISPR System(s) | GW<sup>6</sup> | References<sup>6</sup> and type of study |
|-----------|-------------|-----------|-----------------|-------------|-------------------------------------|
| **Synechococcus sp. UTEX 2973** | Bioproduction | CRISPRi | Fn dCas12a | N | TD: Knoot et al. (2020); MGF: Knoot et al. (2020) |
| **Synechococcus elongatus** | Bioproduction | CRISPRi | Fn dCas12a | N | TD: Choi and Woo, (2020); ME: Choi and Woo, (2020) |
| **Synechococcus sp. PCC 7002** | Bioproduction | CRISPRi | Sp dCas9 | N | TD: Huang et al. (2016); ME: Huang et al. (2016); TRN: Lee and Woo, (2020) |
| **Synechocystis sp. PCC 6803** | Bioproduction, | CRISPRi | Fn dCas12a | N | TD: Gordon et al. (2016); ME: Gordon et al. (2016) |
| **Synechocystis sp. PCC 7002** | | | Sp dCas9 | N | TD: Gordon et al. (2016); ME: Gordon et al. (2016) |
| **Synechocystis sp. PCC 7002** | | | Sp dCas9 | N | TD: Gordon et al. (2016); ME: Gordon et al. (2016) |
| **Firmicutes** | | | | | Y |
| **Bacillus** | | | | | Y |
| **B. amyloliquefaciens** | Bioproduction | CRISPRa | Sp dCas9 | N | TD: Zhao et al. (2020); MFE: Zhao et al. (2020) |
| **B. amyloliquefaciens, B. methanolicus, B. licheniformis** | Bioproduction | CRISPRi | Sp dCas9 | N | TD: Schultenkämper et al. (2020); Zhao et al. (2020); Zhan et al. (2020); Zhao et al. (2020); MGF: Marreddy et al. (2019); Shabestary et al. (2019); MFE: Sha et al. (2020); Zhan et al. (2020) |
| **B. smithii** | Bioproduction | CRISPRi | ThermodCas9 | N | TD: Mougiakos et al. (2020) |
| **Clostridia** | | | | | Y |
| **C. sporogenes, C. spp.** | Bioproduction | CRISPRi | As dCas12a | N | TD: Jiang et al. (2020); MFE: Zhao et al. (2019); MGF: Marreddy et al. (2019) |
| **C. ljungdahlii** | Bioproduction | CRISPRi | Sp dCas9 | N | TD: Jin et al. (2020) |
| **B. smithii** | Bioproduction | CRISPRi | ThermodCas9 | N | TD: Jin et al. (2020) |
| **Enterococcus faecalis** | Pathogen | CRISPRi | Sp dCas9 | N | TD: Shin et al. (2019) |
| **Eubacterium limosum** | Bioproduction, probiotic | CRISPRi | Sp dCas9 | N | TD: Peters et al. (2019); MFE: Zhao et al. (2019); MGF: Afonina et al. (2020) |
| **Hungateiclostridium thermocellum** | Bioproduction | CRISPRi | ThermodCas9 | N | TD: Ganguly et al. (2020) |
| **Lactobacillus plantarum** | Probiotic, bioproduction | CRISPRi | Sp dCas9 | N | TD: Peters et al. (2019); MFE: Zhao et al. (2019); MGF: Afonina et al. (2020) |
| **Lactococcus lactis** | Probiotic | CRISPRi | Sp dCas9 | N | TD: Peters et al. (2019); MFE: Zhao et al. (2019); MGF: Afonina et al. (2020) |
| **Listeria monocytogenes** | Pathogen | CRISPRi | Sp dCas9 | N | TD: Peters et al. (2019); MFE: Zhao et al. (2019); MGF: Afonina et al. (2020) |
| **Paenibacillus polymyxa** | Bioproduction | CRISPRa | As dCas12a | N | TD: Peters et al. (2019); MFE: Zhao et al. (2019); MGF: Afonina et al. (2020) |
| **Paenibacillus sonchi** | Plant symbiote | CRISPRi | Sp dCas9 | N | TD: Peters et al. (2019); MFE: Zhao et al. (2019); MGF: Afonina et al. (2020) |
| **Staphylococcus aureus, S. epidermidis** | Pathogen | CRISPRi | Sp dCas9 | N | TD: Peters et al. (2019); MFE: Zhao et al. (2019); MGF: Afonina et al. (2020) |

