Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Challenges and opportunities with CRISPR activation in bacteria for data-driven metabolic engineering

Jason Fontana[1,4], David Sparkman-Yager[1,4], Jesse G Zalatan[2] and James M Carothers[3]

Creating CRISPR gene activation (CRISPRa) technologies in industrially promising bacteria could be transformative for accelerating data-driven metabolic engineering and strain design. CRISPRa has been widely used in eukaryotes, but applications in bacterial systems have remained limited. Recent work shows that multiple features of bacterial promoters impose stringent requirements on CRISPRa-mediated gene activation. However, by systematically defining rules for effective bacterial CRISPRa sites and developing new approaches for encoding complex functions in engineered guide RNAs, there are now clear routes to generalize synthetic gene regulation in bacteria. When combined with multi-omics data collection and machine learning, the full development of bacterial CRISPRa will dramatically improve the ability to rapidly engineer bacteria for bioproduction through accelerated design-build-test-learn cycles.

Addresses
[1] Molecular Engineering & Sciences Institute and Center for Synthetic Biology, University of Washington. Seattle, WA 98195, United States
[2] Department of Chemistry, University of Washington. Seattle, WA 98195, United States
[3] Department of Chemical Engineering, University of Washington. Seattle, WA 98195, United States

Corresponding authors: Zalatan, Jesse G (zalatan@uw.edu), Carothers, James M (jcaroth@uw.edu)
[4] These authors contributed equally.

Current Opinion in Biotechnology 2020, **64**:190–198

This review comes from a themed issue on **Analytical biotechnology**

Edited by **Yinjie Tang** and **Ludmilla Aristilde**

https://doi.org/10.1016/j.copbio.2020.04.005

Introduction

Bacterial metabolism is made up of complex gene networks that can be engineered to produce medically important and industrially important chemicals. The complexity of these networks means that sophisticated organism engineering efforts are typically needed to optimize the production of high-value compounds [1,2]. In principle, synthetic multi-gene transcriptional programs could be constructed to engineer metabolic networks for efficient industrial chemical production [3,4]. In practice, however, an incomplete understanding of metabolic networks, combined with a limited ability to predictably control the expression of multiple genes, makes achieving this goal difficult [5]. A recurring challenge when engineering strains for chemical production remains the difficulty of predicting the optimal expression level of pathway and non-pathway genes that will result in optimal yields. To overcome this challenge, there is a need for new technologies for rapidly implementing and analyzing combinatorial multi-gene expression programs. These technologies could be combined with advanced capabilities for multi-omics data collection and machine learning to enable accelerated design-build-test-learn cycles (DBTL) [1,6,7].

CRISPR-Cas tools have changed every aspect of microbial engineering, including the speed with which genomes can be edited and the ability to target specific genes for activation and repression [8,9]. CRISPR-Cas tools for programming gene expression use the catalytically inactive Cas9 protein (dCas9) along with guide RNAs that recognize DNA targets through predictable Watson–Crick base pairing [9]. A major strength of CRISPR-based synthetic gene regulation is that new combinatorial multi-gene expression programs that include simultaneous transcriptional activation and repression could be rapidly implemented.

There are well-established approaches for repressing genes (CRISPRi) by targeting dCas9 to physically block RNA polymerase and inhibit transcription [10]. In eukaryotic cells, robust transcriptional activation can be applied using CRISPR-Cas to direct activation domains upstream of target genes (CRISPRa) [8,9]. However, the development of CRISPRa in bacteria has been hindered by the lack of effective activation domains. The recent discovery that at least four different bacterial activators can be linked to programmable CRISPR-Cas DNA binding domains has promised to significantly change the outlook for CRISPRa in bacteria [11–14]. Further, new efforts to uncover practical rules for activating transcription with bacterial CRISPRa may make it possible to build complex multi-gene programs that regulate the expression of both heterologous and endogenous genes. By building on recent efforts, the further development of engineered guide RNAs as flexible platforms for programming CRISPRa may create new capabilities for predictable and metabolite-responsive synthetic gene regulation [15,16]. This review focuses on new advancements in

bacterial CRISPRa technologies that promise to significantly accelerate strain optimization through data-driven metabolic engineering.

CRISPRa for regulating bacterial transcription

In bacteria, the implementation of complex multi-gene CRISPR-Cas expression programs has been limited by a lack of effective gene activators. To address this problem, new synthetic transcriptional activators have been developed in *Escherichia coli* that link activation domains to programmable CRISPR-Cas DNA binding domains [11–14]. The resulting CRISPRa tools have proven capable of driving heterologous gene expression at levels suitable for metabolic engineering. Some successes have also been achieved in activating the expression of endogenous genes from genomic loci. The further development of these capabilities may permit the optimization of metabolic production through the construction of multi-gene programs simultaneously targeting heterologous and endogenous genes.

There are two mechanistic approaches that have been employed to link activation domains to CRISPR-Cas DNA binding domains. Activation domains can be (i) directly fused to dCas9, or (ii) recruited to dCas9 using modified sgRNAs (scaffold RNAs or scRNAs) that bind to RNA binding protein-activation domain fusions [17–19]. Using the first approach, CRISPRa has been achieved in *E. coli* by fusing the ω-subunit of RNA polymerase (*rpoZ*) to dCas9 to obtain 23-fold increases in reporter gene expression from synthetic promoters [11]. These fusions have been applied both in *E. coli* and non-model bacteria. In *E. coli*, dCas9-RpoZ was used activate transcription and identify genes that increase tolerance to the monoterpene pinene, as well as new epistatic interactions between antibiotic resistance genes [20,21]. These tools were successfully ported to *Bacillus subtilis* to obtain threefold activation of reporter gene expression and applied to systematically improve production of amylase BLA by 260-fold compared to a commonly used strong promoter [22]. In *Lysobacter enzymogenes*, dCas9-RpoZ was used to enhance production of anti-MRSA antibiotics up to ninefold [23]. In *Myxococcus xanthus*, dCas9-RpoZ was able to generate eightfold increases in the expression of the epothilone production gene cluster, leading to a 6.8-fold improvement in epothilone A production [24]. Finally, a new portable CRISPRa system where the activation domain AsiA was fused to dCas9 was recently introduced [14]. Using this system, reporter gene expression could be activated by 135-fold in *E. coli*, ~3-fold in *Salmonella enterica*, and ~12-fold in *Klebsiella oxytoca*.

