CRISPR Tools To Control Gene Expression in Bacteria

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Summary

CRISPR-Cas systems have been engineered as powerful tools to control gene expression in bacteria. The most common strategy relies on the use of Cas effectors modified to bind target DNA without introducing DNA breaks. These effectors can either block the RNA polymerase or recruit it through activation domains. We will discuss here the mechanistic details of how Cas effectors can modulate gene expression by blocking transcription initiation or acting as transcription roadblocks. CRISPR-Cas tools can be further engineered to obtain finetuned control of gene expression or target multiple genes simultaneously. Several caveats in using these tools have also been revealed, including off-target effects and toxicity, making it important to understand the design rules of engineered CRISPR-Cas effectors in bacteria. Alternatively, some types of CRISPR-Cas systems target RNA and could be used to block gene expression at the post-transcriptional level. Finally, we will review applications of these tools in high-throughput screens and the progress and challenges in introducing CRISPR knock-down to other species, especially non-model bacteria with industrial or clinical relevance. A deep understanding of how CRISPR-Cas systems can be harnessed to control gene expression in bacteria and build powerful tools will certainly open novel research directions.

Introduction

The ability to precisely control gene expression levels in bacteria is attractive for many reasons. Modifying the concentration of a protein or enzyme is a classical way of understanding gene function, as it allows comparison of the phenotypes of cells at different levels of a gene of interest. This is especially relevant in the case of essential genes where a simple knock-out is impossible. Controlling gene expression is also a way to discover regulatory circuits, by applying a perturbation and measuring how the expression of other genes adapt as a response. Furthermore, recent research in systems biology has aimed to understand the biological significance of gene expression levels in a broader context. Experimenting with gene expression levels has helped our understanding of the evolutionary dynamics of gene regulation (1, 2), and the importance of stochastic processes in protein expression (3, 4). Finally, the fine-tuning of gene expression levels has great potential for the engineering of organisms with the aim of making them more efficient for industrial metabolite production (5, 6) or to create artificial regulatory circuits (7) to be used in biosensors and diagnostic tools (8, 9).

All these applications can benefit from the ability to change gene expression in a programmable way, i.e. the ability to set the expression of any protein of interest to arbitrary levels, according to some user-defined input in the form of a DNA sequence. However, the development of programmable biological devices has remained limited by a longstanding barrier: the complexity of macromolecule folding and their interactions makes it very difficult to design sequences *de novo*

such that they have the desired function *in vivo*. For this reason, biotechnological design has typically been limited to the recycling of components from nature, or the artificial generation of random sequences followed by selection. In particular, inducible promoters are established as the standard tool for the control of gene expression in bacteria, allowing the expression level of a gene to be linked with the concentration of a chemical in the medium. While this method is extremely useful for a range of applications, the recent emergence of programmable methods has brought up new possibilities, especially in conjunction with high-throughput sequencing and DNA-synthesis technologies. Such technologies offer many advantages in principle: as they are programmable, it is easy to create large scale libraries, or to make multiple orthogonal systems to be used all at once in the same cell. The sequence controlling gene expression can itself act as a barcode, allowing for identification by sequencing in large libraries. The field of eukaryotic biology was the first to be revolutionized by programmable control of gene expression, with the development of RNA interference (RNAi) during the 1990s (10). It took a few more years before similar methods were discovered for bacteria: the first tools based on antisense RNA (asRNA) were developed in the 2000s (11–13), followed by prokaryotic small RNA (sRNA) (14) and finally CRISPR-based tools (Clustered Regularly Interspaced Short Palindromic Repeats) in the 2010s (15, 16).

Antisense RNAs act by forming a duplex with the ribosome binding site (RBS) of cognate messenger RNA and preventing ribosomes from initiating translation (13, 17). They can be used on multiple targets at once and be made more stable by using paired termini (13, 18). Small RNAs, on the other hand, use a scaffold to recruit the

Hfq chaperone and lead to mRNA degradation (14). They too can be multiplexed and the strength of knock-down is tunable by changing the binding energy between the sRNA and the target (6). Unfortunately, the coupling between transcription and translation in prokaryotes makes it difficult to interfere with translation, since there is little time for the interfering RNA to find its target, resulting in moderate repression strength (14, 17). They also have limited portability from one species to another (14).

A few years ago, the discovery of the CRISPR bacterial immune systems brought an alternative repression method under the spotlight.

From the CRISPR immune system to artificial transcription factors

Analogous with RNAi, gene knockdown using CRISPR effectors has been dubbed CRISPRi. However, unlike RNAi, which acts at the post-transcriptional level and prevent translation, CRISPRi typically works by stopping transcription. CRISPR-Cas systems are an extremely diverse ensemble of adaptive immune systems found in bacteria and archaea, that can be grouped in two classes, each comprised of many types and subtypes (reviewed in (19)) with variable mechanisms. To grant protection against foreign DNA, CRISPR-Cas systems work in three phases. The first one, called adaptation, is responsible for sampling short DNA sequences from invading genetic elements, and storing them in the form of a series of fixed-length sequences intercalated with a constant motif. This collection of sequences is called

the CRISPR array. In a second phase, the CRISPR array is expressed into a primary transcript, that is subsequently processed into small CRISPR RNA (crRNA) which form ribonucleoprotein complexes with Cas proteins. In the last phase, called interference, crRNAs guide Cas proteins to recognize and destroy invading DNA (or RNA).

The machinery involved in the interference step can be repurposed to artificially control gene expression by affecting either transcription or translation. In nature, the CRISPR array is first transcribed and processed into individual guide RNAs, which are then associated with one or several enzymes (the CRISPR effectors) to form a ribonucleoprotein complex with nuclease activity. Upon phage infection, this RNA-guided nuclease will recognize the invading genome through base pairing and introduce a double-strand break to stop the infection. The nature of CRISPR effectors can be very different depending on the class and type of CRISPR system. In class 2 systems, the effector is one single enzyme, making them very popular for biotechnological applications. Class 2 includes the Type II systems which use the famous Cas9 protein, Type V systems with their effector Cas12a, (formerly known as Cpf1), and Type VI systems with their RNA targeting effector Cas13a (formerly known as C2c2). In class 1 systems, the effector consists of a large multi-protein complex like the DNA-targeting Cascade complex of Type I systems, or the RNA-targeting Csm or Cmr complexes of Type III systems. By engineering type I and II systems to eliminate their nuclease activity, it is possible to create artificial DNA-binding ribonucleoprotein complexes that can be directed to bind any DNA sequence by putting arbitrary addresses in the CRISPR array (15, 16, 20) (figure 1).

