



ELSEVIER

ScienceDirect

Current Opinion in  
Biotechnology

# Hijacking CRISPR-Cas for high-throughput bacterial metabolic engineering: advances and prospects

Ioannis Mougiakos<sup>1,4</sup>, Elleke F Bosma<sup>2,4</sup>, Joyshree Ganguly<sup>3,4</sup>, John van der Oost<sup>1</sup> and Richard van Kranenburg<sup>1,3</sup>

High engineering efficiencies are required for industrial strain development. Due to its user-friendliness and its stringency, CRISPR-Cas-based technologies have strongly increased genome engineering efficiencies in bacteria. This has enabled more rapid metabolic engineering of both the model host *Escherichia coli* and non-model organisms like Clostridia, Bacilli, Streptomyces and cyanobacteria, opening new possibilities to use these organisms as improved cell factories. The discovery of novel Cas9-like systems from diverse microbial environments will extend the repertoire of applications and broaden the range of organisms in which it can be used to create novel production hosts. This review analyses the current status of prokaryotic metabolic engineering towards the production of biotechnologically relevant products, based on the exploitation of different CRISPR-related DNA/RNA endonuclease variants.

## Addresses

<sup>1</sup> Laboratory of Microbiology, Wageningen University, Stippeneng 4, 6708 WE Wageningen, The Netherlands

<sup>2</sup> The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet B220, 2800 Kgs. Lyngby, Denmark

<sup>3</sup> Corbion, Arkelsedijk 46, 4206 AC Gorinchem, The Netherlands

Corresponding author: van Kranenburg, Richard ([richard.vankranenburg@wur.nl](mailto:richard.vankranenburg@wur.nl))

<sup>4</sup> These authors contributed equally to this work.

**Current Opinion in Biotechnology** 2018, **50**:146–157

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

Edited by **Mike Jetten** and **Irene Sánchez Andrea**

<https://doi.org/10.1016/j.copbio.2018.01.002>

0958-1669/© 2018 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Introduction

The transition towards a bio-based economy demands the development of fermentation-based processes economically competitive with the currently employed unsustainable production processes [1]. Unfortunately, only very few natural organisms are suitable for their direct application in an industrial process. Therefore, efficient metabolic engineering via targeted genome engineering is required and the development and use of simple and

high-throughput genome engineering tools generally applicable to many model and non-model organisms is of great importance [2].

Most prokaryotes possess homology directed repair (HDR) systems, which have since long been exploited in a great variety of microorganisms for targeted chromosomal integrations of desired modifications [3–5]. In the HDR-based systems, plasmid-borne homologous recombination templates, which often harbor selection markers for screening purposes, are introduced into the genome through double or sequential single crossover events. The HDR-based approach is usually combined with additional systems, such as the site-specific resolvase based *Cre-lox* or *FLP-FRT* systems, for excision of the markers from the genomes for recycling purposes. However, these systems leave genomic scars that could be the cause of unwanted chromosomal rearrangements [6,7\*\*]. Alternatively, for a small number of bacteria, markerless genomic modifications are possible via recombineering systems [8]. These systems are based on bacteriophage recombinases and ssDNA, dsDNA or plasmid-borne DNA fragments with sequence homology to the genomic target. However, due to the absence of marker-based selection, these systems mostly result in low mutation efficiencies [8]. The construction and screening of mutants in all these HDR-based approaches is time consuming, rendering these tools suboptimal for extensive metabolic engineering, particularly in non-model organisms with low transformability and recombination rates.

A breakthrough moment in the molecular microbiology field was the discovery of bacterial adaptive immune systems that are based on genomic Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs)—the memory of the systems—and CRISPR-associated (Cas) proteins [9–11]. The repurposing of the RNA-guided DNA endonuclease from the type IIa CRISPR-Cas system of *Streptococcus pyogenes* (spCas9) and of other Cas9 orthologues as genome editing tools brought an unprecedented revolution to the life sciences field [2,12,13]. The basis of the Cas9 engineering tools is the simple way in which Cas9 nucleases can be guided to the desired DNA target, denoted as protospacer, by a CRISPR–RNA:trans-activating CRISPR RNA (crRNA:tracrRNA) hybrid complex. For this purpose, the 5'-end of the crRNA module, denoted as spacer, has to be complementary to the selected protospacer [14] and a

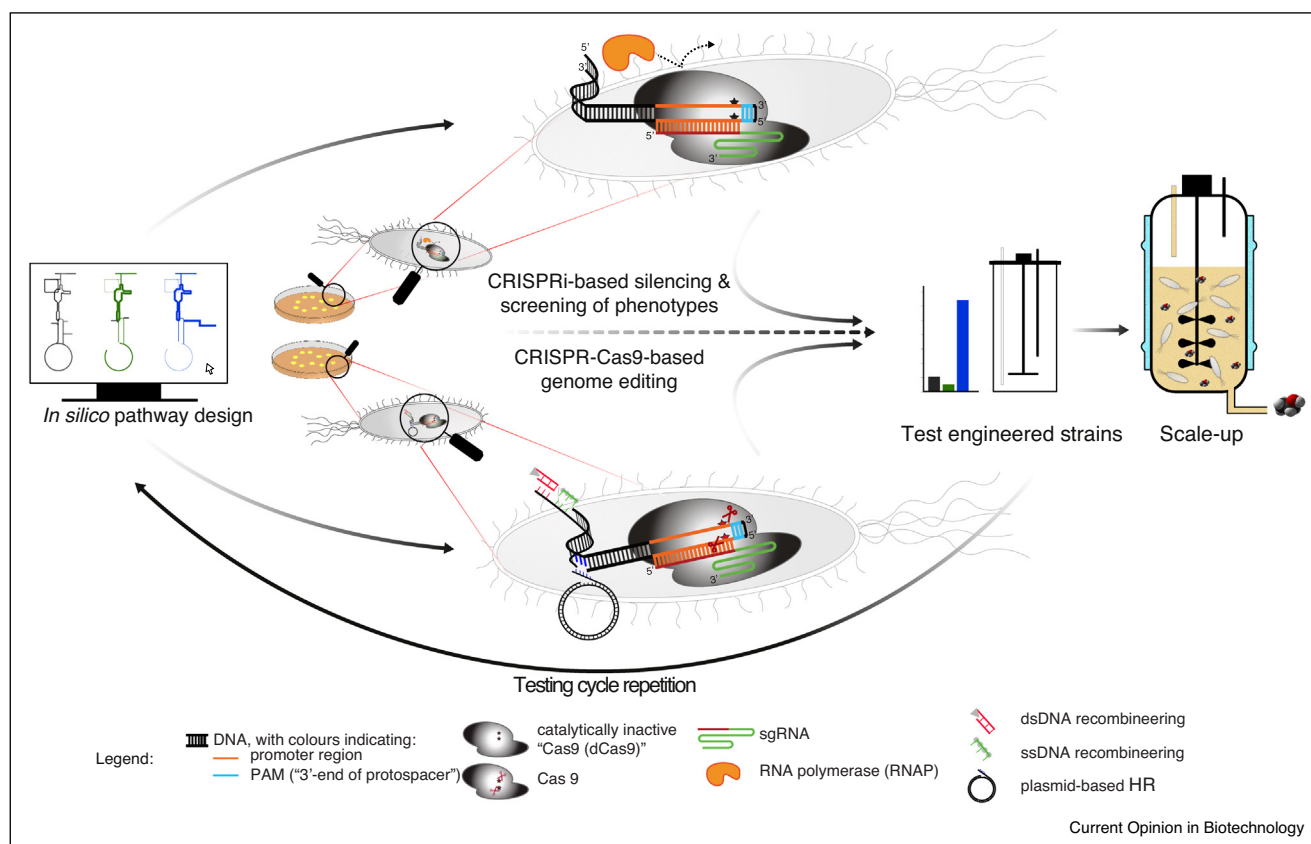
specific short DNA motif, denoted as protospacer adjacent motif (PAM), has to be present at the 3'-end of the selected protospacer [15,16]. For further simplification of the engineering processes, the crRNA:tracrRNA complex can be combined into a chimeric single guide RNA (sgRNA) [17].

