Engineering Microbiology 3 (2023) 100089

Contents lists available at ScienceDirect

Engineering Microbiology

journal homepage: www.elsevier.com/locate/engmic

Review

Genome-scale CRISPRi screening: A powerful tool in engineering microbiology

Letian Sun[a,b], Ping Zheng[a,b,c], Jibin Sun[a,b,c], Volker F. Wendisch[d], Yu Wang[a,b,c,*]

[a] Key Laboratory of Engineering Biology for Low-carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] National Technology Innovation Center of Synthetic Biology, Tianjin 300308, China
[d] Genetics of Prokaryotes, Faculty of Biology and CeBiTec, Bielefeld University, Bielefeld, Germany

ARTICLE INFO

Keywords:
CRISPR interference
Genome-scale library
Pooled screening
Arrayed screening
Genotype-phenotype mapping
Functional genomics

ABSTRACT

Deciphering gene function is fundamental to engineering of microbiology. The clustered regularly interspaced short palindromic repeats (CRISPR) system has been adapted for gene repression across a range of hosts, creating a versatile tool called CRISPR interference (CRISPRi) that enables genome-scale analysis of gene function. This approach has yielded significant advances in the design of genome-scale CRISPRi libraries, as well as in applications of CRISPRi screening in medical and industrial microbiology. This review provides an overview of the recent progress made in pooled and arrayed CRISPRi screening in microorganisms and highlights representative studies that have employed this method. Additionally, the challenges associated with CRISPRi screening are discussed, and potential solutions for optimizing this strategy are proposed.

1. CRISPRi: a CRISPR-derived tool for repression of gene expression

The clustered regularly interspaced short palindromic repeats (CRISPR) system, which was initially identified as a bacterial antiviral defense system, has been widely adopted for genome editing in various organisms. This method typically involves using the CRISPR-associated protein (Cas) in combination with a guide RNA (gRNA) to induce double-strand breaks (DSBs) in the target gene. These DSBs can then be repaired by the cell through homology-directed repair (HDR) or non-homologous end joining (NHEJ), resulting in the deletion or disruption of the target gene, or the insertion of a DNA fragment of interest [38]. By introducing specific mutations to eliminate the DNA cleavage activity of Cas, the CRISPR system can also be repurposed to repress gene expression, a process known as CRISPR interference (CRISPRi) [66].

Over time, with sufficient interest, various types of CRISPRi systems have been developed, including DNA-targeting systems like CRISPR-dCas9 and CRISPR-dCas12a, and RNA-targeting systems like CRISPR-Cas13. The most commonly used system is the class 2 type II-A CRISPR-Cas system, with the *Streptococcus pyogenes* CRISPR-Cas9 reported in 2012 serving as a representative example (Fig. 1a). Cas9 is an endonuclease that can cut both strands of DNA with blunt cleavage, with the RuvC and HNH domains responsible for generating the DSBs [35]. In, Qi et al. introduced amino acid substitutions in the RuvC (D10A) and HNH (H840A) domains to generate a nuclease-deficient Cas9 mutant (dCas9) that retains its ability to bind to target DNA when guided by a gRNA. The CRISPR-dCas9 system can therefore block transcription initiation or elongation, enabling effective repression of gene expression in prokaryotes [66]. In eukaryotes, the fusion of dCas9 with a transcriptional repressor domain is required for effective gene silencing [28].

The class 2 type V-A CRISPR-Cas system is also a popular choice due to its unique features including staggered cleavage. The *Francisella novicida* CRISPR-Cas12a (also known as Cpf1) was reported in 2015 as a representative of this system (Fig. 1b). Unlike Cas9, Cas12a possesses both DNase and RNase activities, which allow it to process CRISPR RNA (crRNA) arrays into mature gRNAs by itself [109]. Cas12a lacks the HNH domain but contains the RuvC domain and a putative novel nuclease domain (Nuc) [102]. To obtain dCas12a variants with loss of cleavage activity against both strands of target DNA but intact RNase activity, certain mutations of the RvuC and Nuc domains (such as D917A or E1006A for *F. novicida* Cas12a) were used [109]. CRISPR-dCas12a has been a promising tool for multiplex gene repression due to the convenience of designing and processing multiple gRNAs from a signal crRNA array [112].

While single-effector class 2 CRISPR-Cas systems have been the most commonly used, multi-subunit class 1 systems comprise about 90% of all identified systems [55]. The engineering of the multi-subunit RNA-

* Corresponding author.
E-mail address: wang_y@tib.cas.cn (Y. Wang).

https://doi.org/10.1016/j.engmic.2023.100089
Received 31 January 2023; Received in revised form 5 April 2023; Accepted 9 April 2023
Available online 20 April 2023

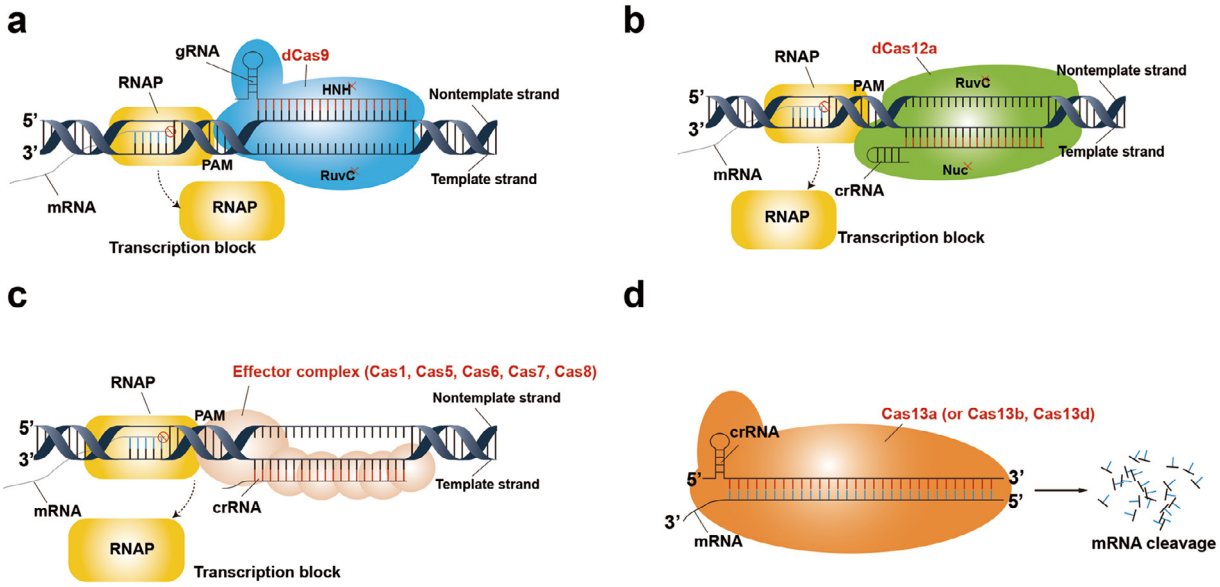

Fig. 1. Different forms of CRISPRi systems. (a) Class 2 type II dCas9-based CRISPRi for transcriptional blocking. (b) Class 2 type V dCas12a-based CRISPRi for transcriptional blocking. (c) Class 1 type I Cascade-based CRISPRi for transcriptional blocking. (d) Class 2 type VI Cas13-based CRISPRi for mRNA cleavage.

guided complex, such as excluding the Cas3 nuclease component, allows class 1 CRISPR-Cas systems to be used for transcriptional inhibition (Fig. 1c). The CRISPRi technologies have been successfully demonstrated in both the native microbial hosts and mammalian cells in 2019 [65].

Not all the CRISPR-Cas systems target DNA. Recent studies have shown that the class 2 type VI CRISPR-Cas systems, such as Cas13a from *Leptotrichia wadei* [1], Cas13b from *Bergeyella zoohelcum* [79], and Cas13d from *Ruminococcus flavefaciens* [39], are RNA-guided and RNA-targeting nucleases. Cas13 has dual RNase activities and can process crRNA arrays into mature gRNAs and cleave target RNAs. This unique feature of Cas13 has been utilized for developing CRISPRi technologies by degrading target mRNA (Fig. 1d).

2. Development of CRISPRi technologies in microorganisms

Because CRISPRi is easily programmed, it has become a popular tool for studying gene function and engineering microbial physiology and metabolism, particularly in medically and industrially relevant microorganisms (Table 1). *Escherichia coli* is a commonly used model microorganism for the design and test of various types of CRISPRi systems. In, Qi et al. first demonstrated that the class 2 type II-A dCas9-gRNA complex from *S. pyogenes* can effectively silence transcription initiation and elongation in an inducible and reversible manner in *E. coli*. Gene expression was repressed was by 10- to 300-fold by gRNAs targeting the non-template DNA strand, while those targeting the template DNA strand showed little repressive effect. Double gene repression was also demonstrated by expressing two independent gRNAs with distinct complementary regions to two reporter genes. CRISPRi-mediated gene silencing was found to be highly specific without detectable off-target effects [66]. Since the initial success of dCas9-based CRISPRi, various types of CRISPRi systems, both class 1 and class 2, have been demonstrated in *E. coli*. *E. coli* has a native class 1 type I-E CRISPR-Cas system consisting of two protein elements for DNA targeting: a multimeric complex of five different Cas proteins responsible for processing crRNA arrays and binding target DNA (designated as Cascade) and a nuclease responsible for cleaving target DNA (designated as Cas3) [5]. In 2015, two research groups repurposed the endogenous CRISPR-Cas system for programmable gene repression independently by deleting the chromosomal *cas3* copy and expressing Cascade and crRNA arrays. These systems proved effective in reducing the mRNA levels of target genes, with reductions ranging from 11-fold to 2200-fold [54,67]. In 2016, the class 2 type V-A CRISPR-dCas12a from *F. novicida* was used as a CRISPRi screening tool to identify the functional protospacer-adjacent motifs (PAMs) that are essential for DNA target recognition [42]. Later, Zhang et al. utilized the CRISPR-dCas12a system from *Acidaminococcus* sp. for multiplex gene regulation, which was able to repress up to six genes simultaneously by expressing a crRNA array with six spacers [112]. In addition to these DNA-targeting CRISPRi systems that inhibit transcription, several RNA-targeting CRISPRi systems have been developed that directly cleave and degrade mRNA. These systems include the class 1 type III-E Cas7–11 from *Desulfonema ishimotonii* [61], the class 2 type VI-A Cas13a from L. *wadei* [1], the class 2 type VI-B Cas13b from *B. zoohelcum* [79], and the class 2 type VI-D Cas13d from *R. flavefaciens* [39]. Researchers have also tested these RNA-targeting CRISPRi systems in *E. coli*. However, while the Cas13d from *R. flavefaciens* has shown efficient RNA interference against target mRNAs in *E. coli*, it has also been observed to cause growth retardation. This may be due to the collateral cleavage activity of Cas13d toward bystander RNAs [111].