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### Table 1 | (Continued) CRISPRi/a studies in non-model bacteria and their key characteristics.

| Bacterium | Application | CRISPRi/a | CRISPR System(s) | GW | References<sup>a</sup> and type of study |
|-----------|-------------|------------|-----------------|----|-----------------------------------------|
| **Streptococcus** | | | | | |
| S. pneumoniae, S. salivarius | Pathogen | CRISPRi | Sp dCas9 | N | TD: Bikard et al. (2013); Liu et al. (2017); MGF: Domenech et al. (2018); Gallay et al. (2021); Knoppers et al. (2022) |
| S. pneumoniae | Pathogen | CRISPRi | Sp dCas9 | N<sup>*</sup> | Y | SS: Liu et al. (2017); SS: Dewachter et al. (2021); Gallay et al. (2021); Liu et al. (2021a); de Bakker et al. (2022) |
| S. agalactiae | Pathogen | CRISPRi | Native dCas9 | N | TD: Dammann et al. (2021); MGF: Dammann et al. (2021) |
| S. mutans | Pathogen | CRISPRi | Native dCas9 | N<sup>*</sup> | TD: Shields et al. (2020); SS: Shields et al. (2020) |
| **Proteobacteria** | | | | | |
| Acidithiobacillus ferrooxidans | Bioresearch, bioremediation | CRISPRi | Sp dCas9 | N | TD: Yamada et al. (2022) |
| Acinetobacter baumannii, A. baylyi | Pathogen | CRISPRi | Sp dCas9 | N | TD: Geng et al. (2019); Peters et al. (2019); Bai et al. (2021); MGF: Bai et al. (2021); Colquhoun et al. (2021); Dai et al. (2021) |
| Aeromonas hydrophila | Bioproduction, bioresearch, bioremediation | CRISPRi | Sp dCas9 | N | TD: Wu et al. (2020); MGF: Wu et al. (2020) |
| Bartonella apis | Bee probiotic | CRISPRi | Sp dCas9 | N | TD: Leonard et al. (2018) |
| Burkholderia cenocepacia, B. multivorans, B. thailandensis | Pathogen | CRISPRi | Sp dCas9 | N | TD: Hogan et al. (2019) |
| Caulobacter crescentus | Bioresearch | CRISPRi | Spa dCas9, Sth1 dCas9 | N | TD: Guzzo et al. (2020) |
| **Chromobacterium violaceum** | Biorecovery | CRISPRi | Sp dCas9 | N | TD: Lai et al. (2019); MGF: Lai et al. (2019); Werner et al. (2020) |
| Enterobacter cloacae | Pathogen | CRISPRi | Sp dCas9 | N | TD: Li et al. (2020); MGF: Li et al. (2020); Werner et al. (2020) |
| Geobacter sulfurreducens | Bioremediation | CRISPRi | Sp dCas9 | N | TD: Liu et al. (2020); MGF: Liu et al. (2020); Werner et al. (2020) |
| Halomonas sp. TD01 | Bioproduction | CRISPRi | Sp dCas9 | N | TD: Tao et al. (2017); MGF: Tao et al. (2017) |
| **Klebsiella** | | | | | |
| K. oxytoca | Pathogen | CRISPRa | Sp dCas9 | N | TD: Ho et al. (2020) |
| K. pneumoniae, K. oxytoca, K. aerogenes | Pathogen, bioproduction | CRISPRi | Sp dCas9 | N | TD: Wang et al. (2018a); Peters et al. (2019); Ho et al. (2020); MGF: Wang et al. (2017); Wang et al. (2018a) |
| Komagataeibacter hansenii, K. xylinus | Bioproduction | CRISPRi | Sp dCas9 | N | TD: Teh et al. (2019); Huang et al. (2020); MGF: Huang et al. (2020); MGF: Huang et al. (2020) |
| Legionella pneumophila | Pathogen | CRISPRi | Sp dCas9 | N | TD: Ellis et al. (2021); MGF: Ellis et al. (2021) |
| Lysobacter enzymogenes | Bioproduction, bioresearch | CRISPRRa | Sp dCas9<sup>ω</sup> | N | TD: Yu et al. (2018); 11; MGF: Yu et al. (2018); 11 |
| Methylocorba extorquens | Bioproduction | CRISPRi | Sp dCas9 | N | TD: Mo et al. (2020); MGF: Mo et al. (2020); MGF: Mo et al. (2020); MGF: Mo et al. (2020) |
| Myxococcus xanthus | Bioproduction | CRISPRa | Sp dCas9<sup>ω</sup> | N | TD: Peng et al. (2018); Wang et al. (2021c); MGF: Peng et al. (2018); Wang et al. (2021c) |
| Photobacterium luminescens | Bioresearch | CRISPRa | Sp dCas9<sup>ω</sup> | N | TD: Ke et al. (2021); MGF: Ke et al. (2021) |
| Proteus mirabilis | Pathogen | CRISPRa | Sp dCas9<sup>ω</sup> | N | TD: Peters et al. (2019) |
| Pseudomonas | Bioproduction, bioremediation | CRISPRa<sup>ω</sup> | Sp dCas9+MCP | N | TD: Koizuma et al. (2020); MGF: Koizuma et al. (2020) |
| P. putida | Bioproduction, bioremediation | CRISPRi | Fn dCas12a | N | TD: Tan et al. (2018); MGF: Gautamm et al. (2021) |
| P. putida, P. fluorescens | Bioproduction, plant symbiote, bioremediation | CRISPRi | Spa dCas9 | N | TD: Sun et al. (2018); Noriot-Gros et al. (2019); Batanis et al. (2020); Kim et al. (2020); MGF: Noriot-Gros et al. (2019); MGF: Kim et al. (2020); MGF: Koizuma et al. (2020); Li and Ye, (2021); TRN: Liu et al. (2020b) |