CRISPR-Cas-based genome editing is now broadly used in a variety of organisms, including human cells, zebrafish, plants, yeast and bacteria [18,19]. The successful application of Cas9-based genome editing in eukaryotic cells is based on the error-prone correction of Cas9-induced double-strand DNA breaks (DSBs) by the efficient eukaryotic Non-Homologous End Joining (NHEJ) mechanism. Contrary to eukaryotes, most prokaryotes do not have an active NHEJ system [3] and Cas9-induced DSBs cannot be repaired, resulting in cell death. Recent studies have shown that engineering efficiencies in prokaryotes were strongly increased (often up to 100%) upon combining the existing homologous recombination and recombineering systems with Cas9-targeting; the Cas9-induced DNA breaks

served, simultaneously, as recombination inducers and counter-selection tools [12,20,21]. Moreover, catalytically inactive variants of Cas9 orthologues and variants fused with transcriptional activating factors have been developed and used for transcriptional regulation. Altogether, CRISPR-Cas9 orthologues and catalytically inactive mutants have accelerated the construction and screening of *in silico* designed strains, facilitating metabolic engineering of a wide range of bacterial species for industrial cell factory development (Figure 1).

In this review, we summarize recent bacterial metabolic engineering studies that focused on the construction and improvement of microbial cell factories, making use of CRISPR-Cas-based technologies. Additionally, we explore newly developed CRISPR-Cas tools and argue on how their application could improve the currently available technologies. Finally, we discuss how screening diverse environments can lead to discovery of new Cas-related variants to extend the repertoire of applications and create novel production hosts.

Figure 1



CRISPR-Cas9-based metabolic engineering of bacterial cell factories. Using Cas9 as counter-selection tool for traditional genome editing processes such as recombineering or plasmid-based homologous recombination has led to efficient metabolic engineering towards a wide range of products. Additionally, dCas9 can be used for CRISPRi to rapidly screen *in silico* predicted phenotypes prior to Cas9-based engineering.

## Bacterial metabolic engineering and CRISPR-Cas technologies

Many chemicals such as terpenoids, alcohols, amino acids, organic acids and antibiotics have high commercial value in the pharmaceutical and nutritional industry, and as fuels and building block chemicals. Most of these compounds result from multi-step metabolic pathways and are often tightly regulated in their natural genomic context [22,23]. Model organisms, like *E. coli*, have well-studied metabolisms, extensive and high-throughput molecular toolboxes and detailed *in silico* metabolic models. Hence, the use of such organisms minimizes the number of required engineering steps and maximizes engineering efficiencies. Nevertheless, the demands for engineering work remain high before efficient production strains are constructed due to the complexity of the metabolic pathways for many commercially interesting products [2]. Moreover, model organisms are often sub-optimal as production hosts and the use of alternative organisms could benefit the production of many valuable chemicals. Cas-based genome engineering and silencing tools have enabled and accelerated complex metabolic engineering and systems-level understanding of metabolic pathways in a wide range of organisms [2,12,13,18] (Figure 1).

### CRISPR-Cas editing

Metabolic engineering strategies include plasmid-based expression or, preferably, chromosomal integration of heterologous metabolic pathways, and/or targeted genome editing and adaptive evolution for flux redistribution through native metabolic pathways (Table 1). In their pioneering work, Li *et al.* [24\*\*] combined Cas9-induced targeted DSBs with ss or ds  $\lambda$ -RED recombineering for the introduction of a heterologous  $\beta$ -carotene biosynthetic pathway into the *E. coli* genome. They further substituted the promoters and ribosome binding sites (RBSs) from the native MEP pathway genes to achieve different levels of overexpression of the corresponding enzymes. Further engineering steps, including numerous deletions and promoter/RBS substitutions of central carbon metabolism genes, improved pyruvate and glyceraldehyde-3P supply and lead to the construction of a highly improved  $\beta$ -carotene producing strain [24\*\*]. This extensive study was possible only due to the development of the Cas9-based tools, revealing their great potential for efficient and diverse manipulation of genomic DNA.

The Cas9-recombineering method was further exploited with the development of the CRISPR-enabled trackable genome engineering (CREATE) tool [25]. Application of this tool in *E. coli* cells allowed their simultaneous transformation with multiple libraries of plasmid-borne recombination templates, each designed to introduce easily trackable mutations at different genomic loci [25]. The CREATE tool was employed to introduce multiple RBS

variations for each of the genes in a genomically integrated isopropanol production pathway in *E. coli*, leading to the time-efficient construction and testing of  $\sim 1000$  strains. The isopropanol titer of the best strain was 1.5-fold higher compared to the initial integration strain, but still lower compared to the plasmid-based overexpression approach [26\*\*]. Cas9-based downregulation or deletion of competing pathways in strains already overexpressing heterologous pathways towards the desired product could further improve titers. This has been proven successful for many compounds, including n-butanol in *E. coli* [27] and *Clostridium saccharoperbutylacetonicum* [28], isopropanol-butanol-ethanol in *Clostridium acetobutylicum* [29], succinic acid in *Synechococcus elongatus* [30],  $\gamma$ -amino-butyric acid (GABA) in *Corynebacterium glutamicum* [31], and 5-aminolevulinic acid in *E. coli* [32] (Table 1). However, the use of natural producers can substantially reduce the complexity of engineering steps towards production and tolerance build-up. The tolerance of *Streptomyces* species to antibiotics has been exploited for the production of antibiotic and antitumor compounds simply by Cas9-facilitated genomic integration of multiple biosynthetic gene cluster (BGC) copies [33,34]. In *C. glutamicum*, the natural proline production was enhanced 6.5-fold through a codon saturation mutagenesis approach to relieve product inhibition [35\*]. Noteworthy, this work was performed using the Cas12a (formerly Cpf1) RNA-guided endonuclease, making it the first application of a non-Cas9-based CRISPR-Cas/recombineering genome editing tool in bacteria for metabolic engineering purposes (Table 1).

Finally, Cas9-based editing tools were successfully employed for membrane engineering purposes in *E. coli*. The  $\beta$ -carotene storage capacity of *E. coli* cell membranes was increased by chromosomal integration of heterologous membrane-bending protein genes using plasmid-borne homologous recombination and Cas9-targeting [36]. Furthermore, the Cas9-recombineering tool proved efficient for the enhancement of the *E. coli* lipid content by simultaneous chromosomal integration of a heterologous fatty acid regulatory transcription factor gene together with a delta9 desaturase and an acetyl-CoA carboxylase gene [37] (Table 1).