According to recent data, CRISPRi systems are now available for over 80 bacterial species across eight bacterial phyla, as well as numerous fungi and archaea [7,53,72,82]. This powerful technology has been applied successfully in a variety of industrially relevant microorganisms such as *Saccharomyces cerevisiae* [16,28], *Corynebacterium glutamicum* [17,44], *Bacillus subtilis* [97], *B. methanolicus* [73], and *Streptomyces* [86] for metabolic engineering of biosynthesis. In pathogenic microorganisms like *Mycobacterium tuberculosis* [15], *Staphylococcus aureus* [12], *S. pneumoniae* [3], and *Listeria monocytogenes* [64], CRISPRi can be used to identify genetic factors responsible for drug resistance and discover novel drug targets [14,90].

3. Genome-scale CRISPRi screening in microorganisms

CRISPRi is a highly efficient gene repression tool that is compatible with a wide range of microbial hosts. Programming the tool is easy as the target-coding region of the gRNA is comprised of only ~20 nucleotides. This feature enables the synthesis of massive oligonucleotides using microarray technology and facilitates monitoring of the gRNA as a barcode with next-generation sequencing (NGS) [92]. As a result, CRISPRi has been readily adopted for constructing genome-scale gRNA libraries

Table 1
Development of CRISPRi technology across representative medically and industrially relevant microorganisms.

Superkingdoms	Orders	Species	Effector	Refs.
Eukaryota	*Eurotiomycetes*	*Saccharomyces cerevisiae*	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[108]
			Class 2 type V-A, dCas12a from *Acidaminococcus* sp., *Lachnospiraceae bacterium*, and *Moraxella bovoculi*	[106]
	Saccharomycetes	*Yarrowia lipolytica*	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[74]
			Class 2 type V-A, dCas12a from *Francisella novicida*	[110]
		Aspergillus niger	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[46]
Bacteria	*Clostridia*	*Clostridium* sp.	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[95]
			Class 2 type V-A, dCas12a from *Francisella novicida*	[36]
	Gammaproteobacteria	*E. coli*	Class 1 type I-E, native Cas complexes from *E. coli*	[54]
			Class 1 type III-E, Cas7–11 from *Desulfonema ishimotonii*	[61]
			Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[66]
			Class 2 type V-A, dCas12a from *Francisella novicida*	[42]
			Class 2 type V-A, dCas12a from *Acidaminococcus* sp.	[112]
			Class 2 type V-A, dCas12a from *Eubacterium eligens*	[37]
			Class 2 type V-A, dCas12a from *Lachnospiraceae bacterium*	[57]
			Class 2 type VI-A, Cas13a from *Leptotrichia shahii*	[2]
			Class 2 type VI-A, Cas13a from *Leptotrichia wadei*	[1]
			Class 2 type VI-B, Cas13b from *Bergeyella zoohelcum*	[79]
			Class 2 type VI-D, Cas13d from *Eubacterium siraeum* and *Ruminococcus* sp.	[103]
			Class 2 type VI-D, Cas13d from *Ruminococcus flavefaciens*	[111]
		Klebsiella pneumoniae	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[91]
		Shewanella oneidensis	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[9]
			Class 2 type V-A, dCas12a from *Acidaminococcus* sp.	[43]
		Pseudomonas sp.	Class 2 type II-A, dCas9 from *Streptococcus pasteurianus*	[85]
		Vibrio alginolyticus	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[84]
		Vibrio natriegens	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[40]
	Alphaproteobacteria	*Zymomonas mobilis*	Class 1 type I-F, native Cas complexes from *Z. mobilis*	[114]
	Cyanophyceae	*Synechococcus elongatus*	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[33]
			Class 2 type V-A, dCas12a from *Francisella novicida*	[13]
		Anabaena sp.	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[31]
		Synechocystis sp.	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[104]
			Class 2 type V-A, dCas12a from *Francisella novicida*	[49]
	Bacilli	*Streptococcus mutans*	Class 2 type II-A, native dCas9 from *Streptococcus mutans* and dCas9 from *Streptococcus pyogenes*	[77]
		Streptococcus pneumoniae	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[51]
		Staphylococus aureus	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[113]
		Bacillus subtilis	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[97]
			Class 2 type V-A, dCas12a from *Francisella novicida*	[100]
	Actinomycetes	*Mycobacterium tuberculosis*	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[15]
			Class 2 type II-A, dCas9 from *Streptococcus thermophilus*	[69]
			Class 2 type V-A, dCas12a from *Francisella novicida*	[24]
		Mycobacterium smegmatis	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[15,101]
			Class 2 type II-A, dCas9 from *Streptococcus thermophilus*	[69]
			Class 2 type V-A, dCas12a from *Francisella novicida*	[24]
		Streptomyces coelicolor	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[86]
		Corynebacterium glutamicum	Class 2 type II-A, dCas9 from *Streptococcus pyogenes*	[17]
			Class 2 type V-A, dCas12a from *Francisella novicida*	[44]

for investigating genotype-phenotype associations at a genome level. Genome-scale libraries can be categorized into pooled and arrayed libraries, depending on their organization form (Fig. 2). Genome-scale CRISPRi screening, based on both pooled and arrayed libraries, has been successfully implemented in a few microorganisms with industrial and medical importance for functional genomics research (Table 2). Because of the simplicity and efficiency of the CRISPRi system based on the *S. pyogenes* class 2 type II-A dCas9, all the reported genome-scale CRISPRi libraries have utilized this system.

3.1. Pooled CRISPRi screening

Pooled CRISPRi screening offers a powerful tool for investigating the connection between genotype and a specific phenotype in a highly efficient and parallel manner. With the aid of sophisticated gRNA design algorithms and chip-based DNA synthesis technologies, it is now possible to create thousands of oligonucleotides targeting most, if not all, genes in the host genome, facilitating the construction of a genome-scale CRISPRi library [8]. The synthesized oligonucleotides are then pooled and integrated into a plasmid for gRNA expression, followed by transformation into the desired strain to identify genes for essentiality, tolerance, biosynthesis, or other phenotypes of interest (Fig. 2a).

3.1.1. *E. coli*

CRISPR-based functional genomics screening, which has been well-established in mammalian cells, was first tested in *E. coli* as a microbial model [75,92]. The success of CRISPRi screening depends heavily on the design of the gRNA pool. To investigate the rules of pooled gRNA library design, like the gRNA position in open reading frame (ORF) where CRISPRi maximally represses gene expression and the minimal gRNA number per gene to maintain reliable hit gene calling, Wang et al. performed a screening test with over 2000 gRNAs targeting 44 genes with known phenotypes. Through exploration of the rules governing pooled gRNA library design, the data from this test were used to develop an algorithm for the design of a comprehensive pooled CRISPRi library (55,671 gRNAs), covering 98.6% of 4140 protein-coding genes and 79.8% of 178 non-coding RNAs (ncRNAs) genes. This library was used to identify essential, auxotrophic, and chemical tolerance-related genes [92]. In comparison to transposon mutant libraries, which typ-

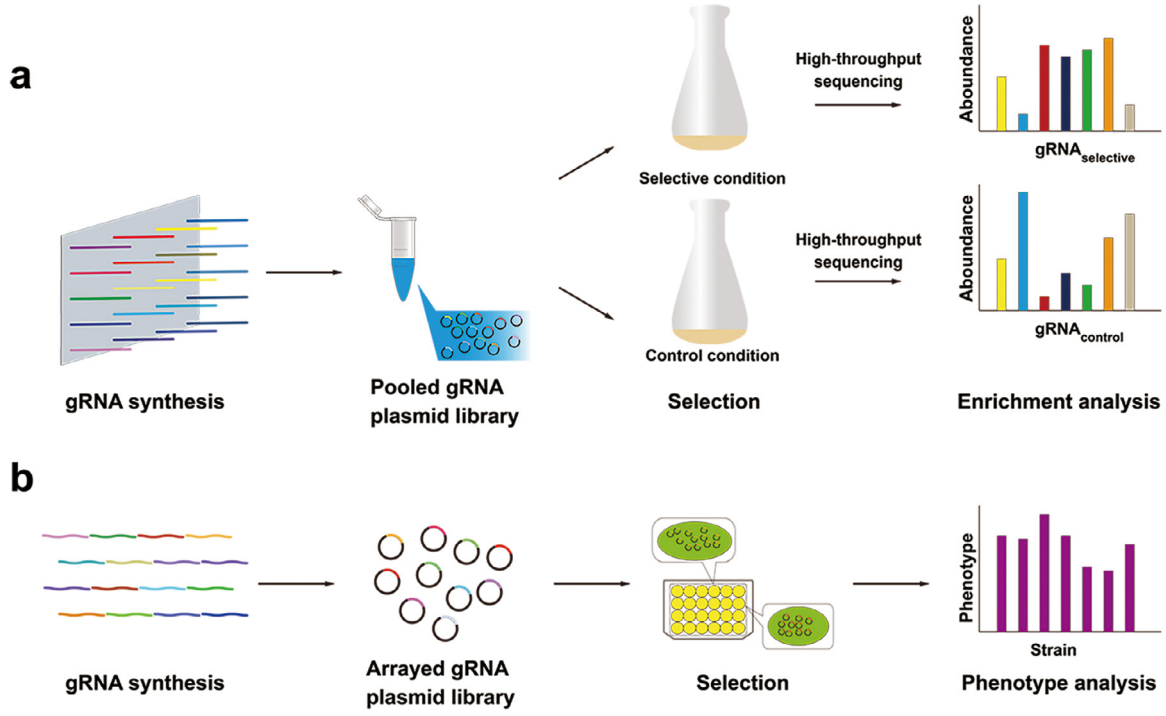

Fig. 2. CRISPRi screening in the pooled (a) and arrayed (b) forms.

ically cover only 80% of protein-coding genes with more densely distributed insertions near the origin of replication [26,98], the CRISPRi method demonstrated higher genome coverage and better distribution uniformity, providing substantial benefits for microbial functional genomics research. Cui et al. also investigated the gRNA design rules of dCas9-based CRISPRi in *E. coli* [18], and designed a pooled library with ~92,000 unique gRNAs targeting random positions throughout the genome of *E. coli*, with only the simple requirement of an "NGG" PAM. The screening and sequencing data of gRNAs targeting essential genes revealed different effects of target strand, orientation, polar effect, distance to the stop codon, and relative position inside the gene on the efficiency of CRISPRi. A machine-learning approach revealed that gRNAs sharing specific 5-nucleotide seed sequences were highly toxic to cells, particularly at high dCas9 concentrations. Moreover, a 9-nt match to the seed sequence was sufficient to produce off-target effects. This information provides important design rules for the safe use of dCas9 in *E. coli* [18]. This pooled CRISPRi library was then used to identify essential genes in *E. coli* during growth in rich medium and genes required by phages to kill their host [71].