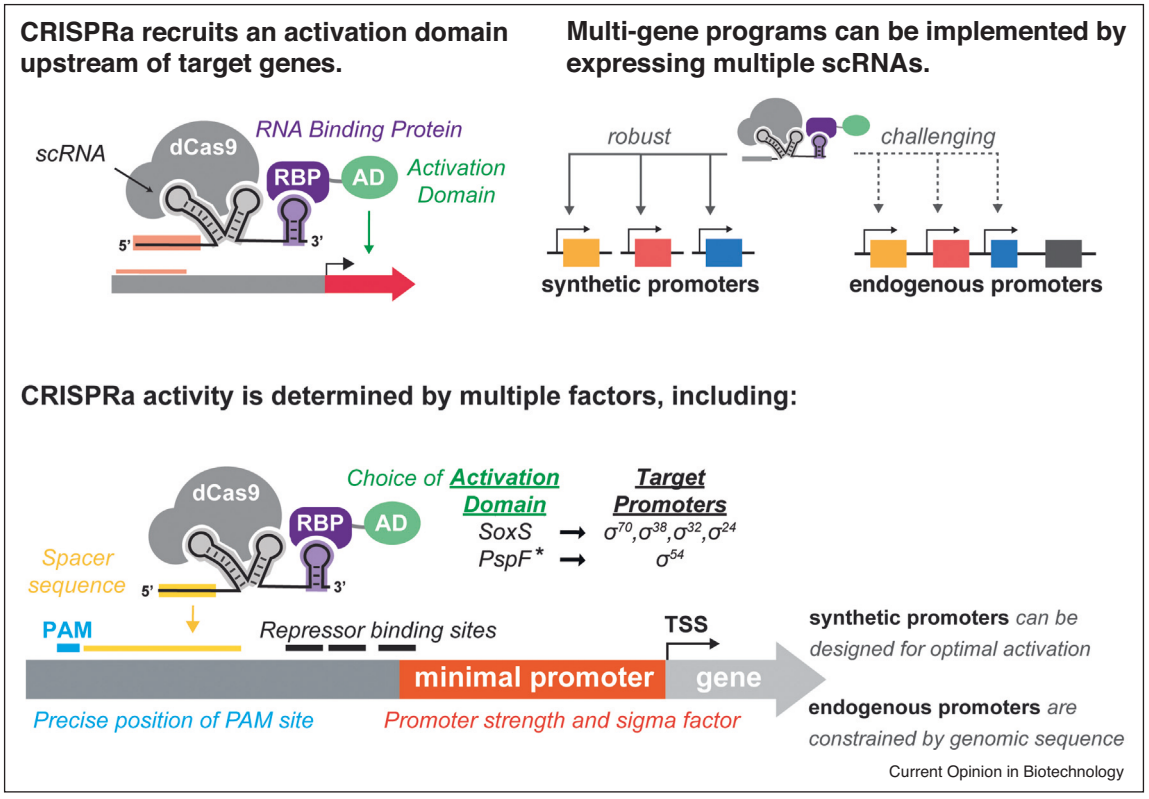
The second approach for bacterial CRISPRa relies on modified gRNAs (scRNAs) that recruit an RNA binding protein fused to an activation domain to the CRISPRa complex. One successful strategy uses the RNA binding protein MCP fused to the SoxS activation domain (MCP-SoxS) (Figure 1) [12]. Using MCP-SoxS and a corresponding MS2 scRNA, 50-fold CRISPRa activation was demonstrated and applied to drive ethanol production in *E. coli* from a *Zymomonas mobilis* gene cluster. MCP-SoxS can activate expression from genes that use σ factors σ^{70} [12] and, at lower levels, σ^{38}, σ^{32}, and σ^{24} [25]. While together these σ^{70}-family promoters cover the majority of the *E. coli* genome, σ^{54} promoters, which drive nitrogen starvation genes, could not be activated by MCP-SoxS. Recently, an alternative bacterial CRISPRa system that is effective at σ^{54} promoters was introduced based on the PspFΔHTH::λN22plus activator [13]. Therefore, PspFΔHTH::λN22plus and MCP-SoxS can be used in combination to target a different, non-overlapping set of promoters in *E. coli*. Further, PspFΔHTH::λN22plus was reported to activate two promoters in the nitrogen fixation pathway of *K. oxytoca* by up to sixfold [13]. In the dCas9-RpoZ and PspFΔHTH::λN22plus systems, obtaining the highest levels of activation requires knocking out the native copy of *rpoZ* [11,26] or *pspF* [13], respectively, to remove the competing, endogenous functions. It is possible, however, to obtain significant activation without using knockout strains. In contrast, the MCP-SoxS and dCas9-AsiA systems do not require any host engineering to achieve their highest levels of activation.