### CRISPRi

Next to the integration or deletion of genes and pathways, an important metabolic engineering strategy is the fine-tuning of gene expression. Whereas in eukaryotic systems siRNA-techniques have since long enabled transcriptional control, for prokaryotes such silencing tools have only recently become available with the CRISPR interference (CRISPRi) tool, which is based on dCas9: the catalytically inactive variant of the Cas9 endonuclease [38]. This tool can be used for complete or partial repression; repression strength can be tuned by altering the position of the selected protospacer within the targeted gene (Figure 2),

Table 1

## CRISPR-Cas-mediated metabolic engineering of bacteria for industrial products

Product	Species	Strategy				Chromosomal modifications made using CRISPR-Cas-editing	Ref.
		Product pathway overexpression	Chromosomal deletions	Chromosomal insertions	Chromosomal substitutions		
Terpenoids	<i>Escherichia coli</i>	Heterologous, chromosomal	Competing pathways	Product pathway	Promoters, RBSs	Knock-in <i>crtE-crtB-crtI-crtY</i> + knock-out <i>ldhA</i> , knock-in <i>gps</i> , combinatorial promoter/RBS replacement of 9 MEP pathway genes, combinatorial overexpressions and deletions of 8 central carbon metabolism genes, knock-in 2nd copies of selected MEP and $\beta$ -carotene pathway genes	[24**]
		Native, plasmid; Heterologous, chromosomal		Product pathway		Knock-in <i>almgs</i> under control of various regulatory parts (in a $\beta$ -carotene production strain)	[36]
Alcohols	<i>Escherichia coli</i>	Heterologous, chromosomal		Product pathway	RBSs	Knock-in and RBS replacement of <i>thl</i> , <i>atoDA</i> , <i>adc</i> , <i>adh</i>	[26**]
		Heterologous, plasmid			5'-UTR of competing pathway gene	Modification of <i>gltA</i> 5'-UTR for expression reduction	[27]
	<i>Clostridium saccharoperbutylaceticum</i>		Competing pathways			Knock-out <i>pta</i> , <i>buk</i>	[28]
Isopropanol-butanol-ethanol	<i>Clostridium acetobutylicum</i>	Heterologous and native, chromosomal		Product pathway		Knock-in <i>ctfAB</i> , <i>adc</i> , <i>adh</i>	[29]

Table 1 (Continued)

Product	Species	Strategy				Chromosomal modifications made using CRISPR-Cas-editing	Ref.
		Product pathway overexpression	Chromosomal deletions	Chromosomal insertions	Chromosomal substitutions		
Amino acids	L-Proline	<i>Corynebacterium glutamicum</i>	–			Codons (to relieve product inhibition)	Codon saturation mutagenesis [35*]
	$\gamma$ -Amino-butyric acid (GABA)		Heterologous, plasmid	Transporters, degradation pathway			Knock-out <i>Ncgl1221</i> , <i>gabT</i> , <i>gabP</i> and various combinations thereof [31]
	5-Amino-levulinic acid	<i>Escherichia coli</i>	Heterologous, plasmid	Competing pathways		Promoter, codons (to relieve product inhibition)	<i>coaA</i> point mutation (R106A), <i>serA</i> promoter replacement and C-terminal residues deletion, knockout <i>sucCD</i> , <i>hemB</i> translational downregulation by start codon substitution [32]
Org. acids	Succinic acid	<i>Synechococcus elongatus</i>	Native, chromosomal	Competing pathways	Product pathway		Knock-out <i>glc</i> and knock-in <i>gltA-ppc</i> (under nitrogen starvation conditions) [30]
Antibiotics/ anti-tumor	Pristinamycin I (PI)	<i>Streptomyces pristinaespiralis</i>	Native, chromosomal	Repressor, competing pathway	Product pathway		Knock-out <i>snaE1</i> , <i>snaE2</i> and <i>papR3</i> , knock-in PI biosynthetic gene cluster [33]
	Pristinamycin II (PII)		Native, chromosomal		Product pathway		Knock-in of artificial bacteriophage attachment/integration ( <i>attB</i> ) sites in which the biosynthetic pathway is subsequently inserted [34]
	Chloramphenicol	<i>Streptomyces coelicolor</i>	Heterologous, chromosomal		Product pathway		
	Anti-tumor compound YM-216391		Heterologous, chromosomal		Product pathway		
Lipid content	Fatty acids	<i>Escherichia coli</i>	Heterologous, chromosomal	Competing pathways	Product pathway		Knock-in <i>fadR</i> , <i>delta9</i> and <i>acc</i> (deletions made previously) [37]

Table 2

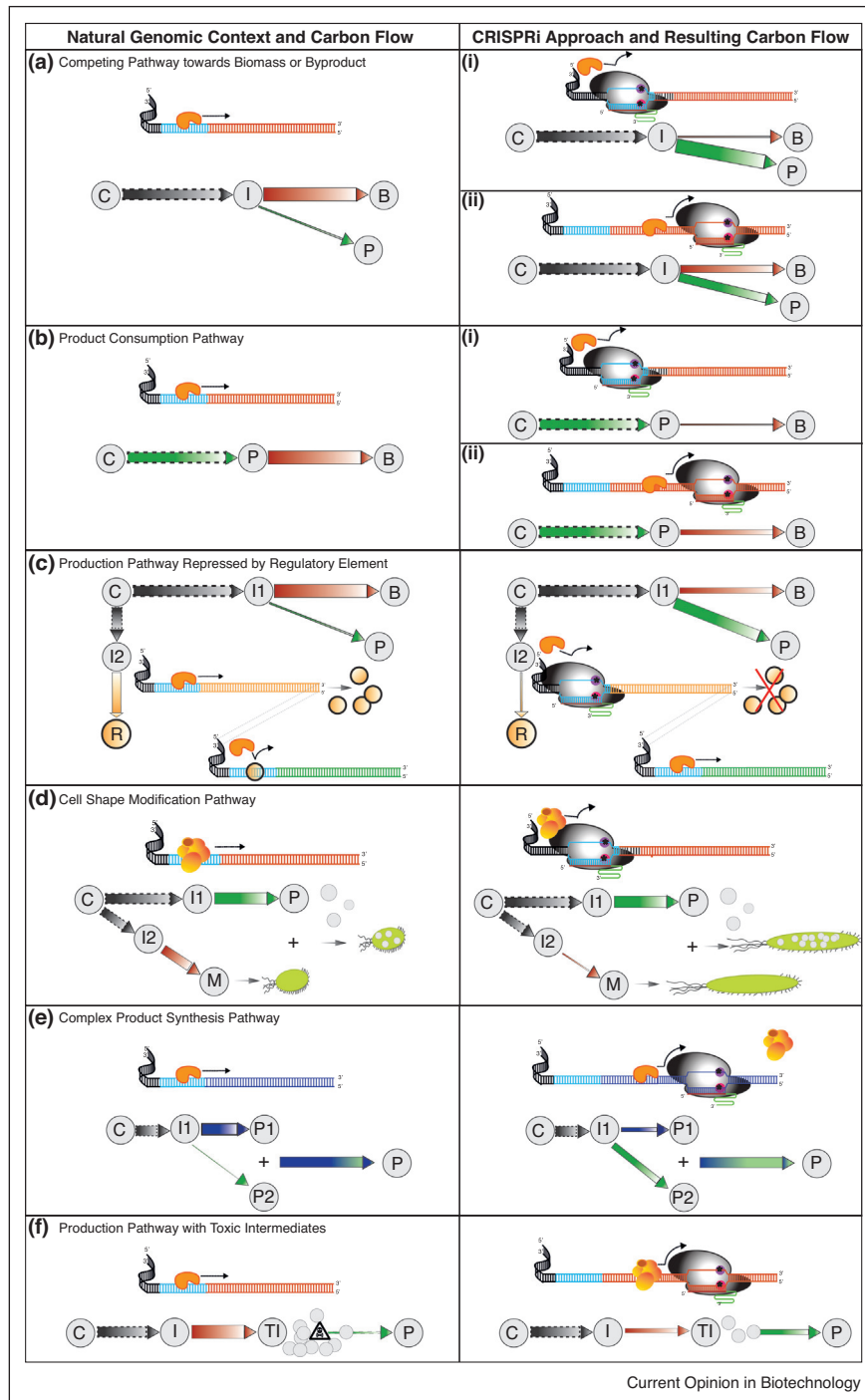
## CRISPRi-based metabolic engineering of bacteria for industrial products