In a similar fashion, effectors from the types III and VI can be turned into programmable RNA-binding complexes (21, 22).

To reprogram a CRISPR system, one can simply replace the native sequences in the CRISPR array with the desired target sequences and rely on natural processing and assembly of the ribonucleoprotein complex. Type II systems as well as some type V systems rely on a ternary complex comprising of the effector (Cas9 or Cas12), the processed guide RNA and a trans-activating CRISPR RNA (tracrRNA) which forms an RNA duplex with repeats in the primary transcript (23, 24). This duplex RNA is recognized by Cas9/12 leading to processing into short CRISPR RNA (crRNA). As an alternative, it is possible to build a chimeric RNA between the crRNA and the tracrRNA, referred to as the single-guide RNA (sgRNA) (25). In some type V systems the Cas12 effector carries RNAse activity enabling it to process the CRISPR primary transcript by itself, without the need for a tracrRNA or a host RNAse (26). While the use of a sgRNA reduces the number of components required and can be advantageous, using CRISPR arrays makes it possible to easily do multiplex targeting (27).

Not all positions can be targeted by CRISPR effectors. The target sequence of type I, II and V complexes must be flanked by a DNA motif called the PAM[1] (28–31). The PAM sequence is different depending on which ortholog of CRISPR is being used, but is typically between 3 and 8 nucleotides long, meaning that there are usually many discrete possible targeting sites throughout a given sequence. Some CRISPR effectors have strict PAM requirements while others recognize a more variable range of motifs. For example, the canonical PAM used in the popular Cas9 from *Streptococcus pyogenes* (SpCas9) is a NGG motif on the 3' side of the target (32), while *Francisella novicida*'s Cas12a prefers NTTN on the 5' end of the target (33, 34). The length of the PAM determines the frequency of possible targets: on average, SpCas9 has one target every 8 bp in *E. coli*'s genome if both DNA strands are considered. This of course depends on the characteristics of the target genome: as SpCas9's canonical PAM is NGG, it is not as common in genomes with a low GC-content. After binding to the DNA, the CRISPR effector starts to unwrap the DNA starting from the side of the PAM (35). As the DNA unwinds, the guide RNA progressively forms a hybrid with the target DNA (36, 37). This structure is called the R-loop. For type II and V effectors, when the entire guide is annealed, a conformational shift occurs and a double-strand break is introduced in the DNA. Cas9 uses two different catalytic domains to nick the two DNA strands, while Cas12a

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[1] PAM stands for Protospacer Adjacent Motif. Protospacer designates the part of phage DNA that will eventually be incorporated into the CRISPR array as a spacer during CRISPR adaptation. The PAM motif is required both for the selection of novel spacers to be acquired and for the identification of target sequences during the interference step.

uses a single domain to cleave each strand sequentially (38, 39). For type I effectors, the Cas3 nuclease is recruited at the bound Cascade complex and unwinds and chops the target strand of DNA (40, 41).

To simply block gene expression without cutting the target DNA, the nuclease activity of the CRISPR effectors must be eliminated. For types II and V systems, this is done by mutating the catalytic residues (15, 16, 34). The resulting enzymes are marked with the letter "d" (for "dead"), for example the inactive variant of Cas9 is called dCas9. For type I, the Cas3 nuclease can simply be deleted (20). Finally, gene knock-down can also be achieved by addressing RNA-binding effectors (from types III or VI) to messenger RNA (21, 22, 42).

Thanks to the remarkable effectiveness of CRISPR-Cas systems, many applications were quickly developed even with a limited knowledge of its exact mechanism. As a result, many rules of thumb were discovered during the testing and optimization of particular applications and research is still on going to develop a unified, predictive biophysical model of how dead Cas effectors block RNA transcription.

The mechanics of nuclease-deficient CRISPR-Cas systems

A roadblock on the way of the RNA-polymerase

To repress a gene, multiple target choices are possible. If the effector (e.g. the dCas9/sgRNA complex) binds to the promoter region of the target, it lowers the number of mRNA transcripts by blocking the initiation of transcription. If the target is located within the coding sequence of the target gene, it interrupts elongation by

standing in the way of the RNA-polymerase (RNAP) (15, 43). After halting, it is unclear how fast the RNAP dissociates from the template. *In vitro* experiments indicated that the stalled RNAP remains bound to the template after encountering dCas9 (44). It is likely that something similar occurs *in vivo*. In fact, sequencing of the incomplete transcripts suggested that after encountering dCas9, the RNAP remains in place for some time, giving birth to trains of stalled polymerases (43). There is also evidence that, depending on the interaction strength between the dCas9/sgRNA complex and the target, the RNAP can actively displace dCas9 and continue elongation to produce a full mRNA transcript (45).

When the target is in the promoter sequence, any DNA strand can be targeted and the repression will be equally strong (figure 2, left). This has been observed for all types of CRISPR used so far: Type I (20), Type II (16, 46) and Type V (33, 47) . On the contrary, when the target is downstream of the promoter, within the coding sequence, target orientation has a crucial importance and the best orientation varies depending on the type of CRISPR system. For dCas9 (type II), repression is effective only if the guide RNA pairs with the non-template strand (i.e. when the guide RNA is homologous to the template strand, figure 2, top-right). If dCas9 is in the wrong orientation, the repressive effect is much weaker (1.5 to 3-fold repression, instead of 10 to 100-fold in the right orientation) (46). Interestingly, an opposite behavior has been observed for dCas12a (type V), which produces a stronger repression when it binds to the template strand (33, 47, 48)(figure 2, middle-right). For the Type-I CRISPR of *E. coli*, the guide RNA must bind to the non-template strand to produce a strong repression (20)(figure 2, bottom-right). Together, these indicate

that no simple rule determines which orientation is most effective for a given type of CRISPR system, be it PAM position, the direction of R-loop extension or the nature of the targeted strand. The exact mechanism of the collision thus remains to be elucidated on a more structural level, especially for effectors other than *S. pyogenes*'s dCas9. Importantly, for dCas9, the ability to stop the RNAP strongly depends on temperature, higher temperatures leading to weaker repression (45, 49). Repression at high temperatures is still possible, using a variant of dCas9 isolated from a thermophilic organism (50). It is not known if other effectors (such a dCas12a) are affected in the same way.