The available CRISPRa tools are uniquely positioned to rapidly implement combinatorial multi-gene expression programs targeting synthetic promoters and identify optimal expression conditions for metabolite production [27]. These tools were recently applied in a proof-of-concept experiment to tune the expression of three genes in the pathway responsible for producing violacein, a pigment with antitumoral properties [13]. Further improving our ability to predictably tune CRISPRa at multiple sites independently could provide a technology for the rapid combinatorial optimization of multi-gene pathways. Dynamically controlled CRISPRi was recently shown to improve production of salicylic acid in engineered *E. coli* through the conditional knock-down of essential genes [28]. Developing dynamically controlled CRISPRa could provide additional avenues to control both the timing and expression levels of multiple genes in engineered metabolic pathways and networks [3].

Promoter design rules improve CRISPRa in bacteria

Recent work has identified multiple features of bacterial promoters that impose stringent requirements on CRISPRa-mediated gene activation [25] (Figure 1). These behaviors suggest an explanation for why CRISPRa and other tools for gene activation in bacteria have lagged far behind comparable tools in eukaryotic systems, where such strict target site requirements are absent. For instance, the activity of CRISPRa using MCP-SoxS is influenced by the strength of the target promoter, the sigma factor regulating the promoter and the sequence composition of the promoter [25]. Most strikingly, when

Figure 1



CRISPR activation (CRISPRa) is a powerful tool for programmable activation of genes in bacteria. A CRISPRa system is shown where an activation domain is recruited to dCas9 using a modified guide RNA (scaffold RNA, scRNA) that binds to an RNA binding protein-activation domain fusion (RBP-AD). While CRISPRa can be used to robustly activate synthetic promoters designed for optimal activation, activating endogenous genes is constrained by the genomic sequence. Factors known to determine CRISPRa activity are indicated. *: PspF activation was demonstrated using a different modified sgRNA design where two BoxB aptamers were incorporated into the Cas9 handle.

activating synthetic promoters in bacteria, CRISPRa is sensitive to the position and periodicity of the scRNA target site relative to the transcription start site (TSS) [13,25]. Activation can only be performed at precisely defined positions in phase with the transcription start site, which are intervened by regions of lower activity or inactivity [13,25]. These requirements are much more stringent than those for activation in eukaryotic cells [17] and constrain CRISPRa to precisely positioned PAM sites which may not be found on every gene. Engineered Cas9 variants and alternative Cas proteins have been introduced that expand the range of PAM sequences that can be targeted and increase the density of available PAM sites up to 6 times [25,29–31]. One of the variants, dxCas9 (3.7), has been used to demonstrate activation of *E. coli* genes previously inaccessible by dCas9 [13,25]. By combining dxCas9(3.7) and newly defined rules for CRISPRa, 3 out of 7 endogenous *E. coli* genes were successfully activated [25]. However, the field still lacks integrated models for predicting effective CRISPRa target sites for arbitrary genes, and explanations for the failure to activate some genes remain elusive. Genome-wide CRISPRa screens of endogenous promoters could more fully elucidate the requirements for CRISPRa targeting. Once predictive rules for targeting endogenous genes are available, combinatorial multi-gene programs for optimizing bioproduction could be extended to endogenous genes, in addition to synthetic promoters.

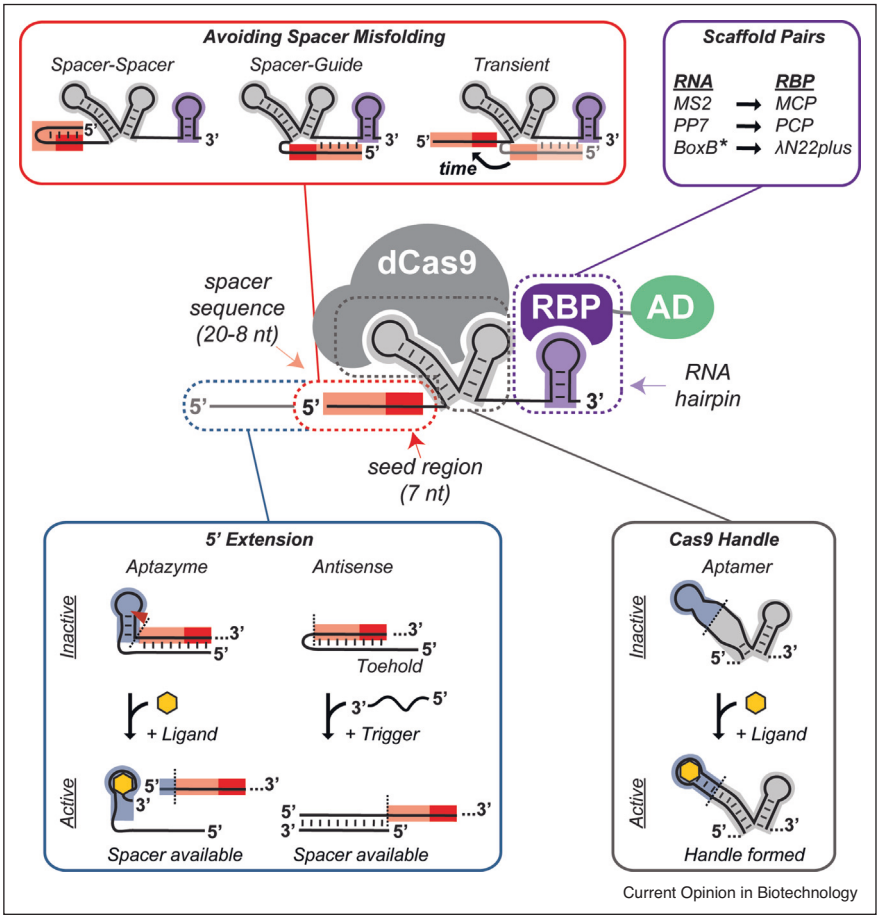
gRNAs can be engineered to program CRISPRa responses

