ACS Synthetic Biology

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Recent Advances in Genetic Engineering Strategies of *Sinorhizobium meliloti*

Xuan Liu,[#] Huina Dong,*[,#] Huiying Wang,[#] Xinyi Ren, Xia Yang, Tingting Li, Gang Fu, Miaomiao Xia, Huan Fang, Guangqing Du, Zhaoxia Jin,* and Dawei Zhang*

Cite This: *ACS Synth. Biol.* 2024, 13, 3497−3506

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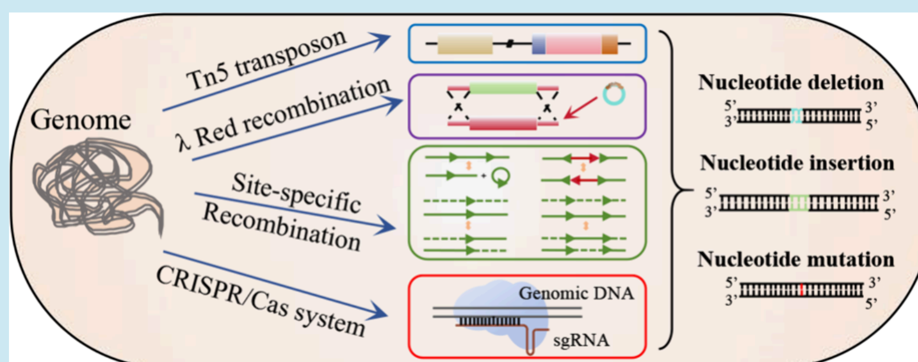


ABSTRACT: *Sinorhizobium meliloti* is a free-living soil Gram-negative bacterium that participates in nitrogen-fixation symbiosis with several legumes. *S. meliloti* has the potential to be utilized for the production of high-value nutritional compounds, such as vitamin B_{12}. Advances in gene editing tools play a vital role in the development of *S. meliloti* strains with enhanced characteristics for biotechnological applications. Several novel genetic engineering strategies have emerged in recent years to investigate genetic modifications in *S. meliloti*. This review provides a comprehensive overview of the mechanism and application of the extensively used Tn5-mediated genetic engineering strategies. Strategies based on homologous recombination and site-specific recombination were also discussed. Subsequently, the development and application of the genetic engineering strategies utilizing various CRISPR/Cas systems in *S. meliloti* are summarized. This review may stimulate research interest among scientists, foster studies in the application areas of *S. meliloti*, and serve as a reference for the utilization of genome editing tools for other Rhizobium species.

KEYWORDS: *Sinorhizobium meliloti, genetic engineering strategies, Tn5 transposon, site-specific recombination, CRISPR/Cas system*

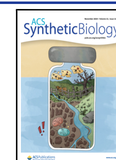
1. INTRODUCTION

Rhizobia, a Gram-negative soil bacterium that is widely distributed in nature, is equipped with the ability to directly convert atmospheric nitrogen (N_2) into ammonium through the use of nitrogenase.[1] A significant proportion of rhizobial species establish symbiotic relationships with their leguminous hosts, which represents the most efficient form of nitrogen fixation in nature.[2] *Sinorhizobium meliloti*, a symbiotic nitrogen-fixing bacteria that forms nitrogen-fixing nodules on the roots of legumes such as *Medicago truncatula*, has been reclassified as *Ensifer meliloti*.

The diverse and abundant metabolic capabilities of *S. meliloti*, including carbohydrates metabolism, enable its adaptability to a wide range of environmental and nutritional conditions, either as a free-living form or as a plant symbiont.[3] Previous studies have demonstrated that *S. meliloti* can be cultured from starch industry wastewater as a feedstock,[4] produce cellulase using waste tobacco as a substrate,[5] and be genetically modified as high-yield strains for vitamin B_6[6] or vitamin B_{12}[7,8] production.

The genome of *S. meliloti* Rm1021 was sequenced and annotated in 2001,[9] which was the first fully sequenced and annotated rhizobia. Subsequently, multiple rhizobia genomes have been sequenced and annotated.[10] The *S. meliloti* genome is tripartite 6.7 megabases (Mb) in size and consists of a 3.65-Mb chromosome and two giant plasmids, 1.35-Mb pSymA and 1.68-Mb pSymB.[9] However, progress in gene characterization studies and functional genomics of *S. meliloti* is relatively slow, primarily due to the lack of powerful gene editing tools.