Consequences of the collision for the RNA transcript

Early experiments on the effect of target position within a gene suggested that targets farther away from the initiation codon tend to be less efficient for target repression (15). More recent studies have since contradicted this phenomenon (46, 51). Targeting within the promoter or in a small sequence window downstream shows strong repression and likely inhibit transcription initiation by either blocking access to the promoter or interfering with the transition of the RNAP from its initiation state into a processive elongation complex. However, when the target is far enough from the promoter to avoid interactions with the initiation step, the position of the target does not systematically affect the repression strength.

When elongation by RNAP is interrupted, an incomplete mRNA is generated. (52). If the target is inside the coding sequence of a protein, the incomplete transcript may have a ribosome binding site and a start codon, but no stop codon. In that case, it

will likely be subject to degradation to avoid ribosome stalling (53), usually relying on a mechanism called trans-translation. As a result, both the non-stop mRNA and the nascent peptide are targeted for degradation (54, 55).

Another crucial aspect of transcription interruption in bacteria is that genes are often co-transcribed in operons. As a result, interfering with one gene will likely have effects on the other genes from the same mRNA molecule, referred to as polar effects[2]. Repression by CRISPR with a target inside an operon has a clear effect on all downstream genes, which are also repressed (57), a property that has been exploited to reveal the presence of cryptic promoters in the middle of operons (57).

There is also some evidence that genes upstream of the target in an operon can be affected, to a variable degree depending on the organism. This "reverse polar effect" appears to be strong in *B. subtilis* (43), but much milder in *S. aureus*, where the level of upstream transcript is reduced only by ≈50% (58). Similarly, in *E. coli*, reverse polar effects are only apparent when the target is within 100 bp from the gene end (46, 51). This is probably due to changes in mRNA stability, however the exact process is not fully understood and might be specific to each operon due to different mRNA regulation motifs (59, 60).

In some cases, polar effects may be troublesome for singling out the effect of one gene. They may also be an advantage, as operons usually consist of genes of related

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[2] It should also be noted that antisense RNA, in spite of acting at the translation step, are also subject to polar effects since they trigger RNA degradation (13, 56), though this effect is only partial.

functions, from the same metabolic pathway or machinery. As a result, polar effects allow the reduction of the number of targets needed in screening libraries.

Other factors may interfere with dCas9 binding to its target. One potentially important contextual factor is transcription on the opposite DNA strand in the case of two convergent promoters (61). If dCas9 is set to block RNAP in one direction, other RNAPs coming in the opposite direction will eject it from the DNA with a very high probability. This could possibly interfere with the desired repression. Positive DNA supercoiling has also been shown to affect dCas9's binding, as it increases the force required to unwind the DNA for target recognition (62). This may be important when using multiple binding sites on the same target, hoping to increase the repression strength: if the targets are too close to each other, they might exhibit anti-cooperative behavior (62).

Taming CRISPR guides with imperfect complementarity

Large data sets have been produced to understand the effect of mismatches between the guide and the target (63–67). A particularly creative strategy was to tether many targets to a sequencing flow cell, locate the different targets by next-generation sequencing, then measure the binding of a fluorescent-labeled CRISPR effectors directly on the same flow cell (68, 69). The tx/tl *in vitro* translation system was also used to measure DNA cleavage and repression for many targets (70). Finally, recent studies employed libraries of CRISPR guides combined with fluorescent-activated cell sorting (FACS) to measure how the target sequence and the presence of mismatches affect the repression strength (65, 67).

In most cases, the seed sequence (first 5-12 bp next to the PAM) is of essential importance for stable binding to the target. This is however not a universal rule, as mismatches in the seed do not necessarily abolish repression when the target is in a promoter (71).

The other end of the guide, the PAM-distal side, is typically important for DNA cleavage, but not necessary for strong binding, and guides with up to 11 mismatches on that side can still have a significant repressive effect (16, 46). For both dCas9 and dCas12a, the complementarity of the PAM-distal region influences the dissociation rate, to the point that it was called the reversibility-defining region (72). This must be taken into account during guide design, in order to avoid off-targets in essential genes that could have a major impact on growth, or that could be related to the phenotype of interest.

Kinetic models have been used to understand the behavior of CRISPR effectors in the presence of a mismatched target. In these models, the system is broken up into a finite number of states, with kinetic parameters associated with the different transitions (73, 74). As the R-loop extends from the PAM-proximal to the PAM-distal end of the guide, mismatches between the guide and the target can be seen as high potential barriers that can be overcome, in agreement with the observation that when the R-loop has extended past a mismatch, it can continue to extend normally (75). Such models make interesting predictions: for example, they have been used to explain the somewhat paradoxical result that Cas9 variants with more relaxed PAM recognition tend to have less off-target cutting sites, despite having a higher number

of potential targets[3] (73, 76). Interestingly, due to the long-lasting binding of Cas9 to the target, the dissociation rate is usually much lower than the catalytic rate (73). The consequence is that, for DNA cleavage, the kinetic equilibrium (where an equal number of molecules bind and unbind at any given moment) will never be reached in most cases. This is a major difference with repression by deactivated nucleases, where the system may eventually reach equilibrium, and is an important reason why repression efficiency cannot be extrapolated from cutting efficiency.

Interfering with translation

In most applications so far, CRISPR knock-down systems were acting on transcription. However, some CRISPR immune systems act by cleaving RNA, making it conceivable to prevent translation rather than transcription. Such an activity is naturally present in type III and VI. In type III systems, the Cas ribonucleoprotein complex binds to target RNA which activates the DNA nuclease activity of Cas10 as well as the production of cyclic oligoadenylate messengers which in turn activate other effectors such as the Csm6 RNAse (77–79). Since the crRNA of type III systems bind RNA targets rather than DNA, there is no risk to target the CRISPR array itself and type III systems do not require a PAM to prevent auto-immunity. Mechanisms have nonetheless been described that enable type III systems to discriminate between target sequences and transcripts generated from the CRISPR array in the

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[3] Briefly, the total frequency of off-target cutting will depend on the number of potential binding sites (as determined by the PAM) and the probability, for each of these sites, that the R-loop will extend until the end. Cas9 mutants selected for relaxed PAM recognition have more potential binding sites but the interaction between Cas9 and the DNA is weaker, so that only guides with high complementarity have a chance of causing cleavage.

antisense direction. This discrimination seems to be achieved by both base pair interactions between the 5' end of the guide RNA and the target, and non-base pair interactions, with possible differences between type III subtypes (79–81). Typically, when the 5'end of the crRNA is fully complementary to the target, the DNAse activity is not triggered. This property was harnessed in the archeon *S. solfataricus* to obtain a 2-fold repression of a reporter using the organism's own type III CRISPR-Cas systems (21). Alternatively, it might be possible to mutate the Cas10 protein and other effectors to maintain a specific RNAse activity while blocking DNA cleavage. Controlling gene expression with type VI systems is an attractive option as these systems target RNA exclusively. However, these effectors indiscriminately degrade the surrounding RNA molecules once they are activated by their target. When the type VI Cas13a was used to repress a reporter in *E. coli*, the growth rate of the bacterium was severely diminished due to the collateral cleavage of other mRNAs in the cell (22). While this problem has not been observed in human cells (82), engineering of Cas13 effectors might therefore be necessary to make this a viable strategy in bacteria.