Feng et al. devised a counter-selection system to explore the effects of gRNA-target DNA mismatches on dCas9 binding activity in *E. coli* [22]. The system coupled binding affinity with cell growth and involved a *sacB* (sucrose lethal gene) cassette that was continuously expressed, and its promoter was modified to include a target DNA for dCas9 binding. To incorporate single- and double-nucleotide mismatches, two libraries were constructed from two distinct gRNAs, each with no off-target hit in *E. coli* genome. NGS analysis of the counter-selected libraries revealed that double mismatches resulted in more significant activity loss. The mismatch-activity landscape was primarily influenced by the thermodynamic properties of base-pairing coupled with strand invasion. This information was then used to create a predictive tool for gRNA design in CRISPRi applications in *E. coli* [22]. As mismatches between gRNAs and target DNA influence dCas9 binding, introducing mismatches is an effective approach for tuning CRISPRi-based transcriptional repression. Hawkins et al. created a comprehensive library of mismatched gRNAs (3201 elements) targeting the green fluorescent protein (GFP) gene and assessed their ability to repress transcription in *E. coli* and *B. subtilis* using fluorescence activated cell sorting (FACS) followed by NGS. [30]. Surprisingly, mismatched gRNAs generated a full range of repression in both bacteria despite their evolutionary distance. The data were then utilized to develop a species-independent linear model for designing mismatched gRNAs with a defined relative repression activity. The study further investigated the expression-fitness relationships of approximately 300 essential genes in *E. coli* and *B. subtilis* using two mismatched gRNA libraries (>30,000 elements each), predicted to span the full range of knockdown levels [30].

The high compatibility allows a developed CRISPRi system to function effectively in most *E. coli* isolates and closely related Enterobacteriaceae species [70]. Rousset et al. constructed an *E. coli* core genome library, consisting of 11,629 gRNAs targeting 60–80% of the protein-coding genes of any *E. coli* strain and 100% of rRNAs, 75–85% of tRNAs, and 15–25% of annotated ncRNAs. This library was used to compare the essentiality of core genes from 18 *E. coli* isolates with different genetic backgrounds, revealing substantial variation in essentiality at the strain level [70].

Pooled CRISPRi libraries require high-throughput screening methods to be effectively utilized. For studying gene essentiality, auxotrophy, and chemical tolerance, the desired phenotype was linked with cell growth rate and thus growth-based screening methods were employed. Fluorescence enrichment analysis has also been combined with CRISPRi for pooled screening. Li et al. constructed a genome-scale gRNA library comprising 12,238 elements targeting ORFs and non-coding genes to screen for genes that impaired biomass formation while maintaining or increasing protein production. They used GFP as a reporter and FACS to facilitate the discovery of growth switches suitable for decoupling growth and production in *E. coli* [45]. Biosensors can transmit the product concentration into easily detectable signals including fluorescence. Wang et al. combined biosensors for *p*-coumaric acid and butyrate with a mismatched CRISPRi library targeting 20 genes involved in central carbon metabolism of *E. coli*. Target genes and specific gRNA variants with mismatches that favored the biosynthesis of *p*-coumaric acid and butyrate were successfully selected using this method [89].

Table 2
Genome-scale CRISPRi screening across microorganisms[a].

Species	Library type	Scope	Application	Refs.
E. coli	Pooled	>30,000 mismatched gRNAs targeting ~300 essential genes	Characterize the expression-fitness relationships of essential genes	[30]
		55,671 gRNAs targeting 4140 protein-coding genes as well as 178 RNA-coding genes	Screen for essential, auxotrophic, chemical tolerance-related, and L-tryptophan biosynthesis genes	[92]
		~ 92,000 gRNAs targeting random positions in the genome	Reveal design rules of gRNA and identify essential genes as well as those providing phage resistance	[18,71]
		11,629 gRNAs targeting 60–80% of the protein-coding genes, 100% of rRNAs, 75–85% of tRNAs, and 15–25% of annotated ncRNAs	Reveal extensive variation in gene essentiality across strains and conditions	[70]
		Random double mismatch gRNAs targeting 20 genes involved in the central carbon metabolism	Combine CRISPRi and biosensors for *p*-coumaric acid and butyrate overproduction phenotype screening	[89]
		12,238 gRNAs targeting 4441 coding sequences and non-coding regions	Screen genes for inhibition of growth while allowing for continued protein production	[45]
		32,992 gRNAs targeting 4457 genes and gene-like elements, 7442 promoters and transcription start sites, and 1060 transcription factor binging sites	Screen essential genes and explore gRNA design rules	[68]
			Screen phage resistance determinants	[60]
		462,382 gRNAs covering 4498 genes with a single average gene targeted by 103 spacers	Validate the efficiency of the CRISPR adaptation-mediated construction of gRNA libraries	[34]
	Arrayed	108 genes related to free fatty acid metabolism	Identify genes beneficial for free fatty acid yield	[21]
		56 phosphatase-encoding genes	Identify genes impacting terpenoid biosynthesis	[93]
		80 NADPH-consuming enzyme-encoding genes and 400 ATP-consuming enzyme-encoding genes	Analyze the effects of genes involved in energy metabolism on 4-hydroxyphenylacetic acid biosynthesis	[76]
		Approximately 500 strains for essential genes	Investigate genes involved in morphology maintenance	[78]
Saccharomyces cerevisiae	Pooled	37,870 gRNAs targeting all ORFs and RNA genes	Combine CRISPRi with CRISPRd and CRISPRa to draw correlations between genotype and phenotype	[48]
		61,094 gRNAs targeting all annotated protein-coding genes	Identify essential genes	[56]
		>51,000 gRNAs targeting essential genes	Screen dosage-sensitive genes and genes involved in the biosynthesis of adenine and arginine	[58]
	Arrayed	9059 arrayed strains from a pooled library of >18,000 gRNAs targeting 1117 essential genes and 514 genes required for respiratory growth	Identify genes which lead to increased tolerance to acetic acid	[59,80]
Corynebacterium glutamicum	Pooled	46,549 gRNAs targeting 99.71% of protein-coding genes and 85.36% of non-coding genes	Combine CRISPRi and a microfluidics system for screening genes related to VHH secretion	[107]
	Arrayed	397 genes encoding membrane transporters	Identify the gene encoding an L-proline exporter	[50]
		74 genes with potential relevance in carotenogenesis	Identify genes responsible for improving carotenoid production	[29]
Vibrio natriegens	Pooled	13,567 gRNAs targeting predicted protein-coding genes	Identify a minimal set of genes required for rapid growth	[40]
V. alginolyticus	Pooled	Dual-gRNA libraries targeting 35 × 35 dual histidine kinase genes (1225 combinations) and 24 × 24 dual glycine-pathway genes (576 combinations)	Decode the interfering impact of histidine kinases on antibiotic resistance and the impact of glycine pathway genes on glycine production	[84]
Synechocystis sp.	Pooled	10,498 gRNAs targeting 3546 ORFs and 1871 non-coding RNA genes	Screen for growth advantage, L-lactate tolerance, and L-lactate production related genes	[105]
Bacillus subtilis	Pooled	>30,000 mismatched gRNAs targeting ~300 essential genes	Characterize expression-fitness relationships between most essential genes	[30]
	Arrayed	289 known or proposed essential genes	Screen essential genes involved in antibiotic resistance and morphological change	[63]
Streptococcus pneumoniae	Pooled	1499 gRNAs targeting 98.4% of all annotated genetic features	Explore bacterial bottlenecks and virulence factors	[52]
	Arrayed	348 essential genes	Identify essential genes	[51]
Staphylococcus aureus	Pooled	129,856 gRNAs targeting all 2666 annotated genes	Identify known and novel pathways of aminoglycoside sensitivity	[34]
Mycobacterium tuberculosis	Pooled	96,700 gRNAs targeting 98.2% of all annotated genes and 1658 non-targeting control gRNAs	Identify highly vulnerable genes in various processes	[4]
	Arrayed	294 essential genes	Mapping the relationship between genes and cell morphology	[20]

[a] The effector used in these studies is the class 2 type II-A dCas9 from *Streptococcus pyogenes*.

3.1.2. *Saccharomyces cerevisiae*

CRISPRi technology has been widely used in prokaryotic organisms such as *E. coli*, but applying it to eukaryotic organisms like *S. cerevisiae* requires special considerations due to differences in transcriptional regulation, chromatin accessibility, and nucleosome occupancy [27]. To enhance the efficiency of gene knockdown, Gilbert et al. fused dCas9 with Mxi1, a mammalian transcriptional repressor domain known to interact with the histone deacetylase Sin3 homolog in yeast. The resulting dCas9-Mxi1 fusion showed a remarkable increase in repression efficiency from 18-fold with dCas9 alone to 53-fold [28]. In another approach, Lian et al. developed an orthogonal tri-functional system called CRISPR-AID by combining CRISPRi with CRISPR activation (CRISPRa, a method that uses nuclease-deficient Cas protein fused to transcriptional activation domains for activating gene transcription [11]), and CRISPR deletion (CRISPRd) [47]. The researchers designed three gRNA libraries consisting of 37,817, 37,870, and 24,806 unique guide sequences (4–6 top-ranked guide sequences for each gene) for the CRISPRa, CRISPRi, and CRISPRd libraries, respectively, to develop a genome-scale CRISPR-

AID system. The approach, named MAGIC, enabled simultaneous upregulation, downregulation, and deletion of multiple targets in a single cell, making it a valuable tool for high-throughput genotype–phenotype mapping. The tri-functional pooled library proved useful in identifying genetic determinants of complex phenotypes, particularly those having synergistic interactions [48].

Lian et al. developed CRISPR systems that are expressed constitutively in *S. cerevisiae*. However, targeting dosage-sensitive genes such as essential genes resulted in cells with lower fitness once they were transformed. To overcome this issue, Momen-Roknabadi et al. developed an anhydrotetracycline-inducible CRISPRi system and carefully designed >51,000 gRNAs (6 to 12 gRNAs per gene) that considered the position upstream of the transcription start site and chromatin accessibility using the method described by Smith et al. [81]. This library accurately identified haploinsufficient genes, as well as enzymatic and regulatory genes involved in adenine and arginine biosynthesis. They also developed a random forest model to explore gRNA optimization factors, recommending rules such as gRNA location, sequence features, and nucleosome occupancy [58]. Similarly, McGlincy et al. presented a comprehensive CRISPRi library consisting of 61,094 gRNAs (most genes having 10 distinct guides) based on empirical design rules. They conducted pooled screening in *S. cerevisiae* to further refine CRISPRi design rules for yeast [56].

