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REVIEW ARTICLE

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

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Abstract

Recently, the clustered regularly interspaced short palindromic repeats (CRISPR) system has been developed into a precise and efficient genome editing tool. Since its discovery as an adaptive immune system in prokaryotes, it has been applied in many different research fields including biotechnology and medical sciences. The high demand for rapid, highly efficient and versatile genetic tools to thrive in bacteria-based cell factories accelerates this process. This review mainly focuses on significant advancements of the CRISPR system in *Bacillus subtilis*, including the achievements in gene editing, and on problems still remaining. Next, we comprehensively summarize this genetic tool's up-to-date development and utilization in other *Bacillus* species, including *B. licheniformis*, *B. methanolicus*, *B. anthracis*, *B. cereus*, *B. smithii* and *B. thuringiensis*. Furthermore, we describe the current application of CRISPR tools in phages to increase *Bacillus* hosts' resistance to virulent phages and phage genetic modification. Finally, we suggest potential strategies to further improve this advanced technique and provide insights into future directions of CRISPR technologies for rendering *Bacillus* species cell factories more effective and more powerful.

KEYWORDS

Bacillus, Cas9, clustered regularly interspaced short palindromic repeats, Cpf1, genetic tools, phages

INTRODUCTION

Bacillus subtilis has long been used in multiple fields, including food, cosmetic, pharmaceutical and agricultural industries (Liu et al., 2013; Song et al., 2015; Westers et al., 2004; Zweers et al., 2008). The extensive applications can be attributed to its (i) generally recognized safe (GRAS) status, (ii) strong protein secretion capacity, (iii) excellent growth characteristics on low-cost carbon

sources and (iv) robustness in large-scale fermentation (Cui, Han, et al., 2018; Su et al., 2020; Ÿztürk et al., 2016). The availability of complete genome sequences and tools for genetic modifications have facilitated the development of *B. subtilis* cell factories to produce biological substances at a commercial scale, such as valuable enzymes, vitamins, antibiotics as well as platform chemicals (Liu, Liu, et al., 2019). Recently, high-throughput multi-omics technologies have provided a high number of novel potential

Yafeng Song and Siqi He contributed equally to this work.

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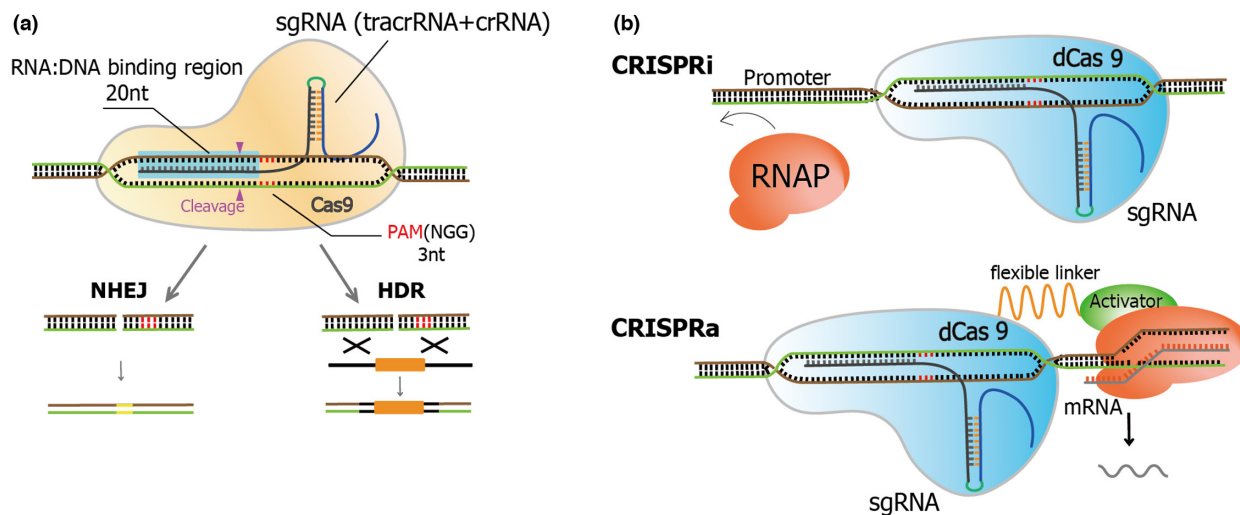


FIGURE 1 The CRISPR-Cas system and CRISPR-based gene regulations. (a) The CRISPR-Cas genome editing technology. PAM, protospacer adjacent motif; NHEJ, non-homologous end joining; HDR, homology-directed repair. (b) CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) systems.

genetic targets for further improvement (Bate et al., 2014; Hess et al., 2013). Hence, to fulfil the increasing demands for gene editing, a series of genetic modification tools have been established (Dong & Zhang, 2014). However, these tools still possess many drawbacks and limitations. For example, limited availability of selection markers, low-genome editing efficiency, genome scars (remnant sequences) being left after modification and laborious selection procedures need to be addressed (Westers, 2003).

In addition, wild-type *B. subtilis* strains and some other industrially relevant *Bacillus* species suffer from a low transformation efficiency caused by restriction-modification systems that further hamper their utilization and scientific research (Zhang et al., 2012). Therefore, multiplexable genetic editing tools with high editing efficiency are urgently needed. Following its discovery, the application of clustered regularly interspaced short palindromic repeat (CRISPR) system has revolutionized the field of genome engineering.

In 1987, Ishino et al. first discovered repetitive DNA sequences in the genome of *Escherichia coli* when they studied phosphate metabolism. Subsequently, the repeats and related sequences were termed clustered regularly interspaced short palindromic repeats (CRISPRs) (Jansen et al., 2002). The intervening 'spacer' displayed high identities with foreign plasmids and bacteriophage sequences, which highly attracted researchers' attention. After that, scientists started to explore and elucidate the native biological functions of CRISPR as the adaptive immune system of bacteria and archaea (Barrangou et al., 2007; Brouns et al., 2008; Deltcheva et al., 2011; Garneau et al., 2010; Marraffini & Sontheimer, 2008). Briefly, in the first acquisition stage, a specific spacer sequence originating from

invading DNA is recognized and incorporated into the CRISPR locus. The spacer-corresponding sequence on a virus' genome is called 'protospacer', and the short conserved nucleotide fragment proximal to the protospacer is called the protospacer adjacent motif (PAM). The CRISPR array, including direct repeats and spacers, is transcribed into the second biogenesis/expression stage. Cas proteins facilitate the maturation 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 complementary to the spacer of crRNA, thus preventing foreign genetic elements invading.