In addition to effectors whose natural target is RNA, certain variants of the DNA-targeting Cas9, such as the ones from *S. aureus*, *C. jejuni* or *N. meningitidis* can also target RNA, with the peculiar property that a PAM is not required. As a result, one can direct Cas9 specifically to RNA by choosing a target that is not flanked by a PAM (42, 83). This also results in gene repression, although it is not clear whether the repression comes from cleavage and degradation of the mRNA or just from interference with the ribosomes. In all cases, the effectiveness of the repression is

strongly influenced by the accessibility of the target, which depends on the secondary structure of the mRNA (22, 42).

Switching CRISPR on and off: a matter of timing

Many points of the mechanism of effector assembly and association to the guide RNA remain to be elucidated. It is likely that the assembly of Cas9 and the guide RNA (crRNA or sgRNA) is quite stable as free Cas9 has a quite flexible structure and is stabilized by the crRNA (84–86).

The process by which the CRISPR effectors find their target has also received a lot of attention. Most known transcriptional regulators, such as those involved in inducible promoters, recognize their binding motif from the side, without unwrapping the two strands of the DNA. They can thus slide along the DNA groove until the operator is found. For CRISPR-Cas systems, however, this is not possible as target recognition involves complementary Watson-Crick base pairing and double-stranded DNA has to be open. Experiments with DNA curtains and fluorescent effectors allow monitoring of the target search process *in vitro*. Such data exists for type II (Cas9) and type V (Cas12a) (35, 37, 87, 88). For both, there is a first step of 3D-diffusion until a DNA molecule is encountered (35). This is followed by 1D-diffusion along DNA (87), though the contact with DNA is only intermittent. For Cas9, the 1D-diffusion is limited to very short distances (along 20 bp) but allows it to jump from one PAM to another. For Cas12a, however, the 1D-diffusion step is dominant (88), and a molecule of Cas12a can appear to diffuse along the DNA molecule for extended periods of time. Another core difference between Cas9 and

Cas12a regarding target search is that binding to the PAM seems to have a limited contribution to DNA unwinding during R-loop formation for Cas12a (59). Cas12a probably only dwells on PAM sequences for very short periods of times if the guide does not match the target, while the transient association of Cas9 to PAM sites can easily be observed (35). Cas9 transiently associates with non-matching sites with a residence time that depends on complementarity. As a result, it spends a few milliseconds on each potential binding site, and a single dCas9 molecule may take several hours to find its target (89). This search time has been evaluated to be about 6 hours for *E. coli*'s 4 Mbp genome. Thus a large number of Cas9 complexes might be required for a quick response, which is of particular importance for anti-phage defense. The importance of this for gene knock-down is still unclear.

The dissociation of dCas9 from the target is also very slow (35), in the order of a few hours *in vitro*. Because of that, the amount of effector that is required for gene silencing is very small. In fact, one single complex per target locus might be sufficient to shut down expression until it gets kicked out by the RNAP or the replication fork. After stopping the expression of dCas9, it takes up to five hours to reach the original expression level of the target gene (15, 90), which presumably corresponds to the time required for dCas9 to be lost through dilution and cell division.

Hacking CRISPR for better control on expression

While the re-purposing of natural CRISPR-Cas systems quickly lead to impressive results, a lot of effort has been undertaken to extend their capabilities. Here we discuss recent advances in improving the repression strength, controlling multiple targets at the same time and extending the application of CRISPR knock-down to a broader range of species, including some organisms that are not standard laboratory models. We also review the efforts that have been made to detect the potential undesirable effects of CRISPR-Cas systems, and strategies to mitigate them.

Tunable gene repression and activation

To obtain partial repression of the gene of interest, multiple approaches have been undertaken. The most straightforward one is to express the CRISPR system from an inducible promoter such as at P_{bad} in *E. coli* (91, 92), or P_{xyl} in *B. subtilis* (43). In that case, the repression strength is controlled by using variable amounts of a chemical in the medium, using a single strain. Changing the amount of sgRNA, rather than of dCas9, may provide better control . Alternatively, by expressing a mismatched guide RNA and a constant amount of effector, it is possible to repress genes by a well-defined fraction (45). While the latter strategy requires one guide RNA design for each repression level, it is easier to multiplex and is less noisy.

Inducible promoters, that are often used to turn CRISPR knock-down on or off, usually produce a base rate of leaky expression even when they are not induced. As previously discussed, due the long residence time of CRISPR effectors on the DNA, a

small number of repressor complexes are enough to silence a target. Thus, even tight promoters can produce significant repression of the target in the absence of an inducer (43, 47, 91). One way to improve the dynamic range is to use a genetically recoded organism, with a non-natural amino acid in dCas9. In this case, the leaky repression is strongly alleviated unless the non-natural amino acid is also present (93). While effective, this method is currently limited to one heavily-modified strain of *E. coli* and cannot be easily adapted to other organisms. Using antisense RNA targeted at the CRISPR guide itself, it is possible to antagonize repression, possibly by impairing the formation of the complex, actively triggering degradation of the guide RNA or blocking binding of dCas9 to the target (94). This provides another layer of regulation to construct genetic circuits, and a way to quickly recover gene expression once the repression is established.

Finally, CRISPR-based programmable transcription factors are not restricted to gene repression. By linking dCas9 to a transcription activator, it is also possible to increase the expression level of a target. Initial studies used the ω sub-unit from the RNA polymerase to induce expression in a *RpoZ* deletion strain (16), however subsequent screening found more potent activators, in particular SoxS (95), an activator normally involved in oxidative stress response, that does not require the *RpoZ* deletion. When SoxS is tethered to dCas9 and the complex is targeted upstream of a promoter, expression of the reporter can be increased by more than 10-fold, making it possible to look at overexpression phenotypes for many genes in an easy way.