gRNA engineering has long been understood to provide routes for tuning CRISPR-Cas functions, and more recently, as a mechanism for encoding dynamic responses to molecular targets. While most of the gRNA design work to date has been performed on guides used for DNA cleavage or CRISPRi, the principles, whether controlling the stability of the guide-Cas9 complex or the entire DNA-guide-Cas9 complex, may be readily applicable to CRISPRa efforts. Guide RNAs are made up of two components: the twenty nucleotide spacer sequence, which hybridizes to the target DNA, and the Cas9-binding handle, which drives the formation of the gRNA-Cas9 complex. Structurally, the only difference between a

gRNA and a scRNA used for CRISPRa is the presence of an additional 3′ RNA hairpin. This hairpin enables the co-localization of the activation domain by binding its cognate RBP tag. While this motif adds additional complexity, it also provides more opportunities for design. Several alternative architectures have also been described that insert the RNA hairpin motif at multiple points within the guide [32,33]. To date, three cognate pairs of RNA binding protein (RBP) and RNA hairpin have been utilized to implement CRISPRa in bacteria (Figure 2) [12,13]. Other pairs have been demonstrated in eukaryotes and may be functional in bacteria as well [18,34]. These orthogonal pairs provide the opportunity to simultaneously implement multiple activators in the same cell.

While the relative simplicity of gRNAs allows the rational specification of target sites, the sequence identity of a chosen site has been demonstrated to have a significant impact on its CRISPR activity. Both the sequence identity and secondary-structure (base-pairing interactions) of gRNA elements are critical for function [35]. There have been several attempts to predict CRISPR activity for novel target sequences, and while these models can be used to increase the probability of selecting a functional guide, they primarily utilize sequence elements, rather than structural information, and have not been applied directly to CRISPRa [36–38]. One key feature that has been demonstrated to influence CRISPR activity is the secondary structure that the guide RNA adopts [39,40]. The degree of secondary structure, whether internal to the spacer or between the spacer and the rest of the guide, has been observed to reduce gRNA effectiveness (Figure 2) [39]. Even the transiently stable structures the guides adopt during transcription have been

Figure 2



Guide RNA (gRNA) structural determinants of CRISPRa activity. gRNA structure can be deleterious to function, as in the case of spacer misfolding, or can be useful for programming dynamic responses. Example dynamic gRNA engineering strategies that may apply to bacterial CRISPRa include extending the 5′ end of the spacer to respond to ligands or RNA trigger strands. Ligand-responsive CRISPRa activities may also be obtainable by inserting ligand-binding aptamers into the Cas9 binding handle. Several cognate pairs of RNA binding protein (RBP) and RNA hairpin have been demonstrated in bacteria, enabling the simultaneous implementation of CRISPRa with different activation domains (AD).
*: CRISPRa using the BoxB:λ22plus pair was demonstrated using two BoxB aptamers incorporated into the Cas9 handle.

demonstrated to impact their activity [39]. As the guides utilized for CRISPRa are actively transcribed from heterologous promoters inside the cell, avoiding transient misfolding may prove important for achieving predictable activity. Thus, developing tools for screening gRNA co-transcriptional folding pathways may aid in the *a priori* selection of highly functional spacer sequences.

To generate differences in the expression levels of multiple genes, it is necessary to develop a general strategy to fine-tune CRISPRa-mediated gene expression at each promoter. To date, two main strategies have been demonstrated to modulate the CRISPR activity for a given target sequence: spacer truncations and 5′ extension. In CRISPRi systems, the level of transcriptional repression applied to target genes has been reduced by truncating the sgRNA target sequence from the 5′ end [10,41]. Practically, spacers shorter than 12 nucleotides may increase off-target activity as the first 12 nt, and even more so the first 7 nt known as the 'seed region', have an especially large impact on the activity [10,42]. Alternatively, it has been demonstrated that adding a 5′ extension onto the guide, which folds back to occlude the spacer, results in monotonic drops in guide activity with increasing stability of the designed interaction; this correspondence even applies to guides from other Cas proteins with different guide architectures [43]. This demonstration provides evidence that computational predictions of gRNA structure may be sufficient to predict guide function. In order to improve the forward engineering of CRISPRa systems and accelerate DBTL cycles, developing quantitatively accurate predictions of CRISPRa activity based on scRNA structure will be essential.

