Developing Bacillus subtilis as a versatile bioproduct platform for agricultural and pharmaceutical applications

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Chapter 6

Development and application of CRISPR-based genetic tools in *Bacillus* species

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Abstract

Recently, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system has been developed into precise and efficient genome editing tools, and been applied in many fields of basic research and bio-industry, ever since it has been discovered as an adaptive immune system in prokaryotes. The high demand for rapid, highly efficient and versatile genetic tools to thrive bacteria-based cell factories accelerates this process. In this review, we mainly focus our attention on developments and applications of the CRISPR system in *Bacillus subtilis*, including the achievements in gene editing and regulation, the advantages and existing problems. Next, we summarize the up-to-date development and utilization of this genetic tool in other *Bacillus* species, including *Bacillus licheniformis*, *Bacillus methanolicus*, *Bacillus anthracis*, *Bacillus cereus*., *Bacillus smithii* and *Bacillus thuringiensis*. Finally, we suggest potential strategies on how to further improve this advanced technique, and provide insights into future directions of CRISPR technologies, aiming at making them as powerful driving forces to fully develop *Bacillus* species cell-factories.

Key words: *Bacillus*, Clustered Regularly Interspaced Short Palindromic Repeats, Cas9, Cpf1, genetic tools

Introduction

Bacillus subtilis has long been used in many fields ranging from food, cosmetic, pharmaceutical and agricultural industrial.[1-4] The extensive applications not only attribute to its generally recognized as safe status and strong protein secretion capacity, but also characters of well growth on low-cost carbon sources and robustness in large-scale fermentations. [5-7] The complete sequencing genome and genetic modifications facilitate the development of *B. subtilis* cell factories to produce biological substances at a commercial scale such as valuable proteins/enzymes, vitamins, antibiotics as well as platform chemicals.[8] Recently, the high-throughput multi-omics technologies provide innumerable potential genetic targets to improve the bacteria performance from holistic views of the biological system. [9, 10] Hence, to fulfill the increasing demands for gene editing, a series of genetic modification tools have been established[11]. However, these tools still possess many drawbacks and limitations. For example, limited number of selection markers are available, low editing efficiency of genome editing, genome scars (remnant sequences) being left after modification and laborious selection procedures etc. Including undomesticated *B. subtilis* strains and some other industrially-important *Bacillus* species, similar obstacles also occurred and hampered the further utilization and scientific research on these species.[12] Therefore, it is urgently demanding for designable and multi-plexable genetic editing tools. Currently, the discovery and application of clustered regularly interspaced short palindromic repeat (CRISPR) system has revolutionized the field of genome engineering.

In 1987, Japanese researchers first discovered the repetitive DNA sequences in the genome of *Escherichia coli* when they studied the phosphate metabolism.[13]Subsequently, the repeats and related sequences were termed as clustered regularly interspaced short palindromic repeats (CRISPRs) in 2002.[14] Since the intervening "spacer" displayed high identities with foreign plasmids and bacteriophage sequences which attracted researchers' attention, soon the native biological functions of CRISPR as the bacteria and archaea adaptive immune system was explored and elucidated thereafter[15-19]. Briefly, in the first acquisition stage, a specific "spacer" sequence originated from invading DNA was recognized and incorporated into the CRISPR locus. The spacer corresponding sequence on the genome of the virus is called "protospacer", and the short conserved nucleotide fragment proximal to the protospacer is called the protospacer adjacent motif (PAM). In the second biogenesis/ expression stage, the CRISPR array including direct repeats and spacers is transcribed. And related Cas proteins facilitate the mature of CRISPR RNA (crRNA); In the third interference stage, the ribonucleoprotein complex of the Cas protein(s) and individual crRNA binds and cleaves target nucleotide sequences which are complementary to the spacer of crRNA, thus preventing foreign genetic elements invading.

After around three decades of study, CRISPR-Cas systems are found to be widely exist in bacteria (50%) and archaea (75%). These diverse systems can be classified into two classes (according to the configuration of effector module relies on multi-protein complex or single-component protein), five types and sixteen subtypes.[20, 21] Among them the type II system from *Streptococcus pyogenes* is the most widely studied, developed and applied system in genome editing. The signature Cas9 protein, a dual RNA-guided DNA endonuclease enzyme, is able to introduce double strand break (DSB) 3bp upstream of PAM (5'-NGG-3') sequence in DNA. (**Figure 1A**) In 2012, Cas9 protein, assisted by the dual-tracrRNA:crRNA, was demonstrated to be able to bind and cleavage DNA *in vitro*.[22] Ever since then, the CRISPR-Cas9 system is widely explored and adapted to powerful genetic tools to modify genomes of various microbial including yeast, *E. coli*, *Lactic acid*, *Corynebacterium* and *Streptomyces*. These have been consistently described by many researchers.[23-28] Significantly increased number of applications of CRISPR-Cas9 system in *B. subtilis* started in 2016, but a systematic overview of these advancements is still lacking. Therefore, in this review, we will introduce high editing efficiency of gene deletion, knock in, mutagenesis and multiplexing editings being achieved and applied to

boost levels of various products in *B. subtilis*. In addition, prosperous applications in gene regulations depending on Cas9 mutants are also discussed. Moreover, the current situations of CRISPR based tools developed in other *Bacillus* species are appreciable and desired more attention. Taken together, the overall progress summarized in this review aims to provide deep insights into *B. subtilis* and other *Bacillus* species' genetic engineering and inspire further investigation.