Studies over several decades show that CRISPR-Cas systems widely exist in bacteria (50%) and archaea (75%). These diverse systems can be classified into two classes, according to the configuration of the effector module relying either on a multi-protein complex or on a single-component protein. Five types and 16 subtypes can be discriminated (Koonin et al., 2017; Makarova et al., 2015). Due to its simplicity, the type II system from *Streptococcus pyogenes* is the most intensively studied, developed and applied system in genome editing. The signature Cas9 protein, a dual RNA-guided DNA endonuclease enzyme, can introduce a double-strand break (DSB) three base pairs (bp) upstream of PAM (5'-NGG-3') (Figure 1a). In 2012, Cas9 protein, assisted by the dual-tracrRNA:crRNA, was demonstrated to bind and cleave DNA in vitro (Jinek et al., 2012). Since then, the CRISPR-Cas9 system has been widely explored and adapted as a powerful genetic tool to modify genomes of various microbes, including yeasts, lactic acid bacteria, *E. coli*, *Corynebacterium* and *Streptomyces* species. Many

researchers have consistently described the advances and applications of CRISPR-based tools for genome engineering (Choi & Lee, 2016; Jakočiunas et al., 2016; Liu et al., 2020; Luo et al., 2016; Peters et al., 2015; Yao et al., 2018). As summarized by Zocca et al., by 2016, the number of CRISPR-Cas9 systems described in *B. subtilis* started to increase. Multiple systems using different plasmids and their editing efficiencies on gene knockout, knockin as well as large fragment deletion were outlined. These authors also thoroughly discussed the engineering of *B. subtilis* to produce industrially relevant compounds using CRISPR tools. The advancements in other *Bacillus* species and bacteriophages were only briefly mentioned (Zocca et al., 2021). This review emphasizes the effects of each CRISPR component on genome editing efficiency and discusses the plasmid-curing strategies aiming for iterative engineering. This gives clear and overall insights into choosing particular elements when researchers establish systems for gene deletion, knockin, mutagenesis and multiplexing editings in *B. subtilis*. In addition, successful 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 systematically and comprehensively described. Furthermore, we discuss how CRISPR systems expanded phage research to facilitate industrial fermentation and phage therapy by elucidating phage genomes and functions.

EFFECTS OF CRISPR COMPONENTS ON GENOME ENGINEERING EFFICIENCY IN *B. SUBTILIS*

Unlike previous genetic technologies, such as 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 (Carroll, 2017; Cho et al., 2018; Maeder & Gersbach, 2016). 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*, *B. smithii* and *B. thuringiensis* (Table 1). Here, we first discuss how the components of the CRISPR-Cas9 system affect the gene editing efficiency, including Cas9, sgRNA and the editing template/donor DNA in *B. subtilis*. Regarding gene regulation, we explain the principle of CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) systems in *B. subtilis* and how these robust systems have been utilized to improve the production or titers of proteins, vitamins, functional sugars or other biochemicals as well as the advantages and disadvantages of these systems (Figure 2).

Cas9 expression and sgRNA transcription

In bacteria, the fine-tuning of the expression level of *S. pyogenes* Cas9 (SpCas9) remains to be critical for CRISPR-Cas9 system application considering its potential toxicity to host strains, as reported in *Clostridium cellulolyticum* and *C. beijerinckii* (Liu et al., 2017; Wang et al., 2016; Xu et al., 2015). Therefore, it is essential to investigate whether the SpCas9 protein should be codon-optimized, which promoters and corresponding concentrations of the inducers should be applied, and which type of vectors should be employed to regulate its expression levels. In *B. subtilis*, the native SpCas9 without codon optimization was engaged in most CRISPR-based genetic tools. The successful employment of native SpCas9 promoter and basal expression level controlled by the inducible promoter P_{grac} implied that the low expression level of SpCas9 protein in the absence of targeting guide RNA was not toxic to *B. subtilis* (Price et al., 2019; Westbrook et al., 2016). The IPTG inducible promoter P_{grac} comprises the *groE* promoter and the *gsiB* Shine-Dalgarno sequence from *B. subtilis*, together with the regulatory element *lacO* operator. The basal expression controlled by P_{grac} without adding IPTG was sufficient for Cas9 expression (Phan et al., 2006; Price et al., 2019).

In vitro work, which discovered that programmable DNA targeting and the following 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 (Jinek et al., 2012). This composition significantly simplified the genetic manipulation process and was rapidly translated into applications for eukaryotic and prokaryotic genome editing, including *Bacillus* species. However, editing rates can vary dramatically (23%–91% for *amyE* in *B. subtilis*) among different PAM sites within the same target gene. Therefore, a number of software tools were developed to assist the design of CRISPR guide sequences in more rational ways, for instance, E-CRISP, CHOPCHOP, ZiFiT, CRISPR Design Tool and sgRNA design tools. Among these tools, multiple scoring systems were applied for predicting the efficiency of sgRNAs combined with high-throughput experimental results and off-target profiles. In-depth analyses and discussions have been presented in other reviews (Ceasar et al., 2016; Wiles et al., 2015).

Editing template/donor DNA

Double-strand breaks are one of the most precarious DNA lesions that can cause genomic instability and cell death. Non-homologous end joining (NHEJ) and endogenous homology-directed repair (HDR) (Figure 1a)

TABLE 1 CRISPR-Cas systems for *Bacillus* species genome editing

Species	Type	Cas protein and promoter	RNA	Promoter of guide	Editing template: type, length and description	Editing efficiency (%)	Cas protein curing	References
<i>Bacillus subtilis</i> 1A751	Knockout	SpCas9 and P _{cas} from <i>Streptococcus pyogenes</i>	Promoter derived from <i>S. pyogenes</i> CRISPR locus		Linear dsDNA; PCR product (1.3 kb; optimal: 1 kb)	82	MazF counter selection	Westbrook et al. (2016)
<i>B. subtilis</i> 1A751	Knockin	SpCas9 and P _{cas} from <i>S. pyogenes</i>	P _{xyIA_SphI+1}		Insert linearized vector with a hyaluronic acid biosynthetic operon (1.3 kb)	69	MazF counter selection	Westbrook et al. (2016)
	Multiplexing				Simultaneous editing double loci: PCR product or plasmid (1.0–1.4 kb)	36–85		
	Mutation				Linear dsDNA; PCR product or linearized plasmid (0.5–1.3 kb; optimal: 1 kb)	23–100		
<i>B. subtilis</i> 168	Knockout	SpCas9 and P _{grc}	P _{ara}		pB0A-derived plasmid (0.4–2 kb)	91–100	Antibiotic-free conditions	So et al. (2017)
	Knockin				38 kb plipastatin-synthesizing operon deletion	16.7–80		
	Mutation				pB0A-derived plasmid (0.5 kb)	66.8–96.8		
					pB0A-derived plasmid (0.5 kb)	33–68.5		
<i>B. subtilis</i> 6051A	Knockout	SpCas9 and P _{amyQ}	Constitutive		Vector: pHYcas9dsrf derived (0.45–0.55 kb)	33–55	Thermo-sensitive replication origin	Zhang et al. (2016)
	Knockin	SpCas9 and mannose-inducible P _{manP}	promoter P ₄₃	Semisynthetic	Vector: pIOE8999 derived (0.7–0.8 kb)	-	Thermo-sensitive replication origin	Watzlawick and Altenbuchner (2019)
	Knockout	SpCas9n (D10A) and P _{xyIA}	Constitutive		pDonor derived plasmid (0.5 kb)	80–90	Thermo-sensitive replication origin	Liu, Huang, et al. (2019)
	Mutation		Promoter P ₄₃		1–8 kb deletion	80–90	gRNA targeting the plasmid replication origin gene <i>rep60</i>	
	Knockin				Single mutation	100		
					Simultaneous double mutation	90–91		
					Simultaneous three-point mutation	49–65		
					1–2 kb gene insertion	92–98		