Towards nucleic acid-responsive gRNAs for CRISPRa

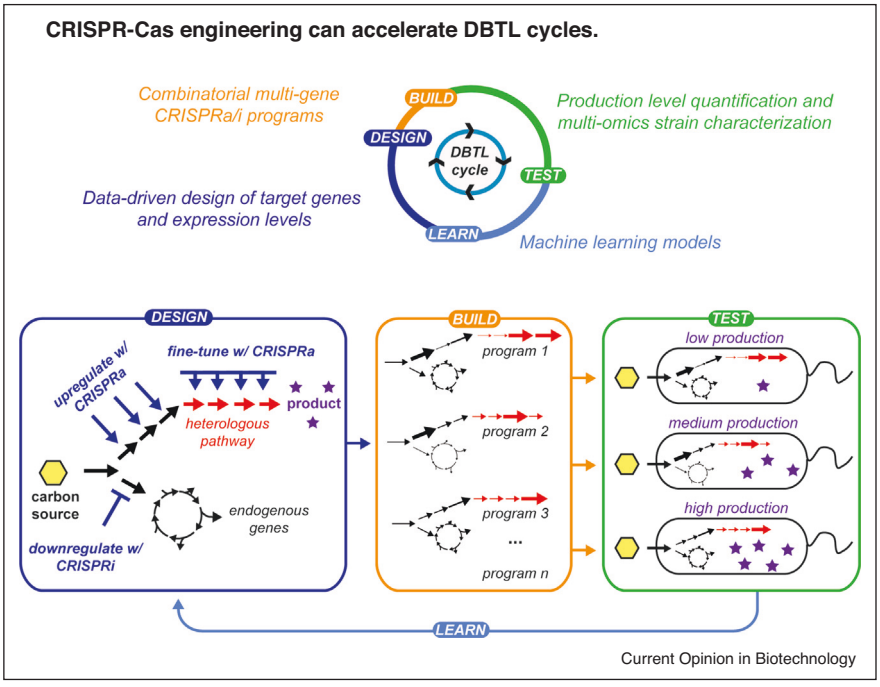
In order to implement complex genetic and metabolic circuits, it becomes necessary to be able to link the intracellular concentration of target molecules to the regulatory circuit being implemented. One such implementation would be to use the levels of cellular RNAs to regulate the activity of CRISPR-based transcriptional programs. To that end, there have been several demonstrations that the activity of a gRNA can be regulated by the presence of target nucleic acid sequences that hybridize to the gRNA [44]. While there have been slightly different implementations, the general principle is that a trans-acting 'trigger' strand is able to bind to a gRNA and either occlude or reveal the spacer sequence, modulating the guide's activity. One of the most common mechanisms is inspired by a previously published 'toehold switch', in which a cis-repressed gRNA is activated upon toehold-mediated hybridization (Figure 2) [45]. Several gRNA switches have even demonstrated the ability to respond to RNA trigger strands within a cell to control gene expression levels [46–49]. For example, 'toehold-gated' sgRNAs (thgRNAs) were capable of inducing CRISPRi in response to endogenous small RNAs (sRNAs) and mRNAs in *E. coli*, with repression up to fivefold [46]. However, half of the thgRNAs responsive to endogenous RNAs resulted in low levels of repression (<2-fold). Unlike short synthetic RNA trigger strands, longer endogenous RNAs may be less effective as trigger strands due to competition for binding from intramolecular RNA structure and cellular proteins. Improving the activity of thgRNAs responsive to cellular RNAs will require advancements in the *a priori* identification of sites within cellular RNAs that can be utilized as highly active trigger strands. Furthermore, in order to apply these mechanisms to CRISPRa, it will be necessary to ensure that the interaction between a trigger-responsive scRNA and a large cellular RNA does not itself interfere with the mechanism of CRISPRa activation.

Metabolite-responsive gRNAs for CRISPRa

In addition to regulation by cellular RNAs, the ability to regulate CRISPR activity in response to real-time concentrations of cellular metabolites would provide many opportunities for implementing and accelerating DBTL cycles for metabolic engineering. Metabolite responsive gRNAs could be used for both readouts of intracellular metabolite concentrations to inform machine learning models, or for implementing model-suggested regulation such as feedback or feedforward motifs. While efforts to design metabolite-responsive gRNAs are fairly new, metabolite-responsive RNAs have become useful tools in metabolic engineering [50,51]. By combining a metabolite-binding RNA aptamer with a control structure, the binding state of the aptamer can be converted into a conditional genetic output.

There have been several demonstrations that small molecule responsive gRNA activity can be dynamically regulated with an aptamer in *cis* [15,16,52,53]. Some demonstrations involve inserting the aptamer at the 3′ end of the guide, where it stabilizes the active gRNA structure in a ligand-responsive manner [52,53]. However, adding both an aptamer and a recruitment hairpin to the 3′ end of the RNA could interfere with gRNA folding and function. Other strategies, which utilize 5′ extension or Cas9 handle insertion, may therefore be preferable. For example, an aptazyme, or ligand-responsive self-cleaving ribozyme, was used to remove a repressive 5′ extension from the guide upon the addition of the target ligand (Figure 2) [16]. This resulted in ligand-responsive control over both Cas9-mediated cleavage and CRISPRa in mammalian cells. In another example, aptamers were inserted into the Cas9-binding handle, or one of two other gRNA hairpins, generating ligand-responsive CRISPRi in *E. coli* (Figure 2) [15]. Depending on the aptamer insertion site within the guide, the addition of ligand can either activate or deactivate CRISPRi.

Figure 3



Developing robust workflows to integrate CRISPRa/i engineering into data-driven workflows will create new capabilities for rapidly optimizing chemical production. In this conception, each Design-Build-Test-Learn (DBTL) cycle uses machine learning and data-driven design to engineer multi-gene CRISPRa/i programs. After each build phase, production titers are measured and the strains are characterized using multi-omics analysis. These data are employed to refine the models and drive the design of CRISPRa/i programs for the next DBTL cycle.

The above successes in identifying ligand-responsive gRNAs provide great confidence that it will be possible to engineer small molecule-responsive scRNAs for conditional CRISPRa. However, there are still hurdles to overcome before metabolite-responsive CRISPRa can be used effectively for metabolic engineering applications. First, design rules that allowing reliable integration of aptamers with diverse sequences and secondary structures into scRNAs must be uncovered. Second, mechanisms for tuning the response to match the desired metabolite concentrations must be developed [54]. Aptamer-regulated kinetic control mechanisms, similar to those found in natural bacterial riboswitches, may provide an approach for engineering metabolite-responsive CRISPRa targeted to specific concentrations of metabolites [55,56]. For example, 10-fold variations in switching concentration among a family of *E. coli* thiamine pyrophosphate (TPP) riboswitches are known to be the result of differences in the amount of time the RNA is available to interact with the ligand [56]. Creating kinetically controlled aptamer-regulated scRNAs may confer the ability to engineer metabolite-responsive CRISPRa systems functional as feedback controllers, or production biosensors useful for optimizing strain performance.