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Zhao et al., 2019), or reduce the expression of the components by substituting genetic parts (Qu et al., 2019). Expansive libraries of genetic parts, including inducible and constitutive promoters, ribosome binding sites, and protein degradation tags, can be used to tune gene expression and characteristics of the CRISPRi/a tool (Depardieu and Bikard, 2020; Fleck and Grundner, 2021; Ouellette et al., 2021). However, the components cannot be expressed so low that it cannot effectively repress or activate the target gene(s), especially during multiplexed gene regulation that relies on a shared dCas protein pool for multiple gRNAs (Zhang and Voigt, 2018; Zhao et al., 2018). A careful balance is required to express the CRISPRi/a components.

### CURRENT CRISPRi/A TOOLS FOR NON-MODEL BACTERIA

Different CRISPRi/a tools have been established in a range of bacteria that span many phyla and have been used for a variety of applications, as summarized here (Table 1). Overwhelmingly, these studies have utilized the Sp dCas9 CRISPRi system. More detailed information for each study can be found in Supplementary Table S1 (Supplementary Data Sheet 1).

#### TABLE 1 | (Continued) CRISPRi/a studies in non-model bacteria and their key characteristics.

| Bacterium              | Application          | CRISPRi/a | CRISPR System(s) | GWb | References and type of study |
|------------------------|----------------------|-----------|------------------|-----|------------------------------|
| *P. aeruginosa*        | Pathogen             | CRISPRi   | Spa dCas9        | N   | TD: McMackin et al. (2019); Gauttam et al. (2021); MGF: McMackin et al. (2019); Gauttam et al. (2021) |
|                        |                      |           | Sp dCas9         | N   | TD: Peters et al. (2019); Xiang et al. (2020); Stolle et al. (2021) |
|                        |                      |           | Sp dCas9, Sth1 dCas9 | N   | TD: Qu et al. (2019); MGF: Qu et al. (2019) |
| *Rhodobacter capsulatus* | Bioproduction        | CRISPRi   | Fn dCas9, AsIA v2.1 | N   | TD: Xu et al. (2021) |
| *Salmonella enterica*  | Pathogen             | CRISPRi/a | Sp dCas9-N-ω    | N   | TD: Zhang and Yuan, (2021) |
|                        |                      | CRISPRi   | Sp dCas9-AsIA v2.1 | N   | TD: Bhokisham et al. (2020); TRN: Bhokisham et al. (2020) |
| *Shewanella oneidensis*| Bioproduction, bioresearch | CRISPRi   | As dCas12a       | N   | TD: Ho et al. (2020) |
|                        |                      |           | Sp dCas9         | N   | TD: Peters et al. (2019); Ho et al. (2020) |
|                        |                      |           | Ec Type I-E      | N   | TD: Rath et al. (2015) |
| *Sorangium cellulosum* | Bioresearch          | CRISPRi/a | Sp dCas9-VP64    | N   | TD: Li et al. (2020a); MGF: Li et al. (2020a) |
| *Vibrio casei*         | Bioproduction        | CRISPRi   | Sp dCas9         | N   | TD: Cao et al. (2017); ME: Yi and Ng, (2021) |
| *Vibrio natriegens*    | Pathogen             | CRISPRi   | Sp dCas9         | N   | TD: Peters et al. (2019) |
| *Vibrio cholerae*      | Pathogen             | CRISPRi   | Sp dCas9         | N   | TD: Lee et al. (2019); SS: Lee et al. (2019) |
| *Yersinia pestis*      | Bioproduction        | CRISPRi   | Sp dCas9         | N   | TD: Caro et al. (2019); Wiles et al. (2020); MGF: Caro et al. (2019); Wiles et al. (2023) |
| *Zymomonas mobilis*    | Bioproduction        | CRISPRi   | Sp dCas9         | N   | TD: Wang et al. (2018) |
|                        |                      |           | Sp dCas9         | N   | TD: Banta et al. (2020); MGF: Banta et al. (2020) |
| *Spirochaetes*         |                      | CRISPRi   | Sp dCas9         | N   | TD: Zheng et al. (2019) |
| *Borreliella burgdorferi* | Bioproduction       | CRISPRi   | Sp dCas9         | N   | TD: Takacs et al. (2020) |
| *Leptospira interrogans, L. biflexa, L. strain LGVFr02* | Bioproduction | CRISPRi | Sp dCas9         | N   | TD: Fernandez et al. (2019); Fernandez et al. (2021); Fernandez et al. (2021b); MGF: Fernandez et al. (2021b) |
| *Tenericutes*          | Synthetic cells      | CRISPRi   | Sp dCas9         | N   | TD: Mariscal et al. (2018) |