3.1.3. Corynebacterium glutamicum

C. glutamicum is a Gram-positive soil bacterium and a popular industrial workhorse for amino acid as well as bulk and fine chemical production [99]. Recently, researchers have utilized *C. glutamicum* as a host for recombinant protein production [41]. To identify the genetic factors responsible for increased recombinant protein production, Yu et al. developed a CRISPRi library comprising 46,549 gRNAs designed to target 99.71% of protein-coding genes and 85.36% of non-coding genes. in *C. glutamicum*. To screen this library, the researchers employed a high-throughput microfluidics platform, capable of converting secreted protein titers into fluorescence signals via an enzyme-independent labeling method within picoliter droplets. The CRISPRi-microfluidics platform was then utilized to screen over 34 million single colonies from the CRISPRi library for improved production of the therapeutic protein VHH. This screening led to the identification of numerous beneficial targets, including previously unknown genes involved in transmembrane transport, amino acid metabolism, and redox regulation [107].

3.1.4. Vibrio sp

The *Vibrio* genus is a rapidly growing bacterial group, that is becoming an increasingly popular host in the field of industrial biotechnology [96]. Despite its potential, the functional genomics research of *Vibrio* lags behind that of more commly used organisms such as *E. coli* and *C. glutamicum*. Nevertheless, the high growth rate of *Vibrio* makes it an attractive research subject. In order to indentfy the minimum set of genes that are essential for rapid growth, Lee et al. constructed a pooled genome-scale CRISPRi library that contained 13,567 unique gRNAs covering 4565 genes (99.7% of predicted protein-coding genes) of *V. natriegens* type strain ATCC 14,048. Through two repeated experiments, they identified 587 overlapping genes as “core genes” that are necessary for rapid growth in rich media, 96% of which are located on the larger chromosome 1. Additionally, they discovered that growth-neutral duplicates of the core genes are mainly found on chromosome 2. This work provided an important foundation for studying and manipulating the biology of *V. natriegens* [40]. Furthermore, Peng et al. isolated three *Vibrio* strains FA1, FA2, and FA3 that grow even faster and have higher biomass than *V. natriegens* ATCC 14,048 in minimal media [62]. The same group developed a CRISPR-dCas9-based multiple gene knockdown method in *V. alginolyticus* FA2 and created two small dual-gRNA libraries targeting 35 × 35 dual histidine kinase genes (1225 combinations) and 24 × 24 dual glycine-pathway genes (576 combinations), respectively. The dual-CRISPRi platform was used to decipher the interfering effect of histidine kinases on antibiotic resistance and that of glycine pathway genes on glycine production [84].

3.1.5. Cyanobacteria

Cyanobacteria have emerged as promising microbial cell factories for the carbon-negative production of chemicals due to their ability to utilize solar energy and carbon dioxide directly as energy and carbon resources, respectively [83]. In order to enhance the industrial properties of cyanobacteria, Yao et al. created a genome-scale CRISPRi library consisting of 10,498 gRNAs for repressing 3546 ORFs and 1871 ncRNAs in *Synechocystis* sp. PCC 6803, a model cyanobacterium. The first screening of genes focused on those related to photoautotrophic growth and L-lactate tolerance. The library was then transformed into a *Synechocystis* strain that produces L-lactate. To screen the library for the secreted L-lactate product, droplet encapsulation and microfluidics were used in combination with a fluorescent L-lactate assay. Clones with gRNAs that targeted nutrient assimilation, central carbon metabolism, and cyclic electron flow showed significant enrichment [105].

3.1.6. Streptococcus pneumoniae

S. pneumonia is a well-known pathogen that poses a significant threat to human health. It is responsible for a range of invasive diseases including pneumonia, meningitis, and acute otitis media, and can have more severe consequences when combined with influenza A virus superinfection [52]. In a recent study, Liu et al. established a doxycycline-inducible CRISPRi system for *S. pneumonia* which allowed for genome-wide fitness testing in a single sequencing step. This system, known as CRISPRi-seq, enabled the investigation of bacterial bottlenecks, an important aspect of pathogenesis imposed by the host during disease progression. A concise CRISPRi library comprising 1499 gRNAs was constructed to target 2111 of the 2146 genetic elements of *S. pneumoniae* D39V, facilitating gene function study at the operon level. The study revealed several previously recognized virulence factors and identified new factors involved in *S. pneumonia* pathogenesis [52]. Moreover, the authors summarized a convenient protocol for the design and application of CRISPRi-seq in *S. pneumonia*, which includes a pipeline for gRNA library design, workflows for pooled library construction, growth assay and sequencing, a read analysis Python tool, and instructions for fitness quantification [19].

3.1.7. Mycobacteria

M. tuberculosis is a serious human pathogen, causing tuberculosis, a leading cause of death due to infectious diseases. To develop effective therapeutic strategies, it is crucial to understand how this bacterium causes tuberculosis and evades antibiotics. Recent research by Rock et al. investigated the efficiency and toxicity of the canonical *S. pyogenes* Cas9-based CRISPRi in *M. tuberculosis*. Their findings showed poor knockdown efficiency and proteotoxicity of this canonical CRISPRi system in this pathogen. The research team tested eleven diverse Cas9 orthologues and identified a highly efficient CRISPRi system based on the dCas9 from *S. thermophilus* ($dCas9_{Sth1}$), which achieved 20- to 100-fold repression of gene expression [69]. Building on these findings, Bosch et al. used $dCas9_{Sth1}$ to create a genome-scale CRISPRi library that could be used to quantify gene vulnerability in *M. tuberculosis*. They used two different strategies to achieve predictable and titratable repression of gene expression. The first involved using non-canonical PAMs recognized by $dCas9_{Sth1}$, and the second involved varying the length of the gRNA targeting sequence. The final library consisted of 96,700 gRNAs targeting 98.2% of all annotated genes and 1658 non-targeting control gRNAs. By conducting pooled CRISPRi screening in two *M. tuberculosis* strains, the researchers identified highly vulnerable and invulnerable genes involved in various processes. This provided novel targets for drug discovery and potential explanations of failed drug discovery efforts, respectively. Using this method, they were able to identify about 93% of essential genes as vulnerable genes. The team also performed the same experiment in the model bacterium *M. smegmatis*, which yielded

broadly consistent results with published datasets. Based on these results, development of new drugs acting on the vulnerability genes may be undertaken [4].

3.2. Arrayed CRISPRi screening

In contrast to a pooled library, in which all the strains are mixed together, an arrayed library consists of individual strains that are created and validated through sequencing (Fig. 2b). However, constructing a genome-scale arrayed library is a more expensive process that typically requires the use of automation and high-throughput equipment, such as a biofoundry [32]. As a result, a complete arrayed CRISPRi library covering all annotated genes has yet to be developed for any microorganism. However, arrayed CRISPRi libraries targeting a subset of genes, such as all the essential genes, biosynthesis genes, and transport genes, have been created for several medically and industrially important microorganisms, providing valuable resources for biological research (Table 2).

3.2.1. Essential genes

Essential genes are a set of genes that are critical for the survival of cells. The study of essential genes and interrelationships is important for comprehending how cell metabolism functions and developing effective treatments for infections. CRISPRi is a suitable method for perturbing and interrogating essential genes as their deletion is lethal to cells. Researchers can use CRISPRi to repress essential genes and interpret slowed growth as an indicator of a gene's essential function. In a study by Peters et al., every essential gene in the Gram-positive model bacterium *B. subtilis* was repressed using a xylose-inducible and titratable CRISPRi framework. They constructed a comprehensive arrayed CRISPRi library covering 289 known or proposed essential genes, and chemical-genomic analysis was used to define essential gene phenotypes by measuring colony size with various chemicals in culture. This approach led to the discovery of new drug targets such as the undecaprenyl pyrophosphate synthetase gene *uppS*, which acts as a target of the antimicrobial MAC-0,170,636. The arrayed CRISPRi library also facilitated the establishment of a functional network of essential gene processes. High-throughput microscopy was used to analyze the morphology of CRISPRi strains, characterizing the effects of essential gene expression on cell morphology [63]. Additional studies have constructed arrayed CRISPRi libraries for essential genes in other bacteria such as *S. pneumoniae* (348 genes) [51], *M. smegmatis* (294 genes) [20], and *E. coli* (372 genes) [78]. These libraries were utilized to investigate the roles of essential genes in controlling cell growth, antibiotic resistance, and cell morphology.

In order to reduce the cost of constructing arrayed libraries, Smith et al. developed an affordable method called Recombinase Directed Indexing (REDI) that enables the production of arrayed strain collections using pooled gRNA libraries [80]. Initially, REDI integrates a pooled gRNA library into the yeast genome by transformation and homologous recombination. Transformants are then arrayed into 1536 formats and mated with another 1536 unique barcoder strains. Through Cre-lox-mediated site-specific recombination in diploids, the gRNA and barcode are linked. Subsequently, barcode-gRNA fragments are amplified by PCR and subjected to NGS. The plate position of gRNA transformants can be easily identified from the sequencing data as the barcode assigned to each barcoder strain is known. Transformants of interest are consolidated into new plates for subsequent phenotype tests. The authors designed over 18,000 gRNAs targeting 1117 essential genes and 514 genes required for respiratory growth of *S. cerevisiae*. Using NGS of approximately 58,300 tagged transformants, REDI produced 9059 strains containing a unique sequence-verified gRNA [80]. Thereafter, Mukherjee et al. used a high-throughput and high-resolution method called the Scan-o-matic system to screen target genes involved in acetate tolerance from the arrayed CRISPRi library consisting of more than 9000 strains. This system assesses growth curves on solid medium [59].

3.2.2. Genes for bioproduction

Arrayed CRISPRi libraries are not only used to interrogate essential genes, but also to study the functions of genes involved in bioproduction of chemicals and fuels. For example, Wang et al. used an arrayed CRISPRi library targeting 56 phosphatase-encoding genes of *E. coli* to test the hypothesis that phosphatase promiscuity could negatively affect terpenoid biosynthesis by redirecting carbon flux away from the biosynthetic pathway via unknown catalytic activities on the phosphorylated intermediates. The arrayed CRISPRi library was constructed and transformed into a lycopene overproducing strain. They found that about half of the tested genes impaired lycopene biosynthesis, providing preliminary evidence for the hypothesis [93]. In another study, two groups constructed arrayed CRISPRi libraries targeting 480 genes encoding NADPH/ATP-consuming enzymes and 108 genes related to free fatty acid metabolism, respectively, to enhance the production of 4-hydroxyphenylacetic acid and free fatty acids in *E. coli*. CRISPRi screening identified several beneficial targets for bioproduction and further combinatorial perturbation of the selected genes led to high-titer producing strains [21,76].