(Continues)

TABLE 1 (Continued)

Species	Type	Cas protein and promoter of guide RNA	Editing template: type, length and description	Editing efficiency (%)	Cas protein curing	References
<i>B. subtilis</i> 168	Knockout depression	SpCas9: P _{grac} P _{veg}	Linear dsDNA: PCR product	76 (63.9–89.2)	Pressure from IPTG-induced high Cas9 expression and antibiotic-free conditions	Price et al. (2019)
<i>B. licheniformis</i> DW2	Knockout	SpCas9n (D10A) and P ₄₃	pGRNA-05 derived plasmid (1 kb)		Antibiotic-free conditions	Li, Cai, et al. (2018)
	Knock in		42.7 kb fragment deletion	79		
	Mutation		Gene insertion	76.5		
			Single mutation	100		
<i>B. licheniformis</i> 2709		SpCas9 and pS	Simultaneous double mutation	11.6		
	Knockout	pLY-2	pWH-cas9-sgRNA-derived plasmid (0.5 kb)			
			Single-gene deletion	97.3	Antibiotic-free conditions	Zhou et al. (2019)
<i>B. licheniformis</i> DSM13	Mutation		Point mutation	99.2		
	Knockout	SpCas9 and Pxy1	pN-sgRNA1-xy1ACas9	47.1–70.9	Thermo-sensitive replication origin and antibiotic-free conditions	Li et al. (2020)
<i>B. amyloliquefaciens</i> NBCSO	Depression	dCas9	Single-gene deletion			
			Low temperature condition (20°C)	97		
<i>B. amyloliquefaciens</i> 205	Knockout	SpCas9	pNX-dCas9, pHY-sgRNA-egfp		Not mentioned	Sha et al. (2020)
	Depression activation	dCas9	Gene depression	-		
			pHT43-cas9			
			pHT43-dcas9-gRNA			Zhao et al. (2020)
		Pgrac	pHT43- ω -dcas9-GN			

TABLE 1 (Continued)

Species	Type	Cas protein and promoter	Promoter of guide RNA	Editing template: type, length and description	Editing efficiency (%)	Cas protein curing	References
<i>B. anthracis</i> A16R	Knockout	SpCas9 and mannose-inducible P_{manP}	Semisynthetic	Vector: pJOE8999-derived plasmid	-	Thermo-sensitive replication origin	Wang et al. (2019)
			Constitutive	lambdaBa03, ~16.8 kb fragment deletion (1.6 kb+0.6 kb)	20		
			Promote P_{vanABK}	lambdaBa01, ~50.5 kb fragment deletion (1.6 kb+1.2 kb)	100		
<i>B. cereus</i> HN001	Mutation	SpCas9 and Mannose-inducible P_{manP}	Semisynthetic	Vector: pJOE8999-derived plasmid: point mutation	-	Thermo-sensitive replication origin	Wang et al. (2019)
			Constitutive				
			Promote P_{vanABK}				
<i>B. smithii</i> ET138	Deletion	SpCas9 and P_{xyrA}	P_{pta}	pWUR_Cas9nt-derived plasmid (1 kb)	90	High temperature and antibiotic-free conditions	Mougiakos, Bosma, et al. (2017)
	Knockout				100		
	Knockin				20		
<i>B. thuringiensis</i> BMB171	Knockout	SpCas9 and mannose-inducible P_{manP}	Semisynthetic	pJOE- Δ HmgA	-	Thermo-sensitive replication	Tan et al. (2019)
			Constitutive promote P_{vanABK}				

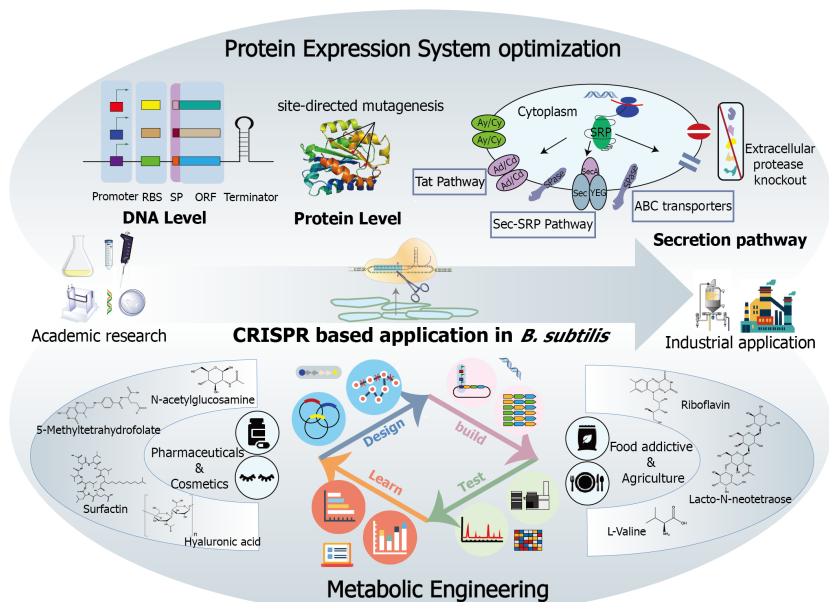


FIGURE 2 Application of CRISPR-based tools for genetic engineering in *Bacillus subtilis*. CRISPR-based engineering tools have been utilized to optimize the protein expression systems in *B. subtilis* to produce high levels of proteins and engineer the metabolic pathways to improve the production levels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

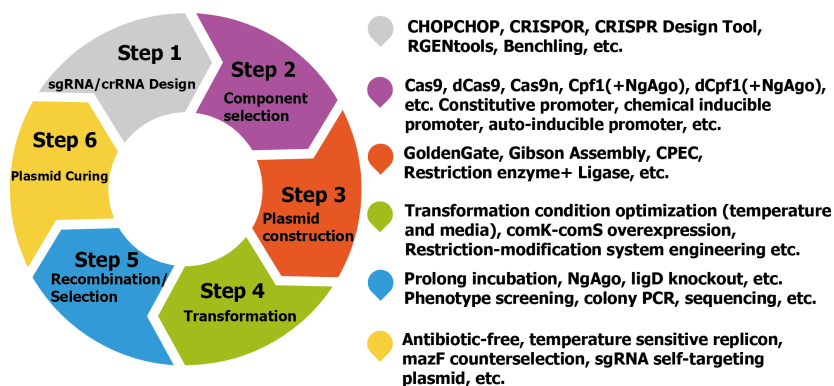


FIGURE 3 Workflow of genetic modification by CRISPR-Cas systems and related strategies in each step.

are two natural DNA repair pathways that have evolved in organisms to maintain genome integrity (Pawelczak et al., 2018). The former is an error-prone repair system, resulting in random inserts or deletions at the repaired sites. In contrast, the HDR system harnesses editing template/donor DNA (homologous templates flanking chromosomal DSBs) to guarantee high-fidelity repair of the damaged loci. NHEJ works inefficiently in prokaryotes and is absent in many bacteria (Bowater & Doherty, 2006; Price et al., 2019). Considering the precise repair of the DSBs by HDR and insufficient capacity of NHEJ in *B. subtilis*, HDR has become the predominant approach to achieve genome editing goals by the CRISPR-Cas9 system.