Product	Species	Product pathway overexpression method	CRISPRi-based repression of <sup>a</sup> :	Genes targeted by CRISPRi	M <sup>b</sup>	Ref.	
Polyhydroxyalkanoates (PHA)	<i>Escherichia coli</i>	P(3HB-co-4HB) with enhanced 4HB content	Heterologous, plasmid	Competing pathways	<i>sad/gadB, sucCD, sdhAB</i>	+	[42]
		Polyhydroxy-butyrates (PHB)	Heterologous, plasmid	Cell morphology	<i>ftsZ, mreB</i>	-	[43]
	<i>Halomonas</i> sp.	PHB	Heterologous, plasmid	Product pathway	<i>phaC</i>	-	[39]
		3-Hydroxybutyrate/3-hydroxyvalerate (PHBV) and PHB	-	Cell morphology Product pathway Competing pathways	<i>ftsZ, prpC, gltA</i>	-	[40]
Phytochemicals	<i>Escherichia coli</i>	Zeaxanthin	Heterologous, plasmid (+sensor for dynamic control) and chromosomal	Cell morphology	<i>ftsZ, mreB, pbp, rodZ</i>	-	[44]
		Naringenin	Heterologous, plasmid	Competing pathways	<i>eno, adhE, mdh, fabB, fabF, sucC, fumC</i>	+	[46]
		Naringenin	Heterologous, plasmid	Competing pathways	<i>fadR, fumC, sucABCD, scpC</i>	+	[47]
		Resveratrol	Heterologous, plasmid	Competing pathways	<i>fabD, fabH, fabB, fabF, fabI</i>	-	[48]
		Pinosylvin	Heterologous, plasmid	Competing pathways	<i>eno, adhE, fabB, sucC, fumC, fabF</i>	+	[49]
		Anthocyanin	Heterologous, plasmid	Repressor of product pathway	<i>metJ</i>	-	[50]
Fatty acids	<i>Escherichia coli</i>	Medium chain fatty acids (MCFAs)	Heterologous and native, plasmid	Competing pathways	<i>rdA, adhE, ldhA, poxB, pta</i>	-	[51]
Terpenoids	<i>Escherichia coli</i>	(-)- $\alpha$ -Bisabolol, isoprene, lycopene	Heterologous, plasmid	Product pathway Competing pathway: biomass	<i>mvaK1, mvaE, ispA</i>	-	[41]
		Mevalonate	Heterologous, plasmid	Competing pathway: biomass	<i>pyrF, oriC, dnaA</i>	-	[53**]
Amino acids	<i>Corynebacterium glutamicum</i>	L-Lysine, L-glutamate	-	Competing pathways	<i>pgi, pck, pyk</i>	-	[45]
Alcohols	<i>Clostridium cellulovorans</i> & <i>Clostridium beijerinckii</i>	Acetone-butanol-ethanol	Native, plasmid	Competing pathways (as well as knock-out)	putative hydrogenase in <i>C. cellulovorans</i>	-	[54]
		n-Butanol	<i>Klebsiella pneumoniae</i>	Heterologous, plasmid	Competing pathways	<i>ilvB, ilvI, metA, alaA</i>	-
	<i>Escherichia coli</i>		Heterologous, plasmid	Competing pathways	<i>pta, frdA, ldhA, adhE</i>	+	[52]
	<i>Synechocystis</i> sp.	Fatty alcohols	Heterologous, plasmid	Competing pathways	<i>plsX, aar, ado, plsC, lplat</i>	+	[60]
Organic acids	<i>Synechococcus elongatus</i>	Succinate	-	Competing pathways (under nitrogen starvation conditions)	<i>glc, sdhA, sdhB</i>	-	[56]
		Lactate	<i>Synechococcus</i> sp.	-	Competing pathway to accumulate activator metabolite	<i>glnA</i>	-

<sup>a</sup> See Figure 2 for a visualization of these strategies. Modifications other than CRISPRi are shown in brackets.

<sup>b</sup> M: multiplexing.

Figure 2



Current Opinion in Biotechnology

Overview of CRISPRi-based metabolic engineering strategies to increase production of the target product (P). Abbreviations: C: carbon source; I: intermediate metabolite; B: byproduct or biomass; P: target product; TI: toxic intermediate; R: repressor; M: morphology. See legend of Figure 1 for graphic legend. Arrows represent intracellular carbon flows and their thickness corresponds to the flow rate. Arrows with dashed outlines represent merged pathways. **(a)** Repression of competing pathway that leads to byproduct or biomass formation with (i) indicating dCas9 targeting the promoter region, resulting in stronger repression than in (ii), where the coding region is targeted. **(b)** Repression of competing pathway that leads to product consumption with (i) indicating dCas9 targeting the promoter region, resulting in stronger repression than in (ii), where the coding region is targeted. **(c)** Repression of repressor of the target product pathway. **(d)** Repression of cell shape/morphology genes to increase cell size and storage capacity for the target product. **(e)** Repression of the product pathway to change product composition or properties. **(f)** Repression of the product pathway to prevent accumulation of toxic intermediates.

or by employing an inducible promoter for the expression of the dCas9 or the sgRNA module and altering the amount of the corresponding inducer. This is crucial when targeting essential genes, competing pathways (which can also be biomass-producing) or regulators for which a basal expression level is required (Figure 2a–d). It can also be used as a quick alternative to the often laborious RBS/promoter-engineering to tune production pathway activity to either modulate amounts and properties of the target product (Figure 2e) [39,40] or prevent accumulation of toxic intermediates [41] (Figure 2f). Although CRISPRi does not lead to the construction of stably genetically modified strains, it is a powerful method for quick evaluation of the possible effects of genetic modifications to the metabolism of a microorganism, allowing to design genome editing approaches and to gain insights into microbial metabolism (Figure 1, Table 2).