*Acronyms for each CRISPR system can be found in Supplementary Table S2 (Supplementary Data Sheet 1).

*Genome-wide (GW) classification for the relative size of the gRNA library: yes (Y) indicates a genome-wide library targeting >90% of coding genes on the genome; no (N) indicates a library of <50 target genes; and a smaller library targeting >50 genes but <90% of genome is indicated (N*).

*Classifications for types of studies: tool development (TD), mapping gene function (MGF), metabolic engineering (ME), screens and/or selections (SS), transcriptional regulatory network (TRN).*

Actinomycetota

CRISPRi has been well established in a wide range of Actinobacteria, including *Mycobacteria*, *Streptomyces*, and *Corynebacterium*, and has been used for metabolic engineering and the elucidation of gene functions in both small studies and genome-wide screens (Table 1). Additionally, several CRISPRi tools are commonly used in *Mycobacteria* (Choudhary et al., 2015; Rock et al., 2017; Agarwal, 2020; Fleck and Grundner, 2021) and *Streptomyces* (Tong et al., 2015; Li et al., 2018; Zhao et al., 2018). CRISPRa has also been recently established in *Corynebacterium* (Liu W. et al., 2019) and *Streptomyces* (Ameruoso et al., 2021).
Cyanobacteria
CRISPRi/a is especially useful in cyanobacteria due to their polyploidal genomes (Kirtania et al., 2019). CRISPRi is relatively well-established in a wide range of cyanobacterial species, including those of research and industrial significance, and has been used for metabolic engineering, transcriptional regulatory networks, and the study of gene functions in small studies and a genome-wide screen/selection (Table 1). Many CRISPRi tools are available in cyanobacteria, each with their own characteristics (Gordon et al., 2016; Yao et al., 2016; Liu D. et al., 2020; Choi and Woo, 2020). CRISPRa has not yet been reported in cyanobacteria.

Firmicutes
CRISPRi is well-established in a wide range of Firmicutes, including Bacilli, Clostridia, Staphylococci, and Streptococci (Table 1). CRISPRi tools have been developed and used for metabolic engineering, elucidation of gene functions, and genome-wide screens and selections. CRISPRa has been reported in Bacillus amyloliquefaciens and Paenibacillus polymyxa in tool development work and some metabolic engineering applications (Schilling et al., 2020; Zhao et al., 2020).

Proteobacteria
CRISPRi and CRISPRa are well established in a wide variety of Proteobacteria, including Klebsiella, Salmonella, Pseudomonas, and Vibrio (Table 1). These tools have been developed and used for metabolic engineering, synthetic transcriptional regulatory networks, and mapping gene function using small gRNA sets and genome-wide screens and selections. Reports of CRISPRi are far more common than CRISPRa.
**Other Bacterial Phyla**

CRISPRi has also been reported in the phyla Chlamydiae, Tenericutes, Spirochaetes, and Bacteroidetes (Table 1). Although these reports have primarily been for tool development, some have used CRISPRi to investigate gene function (Fernandes et al., 2021b; Brockett et al., 2021) or create synthetic genetic circuits (Mimee et al., 2015; Taketani et al., 2020).