Received: May 15, 2024
Revised: October 9, 2024
Accepted: October 21, 2024
Published: October 31, 2024



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https://doi.org/10.1021/acssynbio.4c00348
ACS Synth. Biol. 2024, 13, 3497−3506

In recent years, a variety of approaches have emerged for investigating genetic modification in *S. meliloti*. Thus, it is imperative to summarize recent advancements, current challenges, and future goals in genetic modification of *S. meliloti* to stimulate more research interest and advance studies in the application areas of *S. meliloti*. First, we introduced the mechanism and application of Tn5 transposon-mediated genetic engineering strategies used in *S. meliloti*. Then, we summarized strategies using homologous recombination based on endogenous and exogenous recombinase and discussed several kinds of site-specific recombination strategies. Additionally, we discussed the development and application of different types of CRISPR/Cas-based genome editing tools used in *S. meliloti*. Finally, we described the challenges and limitations of current genetic engineering strategies and suggested future research goals for the development of novel and useful tools for genetic modification of *S. meliloti*.

2. Tn5 TRANSPOSON-MEDIATED GENETIC ENGINEERING STRATEGIES

Tn5, Tn10, Tn3, and Tn7 are among the most extensively studied transposons in bacteria.[11−16] Tn5 is a transposon originally found in *Escherichia coli* (*E. coli*). It contains two inverted insertion sequences, IS50L and IS50R, flanking three passenger genes encoding resistance to kanamycin (*kan*), bleomycin (*bleo*), and streptomycin (*str*) (Figure 1A).[17,18] The 476-amino-acid transposase protein (Tnp), encoded on IS50R, is absolutely required for transposition, while the inhibitor (Inh), consisting of a 421-amino-acid polypeptide lacking the first 55 residues of transposase, exhibits inhibitory activity against Tn5 transposition.[19,20] However, the P3 and P4 encoded on IS50L produce the nonfunctional forms of Tnp and Inh, respectively, and are defective in supplying the transposition-inhibition function.[19] The outer end (OE) sequence contains 19 base pairs with a sequence of 5′-CTGACTCTTATACACAAGT that can be recognized by transposase.[17] The use of two OEs will cause the entire Tn5 element transposition,[21] while the use of OE and inside end (IE) will cause IS50 transposition.[17] Tn5 moves by a cut-and-paste mechanism.

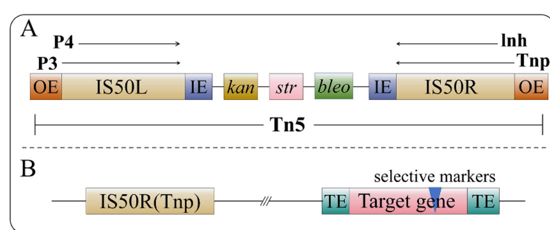

Figure 1. Tn5 transposon-mediated genetic engineering strategies. (A) Genetic organization of Tn5 transposon carries antibiotic resistance genes. (B) Tn5-based transposon delivery vector for mutagenesis of *S. meliloti*.

The Tn5-based transposon method is the most important and widely used in the annals of genetic and genomic research of *S. meliloti*. This method necessitates only one vector containing the transposase (*tnp*), while the transposable element typically consists of two OEs (transposon ends, shown as TE) and selective markers (Figure 1B). The transposable element can be inserted randomly and efficiently into the genomes of *S. meliloti* to generate a larger number of mutants (or a library); thus, the Tn5-based transposon method is a powerful forward-genetic analysis method. Indeed, the method has made great contributions to the functional genomics research on *S. meliloti*.[22,23] For instance, it has identified a few key nodulation and nitrogen fixation genes,[24,25] characterized the β-ketoadipate pathway,[26] and revealed the succinoglycan biosynthetic pathway.[27]

3. λ RED RECOMBINATION-MEDIATED GENETIC ENGINEERING STRATEGIES

The *Bacillus subtilis sacB* gene encodes levansucrase, an enzyme whose activity is lethal to the host in the presence of sucrose. This gene is commonly used as a counter-selection marker to eliminate the donor cells or to create suicide vectors.[28] In earlier years, the counter-selectable marker *sacB*-based gene deletion was once one of the main methods for *S. meliloti* gene deletion.[29] However, this method has relatively low editing efficiency due to the weak homologous recombination ability of *S. meliloti*.