Similar to CRISPR-Cas-based editing, model organisms such as *E. coli* and *C. glutamicum* were the first organisms for which CRISPRi-based metabolic engineering was applied (Table 2). A heterologous polyhydroxyalkanoate (PHA) biosynthesis pathway was introduced into *E. coli* for the production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] [42]. The 4HB fraction of the polymer was enhanced via CRISPRi-based downregulation of multiple TCA cycle genes aiming to increase the supply of the 4HB-precursor succinate semialdehyde [42] (Figure 2e). Subsequent works in *E. coli* and in the natural PHA-producer *Halomonas* focused on silencing cell morphology genes to increase the storage capacity of the cells for PHAs [40,43] (Figure 2d), as well as biosynthetic pathway genes to control PHA content and chemical properties such as molecular weight and polydispersity [39,40] (Figure 2d). Cell morphology engineering, through CRISPRi-based repression of *E. coli* cell division and shape genes, combined with expression of a heterologous production pathway, was also used for the production of the phytochemical zeaxanthin [44] (Table 2). Several other studies used CRISPRi-based repression of competing pathway or repressor genes for the enrichment of precursor pools (Figure 2a,c), aiming at the enhancement of the natural amino acid production by *C. glutamicum* [45] and the *E. coli*-based heterologous production of various phytochemicals including naringenin [46,47], resveratrol [48], pinosylvin [49], anthocyanin [50], as well as medium chain fatty acids (MCFAs) [51] and n-butanol [52] (Table 2). Additionally, CRISPRi-based repression of essential genes was used to minimize carbon loss towards biomass formation by decoupling growth and production [53\*\*] or flux balancing [41] (Table 2). Notably, many of these studies employed multiplex silencing using an sgRNA approach, whereas Cress *et al.* used a dual RNA (crRNA/tracrRNA) approach, developing a rapid CRISPR-array assembly method denoted as CRISPathBrick [47]. This tool could facilitate multiplex CRISPRi-based silencing in non-model organisms with limited genetic toolboxes.

CRISPRi-based repression has already been used for metabolic engineering purposes in non-model organisms such as in *Clostridium cellulovorans* and *Klebsiella pneumoniae* for alcohol production [54,55], *Synechococcus elongatus* for succinate production [56] (Table 2), as well as in *Clostridium acetobutylicum* to relieve carbon catabolite repression for sugar co-utilization [57]. The CRISPRi tool is particularly useful in cyanobacteria, in which genome editing is complicated and time consuming due to slow growth and multiple chromosome copies [56,58]. Furthermore, the ability to fine-tune expression levels using CRISPRi was exploited in *Synechococcus* sp., where repression of nitrogen assimilation gene *glnA* was shown to increase the pool of  $\alpha$ -ketoglutarate [59]. A moderate increase of this metabolite enhanced glycolytic flux and lactate production, whereas a too large increase resulted in a decrease in protein production [59]. CRISPRi-based multiplex gene repression was established in *Synechocystis* sp. [58] and subsequently used to determine optimal gene repression combinations for fatty alcohol production [60] (Table 2). As in all studies using CRISPRi in cyanobacteria, dCas9 and sgRNAs were chromosomally integrated into the genome. This resulted in stable repression strains in the absence of selective pressure for single sgRNAs, but the use of repetitive promoter elements resulted in undesired recombination events when multiplexing was attempted [60], highlighting the potential advantage of using a dual RNA approach. Finally, it was observed that a targeted gene with a very distant transcription start site (TSS) from the start codon could be efficiently repressed by CRISPRi only when employing multiple sgRNAs targeting within the gene or the preceding operon genes [58]. Hence, this study revealed that the efficient application of the CRISPRi tool is strongly connected with the precise identification of TSS for the targeted genes.

### Future perspectives

In prokaryotes, CRISPR-Cas based genome editing has strongly increased engineering efficiencies by adding a powerful counter-selection method to existing engineering systems or by enhancing recombination efficiencies through induction of cellular DNA repair mechanisms [12,20]. In model organism *E. coli* high-throughput tools such as crMAGE [7\*\*] and CREATE [25] enabled multiplex engineering by combining the Cas9 and recombining tools. It is expected that these high-throughput tools will be further developed into automated pipelines for rapid industrial strain development, but the thorough exploitation of their potential requires the additional development of rapid and easy screening and read-out systems.

CRISPR-Cas-based counter-selection tools have increased editing efficiencies in many non-model organisms. However, further improvement of these tools still strongly depends on the development of basics, such as



well-characterized inducible promoters [61]. Tight control of the Cas9-expression would allow for efficient integration of an employed homologous recombination template prior to the counter-selection step. The use of intrinsic Cas9-properties, such as temperature-sensitivity, can substitute the requirement for inducible promoters in organisms that can grow under conditions outside the Cas9 activity range [62].

The use of alternative Class 2 CRISPR systems will further extend the Cas-based engineering toolbox. Recently, the Cpf1 (Cas12) RNA-guided DNA endonucleases from the type V CRISPR-Cas systems of *Francisella novicida* [35<sup>\*</sup>] and *Acidaminococcus sp.* [63] have been repurposed for bacterial genome editing and silencing. Cpf1 does not require a tracrRNA and can process its own precursor crRNA via its intrinsic RNase activity. Hence, the use of Cpf1 for the development of a multiplex engineering tool can prevent the issues encountered when using Cas9 and multiple sgRNAs for multiplex engineering [63]. The use of a Cpf1-recombineering tool in *C. glutamicum*, which tolerates only very low levels of Cas9 expression [31,35<sup>\*</sup>,64], resulted in screenable editing efficiencies [35<sup>\*</sup>], while a DNase-dead Cpf1 (ddCpf1) variant was recently employed for multiplex silencing in *E. coli* [63]. The newly discovered Class 2 Type VI system Cas13 (C2c2) RNA-guided RNA-nuclease can be used for silencing via the degradation of transcripts, or for tracking of transcripts using fluorescent-coupled catalytically inactive variants [65<sup>\*</sup>,66]. The RNA-guided RNA endonuclease from the type VI CRISPR-Cas systems of *Leptotrichia shahii* and *Leptotrichia wadei* have already been successfully repurposed for RNA interference in *E. coli* [67,68]. Furthermore, the repurposing of native CRISPR-Cas systems for genome editing [69<sup>\*</sup>], has been proved efficient and holds promise for organisms with low transformation efficiencies [70]. It is anticipated that the development of easy and rapid characterization techniques [71<sup>\*</sup>,72–74] will accelerate the exploitation of novel CRISPR-Cas systems for the development of prokaryotic engineering tools. These tools will further expand the number of target sites, the range of easy-to-engineer organisms and they will increase the engineering speed by simultaneous usage of different Cas systems for genome editing and plasmid curing [65,75], similar to the recently developed EXIT-circuit approach that combines Cas9-based editing and I-SceI-based plasmid curing [76].