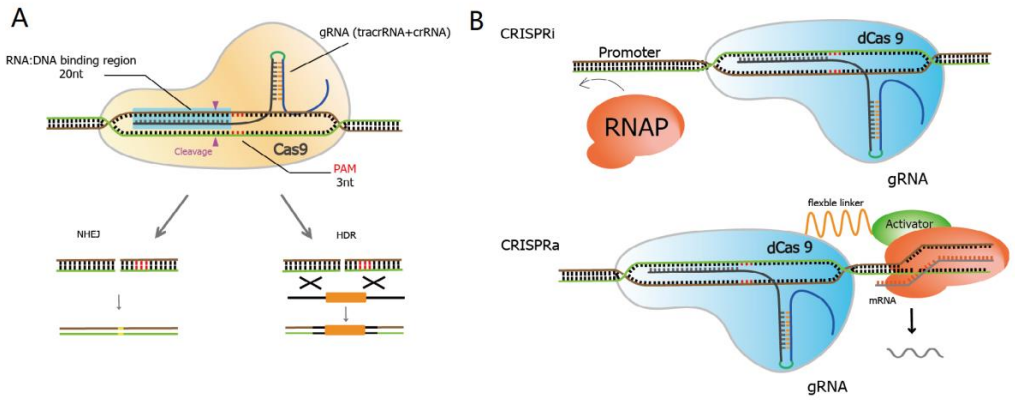

Figure 1. The CRISPR/Cas system and CRISPR-based gene regulations. **A** The CRISPR/Cas genome editing technology. PAM, protospacer adjacent motif; NHEJ, non-homologous end joining; HDR, homology-directed repair. **B** CRISPR activiation (CRISPRa) and CRISPR interference (CRISPRi) systems

Genome engineering in *B. subtilis*

Compare with previous genetic technologies, like zinc finger nucleases and transcription activator-like effector nucleases, the CRISPR-based tools possess the advantages of high editing efficiency, low costs and ease of manipulation.[29-31] Up to now, this novel RNA-guided genome-editing technique has been established in various *Bacillus* species, including *B. subtilis, B. amyloliquefaciens , B. licheniformis, B. methanolicus, B. anthracis, B. cereus* and *B. thuringiensis*. (**Table 1**). Here, we first discuss the development of CRISPR/Cas9, CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) systems in *B. subtilis* and how these robust systems have been utilized to improve production or titers of proteins, vitamins, functional sugars or other biochemicals.(**Figure 2**) Since *B. subtilis* has long been studied as a model Gram-positive bacterium for physiology and metabolism and developed as a universal cell factory, CRISPR-based tools benefit various aspects from scientific researches to industrial applications.

Table1. CRISPR/Cas systems for *Bacillus* species genome editing.

Species	Type	Cas protein and promoter	Promoter of Guide RNA	Editing template: type, length and description	Editing efficiency	Cas protein curing	Ref
Bacillus subtilis 1A751	Knock out	SpCas9 and Pcas from *S. pyogenes*	Promoter derived from S. pyogenes CRISPR locus	Linear dsDNA: PCR product (1.3 kb; optimal: 1 kb)	82%	MazF counter selection	[32]
Bacillus subtilis 1A751	Knockin multiplexing Mutation	SpCas9 and Pcas from *S. pyogenes*	$P_{xylA.\ SphI+1}$	Insert linearized vector with a hyaluronic acid biosynthetic operon(1.3 kb) Simultaneous editing double loci: PCR product or plasmid (1.0–1.4 kb) Linear dsDNA: PCR product or linearized plasmid (0.5-1.3kb; optimal: 1kb)	69% 36–85 % 23-100 %	MazF counter selection	[32]
Bacillus subtilis 168	Knock out Knockin mutation	SpCas9 and P_{grac}	P_{ara}	pB0A derived plasmid (0.4-2kb) 38kb plipastatin-synthesizing operon deletion pB0A derived plasmid (0.5kb) pB0A derived plasmid (0.5kb)	91-100 % 16.7-80 % 66.8-96.8% 33-68.5 %	Antibiotic-free conditions	[33]
Bacillus subtilis 6051A	Knock out	SpCas9 and P_{amyQ}	Constitutive promoter P_{43}	Vector: pHYcas9dsrf derived (0.45-0.55 kb)	33–55 %	Thermo-sensitive replication origin	[34]

Bacillus subtilis REG19	Knockin	SpCas9 and Mannose-inducible P_{manP}	Semisynthetic constitutive promoter P_{vanABK}	Vector: pJOE8999 derived (0.7-0.8 kb)	-	Thermo-sensitive replication origin	[36]
Bacillus subtilis 168	Knock out Mutation Knockin	SpCas9n (D10A) and P_{xylA}	Constitutive Promoter P_{43}	pDonor derived plasmid (0.5kb) 1-8kb deletion Single mutation Simultaneous double mutation Simultaneous three point mutation 1-2kb gene insertion	 80-90% 100% 90-91% 49-65% 92-98%	Thermo-sensitive replication origin gRNA targeting the plasmid replication origin gene *rep60*	[37]
Bacillus subtilis 168	Knockout depression	SpCas9: P_{grac}	P_{veg}	Linear dsDNA: PCR product	76% (63.9%-89.2%)	Pressure form IPTG induced high Cas9 expression and antibiotic-free conditions	[38]
Bacillus licheniformis DW2	Knock out Knock in Mutation	SpCas9n (D10A) and P_{43}	P_{43}	pGRNA-05 derived plasmid (1kb) 42.7kb fragment deletion Gene insertion Single mutation Simultaneous double mutation	 79% 76.5% 100% 11.6%	Antibiotic-free conditions	[39]
Bacillus licheniformis 2709	knockout Mutation	SpCas9 and pS	pLY-2	pWH-cas9-sgRNA derived plasmid (0.5kb) Single gene deletion Point mutation	 97.3% 99.2%	Antibiotic-free conditions	[40]

Bacillus licheniformis DSM13	knockout	SpCas9 and Pxyl	P_{HpaII}	pN-sgRNA1-xylACas9 Single gene deletion Low temperature condition (20°C)	47.1%-70.9% 97%	Thermo-sensitive replication origin and antibiotic-free conditions	41
B. amyloliquefaciens NBCSO	Depression	dCas9	P_{43}	pNX-dCas9, pHY-sgRNAn-egfp gene depression	-	Not mentioned	42
B. amyloliquefaciens 205	Knock out Depression activiation	SpCas9 dCas9 Pgrac	P_{43}	pHT43-cas9 pHT43-dcas9-gRNA pHT43-ω-dcas9-GN	-		43
Bacillus anthracis A16R	Knock out	SpCas9 and Mannose-inducible P_{manP}	Semisynthetic constitutive promote P_{vanABK}	Vector: pJOE8999 derived plasmid: lambdaBa03, ~16.8 kb fragment deletion (1.6kb+0.6kb) lambdaBa01, ~50.5 kb fragment deletion (1.6kb+1.2kb)	20% 100%	Thermo-sensitive replication origin	44
Bacillus cereus HN001	Mutation	SpCas9 and Mannose-inducible P_{manP}	Semisynthetic constitutive promote P_{vanABK}	Vector: pJOE8999 derived plasmid: point mutation	--	Thermo-sensitive replication origin	44
Bacillus smithii ET138	Deletion Knock out Knock in	SpCas9 and P_{xynA}	P_{pta}	pWUR_Cas9nt derived plasmid (1kb)	90% 100% 20%	High temperature and antibiotic-free conditions	45
Bacillus thuringiensis BMB171	knockout	SpCas9 and Mannose-inducible P_{manP}	Semisynthetic Constitutive promote P_{vanABK}	pJOE-ΔHmgA	--	Thermo-sensitive replication	46