An interesting application of CRISPR-based activation was to create a positive link between dCas9 binding and expression of a selection marker, allowing continuous evolution of improved ω-dCas9 fusions and novel dCas9 variants with modified PAM recognition specificity (76). Gene activation is also possible in *B. subtilis* using a similar approach (96). A potential drawback of this method is that it requires a PAM at the right distance from the promoter, as the range of target positions that are effective for activation is very narrow, highlighting the importance of developing CRISPR effectors variants with different PAM specificity. More recently the AsiA anti-σ70 protein from bacteriophage T4 has been identified as a potent activator with a more relaxed binding window to activate target promoters (97). Protein fusions between dCas9 and AsiA could further be evolved to improve their activity leading to a promising tool able to activate endogenous genes and functional in several bacterial species.

A strategy to activate σ54 promoters in bacteria using dCas9 was also demonstrated (98). The σ54 factor binds to its promoters in a closed inactive form. In order to be activated, σ54 promoters require an activator protein to bind an upstream activating sequence (UAS) and remodel the RNAP-σ54–DNA complex in a process involving ATP hydrolysis. The authors were able to recruit σ54 activators to the guide RNA scaffold using aptamers and activate target promoters by directing dCas9 to bind to the UAS (98). Using a Cas9 variant with an extended PAM (xdCas9) also enabled them to increase the range of promoters that can be activated in this manner.

Simultaneous control of multiple targets

Natural CRISPR arrays often contain several dozens, sometimes hundreds, of guides (99). Accordingly, it is possible to express multiple guides with a deactivated effector to repress multiple targets at the same time. This way, more than 20 CRISPR guides have been used simultaneously (100). Multiplex gene repression has been useful to find pairs of synthetic lethal genes and measure epistatic interactions (43, 101), as well as for metabolic engineering. Moreover, activation and repression can be used in the same cell by using a sgRNA-linked aptamer to recruit the activation tag only on certain targets (95). Another approach is to use composite CRISPR arrays: guides with different scaffolds can be co-expressed within the same array, so that after processing they associate with different effectors (27). Co-expressing sgRNAs can be quite cumbersome, as each of them requires its own promoter and transcription terminator. CRISPR arrays, similar to what is found in nature, might be more practical for the expression of many guides (27). dCas12a is particularly attractive for this purpose, owing to its ability to process the primary transcript of the CRISPR array without relying on host factors (27, 48). Due to their very repetitive nature, it is challenging to produce long CRISPR arrays with multiple spacers using standard DNA synthesis. However, by splitting the array into multiple parts to separate the repeats, it is possible to assemble a series of oligonucleotides into CRISPR arrays. Single-pot methods have been developed to create large CRISPR arrays in one step (27). The assembly can also be done iteratively, adding the guides

one by one (20, 102). Finally, by using a set of diverse sgRNA handles, it is possible to avoid repetition and construct long arrays of sgRNA by DNA synthesis (100). As the pool of effectors is shared between all CRISPR guides, having more guides in one array makes each individual complex less abundant, requiring to express the effector at a higher level (20, 100). The number of complexes carrying each guide may also be different depending on the guide's abundance, which is influenced by its position within the array and secondary structure (27). However, by choosing the target in the coding region and having a sufficient dCas9 concentration, it is possible to reach a saturation regime, where the strength of repression is not affected by crRNA abundance (45).

From one bacterium to all others

One reason for the popularity of CRISPR-Cas systems is the fact that they are remarkably portable. Within just a few years after their discovery, CRISPR-based technologies were successfully used in all kingdoms of life (32, 103–107). Systems for gene knock-down are readily available for many bacteria, including species of clinical or industrial interest for which the available genetic tools are limited (108–110). By using easily transferable, modular plasmid systems relying on bacterial conjugation, it is possible to perform genetic screens on non-model bacteria, including many human pathogens (111). Furthermore, a thermostable variant of dCas9 was characterized for use in thermophilic organisms (50). CRISPR activation was also shown to work on non-model bacterial species (98).

Since about 45% of bacterial species possess a native CRISPR immune system (99), it may also be possible to use it for repression, rather than expressing a heterologous system. This has been done with *E. coli*'s endogenous Type I CRISPR system, where a simple deletion of the Cas3 protein, which carries the nuclease activity, allowed the creation of a programmable repressor (20).

Understanding and addressing side effects

When expressed at an excessive level, CRISPR effectors in themselves can have some toxic effects on bacteria. The extent of toxicity depends on the host organism and on the nature of the CRISPR effector. In *E. coli*, growth defects have been reported upon over-expression of *S. pyogenes*'s dCas9 (112) as well as morphological defects (113). SpdCas9 is also toxic to mycobacteria, but this toxicity can be alleviated using a different Cas9 orthologue from *S. thermophilus* (114). Similarly, SpCas9 is toxic to cyanobacteria (115) but not Cas12a from *Francisella novicida* (116).

Aside from direct toxicity, an important source of undesired side effects when using CRISPR knock-down is off-target binding. Even if a good complementarity between the guide and the target is necessary for strong repression, only a few matching bases in the PAM-proximal region can be enough to produce a small repressive effect (46). If this effect happens on an essential gene, a severe growth defect may ensue. For some applications, off-target binding can be greatly reduced by replacing the PAM-binding domain of dCas9 with a binding domain from the PhlF repressor (117). Consequently, dCas9 can only be targeted at regions containing the PhlF

operator sequence, which restricts the application of this system to synthetic biology.

Another unresolved problem is the "bad seed effect" (BSE) (46). Among all the possible seed sequences (the last five nucleotides in 3' of a guide), a few of them systematically cause a strong fitness defect when used with *S. pyogenes*'s dCas9. For instance, when a guide finishing in ACCCA is expressed in K-12 *E. coli*, it causes a near-complete growth arrest of the population. About 130 seed sequences (out of 1024 possible) cause this kind of sickness. A list of the ten most toxic ones is provided in table 1. The origin of this effect is still unknown. It does not appear to be due to off-target binding to a particularly critical locus, but may involve multiple simultaneous binding events, or binding to substrates other than genomic DNA (46). This effect is very pervasive and can go unnoticed: for example, we could find evidence for BSE in data previously published by others (51) (figure 3), showing that it could be an unsuspected source of noise in the data. To date, the best solution to avoid the BSE is simply to avoid bad seeds when designing a CRISPR guide. However, it was also shown that the BSE depends on dCas9's concentration, and that it can be alleviated by using a carefully-chosen expression cassette (46), while still retaining a strong repression capacity. We hope that future work will elucidate the origin of the BSE.