**APPLICATIONS OF CRISPRi/a IN NON-MODEL BACTERIA**

CRISPRi/a tools can be used for a variety of applications in non-model bacteria (Figure 1). The most common application is mapping a gene’s function by altering its gene expression and assaying cellular phenotypic change under some applied selective condition (Figure 1A). CRISPRi is particularly useful for investigating essential genes because its repression can be titrated to prevent full knockdown and cell death (Knooth et al., 2020; Bosch et al., 2021). Additionally, epistatic effects of multiple genes can easily be investigated by simply expressing multiple gRNA within the same cell (Ellis et al., 2021; McNeil et al., 2022). Although not as common as CRISPRi due to stricter design rules (Fontana et al., 2020a), CRISPRa can be used to induce expression of silent genes to investigate their functions and products, including entire silent biosynthetic gene clusters (Ke et al., 2021). Combined, these are the most common use of CRISPRi/a tools in non-model bacteria, with 80 reports across six phyla (Table 1) (Behler et al., 2018; Stamsås et al., 2018; Ke et al., 2021). The recent development of Mobile-CRISPRi (Peters et al., 2019), CRAGE-CRISPR (Ke et al., 2021), and a workflow for introducing genetic manipulation tools into non-model gut bacteria (Jin et al., 2022) will facilitate the expansion of CRISPRi/a tools into new species and strains, including recalcitrant pathogens and novel species without sequenced genomes.

Additionally, CRISPRi/a can be used to control transcription regulatory networks, such as genetic circuits, by designing and expressing gRNA to regulate the output promoter for each logic gate or node (Figure 1B). CRISPRi/a is especially effective for controlling complex synthetic transcription regulatory networks as the gRNA can be designed to target nearly any arbitrary sequence with an appropriate PAM (or equivalent) sequence (Taketani et al., 2020; Ellis et al., 2021). CRISPRi/a circuits can be fully synthetic and auxiliary to the native genetic regulatory networks, such as a heterologous sensor or multi-input circuit that senses and responds to external inputs in complex environments (Mimee et al., 2015; Taketani et al., 2020). Alternatively, CRISPRi/a can be interfaced with native gene regulatory systems to control the host’s metabolism in response to external stimuli, such as cell density, through either heterologous (Liu Y. et al., 2020) or even indigenous sensor systems (Tian et al., 2020). However, caution must be taken to prevent the expression of too many gRNA at once since they compete over the limited dCas protein resource and, thus, can decrease the repression of target genes (Del Vecchio et al., 2008; Li et al., 2018; Zhang and Voigt, 2018). Synthetic CRISPRi/a regulatory networks are rare in non-model bacteria, having been reported in only seven studies across four phyla, and primarily incorporate CRISPRi (Table 1). However, a single CRISPRa genetic circuit in *Salmonella* has been reported (Bhokisham et al., 2020).

CRISPRi/a tools have also been used to redirect carbon and energy flow for metabolic engineering in non-model bacteria (Figure 1C). CRISPRi is often used to repress a native gene(s), including essential genes, to redirect carbon flux towards a desired product (Wang et al., 2017; Shabestary et al., 2018) or bioactive molecule (Yu et al., 2018; Liu et al., 2021b). CRISPRa can be used to activate the desired metabolic pathway to increase biosynthesis of the desired product, such as an anti-cancer drug in a weakly-expressed biosynthetic gene cluster (Peng et al., 2018; Ye et al., 2019). In most examples, the CRISPRi/a components are constitutively expressed, yet some studies employ dynamic metabolic engineering strategies by utilizing inducible systems and/or genetically encoded biosensors to switch between cell growth and product biosynthesis states to improve production (Liu Y. et al., 2020; Tian et al., 2020; Shabestary et al., 2021). These tools can be used to tune endogenous metabolism and/or heterologous metabolic pathways (Peng et al., 2018; Banerjee et al., 2020). CRISPRi/a tools are most often combined with other metabolic engineering techniques, such as the deletion, overexpression, or mutation of select genes and optimization of medium, to further increase titers of the desired product (Park et al., 2019; Dietsch et al., 2021; Kozaeva et al., 2021).