Cas9 expression and sgRNA transcription

In bacteria, the fine-tuning expression level of *S. pyogenes* Cas9 (SpCas9) remains to be critical for CRISPR/Cas9 system application considering its potential toxicity to host strains.[47] Therefore, it's important to well investigate whether the SpCas9 protein should be codon-optimized or not, which promoter could be applied, and what inducer concentration should be used and which type of vectors should be used for its expression etc. In *B. subtilis*, the native SpCas9 without codon optimization was employed in majority of CRISPR-based genetic tools. The successful employment of native SpCas9 promoter, and basal expression level of inducible promoter P_{grac} implied that low expression level of SpCas9 protein, in the absence of targeting guide RNA, was not toxic to *B. subtilis*. [32, 38]

In vitro assay which discovered that programmable DNA targeting and cleavage requires Cas9 protein, crRNA, and trans-crRNA (tracrRNA), led to the subsequent generation of chimeric single guide RNA (sgRNA) as substitution of dual-RNA duplex (crRNA and tracrRNA) through a linker named tetraloop.[22] This composition simplified genetic manipulation process and rapidly translated to applications in eukaryotic and prokaryotic genome editing, including *Bacillus* species. But editing rates can vary dramatically (23% to 91% for *amyE* in *B. subtilis*) among different PAM sites within a single gene. Therefore, an increasing number of software and tools have been developed to rationally design the CRISPR guide sequence, for instance, E-CRISP, CHOPCHOP, ZiFiT, CRISPR Design Tool, sgRNA design tool and so on. Among these tools, multiple scoring systems were applied for predicting efficiency of sgRNAs combined with high-throughput experimental results, and some software also provides off-target profiles. In-depth analysis and discussions could be discovered in reviews by Ceasar *et al.*, and Wiles *et al.*.[48, 49]

Editing template/ Donor DNA

DSBs are one of the most precarious DNA lesions that could cause genomic instability and cell death. Non-homologous end joining (NHEJ) and endogenous homology-directed repair (HDR) are two elaborate DNA repair pathways evolved in organisms to maintain genome integrity.[50] (**Figure 1A**) The former is an error-prone repair system, which normally results random inserts or deletions at the break sites. On the contrary, the latter

system harnesses editing template/ donor DNA with homologous sequences flanking DSBs to guarantee high-fidelity repair of the damaged loci. In prokaryotes, NHEJ either works inefficiently or is absent in many bacteria.[38, 51] Considering the precise repair of the DSBs by HDR and insufficient capacity of NHEJ in *B. subtilis*, HDR become the predominant approach to achieve genome editing goals by CRISPR-Cas9 system.

Generally, the editing template/ donor DNA affects editing efficiency through two aspects, the type of donor DNA and the length of the homologous arm. The donor DNA could be provided either as PCR products or plasmid-borne editing templates, and the latter form remains to be prevalent. Price *et al.* reported an appreciable 76% of site-specific mutation efficiency when donor DNA was supplied as PCR fragment and co-transformed with Cas9 and sgRNA containing plasmid to *B. subtilis*, but the transformation efficiency was very low with only less than 20 CFU/μg.[38] Consistently, Westbrook *et al.* also observed above 90% of *amyE* mutation efficiencies by applying two types of editing templates individually. Whereas, the transformation efficiency of plasmid-borne editing template (1.55×10^3 CFU/μg) was nearly 6-fold higher of PCR editing template.[32] Therefore, the low transformation efficiency of PCR fragments hampered its further application. In addition to the length of editing template, researchers recognized that 400bp to 500bp of each upstream and downstream of homology region was enough to obtain acceptable editing efficiency in *B. subtilis*, and no transformants were observed with only 300bp of each arm.[33] Also, 1kb of homology arm was determined to be the optimal size of Donor DNA.[32]