The arrayed CRISPRi libraries for *C. glutamicum* are important tools for identifying novel targets for bioproduction. In one study, Göttl et al. selected 74 genes involved in carotenogenesis, covering enzymes or regulators in various pathways, such as glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, and methylerythritol phosphate and carotenoid biosynthesis. Through CRISPRi screening, they identified 11 genes that impaired and 3 genes that benefited carotenoid biosynthesis upon repression. These findings provide potential targets for rational metabolic engineering [29]. Transport engineering is a key strategy for improving bioproduction [6,87], but the transporters for many metabolites are still unknown. In another study, Liu et al. constructed an arrayed CRISPRi library consisting of 397 membrane transporter genes of *C. glutamicum*. Using this library, they identified the first L-proline exporter by evaluating the effects of gene repression on extracellular L-proline accumulation. This discovery led to the construction of an L-proline hyperproducing strain and opened the door for investigating the transport of other molecules of interest [50].

4. Concluding remarks and future perspectives

CRISPRi screening, whether in the pooled or arrayed form, has become a valuable tool for functional genomics research in microorganisms. However, several challenges still exist. One such challenge is the difficulty of gRNA design for genes with short coding lengths or extreme GC content due to the PAM requirement and off-target effects of gRNAs. Consequently, the library may fail to cover all the genes of interest [92]. One potential solution is the use of recently engineered Cas variants with broad PAM range and high specificity [10,88]. Another challenge is the difficulty of interrogating the complex connections among genes that form a network with single-spacer gRNA expression, which is the current routine practice for both pooled and arrayed CRISPRi screening. Developing a genome-scale dual-spacer gRNA library is a major challenge due to the extremely large library size. For example, constructing a dual-spacer gRNA library for the *E. coli* genome, which has over 4000 genes, would require at least 1.6×10^7 elements, making manual construction difficult. Automation by using biofoundry platforms and the development of new methods for library construction could be helpful in addressing this challenge. Recently, bacterial cells were repurposed for the production of highly comprehensive combinatorial gRNA libraries *in vivo*, taking advantage of the natural CRISPR adaptation machinery, [34], which could save human labor for library construction.

Although library construction for the pooled form of CRISPRi library is considered relatively simple and convenient, it poses challenges when evaluating phenotypes as it requires a high-throughput screening method, such as growth- and FACS-based screening. Unfortunately, this limits the diversity of phenotype testing [23], and biotechnologically relevant phenotypes may not scale well from microtiter plates to

fermenters, thereby hampering their characterization. To address this limitation, CRISPRi screening has been combined with biosensors capable of translating metabolite concentrations into easily detectable signals to expand the phenotypes that can be characterized [89,107]. Another significant challenge of the pooled method is that since all transformants are pooled, the cells may have different growth conditions, leading to differences in determined gRNA fitness. For instance, some cells may be in late logarithmic phase, while others are not, leading to growth advantages or disadvantages when screening is performed. Unfortunately, these factors have not been comprehensively considered in previous studies. Arrayed CRISPRi screening provides a solution to the above challenges as cell growth conditions can be more finely controlled, and diverse phenotypes can be evaluated for each member of the library. However, the major challenges of this method are the high cost and intensive labor input needed for library construction and phenotype evaluation of individual members. Fortunately, automation and high-throughput equipment can minimize the system required for library construction and evaluation, thereby lowering the overall cost [94]. Furthermore, the use of high-throughput omics approaches such as rapid mass spectrometry, which allows the assay of one sample per second, will largely promote arrayed screening [25].

CRISPRi screening is rapidly gaining popularity and has the potential to become a powerful strategy for understanding and engineering of microbiology. Researchers are continuously constructing genome-scale CRISPRi libraries for more microorganisms, and their applications are still expanding. Emerging technologies are being developed to address the aforementioned challenges with CRISPRi screening. With these advancements, CRISPRi has tremendous potential to significantly contribute to microbiological research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Letian Sun: Visualization, Writing – Original draft preparation. **Ping Zheng:** Writing – Review & Editing. **Jibin Sun:** Writing – Review & Editing. **Volker F. Wendisch:** Writing – Review & Editing. **Yu Wang:** Conceptualization, Writing – Reviewing and Editing.

Acknowledgement

This research was supported by the National Key R&D Program of China (2018YFA0901500), the National Natural Science Foundation of China (32222004 and 32270101), and the Youth Innovation Promotion Association of Chinese Academy of Sciences (2021177).

References

[1] O.O. Abudayyeh, J.S. Gootenberg, P. Essletzbichler, S. Han, J. Joung, J.J. Belanto, V. Verdine, D.B.T. Cox, M.J. Kellner, A. Regev, E.S. Lander, D.F. Voytas, A.Y. Ting, F. Zhang, RNA targeting with CRISPR-Cas13, Nature 550 (2017) 280–284, doi:10.1038/nature24049.

[2] O.O. Abudayyeh, J.S. Gootenberg, S. Konermann, J. Joung, I.M. Slaymaker, D.B. Cox, S. Shmakov, K.S. Makarova, E. Semenova, L. Minakhin, K. Severinov, A. Regev, E.S. Lander, E.V. Koonin, F. Zhang, C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector, Science 353 (2016) 6299, doi:10.1126/science.aaf5573.

[3] D. Bikard, W. Jiang, P. Samai, A. Hochschild, F. Zhang, L.A. Marraffini, Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system, Nucleic. Acids. Res. 41 (2013) 7429–7437, doi:10.1093/nar/gkt520.

[4] B. Bosch, M.A. DeJesus, N.C. Poulton, W. Zhang, C.A. Engelhart, A. Zaveri, S. Lavalette, N. Ruecker, C. Trujillo, J.B. Wallach, S. Li, S. Ehrt, B.T. Chait, D. Schnappinger, J.M. Rock, Genome-wide gene expression tuning reveals diverse vulnerabilities of *M. tuberculosis*, Cell 184 (2021) 4579–4592, doi:10.1016/j.cell.2021.06.033.

[5] S.J.J. Brouns, M.M. Jore, M. Lundgren, E.R. Westra, R.J.H. Slijkhuis, A.P.L. Snijders, M.J. Dickman, K.S. Makarova, E.V. Koonin, J. van der Oost, Small CRISPR RNAs guide antiviral defense in prokaryotes, Science 321 (2008) 960–964, doi:10.1126/science.1159689.

[6] A. Burkovski, R. Krämer, Bacterial amino acid transport proteins: occurrence, functions, and significance for biotechnological applications, Appl. Microbiol. Biotechnol. 58 (2002) 265–274, doi:10.1007/s00253-001-0869-4.

[7] S.N. Call, L.B. Andrews, CRISPR-based approaches for gene regulation in non-model bacteria, Front. Genome Ed. 4 (2022) 892304, doi:10.3389/fgeed.2022.892304.

[8] M.C. Canver, M. Haeussler, D.E. Bauer, S.H. Orkin, N.E. Sanjana, O. Shalem, G.-C. Yuan, F. Zhang, J.-P. Concordet, L. Pinello, Integrated design, execution, and analysis of arrayed and pooled CRISPR genome-editing experiments, Nat. Protoc. 13 (2018) 946–986, doi:10.1038/nprot.2018.005.

[9] Y. Cao, X. Li, F. Li, H. Song, CRISPRi-sRNA: transcriptional-translational regulation of extracellular electron transfer in *Shewanella oneidensis*, ACS Synth. Biol. 6 (2017) 1679–1690, doi:10.1021/acssynbio.6b00374.

[10] P. Chatterjee, N. Jakimo, J. Lee, N. Amrani, T. Rodríguez, S.R.T. Koseki, E. Tysinger, R. Qing, S. Hao, E.J. Sontheimer, J. Jacobson, An engineered ScCas9 with broad PAM range and high specificity and activity, Nat. Biotechnol. 38 (2020) 1154–1158, doi:10.1038/s41587-020-0517-0.

[11] A. Chavez, J. Scheiman, S. Vora, B.W. Pruitt, M. Tuttle, E.P.R. Iyer, S. Lin, S. Kiani, C.D. Guzman, D.J. Wiegand, D. Ter-Ovanesyan, J.L. Braff, N. Davidsohn, B.E. Housden, N. Perrimon, R. Weiss, J. Aach, J.J. Collins, G.M. Church, Highly efficient Cas9-mediated transcriptional programming, Nat. Methods 12 (2015) 326–328, doi:10.1038/nmeth.3312.

[12] W. Chen, Y. Zhang, W.-S. Yeo, T. Bae, Q. Ji, Rapid and efficient genome editing in *Staphylococcus aureus* by using an engineered CRISPR/Cas9 system, J. Am. Chem. Soc. 139 (2017) 3790–3795, doi:10.1021/jacs.6b13317.

[13] S.Y. Choi, H.M. Woo, CRISPRi-dCas12a: a dCas12a-mediated CRISPR interference for repression of multiple genes and metabolic engineering in cyanobacteria, ACS Synth. Biol. 9 (2020) 2351–2361, doi:10.1021/acssynbio.0c00091.

[14] E. Choudhary, R. Sharma, Y. Kumar, N. Agarwal, Conditional silencing by CRISPRi reveals the role of DNA gyrase in formation of drug-tolerant persister population in *Mycobacterium tuberculosis*, Front. Cell. Infect. Microbiol. 9 (2019) 70, doi:10.3389/fcimb.2019.00070.

[15] E. Choudhary, P. Thakur, M. Pareek, N. Agarwal, Gene silencing by CRISPR interference in mycobacteria, Nat. Commun. 6 (2015) 6267, doi:10.1038/ncomms7267.

[16] K. Ciurkot, T.E. Gorochowski, J.A. Roubos, R. Verwaal, Efficient multiplexed gene regulation in *Saccharomyces cerevisiae* using dCas12a, Nucleic. Acids Res. 49 (2021) 7775–7790, doi:10.1093/nar/gkab529.

[17] S. Cleto, J.V.K. Jensen, V.F. Wendisch, T.K. Lu, *Corynebacterium glutamicum* metabolic engineering with CRISPR interference (CRISPRi), ACS Synth. Biol. 5 (2016) 375–385, doi:10.1021/acssynbio.5b00216.

[18] L. Cui, A. Vigouroux, F. Rousset, H. Varet, V. Khanna, D. Bikard, A CRISPRi screen in *E. coli* reveals sequence-specific toxicity of dCas9, Nat. Commun. 9 (2018) 1912, doi:10.1038/s41467-018-04209-5.

[19] V. de Bakker, X. Liu, A.M. Bravo, J.W. Veening, CRISPRi-seq for genome-wide fitness quantification in bacteria, Nat. Protoc. 17 (2022) 252–281, doi:10.1038/s41596-021-00639-6.