Generally, the efficiency of editing by a template/donor DNA is affected by the lengths of the homologous arms and the type of donor DNA. This donor DNA can be PCR products or plasmid-borne editing templates, whereby the latter is more frequently used. Price et al. (2019) reported an appreciable 76% of site-specific mutation efficiency when donor DNA was supplied as PCR fragment and co-transformed with plasmids containing SpCas9 and sgRNA into *B. subtilis*. Still, the transformation efficiency was very

low, producing fewer than 20 colony forming unit (CFU)/ μg (Price et al., 2019). Westbrook et al. (2016) consistently observed above 90% of *amyE* mutation efficiencies by applying two types of editing templates individually. The transformation efficiency of the plasmid-borne editing template (1.55×10^3 CFU/ μg) was nearly 6-fold higher than PCR editing templates. The low transformation efficiency of PCR fragments hampered its further application. With respect to the lengths of editing templates, researchers recognized that 400–500bp homologous sequences upstream and downstream were sufficient to reach acceptable editing efficiency in *B. subtilis*, whereas no transformants were observed with an arm length of only 300bp. In fact, a 1000bp homology arm was determined to be the optimal size of donor DNA under the test conditions (Westbrook et al., 2016).

Types of CRISPR-Cas9 systems in *B. subtilis* and plasmids curing strategy

In *B. subtilis*, different types of CRISPR-Cas9-based systems have been established according to the deployment

strategies of Cas9, sgRNA and editing templates. Hong et al. (2018) briefly reviewed a similar concept, and the employed plasmids and details were updated by Zocca et al. (2021). With several options available, it is still critical to pay attention to specific experimental goals to choose the most suitable strategy. In addition, by introducing λ Red recombinase/RecA into *E. coli* or RecT recombinase of prophage Rac into *Corynebacterium glutamicum*, editing efficiency could be increased (Liu et al., 2017; Zhao et al., 2016). However, whether inherited over-expressing or exogenous recombinases in *B. subtilis* will improve the editing efficiency has not been investigated.

The design-build-test-learn cycle of metabolic engineering in bacteria is a well-accepted paradigm for improving the production of valuable compounds (Petzold et al., 2015). This requires continuous rounds of genome editing in parental strains and therefore effective strategies to cure the cell from sgRNA-containing are needed (Figure 3). At least four strategies have been used to evict unwanted plasmids in *B. subtilis* as summarized in Table 1. Firstly, the bacteria can be incubated under antibiotic-free conditions, whereby the curing efficiency largely depends on plasmid instability. Secondly, for plasmids with a temperature-sensitive replicon, incubation at 50°C can cure 90% of the bacteria (Altenbuchner, 2016). Thirdly, the *mazF* counter-selection marker was applied to evict sgRNA cassettes inserted into the chromosome that had been introduced by integrative plasmids. Still, removal efficiency remained low, ranging from 6% to 31% (Westbrook et al., 2016). Fourthly, some modification plasmids contain both the chromosome-targeting sgRNA and the self-targeting sgRNA, which are regulated by a constitutive promoter and an inducible promoter, respectively. In such a case, following genome editing, the plasmids would be self-targeted and expelled (Lim & Choi, 2019). Collectively, most of these tactics can remove plasmids at variable efficiencies. The less time-consuming and higher efficient strategies are of course preferred.

THE EFFICIENCY OF DIFFERENT GENE EDITING AND POSSIBLE INFLUENCING FACTORS

When describing the different CRISPR systems, the editing efficiencies of gene knockout, knockin or mutation were lightly sketched by Zocca et al. (2021). In this review, we analyse the possible influencing factors and compare the editing efficiencies of different engineering methods. Due to the relatively small genome of prokaryotes, the off-target efficiency of CRISPR-Cas in *Bacillus* species has

been demonstrated to be low (Yu et al., 2020). This issue remains, however, still poorly investigated, such as which factors affect off-target efficiency and the mechanisms behind it.

Knockout and large fragment deletion

Gene disruption can be achieved by deleting entire fragments or introducing premature stop codons or frameshifts. In *B. subtilis*, the general gene knockout efficacy has been demonstrated to be high (86%–100%), except in undomesticated strain *B. subtilis* ATCC 6051a, where it is 33%–53% (Table 1). Nevertheless, Zhang et al. (2016) could disrupt six genes of *B. subtilis* ATCC 6051a sequentially, which has conferred properties advantageous for industrial fermentation. Deletion of a large fragment, such as a 25.1 kb fragment containing the *amyE* gene, reached an efficiency of 89% (Altenbuchner, 2016). However, for deletion of the 38 kb plipastatin-synthesizing *pps* operon, two sgRNAs targeting both the N- and the C-terminus of the fragment were necessary to complete fragment deletion. Notably, the editing efficiency dramatically increased from 16.7% to 80% when transformants experienced prolonged incubation in selective media (So et al., 2017). This indicated that two sgRNA combinations and prolonged incubation are recommended strategies to improve the deletion efficiency of large fragments of *B. subtilis*.

Point mutation and base editing

The introduction of point mutations assists in creating precise site-specific modifications to improve enzyme properties, repair mutagenesis and disrupt gene functions by introducing premature stop codons (So et al., 2017). Up to now, most of the reported efficiencies in introducing point mutations in *B. subtilis* could reach up to 68% under high transformation efficiency, optimized lengths of homology arms and elevated PAM sensitivity (Table 1). Although single nucleotide mutagenesis is accessible, the designed editing templates with higher nucleotide inconsistency with the genome targets, particularly bases proximal to the PAM sequences, are preferred (So et al., 2017). In addition to double- or multiple-gene mutations, simultaneous editing is highly desirable as it is less time-consuming compared to consecutive genetic manipulations. This can be accomplished by the presence of various sgRNAs assembled within one editing plasmid. However, the currently reported simultaneous editing efficiency remains low (36%), which might be caused by the low editing efficiency of one of the target sites (Westbrook et al., 2016).

Currently, cytosine base editor (CBE) and adenine base editor (ABE) are two prominent base editors. CBE and ABE can accomplish single-base changes or substitutions (base editing) from cytosine to thymine (C to T) catalysed by cytosine deaminase and from adenine to guanine (A to G) catalysed by adenine deaminase, respectively (Eid et al., 2018; Molla & Yang, 2019; Xia et al., 2020). For the first time, Yu et al. (2020) achieved C to T conversions using CRISPR-dCas9 (catalytically deactivated Cas9)-mediated cytosine deaminase in *B. subtilis*. The dCas9 and induced cytosine deaminase fusion protein has a 5 nt editing window and enabled up to 100% and 50% editing efficiencies for three and four simultaneous editings, respectively. It was shown that 84.7% of *B. subtilis* genes' function could be disrupted using this gene inactivation method by introducing early stop codons (Yu et al., 2020). However, this technology is not feasible for gene deletions or insertions and ABE has not been explored yet in *B. subtilis*.

Knockin

The structural and segregation instability of plasmids still hampers *Bacillus* strains from continually producing high levels of industrial enzymes, whereas over-expressing genes in the chromosome have been demonstrated to be more stable and productive (Fleming & Patching, 1994; Watzlawick & Altenbuchner, 2019). 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 of developing methods for overexpression of multiple genes or large fragments at genome levels (Abdallah et al., 2019; Liu, Liu, et al., 2019; Xue et al., 2015).