Large-scale CRISPRi screens and selections have been developed to investigate genotype-phenotype relationships through gRNA fitness (Figure 1D). These assays can use small, targeted libraries, such as essential genes or genes in a metabolic pathway (Shields et al., 2020; Göttl et al., 2021), or large genome-wide libraries targeting nearly all genes in the bacterial genome (Lee et al., 2019; Jiang et al., 2020). Additionally, CRISPRi libraries can be constructed in two major forms—pooled libraries, where cells containing different gRNA are mixed during library construction (Bosch et al., 2021; Rahman et al., 2021), a strategy known as multiplexing, or arrayed libraries where different gRNA designs are constructed individually in different clonal populations, typically arrayed in microtiter plates (Liu et al., 2017; Göttl et al., 2021). Pooled competitive selections are more common due to the ease of DNA construction and analysis of large, genome-scale gRNA libraries with >10,000 designs by next-generation sequencing (Lee et al., 2019; Bosch et al., 2021). However, because all cells directly compete in pooled competitive growth assays, “cheaters” may arise that take advantage of different strain interactions, so the results of any individual gRNA design should be verified in isolation (Yao et al., 2020; Liu X. et al., 2021). Additionally, the results from these pooled CRISPRi screens or selections are specific to the gRNA design and not the target gene since confounding effects (i.e., off-target effects) could produce false positives or negatives, so careful design of gRNA libraries is vital (Cui et al., 2018; Wang T. et al., 2018). Genome-wide CRISPRi screens or selections are relatively uncommon (Table 1). While not demonstrated to date, genome-wide bacterial CRISPRa is theoretically possible, provided the design rules for activation are met (Fontana et al., 2020a).
CONCLUSION AND PERSPECTIVES

CRISPRi has been established in non-model bacteria across eight phyla and applied from small, single gene functional studies to large genome-wide screens. The creation of new tools and protocols for introducing CRISPRi/a into non-model bacteria will facilitate the continuation of this rapid expansion. Several novel and exciting CRISPRa tools with greater activation and unique characteristics have been developed recently in both model and non-model bacteria, yet there remains a need for stronger and more versatile bacterial CRISPRa tools, especially for the activation of native genes. These bacterial CRISPRa tools have lagged behind the development of both eukaryotic CRISPRa tools and bacterial CRISPRi tools. However, the recent development of several new CRISPRa systems with less stringent design rules and higher levels of activation (>10-fold) shows great promise for effective, tailored gene activation in bacteria (Liu Y. et al., 2019; Fontana et al., 2020a; Ho et al., 2020; Villegas Kcam et al., 2021). These CRISPRa technologies were created using directed evolution and thorough tool design. Further improvements could be achieved by creating CRISPRa tools from CRISPR systems with more relaxed PAM requirements, directed evolution of CRISPRa components (activator domain, gRNA scaffold(s), and dCas protein) for greater activation, and high-throughput screening of gRNAs and promoters to uncover additional nuanced design rules for a given tool. CRISPRa has the potential to become a more effective and widely used tool for programmable gene activation in both model and non-model bacteria for a variety of industrial and research applications, such as metabolic engineering and elucidation of gene function. While many CRISPRi/a approaches in non-model bacteria have been established using genetic parts that are not well-defined or characterized, the creation of comprehensive genetic part toolboxes for these strains, which are vital for the rational design and precise control of CRISPRi/a tools, will accelerate further development and optimization of the tools. Finally, CRISPRi/a approaches have primarily been developed for more genetically tractable strains of non-model bacteria. There is a need for efficient workflows to domesticate and introduce CRISPRi/a tools to novel bacterial species and strains. Despite these current challenges, CRISPRi/a technology remains a versatile approach for programmable transcriptional regulation in non-model bacteria.

AUTHOR CONTRIBUTIONS

SC and LA conceived of the review and wrote the manuscript. All authors read and approved the manuscript.

FUNDING

This material is based upon work supported by the National Science Foundation under Grant No. DMR-1904901 to LA. Additional funding for this work was provided by startup funds to LA from the University of Massachusetts Amherst and a seed grant award from the UMass ADVANCE program funded by the National Science Foundation (awards #1824090 and #2136150). This work is also supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-1451512 to SC.

ACKNOWLEDGMENTS

The authors would like to thank members of the Andrews research group for their discussions contributing to the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgened.2022.892304/full#supplementary-material

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