[20] T.J. de Wet, K.R. Winkler, M. Mhlanga, V. Mizrahi, D.F. Warner, Arrayed CRISPRi and quantitative imaging describe the morphotypic landscape of essential mycobacterial genes, Elife 9 (2020) e60083, doi:10.7554/eLife.60083.

[21] L. Fang, J. Fan, S. Luo, Y. Chen, C. Wang, Y. Cao, H. Song, Genome-scale target identification in *Escherichia coli* for high-titer production of free fatty acids, Nat. Commun. 12 (2021) 4976, doi:10.1038/s41467-021-25243-w.

[22] H. Feng, J. Guo, T. Wang, C. Zhang, X.-h. Xing, Guide-target mismatch effects on dCas9-sgRNA binding activity in living bacterial cells, Nucleic. Acids Res. 49 (2021) 1263–1277, doi:10.1093/nar/gkaa1295.

[23] H. Feng, Y. Yuan, Z. Yang, X.-H. Xing, C. Zhang, Genome-wide genotype-phenotype associations in microbes, J. Biosci. Bioeng. 132 (2021) 1–8, doi:10.1016/j.jbiosc.2021.03.011.

[24] N. Fleck, C. Grundner, A Cas12a-based CRISPR interference system for multigene regulation in mycobacteria, J. Biol. Chem. 297 (2021) 100990, doi:10.1016/j.jbc.2021.100990.

[25] L. Fu, E. Guo, J. Zhang, K. Li, Y. Chen, T. Si, Towards one sample per second for mass spectrometric screening of engineered microbial strains, Curr. Opin. Biotech. 76 (2022) 102725, doi:10.1016/j.copbio.2022.102725.

[26] L.A. Gallagher, E. Ramage, R. Patrapuvich, E. Weiss, M. Brittnacher, C. Manoil, Sequence-defined transposon mutant library of *Burkholderia thailandensis*, MBio 4 (2013) e00604–e00613, doi:10.1128/mBio.00604-13.

[27] L.A. Gilbert, M.A. Horlbeck, B. Adamson, J.E. Villalta, Y. Chen, E.H. Whitehead, C. Guimaraes, B. Panning, H.L. Ploegh, M.C. Bassik, L.S. Qi, M. Kampmann, J.S. Weissman, Genome-scale CRISPR-mediated control of gene repression and activation, Cell 159 (2014) 647–661, doi:10.1016/j.cell.2014.09.029.

[28] L.A. Gilbert, M.H. Larson, L. Morsut, Z. Liu, G.A. Brar, S.E. Torres, N. Stern-Ginossar, O. Brandman, E.H. Whitehead, J.A. Doudna, W.A. Lim, J.S. Weissman, L.S. Qi, CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes, Cell 154 (2013) 442–451, doi:10.1016/j.cell.2013.06.044.

[29] V.L. Göttl, I. Schmitt, K. Braun, P. Peters-Wendisch, V.F. Wendisch, N.A. Henke, CRISPRi-library-guided target identification for engineering carotenoid production by *Corynebacterium glutamicum*, Microorganisms 9 (2021) 9670, doi:10.3390/microorganisms9040670.

[30] J.S. Hawkins, M.R. Silvis, B.-M. Koo, J.M. Peters, H. Osadnik, M. Jost, C.C. Hearne, J.S. Weissman, H. Todor, C.A. Gross, Mismatch-CRISPRi reveals the co-varying expression-fitness relationships of essential genes in *Escherichia coli* and *Bacillus subtilis*, Cell Syst. 11 (2020) 523–535, doi:10.1016/j.cels.2020.09.009.