CRISPR-Cas9-based gene knockin system has made scarless gene integration accessible and efficient. Westbrook et al. (2016) and So et al. (2017) successfully introduced the hyaluronic acid biosynthetic operon (2.9 kb) and *gfp* cassette into *B. subtilis* chromosome with efficiencies of 69% and 66.8%, respectively. Another study highlighted in this review presents the sequential integration of five copies of *ganA* encoding β -galactosidase into the *B. subtilis* chromosome, which overexpressed an additional copy of competence genes *comK* and *comS* to increase the transformation efficiency (Watzlawick & Altenbuchner, 2019).

GENE REGULATION BY CRISPRa AND CRISPRi

The natural CRISPR-Cas system was harnessed for genome editing and optimized for gene regulation. Both HNH- and RuvC-like nuclease domains of Cas9 are necessary for

introducing DSBs in target DNA. Mutation of the two key residues (D10A and H840A) ultimately resulted in a catalytically deactivated Cas9 (dCas9) (Qi et al., 2013), but the dCas9 maintains its capacity to bind to sgRNA and targeted DNA strand. This allows for developing CRISPRi and CRISPRa tools to modulate gene expression levels (Price et al., 2019, 2020; Figure 1b). The former is intendedly repurposed to repress gene transcription by employing dCas9 to bind to the promoter regions of the target genes, thus blocking the progress of RNA polymerases to the downstream genes and attenuating gene transcription. So far, no evident correlation between the repression efficiency and the targeted loci has been observed (Zhang et al., 2018). On the contrary, the CRISPRa system is implemented by combining dCas9 and a transcriptional activation domain as a fusion protein to enhance endogenous gene transcription (Dominguez et al., 2016). An alternative strategy relies on modified sgRNA by recruiting different RNA-binding proteins fused to activation domains of the CRISPRa complex (Fontana et al., 2020). The high expression level of dCas9 was toxic to *E. coli* and led to reduced cell growth, but this toxicity could be reduced by controlling the expression level of dCas9 (Cui, Vigouroux, et al., 2018; Nielsen & Voigt, 2014). Whereas the toxicity of *B. subtilis* has not been systematically explored and discussed, Zocca et al. (2021) summarize the specific achievements of CRISPR gene expression control, so we mainly focus on the general principle and procedure of how these systems work and their pros and cons.

CRISPRa

A limited number of CRISPRa systems have been explored in *Bacillus* species (Lu et al., 2019; Zhao et al., 2020). Increasing the transcription/expression level of target genes was realized by fusing the ω -subunit of RNA polymerase to the C-terminus of dCas9. Then multiple sgRNAs were designed to guide dCas9- ω targeting several positions upstream of the transcriptional start sites of promoter-gene expression cassettes. However, discrepancies between increased protein expression levels and transcription levels might occur, probably attributed to the post-transcription disparity. In addition, the recruitment of dCas9- α (fusion of dCas9 and α -subunit of RNA polymerase) showed similar activation effects as dCas9- ω . Further, researchers fused the ω -subunit and α -subunit to dCas9 simultaneously in different arrays, producing multiple-activator-mediated transcriptional regulation systems (dCas9- ω - α and dCas9- α - ω). However, no synergistic effects (transcription increase) were observed by fusion proteins of dCas9- ω - α or dCas9- α - ω in *B. subtilis* (Lu et al., 2019). In the future, it is critical to enlarge the profile of effective gene activators (Fontana et al., 2020).

CRISPRi

CRISPRi can investigate the function of essential and unknown genes of *B. subtilis*, due to the reversible halt in the expression of specific genes (Moradpour & Abdulah, 2020; Peters et al., 2016). It has also been recruited to reduce gene expression and fine-tune metabolic pathways to increase the yield of bioproducts that are extensively used in food, cosmetics and pharmaceuticals (Dong et al., 2020; Westbrook et al., 2016; Westbrook, Ren, Moo-Young, et al., 2018; Wu et al., 2018; Figure 2). Normally, branch pathway enzymes are selected and targeted by individually employing a series of sgRNA with different repression efficiencies. The most suitable sgRNAs can then be combined to simultaneously repress several targets to achieve higher production of bioproducts (Dong et al., 2020; Westbrook, Ren, Oh, et al., 2018; Wu et al., 2018). In some cases, the repression scale and time of specific genes require strict controls, especially those involved in cell growth. Therefore, expression levels of dCas9 regulated by inducible promoters should be screened and optimized to control the repression time and strength to guarantee a balance between the biomass and production levels of the products of interest (Yang et al., 2020).

In summary, compared with conventional gene regulation skills, CRISPRi displays multiple advantages (Peters et al., 2015). This technique makes gene repression easy by 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 (Peters et al., 2016). Moreover, CRISPRi can simultaneously suppress the expression of several genes to different levels by utilizing multiple sgRNA within the

same cell. It is essential to regulate biosynthesis pathways within complex metabolic networks. Still, some disadvantages should not be neglected. First, the expression of the downstream genes within one operon might also be affected. Second, the inhibiting effects might fluctuate slightly due to the different expression levels of dCas9 affected by bacterial growth stages. These issues should be carefully considered when using this technology.

OTHER TYPES OF CRISPR-ASSOCIATED NUCLEASES

Cas9 nickase

Two categories of type II CRISPR-Cas9 systems have been reported: Cas9-mediated and Cas9 nickase (Cas9n)-mediated gene editing systems (Figure 4; Trevino & Zhang, 2014; Zhou et al., 2020). Inactivation of the RuvC domain of Cas9 by introducing a D10A mutation leads to Cas9n, which only cleaves the target DNA strand. Conversely, inactivating the HNH domain by an 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 (Davis & Maizels, 2011). Cas9nD10A has been used to fine-tune three ribosome binding site (RBS) regions (*ribB*, *ribA* and *ribH*) of the riboflavin operon for higher riboflavin production in *B. subtilis*, and it demonstrated a better performance in genome cleavage than Cas9nH840A (Liu, Huang, et al., 2019). In addition to achieving extremely high editing rates in large fragment deletion, gene insertion and single point