Current successes in controlling transcription

Genome-wide CRISPR screens in bacteria

For a few model organisms, large collections of knock-out strains have been developed and used successfully. This includes the Keio collection for *E. coli* (118) and two barcoded deletion libraries in *B. subtilis* (119). While these libraries are extremely valuable for the people working on these two model bacteria, creating such libraries is very work intensive, and most importantly, they are limited to non-essential genes.

Early screens for essential genes were conducted using antisense RNA (11, 12, 56) and transposon insertion libraries (120). Recent improvements in sequencing capabilities and molecular biology techniques have enabled to screen very dense transposon insertion libraries more easily, yielding useful data in a lot of bacterial species and conditions (121–125). However, transposon insertion is random and not necessarily homogeneous across the entire chromosome. Long genes are more likely to be disrupted, while short sequences like non-coding RNAs are unlikely to be targeted, requiring very large and dense insertion libraries as well as a lot of sequencing power to reach the desired precision on fitness measurement.

As an alternative, the ability to control the expression of genes in trans using CRISPR knock-down and activation, makes it a promising tool for genome-wide screens. To create a CRISPR guide, one just needs to insert a short sequence (20 bp in the case of SpdCas9) in a well-defined locus. Thus, the construction procedure can

be standardized and streamlined so that a large number of guides can be assembled in parallel, allowing for the creation of customized screening libraries using on-chip oligonucleotide synthesis. Several cloning methods using homologous assembly (102), golden-gate assembly (46, 126) or direct oligo integration coupled with a negative selection (91) have made it possible to assemble CRISPR guides in a single-step. Moreover, strains repressed by a CRISPR effector can easily be genotyped by simply sequencing the CRISPR guide, eliminating the need for a barcode or for a complex amplification protocol to locate an inserted sequence in the genome.

Multiple CRISPR screens have already been used to find essential genes in various conditions (51, 57, 94, 127). The applications of these data include discovery of drug targets, searching for synthetic lethal pairs, or genome minimization. Partial repression can also be useful to find phenotypes for essential genes (43, 67). CRISPR screens are also more versatile than TnSeq, as one can target only a subset of genes of interest, for example by targeting only genes implicated in a function of interest. Guide RNAs can also easily be designed to simultaneously target all copies of a duplicated gene or closely-related paralogs which are likely to be functionally identical (51). If CRISPR guides are expressed from a plasmid, the same library can easily be re-used on many strains and in many conditions, making the method more cost-effective in the long run.

Measuring fitness through growth based enrichment or depletion of specific guides is perhaps the most evident output for a genome-wide CRISPR screening, but it is not the only one. For example, by performing a complete phage replication cycle in a

population repressed by such a screening library, it was possible to identify host factors necessary for the production of infectious phage particles (57). A CRISPR library has been combined with high-content microscopy to find the effect on growth, morphology, and identify the function of unknown genes in the pathogen *S. pneumoniae* (128). In another study, comparing growth of a CRISPR strain library in the presence of a variety of chemicals allowed the reconstruction of genetic networks and identification of the target of antibiotics (43). These last two approaches required isolated cultures of the different library members, greatly limiting the throughput of the screen. Recently, a method was developed to identify the genotype of CRISPR-repressed strains in-situ during a pooled assay, using fluorescence measurements (129, 130). Currently limited to a few hundred strains, this principle could be scaled up to analyze large-scale libraries over many generations by high-content microscopy.

Synthetic biology and metabolic engineering

The programmability of CRISPR has made it popular among synthetic biologists. By expressing multiple guides at the same time and targeting them at each other, it is possible to construct predictable genetic circuits. As these circuits can interface with native chromosomal genes, it makes it easy to build artificial regulatory networks that control the bacteria's natural functions (112). Such genetic circuits have been used to improve the yield of protein expression, by creating a feedback between metabolic burden and transcription (131) so that protein expression is regulated to an optimal level.

Aside from gene repression or activation, dCas9 has been programmed to interfere with various processes in the cell. This includes modifying the spatial structure of *E. coli*'s chromosome to create artificial DNA loops (61), blocking the initiation of replication to take control of the cell cycle (49), and triggering cell filamentation in a reversible manner (132).

Another successful domain of application of CRISPR knock-down is metabolic engineering. CRISPR-based methods allow quick identification of competing pathways and optimize metabolic fluxes (133) for the production of a compound of interest. CRISPRi has been set-up in multiple industrially-relevant organisms, such as *Lactococcus lactis* (108), *Clostridium beijerinckii* (134) or *Corynebacterium glutamicum* (135). A review of other industrial strains where CRISPRi has been used can be found in (5), along with strategies used to improve production yield. Guides targeted at genes involved in rod-shape maintenance were used to diversify the morphology of cells and optimize production of biodegradable plastic (136). Finally, by inhibition of cell growth, one can optimize the balance between the production of biomass and the synthesis of metabolites (90).

Challenges and opportunities

CRISPR-Cas systems have not yet reached their full potential. There is a wide disparity in how much we know about different types of CRISPR-Cas systems. Compared to the vast literature surrounding Type II or V, some other types (like Type IV) are still largely unknown. More generally, the field of bacterial immunity

against phages is in its early stage and will probably uncover more valuable biotechnological tools (137). Additionally, most applications so far used wild-type proteins or simple deactivated mutants, but the artificial evolution of engineered variants offers promising perspectives, as could already be seen for expanded PAM recognition (76). Regarding the control of gene expression, it remains difficult to predict the effect of a given guide *in silico*, at the design stage. Some models are already available for *E. coli* and *B. subtilis* (67) but more research is needed to develop tools that are reliable and easy-to-use. In particular, a major challenge will be to devise predictable repression tools for non-model species, which would require a better understanding of polar effects and the role of host factors. Finally, the use of engineered CRISPR-Cas systems for gene activation in bacteria as so far remained limited to a few proof of concepts. We can expect that these tools will be adapted to high-throughput screens and used to unravel interesting biology in the coming years.

Conclusion

Compared to the usual speed of biotechnological development, CRISPR-Cas systems were repurposed as tools in a particularly short time after their discovery. Due to the multiplicity of their components, many degrees of freedom, like the sequence of the guide RNA scaffold or the stoichiometry of different parts were not fully explored. After a few years, we now know better the importance of each part, and begin to have a clear overview of how CRISPR interference happens, including the assembly of dCas9 complex, the search for target, the extension of the R-loop and

the outcome of collisions with RNA polymerase. While many questions remain open, the control of transcription by CRISPR is becoming a mature technology, that can now be used for more ambitious large-scale projects.