[31] A. Higo, S. Ehira, Spatiotemporal gene repression system in the heterocyst-forming multicellular cyanobacterium *Anabaena* sp. PCC 7120, ACS Synth. Biol. 8 (2019) 641–646, doi:10.1021/acssynbio.8b00496.
[32] N. Hillson, M. Caddick, Y. Cai, J.A. Carrasco, M.W. Chang, N.C. Curach, D.J. Bell, R. Le Feuvre, D.C. Friedman, X. Fu, N.D. Gold, M.J. Herrgård, M.B. Holowko, J.R. Johnson, R.A. Johnson, J.D. Keasling, R.I. Kitney, A. Kondo, C. Liu, V.J.J. Martin, F. Menolascina, C. Ogino, N.J. Patron, M. Pavan, C.L. Poh, I.S. Pretorius, S.J. Rosser, N.S. Scrutton, M. Storch, H. Tekotte, E. Travnik, C.E. Vickers, W.S. Yew, Y. Yuan, H. Zhao, P.S. Freemont, Building a global alliance of biofoundries, Nat. Commun. 10 (2019) 2040, doi:10.1038/s41467-019-10079-2.
[33] C.-H. Huang, C.R. Shen, H. Li, L.-Y. Sung, M.-Y. Wu, Y.-C. Hu, CRISPR interference (CRISPRi) for gene regulation and succinate production in cyanobacterium *S. elongatus* PCC 7942, Microb. Cell Fact. 15 (2016) 196, doi:10.1186/s12934-016-0595-3.
[34] W. Jiang, P. Oikonomou, S. Tavazoie, Comprehensive genome-wide perturbations via CRISPR adaptation reveal complex genetics of antibiotic sensitivity, Cell 180 (2020) 1002–1017, doi:10.1016/j.cell.2020.02.007.
[35] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337 (2012) 816–821, doi:10.1126/science.1225829.
[36] R.C. Joseph, N.R. Sandoval, Single and multiplexed gene repression in solventogenic *Clostridium* via Cas12a-based CRISPR interference, Synth. Syst. Biotechnol. 8 (2023) 148–156, doi:10.1016/j.synbio.2022.12.005.
[37] S.K. Kim, H. Kim, W.-C. Ahn, K.-H. Park, E.-J. Woo, D.-H. Lee, S.-G. Lee, Efficient transcriptional gene repression by type V-A CRISPR-Cpf1 from *Eubacterium eligens*, ACS Synth. Biol. 6 (2017) 1273–1282, doi:10.1021/acssynbio.6b00368.
[38] G.J. Knott, J.A. Doudna, CRISPR-Cas guides the future of genetic engineering, Science 361 (2018) 866–869, doi:10.1126/science.aat5011.
[39] S. Konermann, P. Lotfy, N.J. Brideau, J. Oki, M.N. Shokhirev, P.D. Hsu, Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors, Cell 173 (2018) 665–676, doi:10.1016/j.cell.2018.02.033.
[40] H.H. Lee, N. Ostrov, B.G. Wong, M.A. Gold, A.S. Khalil, G.M. Church, Functional genomics of the rapidly replicating bacterium *Vibrio natriegens* by CRISPRi, Nat. Microbiol. 4 (2019) 1105–1113, doi:10.1038/s41564-019-0423-8.
[41] M.J. Lee, P. Kim, Recombinant protein expression system in *Corynebacterium glutamicum* and its application, Front. Microbiol. 9 (2018) 2523, doi:10.3389/fmicb.2018.02523.
[42] R.T. Leenay, K.R. Maksimchuk, R.A. Slotkowski, R.N. Agrawal, A.A. Gomaa, A.E. Briner, R. Barrangou, C.L. Beisel, Identifying and visualizing functional PAM diversity across CRISPR-Cas systems, Mol. Cell 62 (2016) 137–147, doi:10.1016/j.molcel.2016.02.031.
[43] J. Li, Q. Tang, Y. Li, Y.Y. Fan, F.H. Li, J.H. Wu, D. Min, W.W. Li, P.K.S. Lam, H.Q. Yu, Rediverting electron flux with an engineered CRISPR-ddAsCpf1 system to enhance the pollutant degradation capacity of *Shewanella oneidensis*, Environ. Sci. Technol. 54 (2020) 3599–3608, doi:10.1021/acs.est.9b06378.
[44] M.Y. Li, J.Z. Chen, Y. Wang, J. Liu, J.W. Huang, N. Chen, P. Zheng, J.B. Sun, Efficient multiplex gene repression by CRISPR-dCpf1 in *Corynebacterium glutamicum*, Front. Bioeng. Biotechnol. 8 (2020) 357, doi:10.3389/fbioe.2020.00357.
[45] S. Li, C.B. Jendresen, J. Landberg, L.E. Pedersen, N. Sonnenschein, S.I. Jensen, A.T. Nielsen, Genome-wide CRISPRi-based identification of targets for decoupling growth from production, ACS Synth. Biol. 9 (2020) 1030–1040, doi:10.1021/acssynbio.9b00143.
[46] X.J. Li, L.G. Huang, L.J. Pan, B. Wang, L. Pan, CRISPR/dCas9-mediated epigenetic modification reveals differential regulation of histone acetylation on *Aspergillus niger* secondary metabolite, Microbiol. Res. 245 (2021) 126694, doi:10.1016/j.micres.2020.126694.
[47] J. Lian, M. HamediRad, S. Hu, H. Zhao, Combinatorial metabolic engineering using an orthogonal tri-functional CRISPR system, Nat. Commun. 8 (2017) 1688, doi:10.1038/s41467-017-01695-x.
[48] J. Lian, C. Schultz, M. Cao, M. HamediRad, H. Zhao, Multi-functional genome-wide CRISPR system for high throughput genotype-phenotype mapping, Nat. Commun. 10 (2019) 5794, doi:10.1038/s41467-019-13621-4.
[49] D. Liu, V.M. Johnson, H.B. Pakrasi, A reversibly induced CRISPRi system targeting photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803, ACS Synth. Biol. 9 (2020) 1441–1449, doi:10.1021/acssynbio.0c00106.
[50] J. Liu, M. Liu, T. Shi, G. Sun, N. Gao, X. Zhao, X. Guo, X. Ni, Q. Yuan, J. Feng, Z. Liu, Y. Guo, J. Chen, Y. Wang, P. Zheng, J. Sun, CRISPR-assisted rational flux-tuning and arrayed CRISPRi screening of an L-proline exporter for L-proline hyperproduction, Nat. Commun. 13 (2022) 891, doi:10.1038/s41467-022-28501-7.
[51] X. Liu, C. Gallay, M. Kjos, A. Domenech, J. Slager, S.P. van Kessel, K. Knoops, R.A. Sorg, J.R. Zhang, J.W. Veening, High-throughput CRISPRi phenotyping identifies new essential genes in *Streptococcus pneumoniae*, Mol. Syst. Biol. 13 (2017) 931, doi:10.15252/msb.20167449.
[52] X. Liu, J.M. Kimmey, L. Matarazzo, V. de Bakker, L. Van Maele, J.-C. Sirard, V. Nizet, J.-W. Veening, Exploration of bacterial bottlenecks and *Streptococcus pneumoniae* pathogenesis by CRISPRi-seq, Cell Host Microbe 29 (2021) 107–120, doi:10.1016/j.chom.2020.10.001.
[53] L. Lu, X. Shen, X. Sun, Y. Yan, J. Wang, Q. Yuan, CRISPR-based metabolic engineering in non-model microorganisms, Curr. Opin. Biotech. 75 (2022) 102698, doi:10.1016/j.copbio.2022.102698.
[54] M.L. Luo, A.S. Mullis, R.T. Leenay, C.L. Beisel, Repurposing endogenous type I CRISPR-Cas systems for programmable gene repression, Nucleic. Acids. Res. 43 (2015) 674–681, doi:10.1093/nar/gku971.
[55] K.S. Makarova, Y.I. Wolf, O.S. Alkhnbashi, F. Costa, S.A. Shah, S.J. Saunders, R. Barrangou, S.J.J. Brouns, E. Charpentier, D.H. Haft, P. Horvath, S. Moineau, F.J.M. Mojica, R.M. Terns, M.P. Terns, M.F. White, A.F. Yakunin, R.A. Garrett, J. van der Oost, R. Backofen, E.V. Koonin, An updated evolutionary classification of CRISPR-Cas systems, Nat. Rev. Microbiol. 13 (2015) 722–736, doi:10.1038/nrmicro3569.
[56] N.J. McGlincy, Z.A. Meacham, K.K. Reynaud, R. Muller, R. Baum, N.T. Ingolia, A genome-scale CRISPR interference guide library enables comprehensive phenotypic profiling in yeast, Bmc Genom. 22 (2021) 205 [Electronic Resource], doi:10.1186/s12864-021-07518-0.
[57] C. Miao, H. Zhao, L. Qian, C. Lou, Systematically investigating the key features of the DNase deactivated Cpf1 for tunable transcription regulation in prokaryotic cells, Synth. Syst. Biotechnol. 4 (2019) 1–9, doi:10.1016/j.synbio.2018.11.002.
[58] A. Momen-Roknabadi, P. Oikonomou, M. Zegans, S. Tavazoie, An inducible CRISPR interference library for genetic interrogation of *Saccharomyces cerevisiae* biology, Commun. Biol. 3 (2020) 723, doi:10.1038/s42003-020-01452-9.
[59] V. Mukherjee, U. Lind, R.P. St Onge, A. Blomberg, Y. Nygård, A CRISPR interference screen of essential genes reveals that proteasome regulation dictates acetic acid tolerance in *Saccharomyces cerevisiae*, mSystems 6 (2021) 4, doi:10.1128/mSystems.00418-21.
[60] V.K. Mutalik, B.A. Adler, H.S. Rishi, D. Piya, C. Zhong, B. Koskella, E.M. Kutter, R. Calendar, P.S. Novichkov, M.N. Price, A.M. Deutschbauer, A.P. Arkin, High-throughput mapping of the phage resistance landscape in *E. coli*, PLoS Biol. 18 (2020) 10, doi:10.1371/journal.pbio.3000877.
[61] A. Özcan, R. Krajeski, E. Ioannidi, B. Lee, A. Gardner, K.S. Makarova, E.V. Koonin, O.O. Abudayyeh, J.S. Gootenberg, Programmable RNA targeting with the single-protein CRISPR effector Cas7-11, Nature 597 (2021) 720–725, doi:10.1038/s41586-021-03886-5.
[62] Y. Peng, X. Han, P. Xu, F. Tao, Next-generation microbial workhorses: comparative genomic analysis of fast-growing *Vibrio* strains reveals their biotechnological potential, Biotechnol. J. 15 (2020) 1900499, doi:10.1002/biot.201900499.
[63] J.M. Peters, A. Colavin, H. Shi, T.L. Czarny, M.H. Larson, S. Wong, J.S. Hawkins, C.H.S. Lu, B.M. Koo, E. Marta, A.L. Shiver, E.H. Whitehead, J.S. Weissman, E.D. Brown, L.S. Qi, K.C. Huang, C.A. Gross, A comprehensive, CRISPR-based functional analysis of essential genes in bacteria, Cell 165 (2016) 1493–1506, doi:10.1016/j.cell.2016.05.003.
[64] J.M. Peters, B.-M. Koo, R. Patino, G.E. Heussler, C.C. Hearne, J. Qu, Y.F. Inclan, J.S. Hawkins, C.H.S. Lu, M.R. Silvis, M.M. Harden, H. Osadnik, J.E. Peters, J.N. Engel, R.J. Dutton, A.D. Grossman, C.A. Gross, O.S. Rosenberg, Enabling genetic analysis of diverse bacteria with Mobile-CRISPRi, Nat. Microbiol. 4 (2019) 244–250, doi:10.1038/s41564-018-0327-z.
[65] A. Pickar-Oliver, J.B. Black, M.M. Lewis, K.J. Mutchnick, T.S. Klann, K.A. Gilcrest, M.J. Sitton, C.E. Nelson, A. Barrera, L.C. Bartelt, T.E. Reddy, C.L. Beisel, R. Barrangou, C.A. Gersbach, Targeted transcriptional modulation with type I CRISPR-Cas systems in human cells, Nat. Biotechnol. 37 (2019) 1493–1501, doi:10.1038/s41587-019-0235-7.
[66] L.S. Qi, M.H. Larson, L.A. Gilbert, J.A. Doudna, J.S. Weissman, A.P. Arkin, W.A. Lim, Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression, Cell 152 (2013) 1173–1183, doi:10.1016/j.cell.2013.02.022.
[67] D. Rath, L. Amlinger, M. Hoekzema, P.R. Devulapally, M. Lundgren, Efficient programmable gene silencing by Cascade, Nucleic. Acids. Res. 43 (2015) 237–246, doi:10.1093/nar/gku1257.
[68] Rishi, H.S., Toro, E., Liu, H., Wang, X., Qi, L.S., Arkin, A.P., 2020. Systematic genome-wide querying of coding and non-coding functional elements in *E. coli* using CRISPRi. Biorxiv [Preprint], 2020 [cited 2020 Mar 2010]. doi: 10.1101/2020.03.04.975888.
[69] J.M. Rock, F.F. Hopkins, A. Chavez, M. Diallo, M.R. Chase, E.R. Gerrick, J.R. Pritchard, G.M. Church, E.J. Rubin, C.M. Sassetti, D. Schnappinger, S.M. Fortune, Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform, Nat. Microbiol. 2 (2017) 16274, doi:10.1038/nmicrobiol.2016.274.
[70] F. Rousset, J. Cabezas-Caballero, F. Piastra-Facon, J. Fernandez-Rodriguez, O. Clermont, E. Denamur, E.P.C. Rocha, D. Bikard, The impact of genetic diversity on gene essentiality within the *Escherichia coli* species, Nat. Microbiol. 6 (2021) 301–312, doi:10.1038/s41564-020-00839-y.
[71] F. Rousset, L. Cui, E. Siouve, C. Becavin, F. Depardieu, D. Bikard, Genome-wide CRISPR-dCas9 screens in *E. coli* identify essential genes and phage host factors, PLos Genet. 14 (2018) e1007749, doi:10.1371/journal.pgen.1007749.
[72] K. Schultenkäemper, L.F. Brito, V.F. Wendisch, Impact of CRISPR interference on strain development in biotechnology, Biotechnol. Appl. Bioc. 67 (2020) 7–21, doi:10.1002/bab.1901.
[73] K. Schultenkämper, L.F. Brito, M.G. Lopez, T. Brautaset, V.F. Wendisch, Establishment and application of CRISPR interference to affect sporulation, hydrogen peroxide detoxification, and mannitol catabolism in the methylotrophic thermophile *Bacillus methanolicus*, Appl. Microbiol. Biotechnol. 103 (2019) 5879–5889, doi:10.1007/s00253-019-09907-8.
[74] C. Schwartz, K. Frogue, A. Ramesh, J. Misa, I. Wheeldon, CRISPRi repression of nonhomologous end-joining for enhanced genome engineering via homologous recombination in *Yarrowia lipolytica*, Biotechnol. Bioeng. 114 (2017) 2896–2906, doi:10.1002/bit.26404.
[75] O. Shalem, N.E. Sanjana, F. Zhang, High-throughput functional genomics using CRISPR-Cas9, Nat. Rev. Genet. 16 (2015) 299–311, doi:10.1038/nrg3899.
[76] Y.-P. Shen, Y.-L. Liao, Q. Lu, X. He, Z.-B. Yan, J.-Z. Liu, ATP and NADPH engineering of *Escherichia coli* to improve the production of 4-hydroxyphenylacetic acid using CRISPRi, Biotechnol. Biofuels 14 (2021) 100, doi:10.1186/s13068-021-01954-6.
[77] R.C. Shields, A.R. Walker, N. Maricic, B. Chakraborty, S.A.M. Underhill, R.A. Burne, Repurposing the *Streptococcus mutans* CRISPR-Cas9 system to understand es-