Conclusions

The relationships between the expression levels and reaction kinetics for enzymes in both endogenous and engineered metabolic networks are poorly understood. This incomplete knowledge constitutes a major limitation for the field of metabolic engineering [1]. Because of these gaps, data-driven methods relying on cycles of genetic engineering, high-throughput production screening, multi-omics analysis, and machine learning have become increasingly central to strain optimization [57,58]. To accelerate data-driven metabolic engineering, methods to independently target and predictably manipulate the expression levels of multiple genes are needed. By coupling new tools for CRISPRa with existing approaches for CRISPRi, it should be possible to more efficiently search gene expression spaces and optimize bioproduction in engineered bacteria through accelerated DBTL cycles (Figure 3).

CRISPRa can now be used to selectively activate synthetic promoters with large dynamic ranges and in a way that is relatively straightforward to implement. Recent advances in gRNA design have enabled the identification of small-molecule responsive gRNAs able to dynamically regulate gene expression in *E. coli*, opening the door for the development of CRISPRa-based metabolite biosensors and

circuit controllers. The ability to use CRISPRa to activate endogenous genes remains limited by the sequence constraints of the native genomic loci, where less-than-optimal position of PAM site or the inherent features of the promoters can significantly impact the activation that can be achieved. Predictive models are needed to identify which endogenous genes can be activated and which target sites are the most effective. The refinement of sequence and structure-based rules for constructing synthetic promoters and cognate scRNAs for expressing heterologous genes will improve the ability to precisely tune multi-gene pathways. CRISPRa has been demonstrated in *E. coli* [11–14,20,21,25] and other industrially and medically relevant bacteria including *B. subtilis* [22], *K. oxytoca* [13,14], *L. enzymogenes* [23], *Myxococcus xhantus* [24] and *S. enterica* [14]. Porting these tools to other non-model bacteria with diverse substrate utilization, a range of metabolic capabilities, and resistance to harsh bioprocessing conditions could accelerate the development of efficient bioproduction processes. Collectively, these strategies lay the groundwork for more widespread use of bacterial CRISPRa in basic research and advanced applications in data-driven metabolic engineering.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Jason Fontana: Conceptualization, Visualization, Writing - original draft, Writing - review & editing. **David Sparkman-Yager:** Conceptualization, Visualization, Writing - original draft, Writing - review & editing. **Jesse G Zalatan:** Supervision, Funding acquisition, Writing - review & editing. **James M Carothers:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

Acknowledgements

The authors thank Joely Nelson and members of the Zalatan and Carothers groups for technical assistance and helpful discussions. This work was supported by NSF Award 1817623 (J.G.Z., J.M.C.) and NSF Award 1844152 (J.M.C.).

References

1. Nielsen J, Keasling JD: **Engineering cellular metabolism**. *Cell* 2016, **164**:1185-1197.

2. Lee SY, Kim HU, Chae TU, Cho JS, Kim JW, Shin JH, Kim DI, Ko Y-S, Jang WD, Jang Y-S: **A comprehensive metabolic map for production of bio-based chemicals**. *Nat Catal* 2019, **2**:18-33.

3. Fontana J, Voje WE, Zalatan JG, Carothers JM: **Prospects for engineering dynamic CRISPR–Cas transcriptional circuits to improve bioproduction**. *J Ind Microbiol Biotechnol* 2018, **45**:481-490.

4. Choi KR, Jang WD, Yang D, Cho JS, Park D, Lee SY: **Systems metabolic engineering strategies: integrating systems and synthetic biology with metabolic engineering**. *Trends Biotechnol* 2019, **37**:817-837.

5. Liu R, Bassalo MC, Zeitoun RI, Gill RT: **Genome scale engineering techniques for metabolic engineering**. *Metab Eng* 2015, **32**:143-154.

6. Carbonell P, Jervis AJ, Robinson CJ, Yan C, Dunstan M, Swainston N, Vinaixa M, Hollywood KA, Currin A, Rattray NJW *et al.*: **An automated Design-Build-Test-Learn pipeline for enhanced microbial production of fine chemicals**. *Commun Biol* 2018, **1**:1-10.

7. Brunk E, George KW, Alonso-Gutierrez J, Thompson M, Baidoo E, Wang G, Petzold CJ, McCloskey D, Monk J, Yang L *et al.*: **Characterizing strain variation in engineered *E. coli* using a multi-omics-based workflow**. *Cell Syst* 2016, **2**:335-346.

8. Wang H, La Russa M, Qi LS: **CRISPR/Cas9 in genome editing and beyond**. *Annu Rev Biochem* 2016, **85**:227-264.

9. Dominguez AA, Lim WA, Qi LS: **Beyond editing: repurposing CRISPR–Cas9 for precision genome regulation and interrogation**. *Nat Rev Mol Cell Biol* 2016, **17**:5-15.

10. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA: **Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression**. *Cell* 2013, **152**:1173-1183.

11. Bikard D, Jiang W, Samai P, Hochschild A, Zhang F, Marraffini LA: **Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system**. *Nucleic Acids Res* 2013, **41**:7429-7437.

12. Dong C, Fontana J, Patel A, Carothers JM, Zalatan JG: **Synthetic CRISPR-Cas gene activators for transcriptional reprogramming in bacteria**. *Nat Commun* 2018, **9**:2489.
This paper introduced a new bacterial CRISPRa system capable of generating high levels of activation at σ^{70} promoters. This work demonstrated the first use of scaffold RNAs in bacteria and their use for simultaneous CRISPRa and CRISPRi.

13. Liu Y, Wan X, Wang B: **Engineered CRISPRa enables programmable eukaryote-like gene activation in bacteria**. *Nat Commun* 2019, **10**:3693.
This work describes a bacterial CRISPRa system that activates σ^{54} promoters using a new activation domain based on the PspF activator, expanding the range of promoters that can be targeted with CRISPRa.

14. Ho H-I, Fang J, Cheung J, Wang HH: **Programmable and portable CRISPR-Cas transcriptional activation in bacteria**. *bioRxiv* 2020 http://dx.doi.org/10.1101/2020.01.03.882431.