Modern biology is in need for precise and quantitative results, as opposed to qualitative differences that simply pass the test for significance. Hence, it is crucial to identify all potential artifacts and false-positive that are likely to arise when using CRISPR-Cas systems. Off-target binding, time- and concentration-dependence, and the bad seed effect are all important challenges that need to be understood better to take full advantage of CRISPR's capabilities.

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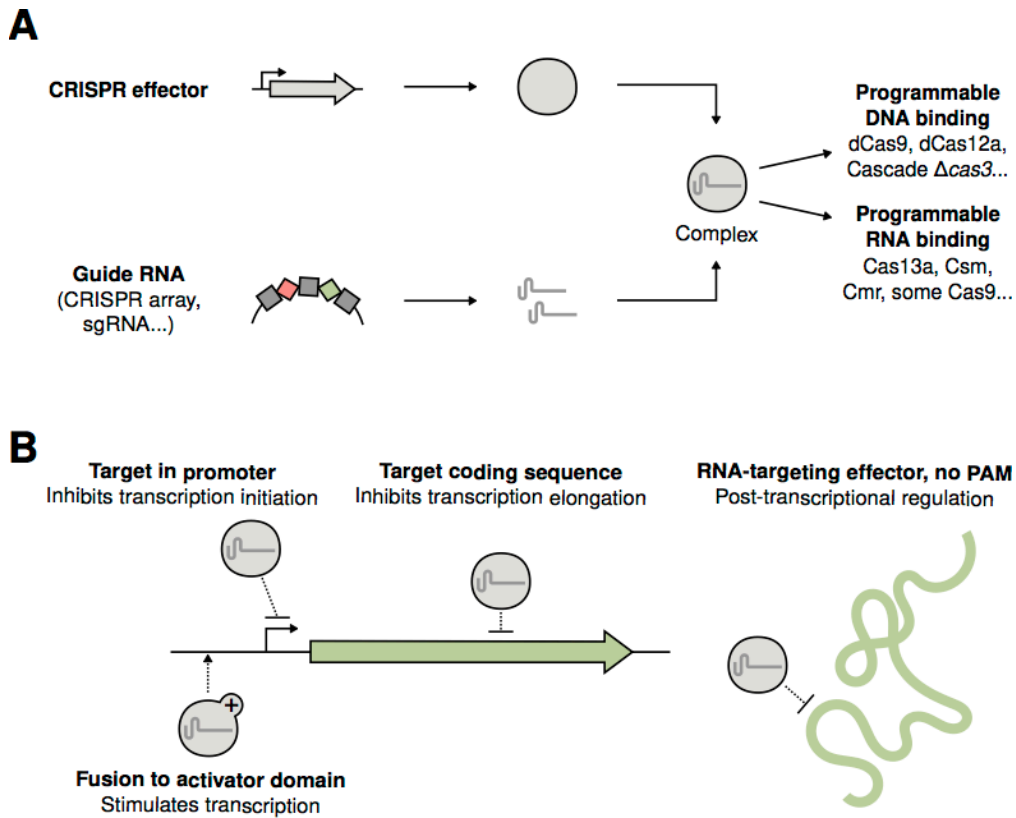
Figure 1. General principle for the control of bacterial gene expression using reprogrammed CRISPR effectors. A. Guide RNAs form complexes with natural or engineered CRISPR effectors. This results in a programmable ribonucleoprotein complex that will either bind to homologous DNA or RNA depending on the effector type. **B.** Gene repression is possible by targeting either the promoter, coding sequence, or messenger RNA. Gene activation is possible by fusing dCas9 to a transcriptional activator (such as ω, SoxS or AsiA) and addressing it to a precise location upstream of the promoter.
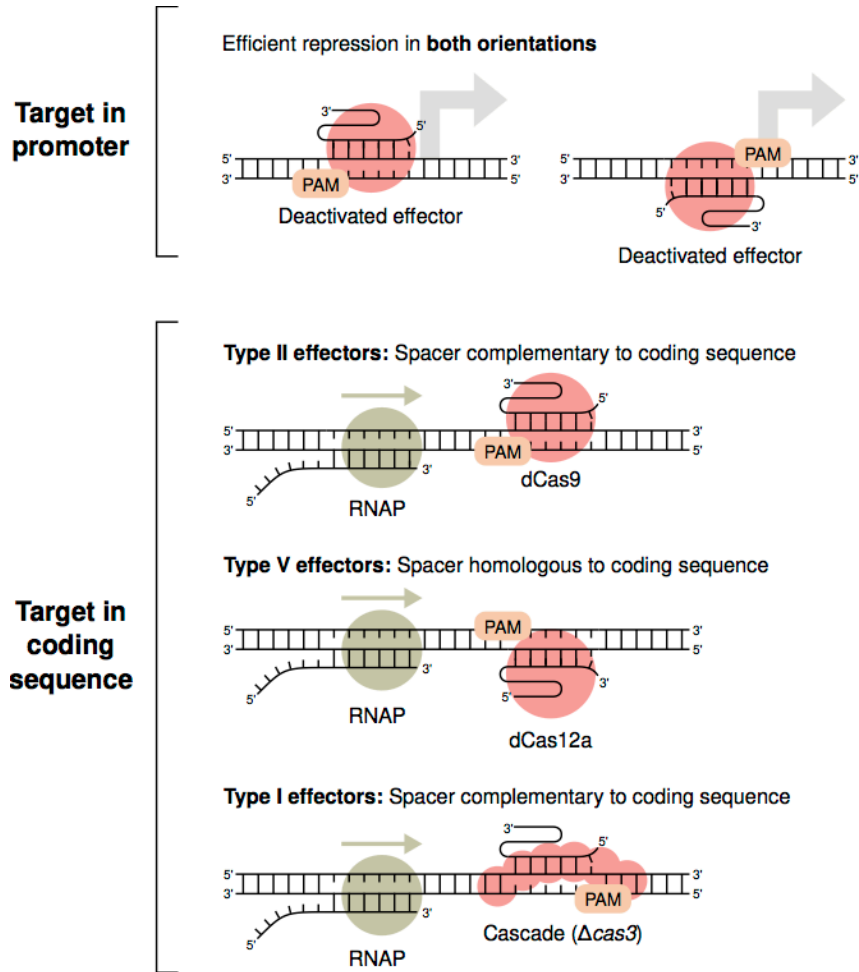
Figure 2. Targeting the right strand for efficient repression. When the target is within the promoter sequence, any strand can be targeted for strong repression. When the target is within the coding sequence, one orientation is typically much more effective than the other. The configuration depicted here is the one that leads to the strongest repression, for each of the CRISPR types that have been tested.
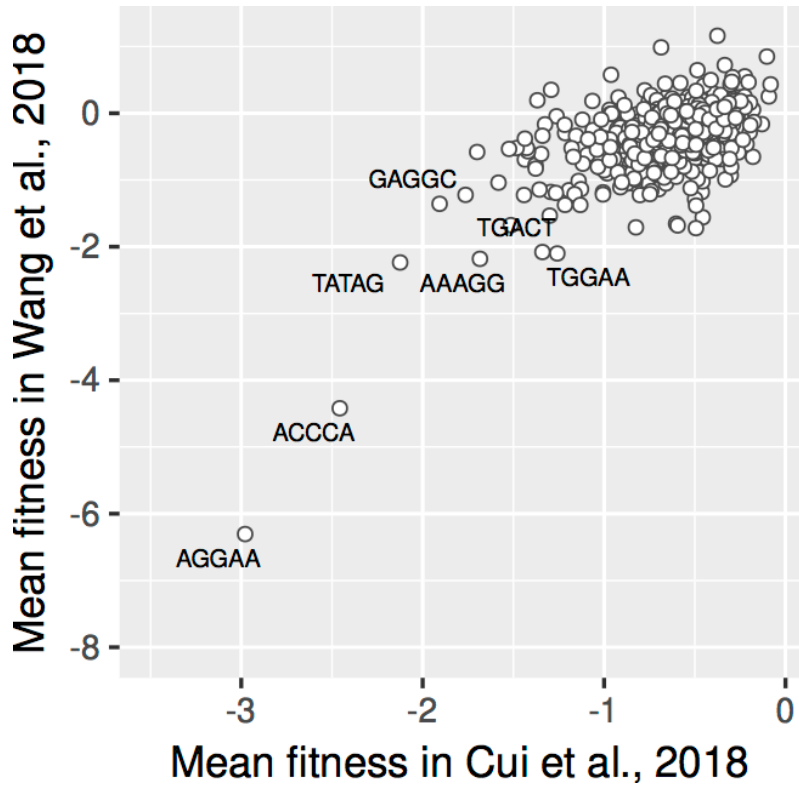