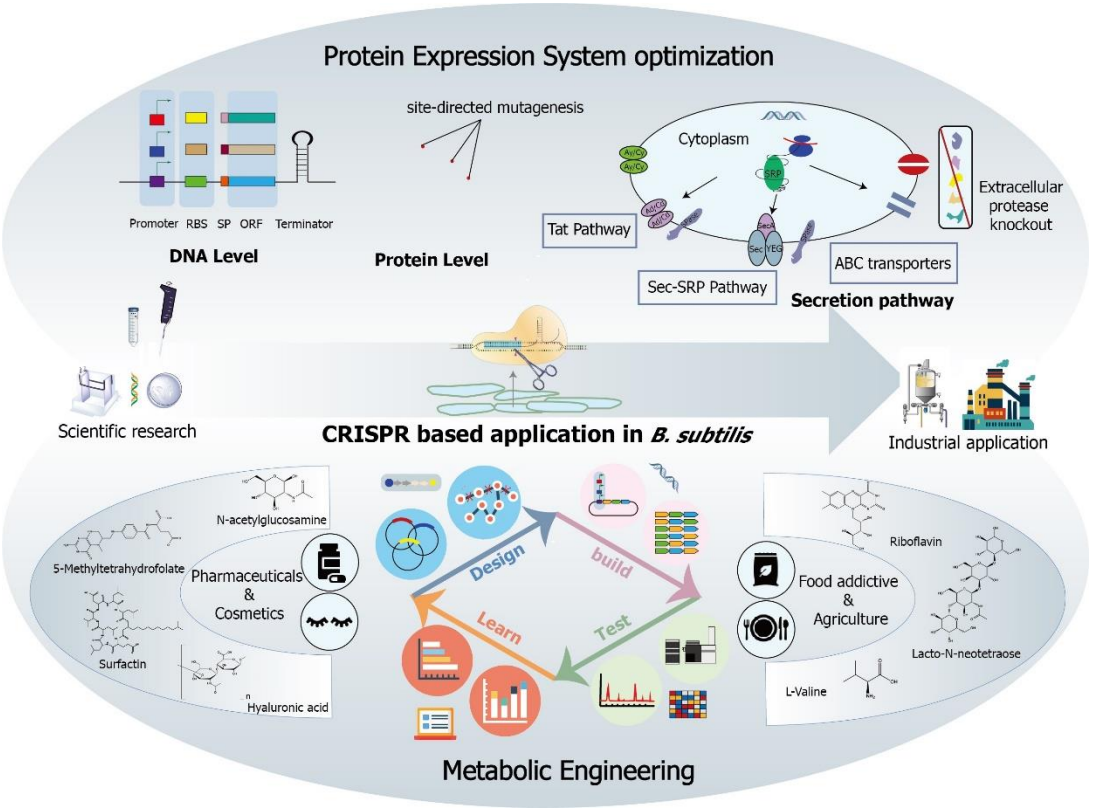

Figure 2. **Application of CRISPR-based tools in *B. subtilis.*** CRISPR-based engineering tools have been utilized to optimize the protein expression systems in *B. subtilis* to produce high levels of proteins and engineer the metabolic pathways to improve the production levels of different compounds that are widely used in food, cosmetics, agricultural and pharmaceutical industries.

Different type of CRISPR-Cas9 systems in *B. subtilis*

In *B. subtilis*, different types of CRISPR-Cas9 based systems have been established according to the deployment strategy of Cas9, sgRNA and editing template. A similar concept has been reviewed by Hong *et al.*[52] Firstly, Westbrook *et al.* designed the chromosome integrated system, in which Cas9 integrated into the genome of *B. subtilis* and was not evicted after the editing process. While sgRNA cassette accompanied with the *mazF* counter-selection marker inserted into *thrC* loci of *B. sutbilis* through the second integrative plasmid, which also carried the editing template. Later after performing the gene editing, the sgRNA cassettes along with the *mazF* counter-selectable marker were evicted by *thrC* homology recombination.[32] Secondly, So *et al*, applied two replicative plasmids, one carried Cas9, and the other carried sgRNA and editing template. After genetic modification, antibiotic-free conditions were used to curing the plasmids to prepare for next round of genome modification.[33] Thirdly, both the Cas9 and sgRNA were

maintained within one plasmid, and editing templates were either assembled in the plasmid or be provided as PCR fragments.[35, 38] Taken together, all the systems as they reported, could achieve high gene-editing efficiency after optimization in *B. subtilis*. With several options available, it is still critical to pay attention to specific experimental interests to choose the most suitable strategy. In addition, introducing λRed recombinase/ RecA into *E. coli* or RecT recombinase of prophage Rac into *C. glutamicum* could increase editing efficiency.[47, 53] Whether over-expressing inherit or exogenous recombinases in *B. subtilis* will improve the editing efficiency has not been investigated.

Knock-out and large fragment deletion

Gene disruption could be achieved either by deleting the entire fragment or introducing premature stop codons or open reading frame shifts. In *B. subtilis*, the general gene knockout efficacy has been demonstrated to be high (86%-100%), except in undomesticated strains with only 33% to 53%. (**Table 1**) But Zhang *et al.* were still able to sequentially disrupt six genes of *B. subtilis* ATCC 6051a and conferred bacteria advantageous properties in industrial fermentation.[34] For the scenario of large fragment deletion, the deletion of a 25.1 kb fragment containing the *amyE* gene reached an efficiency of 89%.[35] However, when came to the deletion of the 38 kb plipastatin-synthesizing pps operon, two sgRNAs targeting both the N- and C-terminus of the fragment are necessary to generate the entire fragment deletion. And this editing efficiency dramatically increased from 16.7% to 80% when transformants experienced prolonged incubation in selective media.[33] This indicated two sgRNAs combination and prolonged incubation as recommended strategies to improve deletion efficiency of large fragments of *B. subtilis*.

Point mutation and base editing

Point mutation assists to create precise site-specific modifications for improved enzyme properties, repair mutagenesis, as well to disrupt gene functions by introducing stop codons.[33] Up to now, most of the reported point mutation efficiency in *B. subtilis* could reach more than 68%, under the condition of high transformation efficiency, optimized length of homology arms and elevated PAM sensitivity. (**Table 1**) Although one nucleotide mutagenesis is accessible, in many cases, higher nucleotide inconsistency between genome target and editing template, particularly bases proximal to the PAM

sequence, are being appreciated. [33] In addition to double or multiple gene mutations, simultaneous editing is appreciated due to its less time-consuming compared to continuous genetic manipulations. This could be accomplished by the presence of multiple sgRNAs assembled within one knockout plasmid. But the currently reported simultaneous editing efficiency remains to be low (36%) which might be caused by the low editing efficiency of one target site.[32]

Currently, two main base editors include cytosine base editor (CBE) and adenine base editor (ABE), which accomplish precise single-base changes or substitutions (base editing) from cytosine to thymine (C to T) and adenine to guanine (A to G), catalyzed by cytosine deaminase and adenine deaminase, respectively.[54-56] For the first time, Yu *et al.*, achieved Cs to Ts conversion based on CRISPR-dCas9 (catalytically deactivated Cas9) mediated cytosine deaminase base editing in *B. subtilis*. The dCas9 and induced cytidine deaminase fusion protein has a 5 nt editing window, and enables up to 100% and 50% editing efficiencies for three and four loci simultaneous editing. With only two rounds of multiplex editing, they successfully inactivated eight extracellular proteases by introducing premature stop codons. It is assessed that 84.7% of *B. subtilis* genes are editable by using this gene inactivation method.[57] However, this technology is not feasible for gene deletions or insertions and ABE has not been explored yet in *B. subtilis*.