15. Kundert K, Lucas JE, Watters KE, Fellmann C, Ng AH, Heineike BM, Fitzsimmons CM, Oakes BL, Qu J, Prasad N *et al.*: **Controlling CRISPR-Cas9 with ligand-activated and ligand-deactivated sgRNAs**. *Nat Commun* 2019, **10**:1-11.
This paper demonstrates a strategy for isolating ligand-activated and ligand-repressed guide RNAs in bacteria. The authors show that different aptamers and different insertion sites within the guide can be successfully utilized in the same screening methodology. It provides a potential roadmap for designing metabolite-responsive guides for CRISPR activation.

16. Tang W, Hu JH, Liu DR: **Aptazyme-embedded guide RNAs enable ligand-responsive genome editing and transcriptional activation**. *Nat Commun* 2017, **8**:15939.

17. Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH, Guimaraes C, Panning B, Ploegh HL, Bassik MC *et al.*: **Genome-scale CRISPR-mediated control of gene repression and activation**. *Cell* 2014, **159**:647-661.

18. Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS *et al.*: **Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds**. *Cell* 2015, **160**:339-350.

19. Mali P, Esvelt KM, Church GM: **Cas9 as a versatile tool for engineering biology**. *Nat Methods* 2013, **10**:957-963.

20. Niu F-X, Huang Y-B, Ji L-N, Liu J-Z: **Genomic and transcriptional changes in response to pinene tolerance and overproduction in evolved *Escherichia coli***. *Synth Syst Biotechnol* 2019, **4**:113-119.

21. Otoupal PB, Erickson KE, Escalas-Bordoy A, Chatterjee A: **CRISPR perturbation of gene expression alters bacterial**

fitness under stress and reveals underlying epistatic constraints. *ACS Synth Biol* 2017, **6**:94-107.

22. Lu Z, Yang S, Yuan X, Shi Y, Ouyang L, Jiang S, Yi L, Zhang G: **CRISPR-assisted multi-dimensional regulation for fine-tuning gene expression in *Bacillus subtilis***. *Nucleic Acids Res* 2019, **47** e40–e40.

23. Yu L, Su W, Fey PD, Liu F, Du L: **Yield improvement of the Anti-MRSA antibiotics WAP-8294A by CRISPR/dCas9 combined with refactoring self-protection genes in *Lysobacter enzymogenes* OH11**. *ACS Synth Biol* 2018, **7**:258-266.

24. Peng R, Wang Y, Feng W, Yue X, Chen J, Hu X, Li Z, Sheng D, Zhang Y, Li Y: **CRISPR/dCas9-mediated transcriptional improvement of the biosynthetic gene cluster for the epothilone production in *Myxococcus xanthus***. *Microb Cell Fact* 2018, **17**:15.

25. Fontana J, Dong C, Kiattisewee C, Chavali VP, Tickman BI, Carothers JM, Zalatan JG: **Effective CRISPRa-mediated control of gene expression in bacteria must overcome strict target site requirements**. *Nat Commun* 2020, **11**:1618 http://dx.doi.org/10.1101/770891.

26. Dove SL, Hochschild A: **Conversion of the ω subunit of *Escherichia coli* RNA polymerase into a transcriptional activator or an activation target**. *Genes Dev* 1998, **12**:745-754.

27. Tian T, Kang JW, Kang A, Lee TS: **Redirecting metabolic flux via combinatorial multiplex CRISPRi-mediated repression for isopentenol production in *Escherichia coli***. *ACS Synth Biol* 2019, **8**:391-402.
The authors show that a system of multiplexed CRISPRi repression can be applied to endogenous genes to increase isopentanol precursor availability. The framework described in this paper could be integrated with CRISPRa to apply multiplexed up and down-regulation of genes for strain optimization.

28. Dinh CV, Prather KLJ: **Development of an autonomous and bifunctional quorum-sensing circuit for metabolic flux control in engineered *Escherichia coli***. *Proc Natl Acad Sci U S A* 2019, **116**:25562-25568.

29. Hu Johnny H, Shannon DRL, Miller M, Geurts Maarten H, Tang Weixin, Chen Liwei, Sun Ning, Zeina Christina M, Gao Xue, Rees Holly A, Lin Zhi: **Evolved Cas9 variants with broad PAM compatibility and high DNA specificity**. *Nature* 2018, **556**:57-63.

30. Chatterjee P, Jakimo N, Jacobson JM: **Robust genome editing of single-base PAM targets with engineered ScCas9 variants**. *bioRxiv* 2019 http://dx.doi.org/10.1101/620351.

31. Martella A, Firth M, Taylor BJM, Göppert A, Cuomo EM, Roth RG, Dickson AJ, Fisher DI: **Systematic evaluation of CRISPRa and CRISPRi modalities enables development of a multiplexed, orthogonal gene activation and repression system**. *ACS Synth Biol* 2019, **8**:1998-2006.

32. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H *et al.*: **Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex**. *Nature* 2015, **517**:583-588.

33. Shechner DM, Hacisuleyman E, Younger ST, Rinn JL: **Multiplexable, locus-specific targeting of long RNAs with CRISPR-display**. *Nat Methods* 2015, **12**:664-670.

34. Cheng AW, Jillette N, Lee P, Plaskon D, Fujiwara Y, Wang W, Taghbalout A, Wang H: **Casilio: a versatile CRISPR-Cas9-Pumilio hybrid for gene regulation and genomic labeling**. *Cell Res* 2016, **26**:254-257.

35. Briner AE, Donohoue PD, Gomaa AA, Selle K, Slorach EM, Nye CH, Haurwitz RE, Beisel CL, May AP, Barrangou R: **Guide RNA functional modules direct Cas9 activity and orthogonality**. *Mol Cell* 2014, **56**:333-339.