Screening natural resources for novel CRISPR-Cas systems will further expand the applications and range of organisms and environments in which CRISPR-Cas-based editing can be applied [77–79]. A recent example of this approach is the identification of a thermostable Cas9-orthologue in the genome of a *Geobacillus thermodenitrificans* strain isolated from a compost sample, which was further characterized and employed to establish the

first Cas9-based engineering tool for thermophilic bacteria [80<sup>\*</sup>]. The robustness of thermostable Cas9-based tools can be further exploited for applications in extreme environments, as was recently shown for another thermostable Cas9-orthologue with prolonged life time in blood plasma [81]. The discovery of novel Cas nucleases with different properties, such as tolerance to alkaline or acidic pH and high saline concentrations, would be possible by screening selected environmental samples and metagenomic libraries. The characterization of these nucleases could lead to the development of engineering tools with wide applicability to biotechnologically relevant but currently unexploited extremophilic organisms.

## Conclusions

Altogether, the developments in CRISPR-Cas-based bacterial genome engineering increase insight into metabolism on a systems level and enable more rapid strain engineering, which is crucial for the development of a bio-based economy using microbial cell factories. Rapid current developments and future applications, which will further expand the range of organisms and applications of CRISPR-Cas-based editing for metabolic engineering, consist of fine-tuning of the tools, their adaptation to different hosts, their extension into combinations with other active components such as proteases, markers or activators, as well as the discovery and development of novel Cas-like systems.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

This work was supported by Dutch Technology Foundation STW, which is part of The Netherlands Organization for Scientific Research (NWO) and which is partly funded by the Ministry of Economic Affairs (Grant Number 13938); Marine Biotechnology ERA-NET ‘ThermoFactories’ (Grant Number 5178-00003B) and the Novo Nordisk Foundation, Denmark; and Corbion BV, The Netherlands; The European Union Marie Skłodowska-Curie Innovative Training Networks (ITN) [contract number 642068].

## References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
  - of outstanding interest
1. Hillmyer MA: **The promise of plastics from plants.** *Science* 2017, **358**:868–870.
  2. Donohoue PD, Barrangou R, May AP: **Advances in industrial biotechnology using CRISPR-Cas systems.** *Trends Biotechnol* 2017.
  3. Bowater R, Doherty AJ: **Making ends meet: repairing breaks in bacterial DNA by non-homologous end-joining.** *PLoS Genet* 2006, **2**:e8.
  4. Cromie GA, Connelly JC, Leach DR: **Recombination at double-strand breaks and DNA ends: conserved mechanisms from phage to humans.** *Mol Cell* 2001, **8**:1163–1174.
  5. Court DL, Sawitzke JA, Thomason LC: **Genetic engineering using homologous recombination.** *Annu Rev Genet* 2002, **36**:361–388.

6. Olorunniji FJ, Rosser SJ, Stark WM: **Site-specific recombinases: molecular machines for the Genetic Revolution.** *Biochem J* 2016, **473**:673-684.
7. Ronda C, Pedersen L, Sommer M, Nielsen A: **CRIMAGE: CRISPR Optimized MAGE Recombineering.** 2016.  
The authors greatly increased recombineering editing efficiencies by combining Cas9-counter-selection with MAGE recombineering. The developed tool can facilitate high-throughput metabolic engineering studies.
8. Pines G, Freed EF, Winkler JD, Gill RT: **Bacterial recombineering: genome engineering via phage-based homologous recombination.** *ACS Synth Biol* 2015, **4**:1176-1185.
9. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P: **CRISPR provides acquired resistance against viruses in prokaryotes.** *Science* 2007, **315**:1709-1712.
10. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV, Van Der Oost J: **Small CRISPR RNAs guide antiviral defense in prokaryotes.** *Science* 2008, **321**:960-964.
11. Garneau JE, Dupuis M-E, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadan AH, Moineau S: **The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA.** *Nature* 2010, **468**:67-71.
12. Mougiakos I, Bosma EF, de Vos WM, van Kranenburg R, van der Oost J: **Next generation prokaryotic engineering: the CRISPR-Cas toolkit.** *Trends Biotechnol* 2016, **34**:575-587.
13. Barrangou R: **Cas9 targeting and the CRISPR revolution.** *Science* 2014, **344**:707-708.
14. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao YJ, Pirzada ZA, Eckert MR, Vogel J, Charpentier E: **CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III.** *Nature* 2011, **471**:602.
15. Deveau H, Barrangou R, Garneau JE, Labonte J, Fremaux C, Boyaval P, Romero DA, Horvath P, Moineau S: **Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*.** *J Bacteriol* 2008, **190**:1390-1400.
16. Mojica F, Diez-Villasenor C, Garcia-Martinez J, Almendros C: **Short motif sequences determine the targets of the prokaryotic CRISPR defence system.** *Microbiology* 2009, **155**:733-740.
17. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: **A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity.** *Science* 2012, **337**:816-821.
18. Komor AC, Badran AH, Liu DR: **CRISPR-based technologies for the manipulation of eukaryotic genomes.** *Cell* 2016.
19. Barrangou R, Horvath P: **A decade of discovery: CRISPR functions and applications.** *Nat Microbiol* 2017, **2**:17092.
20. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA: **RNA-guided editing of bacterial genomes using CRISPR-Cas systems.** *Nat Biotechnol* 2013, **31**:233-239.
21. Oh J-H, van Pijkeren J-P: **CRISPR-Cas9-assisted recombineering in *Lactobacillus reuteri*.** *Nucleic Acids Res* 2014, **42**:e131.
22. Mewalal R, Rai DK, Kainer D, Chen F, K ulheim C, Peter GF, Tuskan GA: **Plant-derived terpenes: a feedstock for specialty biofuels.** *Trends Biotechnol* 2017, **35**:227-240.
23. Delmulle T, De Maeseneire SL, De Mey M: **Challenges in the microbial production of flavonoids.** *Phytochem Rev* 2017:1-19.
24. Li YF, Lin ZQ, Huang C, Zhang Y, Wang ZW, Tang YJ, Chen T, Zhao XM: **Metabolic engineering of *Escherichia coli* using CRISPR-Cas9 mediated genome editing.** *Metab Eng* 2015, **31**:13-21.  
This pioneering study was the first in which extensive metabolic engineering was performed with Cas9-counter selection and recombineering, showing the great improvements in engineering throughput enabled by Cas9. By using this tool, a heterologous  $\beta$ -carotene pathway was inserted to the *E. coli* genome and numerous RBSs and promoters were replaced.
25. Garst AD, Bassalo MC, Pines G, Lynch SA, Halweg-Edwards AL, Liu R, Liang L, Wang Z, Zeitoun R, Alexander WG *et al.*: **Genome-wide mapping of mutations at single-nucleotide resolution for protein, metabolic and genome engineering.** *Nat Biotechnol* 2017, **35**:48-55.
26. Liang LY, Liu RM, Garst AD, Lee T, Nogue VSI, Beckham GT, Gill RT: **CRISPR EnAble Trackable genome Engineering for isopropanol production in *Escherichia coli*.** *Metab Eng* 2017, **41**:1-10.  
Elegant example of how using CRISPR-Cas-systems can increase engineering and screening throughput. By creating trackable edited genotypes the selection of mutants from a mutant library is simplified and engineering efficiencies and throughput are improved.
27. Heo MJ, Jung HM, Um J, Lee SW, Oh MK: **Controlling citrate synthase expression by CRISPR/Cas9 genome editing for n-butanol production in *Escherichia coli*.** *ACS Synth Biol* 2017, **6**:182-189.
28. Wang SH, Dong S, Wang PX, Tao Y, Wang Y: **Genome editing in *Clostridium saccharoperbutylacetonicum* N1-4 with the CRISPR-Cas9 system.** *Appl Environ Microbiol* 2017, **83**.
29. Wasels F, Jean-Marie J, Collas F, L opez-Contreras AM, Lopes Ferreira N: **A two-plasmid inducible CRISPR/Cas9 genome editing tool for *Clostridium acetobutylicum*.** *J Microbiol Methods* 2017, **140**:5-11.
30. Li H, Shen CR, Huang C-H, Sung L-Y, Wu M-Y, Hu Y-C: **CRISPR-Cas9 for the genome engineering of cyanobacteria and succinate production.** *Metab Eng* 2016, **38**:293-302.
31. Cho JS, Choi KR, Prabowo CPS, Shin JH, Yang D, Jang J, Lee SY: **CRISPR/Cas9-coupled recombineering for metabolic engineering of *Corynebacterium glutamicum*.** *Metab Eng* 2017, **42**:157-167.
32. Ding WW, Weng HJ, Du GC, Chen J, Kang Z: **5-Aminolevulinic acid production from inexpensive glucose by engineering the C4 pathway in *Escherichia coli*.** *J Ind Microbiol Biotechnol* 2017, **44**:1127-1135.
33. Meng J, Feng R, Zheng G, Ge M, Mast Y, Wohlleben W, Gao J, Jiang W, Lu Y: **Improvement of pristinamycin I (PI) production in *Streptomyces pristinaespiralis* by metabolic engineering approaches.** *Synth Syst Biotechnol* 2017, **2**:130-136.
34. Li L, Zheng GS, Chen J, Ge M, Jiang WH, Lu YH: **Multiplexed site-specific genome engineering for overproducing bioactive secondary metabolites in actinomycetes.** *Metab Eng* 2017, **40**:80-92.
35. Jiang Y, Qian FH, Yang JJ, Liu YM, Dong F, Xu CM, Sun BB, Chen B, Xu XS, Li Y *et al.*: **CRISPR-Cpf1 assisted genome editing of *Corynebacterium glutamicum*.** *Nat Commun* 2017:8.  
Example of how the use of alternative CRISPR-Cas-systems can enable more efficient genome engineering. Unlike Cas9 expression in *C. glutamicum*, which was proved to be toxic, Cpf1 expression resulted in an efficient engineering tool that was applied for codon saturation mutagenesis of  $\gamma$ -glutamyl kinase aiming to relieve L-proline inhibition.
36. Wu T, Ye L, Zhao D, Li S, Li Q, Zhang B, Bi C, Zhang X: **Membrane engineering—a novel strategy to enhance the production and accumulation of  $\beta$ -carotene in *Escherichia coli*.** *Metab Eng* 2017, **43**:85-91.
37. Xia J, Wang L, Zhu JB, Sun CJ, Zheng MG, Zheng L, Lou YH, Shi L: **Expression of *Shewanella frigidimarina* fatty acid metabolic genes in *E-coli* by CRISPR/cas9-coupled lambda Red recombineering.** *Biotechnol Lett* 2016, **38**:117-122.
38. 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.
39. Li D, Lv L, Chen JC, Chen GQ: **Controlling microbial PHB synthesis via CRISPRi.** *Appl Microbiol Biotechnol* 2017, **101**:5861-5867.
40. Tao W, Lv L, Chen GQ: **Engineering *Halomonas* species TD01 for enhanced polyhydroxyalkanoates synthesis via CRISPRi.** *Microb Cell Fact* 2017, **16**.