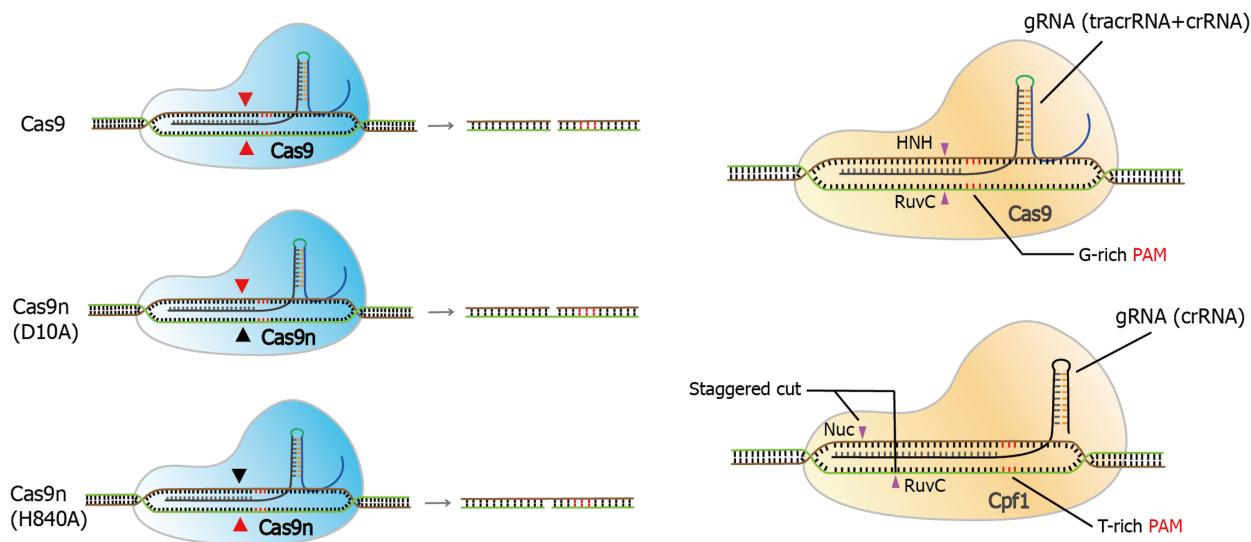


FIGURE 4 Cas9 nickases and comparison of Cas9 and Cpf1.

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 breaks by a two-component Ku-ligase break repair complex, was knocked out in this way (de Vega, 2013). The disruption of *ligD* facilitated the repair of strand break mediated by HDR, thus increasing the efficiency for simultaneous modulation of three sites from 49% to 65%.

CRISPR-Cpf1 system

In the 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 to crRNA length (Figure 4; Bayat et al., 2018). Unlike SpCas9 which recognizes G-rich PAMs (5'-NGG-3'), Cpf1, a single RNA-guided endonuclease, recognizes T-rich PAMs. FnCpf1 originating from *Francisella novicida* U112 recognizes 5'-TTN-3'; AsCpf1 from *Acidaminococcus* sp. BV3L6 and LbCpf1 from *Lachnospiraceae bacterium* prefer the 5'-TTTV-3' PAM with V representing A, G or C (Safari et al., 2019; Zetsche et al., 2015). Compared with SpCas9, Cpf1 shows higher sensitivity to mismatches between guide sequence and target genome. The Cpf1:crRNA complex alone (without tracrRNA) is sufficient to execute DNA cleavage (Safari et al., 2019). Also, Cpf1 introduces the DSB at the distal end of the protospacer, thus preserving the target site for the subsequent generation of cleavage. These features confer the CRISPR-Cpf1 tool as an ideal addition to the CRISPR-Cas9 system, which shows a preference for editing GC-rich regions.

Recently, the feasibility of this CRISPR-FnCpf1-assisted multiple-gene editing and regulation system (CAMERS-B) has been employed to significantly increase the production levels of *N*-acetylglucosamine and acetoin in *B. subtilis* (Wu et al., 2020). Remarkably, 100% efficiency was achieved in either gene insertion, multiple (up to six) site mutagenesis, or two-gene simultaneous deletion after conducting the prolonged incubation of transformation reaction mixture in a liquid medium with selective pressure. Also, it is worth pointing out that the modified Argonaute (NgAgo) originating from *Natronobacterium gregoryi* was concurrently employed to facilitate Cpf1-mediated gene editing (Fu et al., 2019), as the mutated NgAgo (retaining 650–887 residues, D663A, D738A) could strengthen the RecA-mediated HDR, but it is unable to cleave DNA or RNA. Moreover, the CRISPR-Cpf1 could also be used for multiple-gene repression and transcriptional dual-control (simultaneous activation and repression) using a DNase deactivated Cpf1 (D917A, dCpf1). When the transcription

activator RemA was fused to the C-terminus of dCpf1, the gene repression ability of dCpf1-RemA was still preserved because the dCpf1-RemA was able to repress and activate the downstream gene in a position-dependent manner (Wu et al., 2020). In addition, Cpf1-mediated large fragments deletion was more efficient than Cas9-mediated editing (Hao et al., 2020).

Considering that CRISPR-Cpf1 utilizes T-rich PAMs, its editing targets in the genome of *B. subtilis* (low GC content, 43%) are supposed to be more frequent than those of SpCas9 (Akashi & Yoshikawa, 2013). Further developing a facile yet robust CRISPR-Cpf1-mediated genome editing toolbox is urgently needed. After all, the editing rate of simultaneously deleting two genes by CRISPR-Cpf1 without prolonged incubation reached 43.1%, and completely knocking out three genes simultaneously remains unattainable (Bayat et al., 2018). Furthermore, eight identified Cpf1 orthologs are promising candidates to expand the versatility and feasibility of the CRISPR-Cpf1 system, as they could induce efficient DNA cleavages with identified PAM sequences, particularly those that possess conformational flexibility in recognizing both canonical (TTTV) and non-canonical (CTTV, TCTV and TTCV) PAMs, for example LbCpf1 (Bayat et al., 2018). Meanwhile, exploring novel Cpf1 variants can also broaden the utilization of this genetic tool by scaling up the targeting range of Cpf1 and maintaining high DNA-targeting accuracy.

GENOME ENGINEERING IN OTHER BACILLUS SPECIES

Besides *B. subtilis*, many other *Bacillus* species have significant applications in medical, pharmaceutical, food, agricultural and other industries. However, their genetic manipulation remains challenging because of low transformation efficiencies, and only a small repertoire of genetic tools is available for these species (Dong & Zhang, 2014). To take full advantage of producing enzymes, nucleotides, amino acids, vitamins, poly- γ -glutamate and biosurfactants, efficient and straightforward genetic tools are urgently needed to advance the promising strains' metabolic engineering. The application of CRISPR-based systems on these strains will lead to future breakthroughs in genome editing and regulation in these *Bacillus* microbial workhorses.

Genome engineering in *B. licheniformis*

In *B. licheniformis*, the Cas9-, dCas9- and Cas9n-mediated genetic tools have been developed (Li et al., 2020; Li, Cai, et al., 2018; Zhan et al., 2020; Zhou et al., 2019). In species

with a low transformation rate, two main factors should be considered: the vector size and the expression of the genes these vectors carry (Muth et al., 2017; Waschkau et al., 2008). When a genome-integrated Cas9n was employed, this decreased the gene editing plasmid capacity (Li, Cai, et al., 2018). Since the expression of chromosomally encoded Cas9n did not remarkably influence the growth of the bacteria, indicating the neglectable lethality effects, the Cas9n encoding gene could be retained in the genome-modified strains. However, the toxicity of heterologous Cas9 endonuclease resulted in a 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 (Zhan et al., 2020; Zhou et al., 2019). Generally, the single-gene deletion, large fragment (~42.7 kb) deletion and gene integration efficiency could reach as high as 100%, 79% and 76.5%, respectively (Li, Cai, et al., 2018). Simultaneously, disrupting multiple genes remain inefficient (double gene deletion was only 11%), probably due to the competition of different sgRNAs or the low editing efficiency among one of the target genes. In contrast, the multiple-gene repression by dCas9 has been reported to be efficient in *B. licheniformis* (Zhan et al., 2020). Low incubation temperatures enhance genome editing efficiency (Li et al., 2020). Moreover, the single-gene deletion efficiency can be as high as 51.4% with a short homologous arm size (100 bp homology flanks) (Li, Cai, et al., 2018). Different available strategies offer several choices for the most suitable approach to specific experimental demands.