36. Moreno-Mateos MA, Vejnar CE, Beaudoin J-D, Fernandez JP, Mis EK, Khokha MK, Giraldez AJ: **CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo**. *Nat Methods* 2015, **12**:982-988.

37. Haeussler M, Schönig K, Eckert H, Eschstruth A, Mianné J, Renaud J-B, Schneider-Maunoury S, Shkumatava A, Teboul L, Kent J *et al.*: **Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR**. *Genome Biol* 2016, **17**:148.

38. Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, Smith I, Tothova Z, Wilen C, Orchard R *et al.*: **Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9**. *Nat Biotechnol* 2016, **34**:184-191.

39. Thyme SB, Akhmetova L, Montague TG, Valen E, Schier AF: **Internal guide RNA interactions interfere with Cas9-mediated cleavage**. *Nat Commun* 2016, **7**:1-7.
This paper illuminates the significant role of RNA structure in determining guide RNA activity. The authors demonstrate that even nonfunctional guide RNAs can degrade the performance of co-expressed guides, and highlight the rarely discussed impact that co-transcriptional folding can have on guide activity.

40. Smith JD, Schlecht U, Xu W, Suresh S, Horecka J, Proctor MJ, Aiyar RS, Bennett RAO, Chu A, Li YF *et al.*: **A method for high-throughput production of sequence-verified DNA libraries and strain collections**. *Mol Syst Biol* 2017, **13**:913.

41. Vigouroux A, Oldewurtel E, Cui L, Teeffelen S, van, Bikard D: **Engineered CRISPR-Cas9 system enables noiseless, fine-tuned and multiplexed repression of bacterial genes**. *bioRxiv* 2017 http://dx.doi.org/10.1101/164384.

42. Cui L, Vigouroux A, Rousset F, Varet H, Khanna V, Bikard D: **A CRISPRi screen in *E. coli* reveals sequence-specific toxicity of dCas9**. *Nat Commun* 2018, **9**:1-10.

43. Kocak DD, Josephs EA, Bhandarkar V, Adkar SS, Kwon JB, Gersbach CA: **Increasing the specificity of CRISPR systems with engineered RNA secondary structures**. *Nat Biotechnol* 2019, **37**:657-666.

44. Jin M, Garreau de Loubresse N, Kim Y, Kim J, Yin P: **Programmable CRISPR-Cas repression, activation, and computation with sequence-independent targets and triggers**. *ACS Synth Biol* 2019, **8**:1583-1589.

45. Green AA, Silver PA, Collins JJ, Yin P: **Toehold switches: de-novo-designed regulators of gene expression**. *Cell* 2014, **159**:925-939.

46. Siu K-H, Chen W: **Riboregulated toehold-gated gRNA for programmable CRISPR–Cas9 function**. *Nat Chem Biol* 2019, **15**:217-220.

47. Li Y, Teng X, Zhang K, Deng R, Li J: **RNA strand displacement responsive CRISPR/Cas9 system for mRNA sensing**. *Anal Chem* 2019, **91**:3989-3996.

48. Oesinghaus L, Simmel FC: **Switching the activity of Cas12a using guide RNA strand displacement circuits**. *Nat Commun* 2019, **10**:2092.

49. Hanewich-Hollatz MH, Chen Z, Hochrein LM, Huang J, Pierce NA: **Conditional guide RNAs: programmable conditional regulation of CRISPR/Cas function in bacterial and mammalian cells via dynamic RNA nanotechnology**. *ACS Cent Sci* 2019, **5**:1241-1249.

50. Abatemarco J, Sarhan MF, Wagner JM, Lin J-L, Liu L, Hassouneh W, Yuan S-F, Alper HS, Abate AR: **RNA-aptamers-in-droplets (RAPID) high-throughput screening for secretory phenotypes**. *Nat Commun* 2017, **8**:1-9.

51. Porter EB, Polaski JT, Morck MM, Batey RT: **Recurrent RNA motifs as scaffolds for genetically encodable small-molecule biosensors**. *Nat Chem Biol* 2017, **13**:295-301.

52. Liu Y, Zhan Y, Chen Z, He A, Li J, Wu H, Liu L, Zhuang C, Lin J, Guo X *et al.*: **Directing cellular information flow via CRISPR signal conductors**. *Nat Methods* 2016, **13**:938-944.

53. Lin B, An Y, Meng L, Zhang H, Song J, Zhu Z, Liu W, Song Y, Yang C: **Control of CRISPR-Cas9 with small molecule-activated allosteric aptamer regulating sgRNAs**. *Chem Commun* 2019, **55**:12223-12226 http://dx.doi.org/10.1039/C9CC05531B.

54. Ricci F, Vallée-Bélisle A, Simon AJ, Porchetta A, Plaxco KW: **Using nature's "Tricks" to rationally tune the binding properties of biomolecular receptors**. *Acc Chem Res* 2016, **49**:1884-1892.

55. Chen X, Ellington AD: **Design principles for ligand-sensing, conformation-switching ribozymes**. *PLoS Comput Biol* 2009, **5**: e1000620.

56. Guedich S, Puffer-Enders B, Baltzinger M, Hoffmann G, Da Veiga C, Jossinet F, Thore S, Bec G, Ennifar E, Burnouf D *et al.*: **Quantitative and predictive model of kinetic regulation by *E. coli* TPP riboswitches**. *RNA Biol* 2016, **13**:373-390.

57. Leavell MD, Singh AH, Kaufmann-Malaga BB: **High-throughput screening for improved microbial cell factories, perspective and promise**. *Curr Opin Biotechnol* 2020, **62**:22-28.

58. Costello Z, Martin HG: **A machine learning approach to predict metabolic pathway dynamics from time-series multiomics data**. *npj Syst Biol Appl* 2018, **4**:1-14.