41. Kim SK, Han GH, Seong W, Kim H, Kim SW, Lee DH, Lee SG: **CRISPR interference-guided balancing of a biosynthetic mevalonate pathway increases terpenoid production.** *Metab Eng* 2016, **38**:228-240.
42. Lv L, Ren YL, Chen JC, Wu Q, Chen GQ: **Application of CRISPRi for prokaryotic metabolic engineering involving multiple genes, a case study: controllable P(3HB-co-4HB) biosynthesis.** *Metab Eng* 2015, **29**:160-168.
43. Elhadi D, Lv L, Jiang XR, Wu H, Chen GQ: **CRISPRi engineering *E. coli* for morphology diversification.** *Metab Eng* 2016, **38**:358-369.
44. Shen HJ, Cheng BY, Zhang YM, Tang L, Li Z, Bu YF, Li XR, Tian GQ, Liu JZ: **Dynamic control of the mevalonate pathway expression for improved zeaxanthin production in *Escherichia coli* and comparative proteome analysis.** *Metab Eng* 2016, **38**:180-190.
45. Cleto S, Jensen JVK, Wendisch VF, Lu TK: ***Corynebacterium glutamicum* Metabolic Engineering with CRISPR Interference (CRISPRi).** *ACS Synth Biol* 2016, **5**:375-385.
46. Wu JJ, Du GC, Chen J, Zhou JW: **Enhancing flavonoid production by systematically tuning the central metabolic pathways based on a CRISPR interference system in *Escherichia coli*.** *Sci Rep* 2015, **5**.
47. Cress BF, Toparlak OD, Guleria S, Lebovich M, Stieglitz JT, Englaender JA, Jones JA, Linhardt RJ, Koffas MAG: **CRISPathBrick. Modular combinatorial assembly of type II-A CRISPR Arrays for dCas9-mediated multiplex transcriptional repression in *E. coli*.** *ACS Synth Biol* 2015, **4**:987-1000.
48. Wu JJ, Zhou P, Zhang X, Dong MS: **Efficient de novo synthesis of resveratrol by metabolically engineered *Escherichia coli*.** *J Ind Microbiol Biotechnol* 2017, **44**:1083-1095.
49. Wu JJ, Zhang X, Zhu YJ, Tan QY, He JC, Dong MS: **Rational modular design of metabolic network for efficient production of plant polyphenol pinosylvin.** *Sci Rep* 2017, **7**.
50. Cress BF, Leitz QD, Kim DC, Amore TD, Suzuki JY, Linhardt RJ, Koffas MAG: **CRISPRi-mediated metabolic engineering of *E. coli* for O-methylated anthocyanin production.** *Microb Cell Fact* 2017, **16**.
51. Wu JJ, Zhang X, Xia XD, Dong MS: **A systematic optimization of medium chain fatty acid biosynthesis via the reverse beta-oxidation cycle in *Escherichia coli*.** *Metab Eng* 2017, **41**:115-124.
52. Kim SK, Seong W, Han GH, Lee D-H, Lee S-G: **CRISPR interference-guided multiplex repression of endogenous competing pathway genes for redirecting metabolic flux in *Escherichia coli*.** *Microb Cell Fact* 2017, **16**:188.
53. Li SY, Jendresen CB, Grunberger A, Ronda C, Jensen SI, Noack S, Nielsen AT: **Enhanced protein and biochemical production using CRISPRi-based growth switches.** *Metab Eng* 2016, **38**:274-284.
- Elegant example of how CRISPRi has enabled novel metabolic engineering strategies. Full deletion of essential genes is not possible, but down-regulation by CRISPRi resulted in decoupling of growth and production and redirection of carbon flux from biomass to product.
54. Wen ZQ, Minton NP, Zhang Y, Li Q, Liu JL, Jiang Y, Yang S: **Enhanced solvent production by metabolic engineering of a twin-clostridial consortium.** *Metab Eng* 2017, **39**:38-48.
55. Wang MM, Liu LT, Fan LH, Tan TW: **CRISPRi based system for enhancing 1-butanol production in engineered *Klebsiella pneumoniae*.** *Process Biochem* 2017, **56**:139-146.
56. Huang CH, Shen CR, Li H, Sung LY, Wu MY, Hu YC: **CRISPR interference (CRISPRi) for gene regulation and succinate production in cyanobacterium *S. elongatus* PCC 7942.** *Microb Cell Fact* 2016, **15**.
57. Bruder MR, Pyne ME, Moo-Young M, Chung DA, Chou CP: **Extending CRISPR-Cas9 technology from genome editing to transcriptional engineering in the genus *Clostridium*.** *Appl Environ Microbiol* 2016, **82**:6109-6119.
58. Yao L, Cengic I, Anfelt J, Hudson EP: **Multiple gene repression in cyanobacteria using CRISPRi.** *ACS Synth Biol* 2015, **5**:207-212.
59. Gordon GC, Korosh TC, Cameron JC, Markley AL, Begemann MB, Pfeleger BF: **CRISPR interference as a titratable, trans-acting regulatory tool for metabolic engineering in the cyanobacterium *Synechococcus* sp. strain PCC 7002.** *Metab Eng* 2016, **38**:170-179.
60. Kaczmarzyk D, Cengic I, Yao L, Hudson EP: **Diversion of the long-chain acyl-ACP pool in *Synechocystis* to fatty alcohols through CRISPRi repression of the essential phosphate acyltransferase PlsX.** *Metab Eng* 2017.
61. Yan Q, Fong SY: **Challenges and advances for genetic engineering of non-model bacteria and uses in consolidated bioprocessing.** *Front Microbiol* 2017, **8**:2060.
62. Mouggiakos I, Bosma EF, Weenink K, Vossen E, Goijvaerts K, van der Oost J, van Kranenburg R: **Efficient genome editing of a facultative thermophile using mesophilic spCas9.** *ACS Synth Biol* 2017.
63. Zhang H, Cheng QX, Liu AM, Zhao GP, Wang J: **A novel and efficient method for bacteria genome editing employing both CRISPR/Cas9 and an antibiotic resistance cassette.** *Front Microbiol* 2017, **8**.
64. Peng F, Wang X, Sun Y, Dong G, Yang Y, Liu X, Bai Z: **Efficient gene editing in *Corynebacterium glutamicum* using the CRISPR/Cas9 system.** *Microb Cell Fact* 2017, **16**:201.
65. Nakade S, Yamamoto T, Sakuma T: **Cas9, Cpf1 and C2c1/2/3—what's next?** *Bioengineered* 2017, **8**:265-273.
- Comprehensive overview of improvements and alternatives of CRISPR-Cas systems such as novel Cas9s, Cas9-like molecules and engineered Cas9-variants to broaden the range of genome editing possibilities.
66. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DBT, Shmakov S, Makarova KS, Semenova E, Minakhin L *et al.*: **C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector.** *Science* 2016, **353**.
67. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DB, Shmakov S, Makarova KS, Semenova E, Minakhin L: **C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector.** *Science* 2016, **353**: aaf5573.
68. Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox DB, Kellner MJ, Regev A: **RNA targeting with CRISPR-Cas13.** *Nature* 2017, **550**:280-284.
69. Li Y, Pan S, Zhang Y, Ren M, Feng M, Peng N, Chen L, Liang YX, She Q: **Harnessing Type I and Type III CRISPR-Cas systems for genome editing.** *Nucleic Acids Res* 2016, **44**:e34.
- This work is one of the first examples of employing native CRISPR-Cas systems for genome editing, showing that both the native Type I-A and Type III-B CRISPR systems of the archaeon *Sulfolobus islandicus* can be used for genome editing.
70. Stout E, Klaenhammer T, Barrangou R: **CRISPR-Cas technologies and applications in food bacteria.** *Annu Rev Food Sci Technol* 2017, **8**:413-437.
71. Jenkinson E, Krabben P: **Targeted mutations.** . GB2530831 2015.
- ¶This patent is one of the first works to describe genome editing using native CRISPR-Cas systems. In Clostridia, the authors use the organisms' native Type I systems in combination with plasmid-borne homologous recombination templates for genome editing. It also describes an easy way to identify PAM sequences of the systems and test their functionality.
72. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA *et al.*: **Multiplex genome engineering using CRISPR/Cas systems.** *Science* 2013, **339**:819-823.
73. Esvelt KM, Mali P, Braff JL, Moosburner M, Yaung SJ, Church GM: **Orthogonal Cas9 proteins for RNA-guided gene regulation and editing.** *Nat Methods* 2013, **10**:1116-1121.