Knock-in

As important industrial microorganisms, the structural and segregation instability of plasmids still hamper the *Bacillis* strains from continually producing high levels of industrial enzymes.[58] In addition, with the development of synthetic biology and system biology, reconstruction and de novo construction of metabolic pathways in *B. subtilis* have also raised the challenges to develop methods for gene over-expression at genome level.[8] To deal with this dilemma, CRISPR-Cas9 based gene knock-in has made the scar-less gene integration accessible and efficient. Westbrook *et al.* and So *et al.* successfully introduced the hyaluronic acid biosynthetic operon (2.9 kb) and *gfp* into *B. subtilis* chromosome with the efficiency of 69% and 66.8%, respectively.[32, 33] Another study highlighted in this review presents the sequential integration of five copies of *ganA* encoding β-galactosidase into the *B. subtilis* chromosome, which over-expressed an additional copy of competence genes *comK* and *comS* to increase the transformation efficiency.[36] The resultant strain continuously produced approximately the same level of

GanA within at least 40 generations in antibiotic-free conditions. This study notably demonstrated the possibility that tandem copies of genes at the chromosome are relatively stable, whereas the upper limitation of gene copies still requires further exploration.

Gene regulation

The natural CRISPR-Cas system was originally harnessed for genome editing and was later optimized to accomplish gene regulation. HNH- and RuvC-like nuclease domains of Cas9 are all necessary for producing double strand break. Two key residues in the nuclease domain are involved in Cas9 endonuclease activity, and their mutation (D10A and H840A) could ultimately result in a catalytically deactivated Cas9 (dCas9).[59] But the dCas9 still remains the capacity to bind to sgRNA and targeting DNA strand. This allows for the development of CRISPRi and CRISPRa tools to modulate gene expression levels.[38, 60] (**Figure 1B**) The former is intensively repurposed to repress gene transcription by employing dCas9 to bind to the promoter region of the target gene, thus blocking the progress of RNA polymerase to the downstream gene and attenuating gene transcription. And no evident correlation between the repression efficiency and the targeting loci has been observed.[61] On the contrary, the latter is implemented by the combination of dCas9 and a transcriptional activation domain as a fusion protein to enhance endogenous gene transcription.[62] An alternative strategy relies on modified sgRNA by recruiting different types of RNA binding proteins fused to activation domains to the CRISPRa complex.[63] The high expression level of dCas9 was toxic to *E. coli* and led to reduced cell growth, but this toxicity could be released by controlling the expression level of dCas9.[64, 65] This toxicity has not been systematically explored and discussed in *B. subtilis*.

CRISPRa and CRISPRi

Less CRISPRa systems have been explored in *Bacillus species*.[43, 66] Increasing the transcription/expression level of target genes was realized by fusing the ω-subunit of RNA polymerase to C-terminus of dCas9, and then multiple sgRNAs were designed to guide dCas9-ω targeting several positions upstream of the transcriptional start sites of promoter-gene expression cassettes. But the discrepancy between increased protein expression level and transcription level might occur attributed probably to the post-transcription disparity.[66] In addition, the recruitment of dCas9-α subunit showed similar activation effects as dCas9-ω. By recruiting the sunCas9 system, which was the

fusion of dCas9 and multiple copies of activating effector domain, enables transcription levels amplified obviously in eukaryotic cells.[67] However, no synergistic effect of multiple activators mediated transcription increase was observed by fusion proteins of dCas9-ω-α and dCas9-α-ω in *B. subtilis*.[66] In the future, it's critical to enlarge the profile of effective gene activators.[63]

CRISPRi is capable of investigating the function of essential and unknown genes of *B. subtilis*, due to the reversible breakdown expression of specific genes.[68, 69] Also, it has been recruited to reducing gene expression and fine-tuning metabolic pathways to obtain more bioproducts that are extensively used in food, cosmetic and pharmaceuticals.[32, 70-73] (**Figure 2**) Normally, the branch pathway enzymes would be carefully selected to be reducing targets by individually employing a series of sgRNA with different repression efficiencies. Then, the most suitable sgRNAs were combined to simultaneously repressing several targets to achieve higher productions of target goods.[71, 72, 74] While in some cases, the repression scale and time of certain genes requires strict controls, especially those genes involved in cell growth. Therefore, the inducable promoter controlled expression of dCas9 should be screened and optimized to control the repression time and strength to guarantee the balance between production levels of the interesting products and the biomass.[73]

In summary, compared with conventional gene regulating skills, CRISPRi displays multiple advantages.[24] Firstly, this technique makes gene repression easy by simply inserting a 20-nt sgRNA into cells. In addition, it facilitates deciphering the functions of essential genes or critical genes by using this inducible and partial gene repression strategy.[68] Moreover, CRISPRi can simultaneously suppress the expression of several genes within the same cell to different levels by utilizing multiple sgRNA, and this is extremely important to regulate biosynthesis pathways within complicated cellular regulatory networks. Still, there are some disadvantages that shouldn't be neglect. First, expression of the downstream gene within one operon might also be affected. Second, the inhibiting effects might be slightly fluctuant due to the different expression levels of dCas9 affected by bacteria growth stages. These issues should be considered when using this technology.

Plasmids curing strategy

The design–build–test–learn cycle of metabolic engineering in bacteria is well accepted paradigm to improve the production of valuable compounds.[75] This requires continuous rounds of genome editing in parental strains. To facilitate iterative genome editing, several main strategies have been employed to cure the modification plasmids after genome editing (**Figure 3**). There are at least four strategies having been used to evict unwanted plasmids in *B. subtilis*. First, the bacteria were incubated under antibiotic-free conditions and the curing efficiency largely depends on plasmid stability. (**Table 1**) Second, for the plasmids with temperature-sensitive replicon, performing the bacteria incubation at 50^{o}C could normally lead to 90% of bacteria losing plasmid.[35] Third, the *mazF* counter-selection marker was also applied to evict sgRNA cassettes inserted into the chromosome introduced by integrative plasmids, but the efficiency remained low, ranging from 6% to 31%.[32] Fourth, except for the sgRNA targeting the genome loci, some modification plasmids also carry another sgRNA scaffold that could target the self plasmid fragments under inducible-promoter control. Followed by genome editing, the plasmids would be self-targeted and expelled.[76] Collectively, most of these tactics are competent to remove plasmids, and the less time-consuming and high efficient strategies are more prevalent.