sential gene function, PLoS Pathog. 16 (2020) e1008344, doi:10.1371/journal.ppat.1008344.
[78] M.R. Silvis, M. Rajendram, H. Shi, H. Osadnik, A.N. Gray, S. Cesar, J.M. Peters, C.C. Hearne, P. Kumar, H. Todor, K.C. Huang, C.A. Gross, Morphological and transcriptional responses to CRISPRi knockdown of essential genes in *Escherichia coli*, MBio 12 (2021) 5, doi:10.1128/mBio.02561-21.
[79] A.A. Smargon, D.B.T. Cox, N.K. Pyzocha, K. Zheng, I.M. Slaymaker, J.S. Gootenberg, O.A. Abudayyeh, P. Essletzbichler, S. Shmakov, K.S. Makarova, E.V. Koonin, F. Zhang, Cas13b is a type VI-B CRISPR-associated RNA-guided RNase differentially regulated by accessory proteins Csx27 and Csx28, Mol. Cell 65 (2017) 618–630, doi:10.1016/j.molcel.2016.12.023.
[80] J.D. Smith, U. Schlecht, W. Xu, S. Suresh, J. Horecka, M.J. Proctor, R.S. Aiyar, R.A.O. Bennett, A. Chu, Y.F. Li, K. Roy, R.W. Davis, L.M. Steinmetz, R.W. Hyman, S.F. Levy, R.P. St Onge, A method for high-throughput production of sequence-verified DNA libraries and strain collections, Mol. Syst. Biol. 13 (2017) 913, doi:10.15252/msb.20167233.
[81] J.D. Smith, S. Suresh, U. Schlecht, M. Wu, O. Wagih, G. Peltz, R.W. Davis, L.M. Steinmetz, L. Parts, R.P. St Onge, Quantitative CRISPR interference screens in yeast identify chemical-genetic interactions and new rules for guide RNA design, Genome Biol. 17 (2016) 45, doi:10.1186/s13059-016-0900-9.
[82] A.-E. Stachler, T.S. Schwarz, S. Schreiber, A. Marchfelder, CRISPRi as an efficient tool for gene repression in archaea, Methods 172 (2020) 76–85, doi:10.1016/j.ymeth.2019.05.023.
[83] C. Tan, P. Xu, F. Tao, Carbon-negative synthetic biology: challenges and emerging trends of cyanobacterial technology, Trends Biotechnol. 40 (2022) 1488–1502, doi:10.1016/j.tibtech.2022.09.012.
[84] C. Tan, P. Xu, F. Tao, Harnessing interactional sensory genes for rationally reprogramming chaotic metabolism, Research 2022 (2022) 0017, doi:10.34133/research.0017.
[85] S.Z. Tan, C.R. Reisch, K.L.J. Prather, A robust CRISPR interference gene repression system in *Pseudomonas*, J. Bacteriol. 200 (2018) 7, doi:10.1128/jb.00575-17.
[86] Y. Tong, P. Charusanti, L. Zhang, T. Weber, S.Y. Lee, CRISPR-Cas9 based engineering of actinomycetal genomes, ACS Synth. Biol. 4 (2015) 1020–1029, doi:10.1021/acssynbio.5b00038.
[87] A. Vahedi, M.M.S. Dallal, M. Douraghi, F. Nikkhahi, Z. Rajabi, M. Yousefi, M. Mousavi, Isolation and identification of specific bacteriophage against enteropathogenic *Escherichia coli* (EPEC) and *in vitro* and *in vivo* characterization of bacteriophage, FEMS Microbiol. Lett. 365 (2018) fny136, doi:10.1093/femsle/fny136.
[88] R.T. Walton, K.A. Christie, M.N. Whittaker, B.P. Kleinstiver, Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants, Science 368 (2020) 290–296, doi:10.1126/science.aba8853.
[89] J. Wang, C. Li, T. Jiang, Y. Yan, Biosensor-assisted titratable CRISPRi high-throughput (BATCH) screening for over-production phenotypes, Metab. Eng. 75 (2023) 58–67, doi:10.1016/j.ymben.2022.11.004.
[90] J. Wang, P. Zhao, Y. Li, L. Xu, P. Tian, Engineering CRISPR interference system in *Klebsiella pneumoniae* for attenuating lactic acid synthesis, Microb. Cell Fact. 17 (2018) 56, doi:10.1186/s12934-018-0903-1.
[91] M.M. Wang, L.T. Liu, L.H. Fan, T.W. Tan, CRISPRi based system for enhancing L-butanol production in engineered *Kiebsiella pneumoniae*, Process Biochem. 56 (2017) 139–146, doi:10.1016/j.procbio.2017.02.013.
[92] T. Wang, C. Guan, J. Guo, B. Liu, Y. Wu, Z. Xie, C. Zhang, X.H. Xing, Pooled CRISPR interference screening enables genome-scale functional genomics study in bacteria with superior performance, Nat. Commun. 9 (2018) 2475, doi:10.1038/s41467-018-04899-x.
[93] T. Wang, J. Guo, Y. Liu, Z. Xue, C. Zhang, X.H. Xing, Genome-wide screening identifies promiscuous phosphatases impairing terpenoid biosynthesis in *Escherichia coli*, Appl. Microbiol. Biotechnol. 102 (2018) 9771–9780, doi:10.1007/s00253-018-9330-9.
[94] Y. Wang, Y. Liu, J. Liu, Y.M. Guo, L.W. Fan, X.M. Ni, X.M. Zheng, M. Wang, P. Zheng, J.B. Sun, Y.H. Ma, MACBETH: multiplex automated *Corynebacterium glutamicum* base editing method, Metab. Eng. 47 (2018) 200–210, doi:10.1016/j.ymben.2018.02.016.
[95] Y. Wang, Z.T. Zhang, S.O. Seo, P. Lynn, T. Lu, Y.S. Jin, H.P. Blaschek, Gene transcription repression in *Clostridium beijerinckii* using CRISPR-dCas9, Biotechnol. Bioeng. 113 (2016) 2739–2743, doi:10.1002/bit.26020.
[96] M.T. Weinstock, E.D. Hesek, C.M. Wilson, D.G. Gibson, *Vibrio natriegens* as a fast-growing host for molecular biology, Nat. Methods 13 (2016) 849–851, doi:10.1038/nmeth.3970.
[97] A.W. Westbrook, M. Moo-Young, C.P. Chou, Development of a CRISPR-Cas9 tool kit for comprehensive engineering of *Bacillus subtilis*, Appl. Environ. Microbiol. 82 (2016) 4876–4895, doi:10.1128/AEM.01159-16.
[98] K.M. Wetmore, M.N. Price, R.J. Waters, J.S. Lamson, J. He, C.A. Hoover, M.J. Blow, J. Bristow, G. Butland, A.P. Arkin, A. Deutschbauer, Rapid quantification of mutant fitness in diverse bacteria by sequencing randomly bar-coded transposons, MBio 6 (2015) e00306–e00315, doi:10.1128/mBio.00306-15.
[99] S. Wolf, J. Becker, Y. Tsuge, H. Kawaguchi, A. Kondo, J. Marienhagen, M. Bott, V.F. Wendisch, C. Wittmann, Advances in metabolic engineering of *Corynebacterium glutamicum* to produce high-value active ingredients for food, feed, human health, and well-being, Essays Biochem. 65 (2021) 197–212, doi:10.1042/ebc20200134.
[100] Y.K. Wu, Y.F. Liu, X.Q. Lv, J.H. Li, G.C. Du, L. Liu, CAMERS-B: CRISPR/Cpf1 assisted multiple-genes editing and regulation system for *Bacillus subtilis*, Biotechnol. Bioeng. 117 (2020) 1817–1825, doi:10.1002/bit.27322.
[101] J. Xiao, H.Y. Jia, L.P. Pan, Z.H. Li, L.N. Lv, B.P. Du, L.Y. Zhang, F.J. Du, Y.X. Huang, T.M. Cao, Q. Sun, R.R. Wei, A.Y. Xing, Z.D. Zhang, Application of the CRISPRi system to repress *sepF* expression in *Mycobacterium smegmatis*, Infect. Genet. Evol. 72 (2019) 183–190, doi:10.1016/j.meegid.2018.06.033.
[102] T. Yamano, H. Nishimasu, B. Zetsche, H. Hirano, I.M. Slaymaker, Y. Li, I. Fedorova, T. Nakane, K.S. Makarova, E.V. Koonin, R. Ishitani, F. Zhang, O. Nureki, Crystal structure of Cpf1 in complex with guide RNA and target DNA, Cell 165 (2016) 949–962, doi:10.1016/j.cell.2016.04.003.
[103] W.X. Yan, S. Chong, H. Zhang, K.S. Makarova, E.V. Koonin, D.R. Cheng, D.A. Scott, Cas13d is a compact RNA-targeting type VI CRISPR effector positively modulated by a WYL-domain-containing accessory protein, Mol. Cell 70 (2018) 327–339, doi:10.1016/j.molcel.2018.02.028.
[104] L. Yao, I. Cengic, J. Anfelt, E.P. Hudson, Multiple gene repression in cyanobacteria using CRISPRi, ACS Synth. Biol. 5 (2016) 207–212, doi:10.1021/acssynbio.5b00264.
[105] L. Yao, K. Shabestary, S.M. Bjork, J. Asplund-Samuelsson, H.N. Joensson, M. Jahn, E.P. Hudson, Pooled CRISPRi screening of the cyanobacterium *Synechocystis* sp PCC 6803 for enhanced industrial phenotypes, Nat. Commun. 11 (2020) 1666, doi:10.1038/s41467-020-15491-7.
[106] L. Yu, M.A. Marchisio, *Saccharomyces cerevisiae* synthetic transcriptional networks harnessing dCas12a and type V-A anti-CRISPR proteins, ACS Synth. Biol. 10 (2021) 870–883, doi:10.1021/acssynbio.1c00006.
[107] X. Yu, S. Li, H. Feng, X. Liao, X.-H. Xing, Z. Bai, X. Liu, C. Zhang, CRISPRi-microfluidics screening enables genome-scale target identification for high-titer protein production and secretion, Metab. Eng. 75 (2023) 192–204, doi:10.1016/j.ymben.2022.12.004.
[108] J.G. Zalatan, M.E. Lee, R. Almeida, L.A. Gilbert, E.H. Whitehead, M. La Russa, J.C. Tsai, J.S. Weissman, J.E. Dueber, L.S. Qi, W.A. Lim, Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds, Cell 160 (2015) 339–350, doi:10.1016/j.cell.2014.11.052.
[109] B. Zetsche, J.S. Gootenberg, O.O. Abudayyeh, I.M. Slaymaker, K.S. Makarova, P. Essletzbichler, S.E. Volz, J. Joung, J. van der Oost, A. Regev, E.V. Koonin, F. Zhang, Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, Cell 163 (2015) 759–771, doi:10.1016/j.cell.2015.09.038.
[110] J.L. Zhang, Y.Z. Peng, D. Liu, H. Liu, Y.X. Cao, B.Z. Li, C. Li, Y.J. Yuan, Gene repression via multiplex gRNA strategy in *Y. lipolytica*, Microb. Cell Fact. 17 (2018) 62, doi:10.1186/s12934-018-0909-8.
[111] K. Zhang, Z.H. Zhang, J.N. Kang, J.Z. Chen, J. Liu, N. Gao, L.W. Fan, P. Zheng, Y. Wang, J.B. Sun, CRISPR/Cas13d-mediated microbial RNA knockdown, Front. Bioeng. Biotechnol. 8 (2020) 56, doi:10.3389/fbioe.2020.00856.
[112] X. Zhang, J. Wang, Q. Cheng, X. Zheng, G. Zhao, J. Wang, Multiplex gene regulation by CRISPR-ddCpf1, Cell Discov. 3 (2017) 17018, doi:10.1038/celldisc.2017.18.
[113] C.L. Zhao, X.Q. Shu, B.L. Sun, Construction of a gene knockdown system based on catalytically inactive ("Dead") Cas9 (dCas9) in *Staphylococcus aureus*, Appl. Environ. Microb. 83 (2017) 12, doi:10.1128/aem.00291-17.
[114] Y.L. Zheng, J.M. Han, B.Y. Wang, X.Y. Hu, R.X. Li, W. Shen, X.D. Ma, L.X. Ma, L. Yi, S.H. Yang, W.F. Peng, Characterization and repurposing of the endogenous type I-F CRISPR-Cas system of *Zymomonas mobilis* for genome engineering, Nucleic. Acids. Res. 47 (2019) 11461–11475, doi:10.1093/nar/gkz940.