Genome engineering in *B. methanolicus*

The thermophilic *B. methanolicus* can utilize methanol and other carbon sources for growth to produce amino acids, which makes it a promising candidate for biotechnological applications (Brautaset et al., 2007). Unfortunately, genetic tools for genomic gene alteration are not yet available. To solve this problem, Schultenkämper et al. (2019) established a CRISPRi-dCas9-based system to repress gene transcription. The promoter of mannitol activator gene *mtlR* from *B. methanolicus* was employed to regulate transcription of both dCas9 and sgRNA. This promoter was combined with a LacI operator (*lacO*) from *E. coli* that was placed upstream of dCas9 to repress the *mtlR* promoter, thus avoiding the toxicity triggered by dCas9 to *E. coli* during plasmid construction. By this approach, three putative genes involved in spore formation (*spo0A*), mannitol metabolism (*mtlD*, 50%) and hydrogen peroxide conversion (*katA*, 20%) were successfully repressed, which confirmed their functions in these processes experimentally.

In contrast to spCas9, which is not active at or above 42°C in *B. smithii*, dCas9 function was not affected at 50°C in *B. methanolicus* (Schultenkämper et al., 2019).

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

The anthrax causative pathogen *B. anthracis* and foodborne pathogen *B. cereus* belong to the *B. cereus sensu lato* group and both are spore-forming bacteria. Previously developed genome modification methods in these pathogens mainly focus on gene deletion based on homologous recombination (Pomerantsev et al., 2006). Recently, Wang et al. (2019) introduced a precise point mutation in *B. cereus* and deleted two large fragments in *B. anthracis*. The plasmid pJOE8999 used to implement gene modifications was initially constructed by Altenbuchner (2016) for *B. subtilis*, but the experimental details were adapted to *B. cereus sensu lato* species. The pioneer pJOE8999 and its derivatives have since been extensively used in multiple species, including *B. subtilis*, *B. anthracis*, *B. cereus*, *B. thuringiensis* and *Bacillus* phages, since this system worked efficiently and possessed several advantages (Wang et al., 2019). The SpCas9 was under the control of a mannose-inducible promoter, which was active in the presence of mannose and absence of glucose in *B. subtilis*, but inactive in *E. coli* to avoid any toxicity. sgRNA transcription was controlled by a strong constitutive promoter. The small selective marker, a kanamycin resistance gene, worked well in both organisms. The temperature-sensitive replication origin guaranteed efficient plasmid curing. The N20 sequences of sgRNA and DNA templates could be conveniently substituted through restriction enzyme sites *BsaI* and *SfiI*, respectively.

The antibiotic selection was specific for *B. anthracis* and *B. cereus*, and was only applied after transformation, to guarantee the plasmids were absorbed. Then an inducer was supplied to accumulate plasmid, allow sufficient Cas9 protein expression and achieve subsequent homologous recombination. Thus antibiotic selection and Cas9-mediated genome editing were combined within one manipulation step. Nevertheless, the final site-specific mutagenesis of the *plcR* gene in *B. cereus* merely reached 10% efficiency. This might be attributed to the likely indispensable role of PlcR in bacterial survival as an essential regulatory protein. Moreover, the deletion rates for the shortest (17 kb) and longest (51 kb) prophages in *B. anthracis* displayed a distinct 100% and 20%, respectively. This indicated relatively lower effectiveness for deletion of longer fragments (i.e. ~50 kb), compared with >90% efficiency of 25 kb 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 functional analysis of essential genes.

Genome engineering in *B. smithii*

The facultatively thermophilic *B. smithii* can utilize many carbon sources for lactate production and other green building block chemicals and grows between 37 and 65°C (Bosma et al., 2015). Recently, for the first time, Mougiakos, Bosma, et al. (2017) established a CRISPR-Cas9-based temperature-controlled recombination/counter-selection tool to modify genes of *B. smithii* ET 138. This approach achieved 90%, 100% and 20% efficiencies for gene deletion, knockout and insertion, respectively. More importantly, an inducible promoter was not indispensable in this system as the target activity of spCas9 could be precisely regulated by temperature. According to presented in vitro and in vivo tests, spCas9 was not active enough to introduce lethal DSBs at 42°C and above (Mougiakos, Mohanraju, et al., 2017). Therefore, culturing at 37°C is necessary to allow sufficient Cas9 targeting. Then the temperature should be gradually increased to 55°C to guarantee normal metabolism, during which plasmid-borne editing templates enable homologous recombination. For the subsequent plasmid eviction, the temperature is then increased to 65°C. This is the first CRISPR-Cas9 system being employed in a thermophile. It is also potentially applicable for other microorganisms that grow at or above 42°C, for which only a limited genetic toolbox is available.

Genome engineering in *B. thuringiensis*

Bacillus thuringiensis is a well-known biological pesticide that produces toxins specifically toxic to lepidopteran, dipteran, coleopteran and hemipteran insects. To further improve its ability to produce stable and functional crystal and vegetative insecticidal proteins at a large scale, precise gene engineering is required. The CRISPR-Cas9 strategy has been successfully developed to delete genes in this species, in which double crossover recombination was rarely occurring (Soonsanga et al., 2020; Tan et al., 2019). Though the obtained transformant numbers were very low, the editing efficiency was still acceptable (12.5%–62.5%). Low copy number plasmids were needed to reduce the toxicity of Cas9, whereas high copy number plasmids were necessary to modify plasmid genes with longer homology arms (around 1 kb) than genome fragments editing (around 0.5 kb).

INSIGHT INTO *BACILLUS*–PHAGES RELATIONS WITH NOVEL ROLES FOR CRISPR-CAS SYSTEMS

Bacillus and associated phage relations

Bacteriophages are considered one of the most abundant biological entities on this planet and outnumber their bacterial hosts. The interactions of *Bacillus* with their phages are based on the fundamental properties of bacteria (Wünsche, 1989). Recent investigations show that bacteriophages can be used to control food-borne pathogens (Abraha et al., 2021). This might also be a strategy to tackle *B. cereus*, which causes diarrhoea and emetic types of diseases.

Some phages can modify their restriction sites and bypass *Bacillus* antiviral systems (Samson et al., 2013). Specifically, the phage genome is modified by a host methyltransferase (MTase) and is able to multiply as it is now protected against restriction endonucleases (REases). This discovery provides the potential for the use of therapeutic temperate phages (Torres-Barceló et al., 2022). Recently discovered phage-encoded products can trigger the lytic cycles of prophages. The ‘Rapφ-Phrφ’ peptide-based systems have been used to control the decision of *Bacillus*-infecting phages towards lysis or lysogeny (Bernard et al., 2020).