Cas9 nickase

Two categories of type II-CRISPR/Cas9 system have been reported: Cas9-mediated and Cas9 nickase (Cas9n)–mediated (**Figure 4**).[77, 78] Inactivation of the RuvC domain of Cas9 by creating D10A mutation leads to Cas9n which only cleaves the target strand. Conversely, inactivation the HNH domain by H840A mutation creates a non-target strand-cleaving nickase. The Cas9n generated single-strand break will trigger HDR which is easier to be repaired than DSB thus causing less toxicity for the bacteria.[79] Cas9nD10A has been used to fine-tune three RBS regions of riboflavin operon for higher riboflavin production in *B. subtilis*, considering its better performance in genome cleavage than Cas9nH840A.[37] Except for achieving extremely high editing rates in large fragment deletion, gene insertion and single point mutation in *B. subtilis*, this system also achieved the highest efficiency (65%) for simultaneous triple-mutation by inhibiting the nicks re-ligation process. The *ligD* gene, whose corresponding protein was supposed to repair strand break by a two-component Ku-ligase break repair complex, was knocked out.[80] The disruption of *ligD* facilitated the repair of strand break mediated by HDR, thus leading to

the efficiency for simultaneous modulation of three sites increased from 49 to 65%.

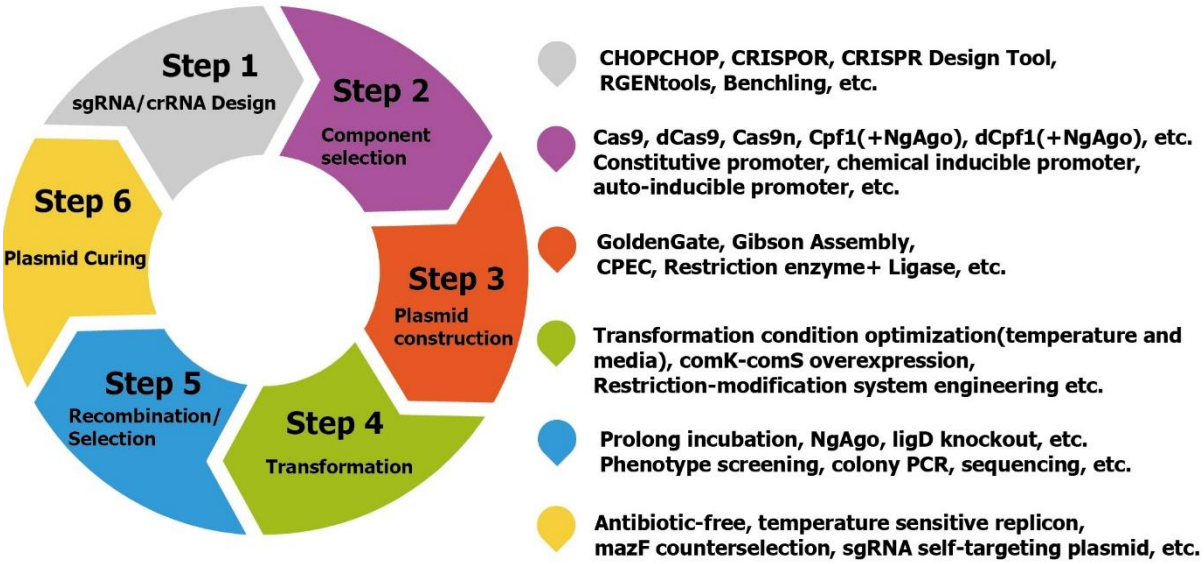

Figure 3. Workflow of genetic modification by CRISPR/Cas system and related strategies in each step.

CRISPR/Cpf1 system

In class II CRISPR-Cas system, type V CRISPR-Cpf1 (also known as Cas12a) could also mediate robust DNA interference by introducing DNA DSBs with 5- or 8-nucleotides staggered 5' overhang respect to crRNA length (**Figure 4**).[81] Unlike SpCas9 recognizes G-rich PAMs (5'-NGG-3'), Cpf1, a single RNA-guided endonuclease, recognizes T-rich PAMs. FnCpf1 originated from *Francisella novicida U112* recognizes 5'-TTN-3'; AsCpf1 from *Acidaminococcus sp. BV3L6* and LbCpf1 from *Lachnospiraceae bacterium* prefer the 5'-TTTV3' PAM with V can be A, G or C.[82, 83] Compared with SpCas9, Cpf1 not only shows higher sensitivity to mismatches between guide sequence and target genome, but also the Cpf1:crRNA complex alone without tracrRNA is sufficient to execute DNA cleavage.[82] Also, Cpf1 introduces the DSB at the distal end of the protospacer, thus preserving the target site for subsequent generation of cleavage. These features confer CRISPR-Cpf1 tool an ideal compensation for the CRISPR-Cas9 system which shows preference in GC-rich fragment editing.

Recently, the feasibility of this CRISPR/FnCpf1 assisted multiple-genes editing and regulation system (CAMERS-B) has been assessed and it significantly increased the production of N-acetylglucosamine and acetoin in *B. subtilis*.[84] Remarkably, maximum 100% efficiency has been achieved in either gene insertion, multiple (up to six) point

mutagenesis, or two-gene simultaneous deletion after conducting the prolonged incubation (post-culture). Also, it is worth stating that the modified Argonaute (NgAgo) originated from *Natronobacterium gregoryi* was concurrently employed to facilitate Cpf1-mediated gene editing.[85] Since the mutated NgAgo (retaining 650–887 residues, D663A, D738A) could strengthen the RecA mediated HDR but unable to cleave DNA or RNA. Moreover, the CRISPR-Cpf1 could also fulfill multiple genes repression and transcriptional dual-control (activation and repression at the same time) using the DNase deactivated Cpf1 (D917A, dCpf1). When the transcription activator RemA was fused to the C-terminus of dCpf1, gene repression ability of dCpf1-RemA still preserved. Because the dCpf1-RemA was able to repress and activate the downstream gene in a position-dependent manner.[84] In addition, Cpf1-mediated large fragments deletion was more efficient than Cas9-mediated editing.[86]

Considering CRISPR/Cpf1 utilizes T-rich PAMs, its editing targets in low GC content (43%) *B. subtilis* genome are supposed to be more than SpCas9.[87] Further developing facile yet robust CRISPR/Cpf1-mediated genome editing toolbox is still urgently demanded. After all, the editing rate of simultaneously deleting two genes without prolonged incubation by CRISPR/Cpf1system only reached 43.1%, and completely knock out three genes simultaneously remains unattainable. To expand the versatility and feasibility of CRISPR/Cpf1 system, eight Cpf1 orthologs members which could induce efficient targeted DNA cleavages with identified PAM sequences could be promising candidates, particularly those possess conformational flexibility in recognizing both canonical (TTTV) and non-canonical (CTTV, TCTV, TTCV) PAMs, e.g. LbCpf1.[81] Meanwhile, exploring novel Cpf1 variants can also broaden the utilization of this genetic tool by scaling up the targeting range of Cpf1and remaining high DNA-targeting accuracy.