74. Mitsunobu H, Teramoto J, Nishida K, Kondo A: **Beyond native Cas9: manipulating genomic information and function.** *Trends Biotechnol* 2017, **35**:983-996.
75. Yan MY, Yan HQ, Ren GX, Zhao JP, Guo XP, Sun YC: **CRISPR-Cas12a-assisted recombineering in bacteria.** *Appl Environ Microbiol* 2017, **83**.
76. Tang Q, Lou C, Liu S-J: **Construction of an easy-to-use CRISPR-Cas9 system by patching a newly designed EXIT circuit.** *J Biol Eng* 2017, **11**:32.
77. Murugan K, Babu K, Sundaresan R, Rajan R, Sashital DG: **The revolution continues: newly discovered systems expand the CRISPR-Cas toolkit.** *Mol Cell* 2017, **68**:15-25.
78. Karvelis T, Gasiunas G, Siksnys V: **Harnessing the natural diversity and in vitro evolution of Cas9 to expand the genome editing toolbox.** *Curr Opin Microbiol* 2017, **37**:88-94.
79. Koonin EV, Makarova KS, Zhang F: **Diversity, classification and evolution of CRISPR-Cas systems.** *Curr Opin Microbiol* 2017, **37**:67-78.
80. Mougiakos I, Mohanraju P, Bosma EF, Vrouwe V, Finger Bou M, Naduthodi MIS, Gussak A, Brinkman RBL, van Kranenburg R, van der Oost J: **Characterizing a thermostable Cas9 for bacterial genome editing and silencing.** *Nat Commun* 2017, **8**:1647.
- This work describes the identification and characterization of a novel thermophilic Cas9 (ThermoCas9) from a *Geobacillus thermodenitrificans* strain. ThermoCas9 was shown to be active over a broad temperature range (*in vitro* between 20 and 70°C) and further developed into the first Cas9-based engineering tool for thermophilic bacteria.
81. Harrington L, Paez-Espino D, Chen JS, Ma E, Staahl BT, Kyrpides NC, Doudna JA: **A thermostable Cas9 with increased lifetime in human plasma.** *Nat Commun* 2017, **8**:1424.