Bacteria can develop resistance to their phages, and the mechanisms behind this resistance are continuing to attract interest. The CRISPR-Cas system is an important component of bacterial phage resistance (Azam & Tanji, 2019), while it also provides us with an efficient and adaptable tool for editing genomes of virulent phages and better understanding phage–host interactions. This approach can be easily adapted to engineer any virulent phage infecting other bacterial species (Martel & Moineau, 2014). The discovery of the anti-phage defense system CRISPR-Cas has thrived the research in bacteriophage science. *Bacillus* species with an available CRISPR-Cas toolset also offer the potential to study their associated phages genetically.

CRISPR-Cas and anti-CRISPR-Cas proteins (ACRs) in *Bacillus* and its phages

The CRISPR-Cas systems may act as barriers to horizontal gene transfer. However, this comes to a cost, as introducing a functional CRISPR-Cas system leads to decreased adaptability to stresses and declined pathogenicity of the host strains. The inactivation of CRISPR-Cas systems in *B. cereus* group organisms is often correlated with the acquisition of mobile genetic elements (MGEs), including prophage elements (Zheng et al., 2020).

Bacteriophages and other MGEs often express anti-CRISPR proteins (Acrs) to provide protection against eradication by CRISPR-Cas systems. *Bacillus* phages have been found to encode more than one anti-CRISPR protein (Gussow et al., 2020). For instance, DUF1874 proteins belong to the type III anti-CRISPR (AcrIII-1) family, among which Yddf protein encoded by *B. subtilis* integrative and conjugative elements *BS1*. This protein was cloned and expressed to explore the mechanism of action of AcrIII-1 protein (Gussow et al., 2020). Yddf behaves as a new viral anti-CRISPR enzyme, which shows a strong preference for cyclic tetra-adenylate (cA₄) degradation. The *acrIII-1* gene can be a part of an integrated MGE, which highlights the crucial role of cyclic nucleotide signalling in the conflict between viruses and their hosts (Athukoralage et al., 2020). Meanwhile, numerous phage technologies have been developed that enable the screening of protein–protein interactions. Among these, the machine-learning approach facilitates the expansion of the anti-CRISPR protein families.

In one particular case, it was shown that a protein from a phage could facilitate the editing efficiency of the CRISPR-Cas system. Uracil DNA glycosylase inhibitor (UGI) from *B. subtilis* bacteriophage PBS1 can effectively block human UDG. However, to subvert base editing repair at the site of base editing, UGI was fused to the C-terminus of the first-generation base editor (BE1, APOBEC1–XTEN–dCas9) to create a second-generation base editor (BE2, APOBEC–XTEN–dCas9–UGI), and this resulted in editing efficiencies in human cells that were on average threefold higher with BE2 than with BE1 (Komor et al., 2016).

CRISPR-Cas applications on *Bacillus* phages

The boundless diversity of bacteriophages makes them a limitless resource for tackling antibiotic-resistant bacteria. However, the biology of bacteriophages is poorly known and only a fraction of their genes have been annotated with functions.

Bacillus phages are very relevant for *Bacillus*-based fermentation processes, as they can potentially lyse production strains and destroy the fermentation process. Unwanted phage infections can be avoided if resistance is increased. For this, a type II CRISPR-Cas system of *Streptococcus thermophilus* DGCC7710 can be exploited to edit the genome of virulent phages at very high efficiencies through point mutations, small and large deletions as well as complete gene replacement. Such altered, avirulent phages can then be used to infect industrious *B. subtilis* strains to infer resistance to lytic bacteriophage SPP1 (Jakutyte-Giraitiene & Gasiunas, 2016).

Bacillus species with an available CRISPR-Cas toolset also offer the potential to study their associated phages genetically. A toolkit for easy and quick genetic engineering of *B. subtilis* phages has been designed, whereby the model phage vB_BsuP-Goe1 serves as a template for clean gene deletions and insertions with high efficiency and reliability (Schilling et al., 2018). Furthermore, a new CRISPR-Cas9 vector pRH030 was explicitly designed for the use of *Bacillus* phages modification. This vector can achieve constitutive expression of Cas9 and lead to inactivation of viral attackers and enhanced mutagenic efficiency of phages (Otte et al., 2020). Consequently, this Cas9-based mutagenesis vector pRH030 was used as a genetic tool on *B. subtilis* model phage Goe1 to specifically disrupt all phage genes to verify essential or non-essential genes for phage survival (Kohm et al., 2021).

FUTURE PERSPECTIVE

Ever since the CRISPR tools were established in *Bacillus*, they have been simplified, improved and combined with existing resources to facilitate genome manipulations (Koo et al., 2017; Sachla et al., 2021). An example worth highlighting is the engineering of the BKE collection of strains by recruiting the CRISPR-Cas9 system without designing specific sgRNAs. The BKE collection comprises nearly 4000 *B. subtilis* single-gene knockout strains with each gene replaced by the erythromycin (*erm*)-resistant gene (Sachla et al., 2021). By employing pJOE8999-derived plasmids with an anti-*erm* sgRNA, genome editing can be targeted to any BKE strain with the repair templates integrated into plasmids or either as PCR products or genomic DNA. Efficient gene replacements, site-specific mutations, intergenic region modification and the introduction of gene–reporter fusions have all been demonstrated (Sachla et al., 2021). This illustrates progress, but the research on the CRISPR-based tools in *Bacillus* species is still at an early stage and far from the optimal state. It is urgent to pursue higher editing efficiency and develop more strategies to fulfil different genetic requirements (Choi & Lee, 2016).

Figure 3 summarizes the workflow and key factors and approaches to effectively using current CRISPR genetic tools. Also, the advanced CRISPR tools established in eukaryotes and other bacterial species provide guidance for further directions of improving editing specificity and efficiency, such as engineering existing Cas9 protein, developing innovative systems and modification of sgRNA (Dang et al., 2015; Hendel et al., 2015; Slaymaker et al., 2016; Walton et al., 2020). The multiple CRISPR loci identified in bacteria and archaea all have the potential to be

transformed into novel and facile genome editing platforms (Makarova et al., 2015). Moreover, introducing a heterologous NHEJ system originated from *Mycobacterium tuberculosis* to *Bacillus* species to form a CRISPR-assisted NHEJ strategy could be explored for gene-function disruptions and even evolutionary engineering (Li, Wei, et al., 2018; Su et al., 2016). Furthermore, regarding gene expression regulation, the repression efficiency of given sgRNAs can now be predicted. Combining more experimental results with deep learning strategies will further facilitate the development of prediction tools to reduce laboratory work. Also, the discovery of more effective activation domains is highly recommended since their lacking has strongly hindered the development of CRISPRa in bacteria (Fontana et al., 2020). Adapting the diversity of CRISPR-based systems to the industrially and medically essential *Bacillus* species can be expected in the near future.

Nowadays, re-sequencing the entire genome after gene modification can be employed to detect undesired mutations that might influence the bacteria's performance in industrial production and to investigate the underlying mechanisms. Utilization of previously constructed tools among different *Bacillus* species would be an option to explore the feasibility of genome editing (Toymentseva & Altenbuchner, 2019). With the discovery of novel CRISPR-Cas mechanisms and optimization of current Cas9 proteins to expand editing realms, the highly diverse applications of *Bacillus* species will undoubtedly benefit from these powerful CRISPR biotechnologies.

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CONFLICT OF INTEREST

Authors declare that no conflicts of interest have been identified.

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