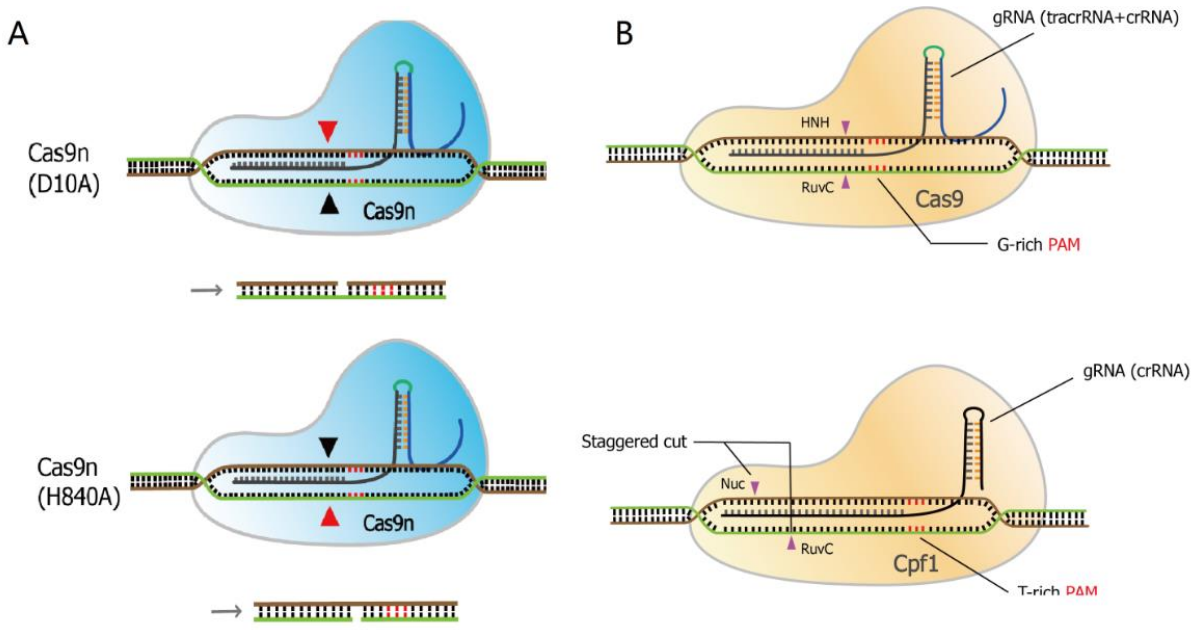

Figure 4. Cas9 nickases and comparison of Cas9 and Cpf1.

Genome engineering in other *Bacillus* species

Except for *B. subtilis*, many other *Bacillus* species have significant applications in the fields of medical, pharmaceutical, food, agricultural and other industrials. However, because of their low transformation efficiency, genetic manipulation remains challenging, and only a small repertoire of genetic tools are available.[11] To take their full advantages in producing enzymes, nucleotides, amino acids, vitamins, poly-γ-glutamate and biosurfactant, simple and efficient genetic tools are urgently needed for further metabolic engineering of their promising strains. Application of CRISPR-based system on these strains will lead to more breakthroughs in genome editing and regulation in these *Bacillus* microbial workhorses.

Genome engineering in *B. licheniformis*

In *B. licheniformis*, the Cas9-mediated, dCas9-mediated and Cas9n-mediated genetic tools have all been developed.[39-41, 88] In this low transformation rate bacterium, the vector size and expression of carrying genes are two main factors that require consideration. Hence the genome integrated Cas9n was employed for the sake of decreasing the gene-editing-plasmid capacity.[39] Since expression of chromosome Cas9n did not remarkably influence the growth of bacteria due to the neglectable lethality effects, it

could be reserved in the genome modified strains. However, the toxicity of heterologous Cas9 endonuclease resulted significantly decreased number of transformants, thus the editing plasmids had to be cured or the expression of Cas9 should be strictly controlled by inducible promoters.[40, 88] Generally, the single gene deletion, large fragment (~42.7kb) deletion and gene integration efficiency could reach as high as 100%, 79%, and 76.5%, respectively.[39] But simultaneously disrupting multiple genes remains to be inefficient (double gene deletion was only 11%), which was probably due to the competition of different sgRNAs in bacteria or the low editing efficiency among one of the target genes. However, the multiple gene repression by dCas9 has been reported to be efficient.[88] And low temperatures enables enhanced genome editing efficiency.[41] What's more, the single gene deletion efficiency can be as high as 51.4% with a very small homologous-arm size (0.1kb homology flanks).[39] Taken together, critical attention is needed to choose suitable strategies when more than one approach is available, regarding experimental interests.