Figure 3. Unnoticed "bad seed" effect in Wang et al.'s (51) **CRISPR screen.** Using only CRISPR guides that target non-essential genes, we calculated the average fitness score ($\log_2$ of fold-change) of guides depending on their seed sequence. The sequences AGGAA and ACCCA are the two most toxic "bad seeds" discovered in (46). In (51), guides carrying a "bad seed" also have a strong average fitness defect, regardless of the genes they target.
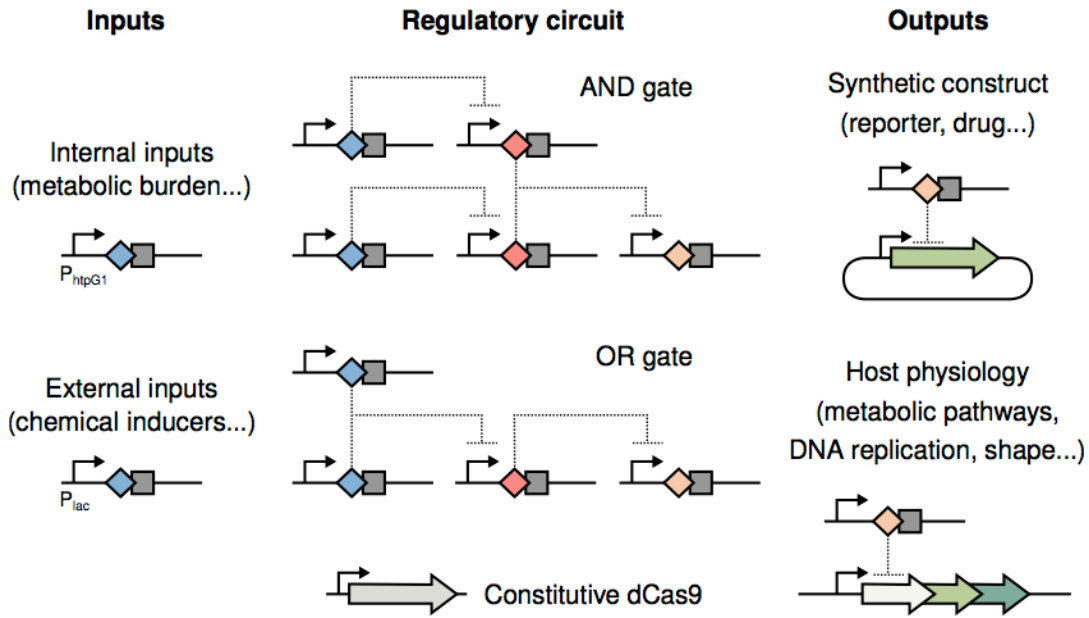
Figure 4. Overview of regulatory circuits that can be built using CRISPR repressors. As inputs, chemical inducers were used in (112). Metabolic burden was used in (131). The logic gates are from (112). Since sgRNAs act as a NOT gate and two identical sgRNAs act as a NOR gates, all other basic logic gates like AND and OR can be constructed. As outputs, metabolic production is discussed in (131), replication in (49), filamentation in (132), biofilm formation in (112) and cell shape in (136).

Table 1. The 10 seed sequences that have the highest toxic effect on *E. coli*, from (46). Using an optimized strain with low dCas9 expression greatly reduces the bad seed effect (right column), but does not completely abolish it. CRISPR guides with these seed sequences should be avoided if possible.

Seed sequence	Mean fitness effect (average log$_2$ transformed fold-change in guide abundance through 17 generations, measured by high-throughput sequencing)	
	High dCas9 expression	Low dCas9 expression
AGGAA	-6.50	-2.98
TGACT	-5.90	-1.34
ACCCA	-5.87	-2.46
AAAGG	-5.63	-1.68
GAGGC	-5.41	-1.91
CGGAA	-5.40	-1.51
ATATG	-5.37	-1.08
AACTA	-5.10	-0.88
TGGAA	-5.07	-1.26
CACTC	-5.01	-1.76