Genome engineering in *B. methanolicus*

The thermophilic *B. methanolicus* has the capacity to utilize methanol and other carbon source for growth to produce amino acids, which gives it priority to be a promising candidate for biotechnological applications.[89] Unfortunately, genetic tools for genomic gene alteration are unavailable. To solve this problem, Schultenkämper *et al.* established a CRISPRi-dCas9 based system to repress gene transcription.[90] The promoter of mannitol activator gene mtlR from *B. methanolicus* was employed to regulate transcription of both dCas9 and sgRNA. But LacI operator fragment (lacO) from *E. coli* was placed upstream of dCas9 to repress mtlR promoter in *E. coli*, thus avoiding the toxicity triggered by dCas9 to *E. coli* during the plasmid construction process. Here, three putative genes related to spore formation (*spo0A*), mannitol metabolism (*mtlD*, 50%), and hydrogen peroxide conversion (*katA*, 20%) were successfully repressed, which confirmed their functions at experiment level. In contrast to spCas9 which is not active at 42^{o}C or above in *B. smithii*, dCas9 function was not affected at 50^{o}C in *B. methanolicus*.[90]

Genome engineering in *B. anthracis* and *B. cereus*

Both the anthrax causative pathogen *B. anthracis* and foodborne pathogen *B. cereus* belong to *B., cereus sensu lato* group, a group of spore-forming bacteria. Previously developed genome modification methods in these pathogenic microbes mainly focus on

gene deletion based on homologous recombination.[91] Recently, Wang *et al.* initially managed the introduction of precise point mutation in *B. cereus* and two large fragments deletion in *Bacillus anthracis*.[44]

Generally, the plasmids they used to implement gene modifications were constructed by Altenbuchner *et al.* for *B. subtilis*, but the execution process details were adapted to these two *B. cereus sensu lato* species. Specifically, the antibiotic selection was first applied after transformation to guarantee the plasmids being absorbed and resultant *B. anthracis*/ *B. cereus* transformants were followed to propagate in liquid medium. Then inducer was supplied upon enough plasmids being accumulated, to allow for Cas9 protein expression and subsequent homology recombination. Whereas, antibiotic selection and Cas9-mediated genome editing were combined within one manipulation step. Finally, site-specific mutagenesis of plcR gene in *B. cereus* acquired merely less than10%, and this might be attributed to the likely indispensable role of PlcR in bacterial survival as a fundamental regulatory protein. Moreover, the deletion rates for the shortest (17 kb) and longest (51 kb) prophages in the *B. anthracis* displayed a distinct 100% and 20%, respectively. Though this indicated relatively less effectiveness on very large fragment (i.e. ~50kb) deletion. Compared with >90% efficiency of 25kb fragment deletion in *B. subtilis*, however, no significant differences in editing efficiency were observed between *B. anthracis and B. subtilis.* Overall, the successful deletion of prophages in *B. anthracis* will ultimately benefit the generation of marker-free live anthrax vaccines and other fundamental gene studies.

Genome engineering in *B. smithii*

The facultatively thermophilic bacterium *B. smithii*, is able to utilize wide range of carbon sources for lactate production and other green building block chemicals, and grows between 37ºC and 65ºC.[92] Recently, Mougiakos *et al.* for the first time established a CRISPR-Cas9 based temperature-controlled recombination/counterselection tool to modify genes of *B. smithii* ET 138.[45] This approach successfully achieved 90%, 100%, and 20% efficiencies for gene deletion, knockout, and insertion, respectively. More importantly, inducible or well-characterized promoter was not indispensable in this system as target activity of spCas9 could be precisely regulated by temperatures. According to their *in vitro* and *in vivo* tests, spCas9 was not active enough to introduce lethal DSBs at 42°C and above.[93] Therefore, culturing at 37°C is necessary to allow for sufficient Cas9

targeting. Then the temperature should be gradually increased to 55°C to guarantee bacteria normal metabolism and cellular homologous recombination by plasmid-borne editing templates. For the subsequent plasmid eviction, further increasing to 65ºC was required. Nevertheless, this is the first CRISPR-Cas9 system being employed in a thermophile and is also potentially applicable for other non-model microorganisms that grow at or above 42°C and even with limited genetic toolbox.

Genome engineering in *B. thuringiensis*

B. thuringiensis has been well-known as a biological pesticide being specifically toxic against lepidopteran, dipteran, coleopteran, and hemipteran insects.[94] To further improve its ability to produce stable and functional crystal and vegetative insecticidal proteins at large-scale, precise gene engineering is earnestly required. CRISPR-Cas9 strategy has been successfully developed to delete genes in this bacterium, in which double crossover recombination was rarely happening.[46, 95] However, the obtained transformant numbers remain very low with the editing efficiency still acceptable (12.5%-62.5%). Low copy number plasmids were demanded to reduce the toxicity of Cas9. Whereas, high copy number plasmids were necessary to modify plasmid genes with longer homology arms (around 1kb) than genome fragments editing (around 0.5kb).

Future perspective

Despite some progress, the research on the CRISPR-based tools in *Bacillus* species is at an early stage and far from the optimal state, it is urgent to pursue higher editing efficiency.[27] **Figure 3** summarizes the workflow and key factors and approaches to effectively use current CRISPR genetic tools. Also, the advanced CRISPR tools established in eukaryotes and other bacteria species provide guidelines for further directions of improving editing specificity or efficiency of genetic tools in prokaryotes. For example, engineering of existing Cas9 protein, developing new systems and modification of sgRNA[96-99] Remember, CRISPR loci have been identified in around 50% of bacteria and around 87% of archaea.[20] These systems all have the chance to be transformed into novel and facile genome editing platforms. Moreover, introducing heterologous NHEJ system to *Bacillus* species to form CRISPR assisted NHEJ strategy could also be explored and achieve the goal of gene function disruptions and even evolutionary engineering.[100, 101] Adapting diversity of CRISPR-based systems to the industrially and medically important *Bacillus*

species will be highly expected.

As dominant bacterial workhorses, *Bacillus* species play important roles in producing enzymes and chemicals in industrial fermentations and fundamental scientific researches for cell division and sporulation process studies. [1, 9, 59, 102, 103] The robust restriction-modification systems of some species result extremely low transformation efficiency thus showing resistance to traditional molecular biology tools.[12, 43] The emerged CRISPR-based tools significantly improve this situation, and enable high-throughput gene editing and multiple-site genome editing accessible.[68] Due to the relatively small genome of prokaryotes, the off-target efficiency of CRISPR-Cas in *Bacillus* species has been demonstrated to be low.[57] But this issue remains poorly investigated. In the future, re-sequencing the entire genome after gene modification seems necessary to screen undesired mutations which might potentially influence the bacteria's performance in industrial production and the underlying mechanisms. Utilization of previously constructed tools among different *Bacillus* species would be an option to explore the feasibility of genome editing at the beginning.[104] With the discovery of novel CRISPR-Cas mechanisms and optimization of current Cas9 proteins to expand editing realms, the *Bacillus* species will undoubtedly benefit from these powerful CRISPR biotechnologies.

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