Yang et al. introduced λ Red recombineering, which relies on the recombinase-catalyzed homologous recombination between the targeting substrate DNA and its allele in the chromosome or episome, in the *sacB*-based gene deletion method.[30] The λ Red operon is composed of three genes, *redα*, *redβ*, and *redγ*.[31,32] Redα, encoded by *redα*, is an exonuclease that acts on double-stranded DNA from 5′ to 3′ to produce a fully single-stranded DNA as a substrate for the single-stranded DNA-binding protein Redβ. Redβ, encoded by *redβ*, functions as a recombinase that facilitates the annealing of two DNA molecules. Meanwhile, Redγ, encoded by *redγ*, acts as a targeted DNA molecular stabilizer, protecting DNA from degradation by the host's endonucleases, primarily RecBCD and SbcCD. This gene deletion strategy employs overlap-extension PCR-generated linear targeting DNA instead of plasmid, which eliminates the plasmid construction process. The deletion efficiency of the chromosomal *visNR* operon is up to 100%, compared to 40% for the *SMa2341* gene in pSymA and 35% for the *bacA* gene in pSymB.[30] The reduced deletion efficiency of genes in the two giant plasmids relative to those in the genome, which has been observed, may be attributed to a number of factors, including differences in replication mechanisms and recombination conditions, or differences in the copy number of chromosomes and giant plasmids. Despite the reduced efficiency of λ Red recombination in mediating gene deletions in giant plasmids, the PCR screening of a mere 20 clones yielded the desired mutants, indicating that this recombination approach is sufficiently robust to be a viable method for gene deletion.

4. SITE-SPECIFIC RECOMBINATION-MEDIATED GENETIC ENGINEERING STRATEGIES

Site-specific recombination systems mediate recombination reactions between two specific and identical or different DNA recognition sites, which generates a desired DNA integration, excision (sometimes called resolution), or inversion events. The site-specific recombinases in site-specific recombination systems fall into one of two evolutionarily and mechanistically distinct families:[33] the tyrosine recombinases (also known as the λ integrase family)[34] and the serine recombinases (also known as the resolvase family).[35] In the tyrosine recombinases, the 5′ bridging oxygen of DNA is displaced to form a phosphotyrosyl bond with the 3′ end of the broken DNA

https://doi.org/10.1021/acssynbio.4c00348
ACS Synth. Biol. 2024, 13, 3497−3506

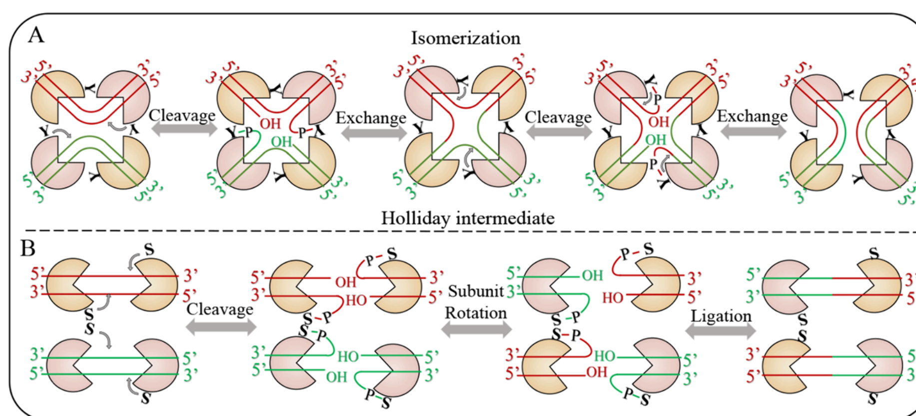

Figure 2. Site-spectic recombinases as tools for heterologous gene integration. (A) Recombination mechanism catalyzed by Tyrosine-based recombinase (based on λ phage integrase-mediated recombination). The hydroxyl groups of the tyrosine residues that act as catalysts in the two recombinase molecules act as nucleophiles, attacking the strand at the top of the substrate DNAs in a cis direction to form a 3′ phosphotyrosine linkage. The free 5′-OH on each cleaved DNA strand then attacks the 3′ phosphotyrosine linkage of the opposite nucleoprotein complex, resulting in a strand exchange to form a Holliday linkage intermediate. After the first strand exchange, isomerization of the Holliday linkage intermediate initiates a second strand exchange catalyzed by another pair of recombinases. The second exchange produces the recombination product. Other Tyrosine-type recombinase-catalyzed recombination events (e.g., trans cleavage by Flp recombinase) may have some differences. (B) Mechanism of recombination catalyzed by Serine-type recombinases. The hydroxyl group of its catalytic serine residue in all four recombinase molecules acts as a nucleophile, attacking the substrate DNAs and forming a 5′ phosphoserine linkage. Two of the four recombinase molecules covalently linked to different substrate DNAs then rotate with respect to the other pair of recombinases and recombine their linked 5′ phosphoryl groups with the 3′-OH of the unrotated DNA, resulting in recombination products.

strand, while in the case of serine recombinases, the 3′ bridging oxygen is displaced to create a 5′ phosphoserine linkage (Figure 2A and B).[36] Although most site-specific recombination systems occur naturally in bacteria and their viruses, many site-specific recombinases can be used to manipulate or engineer the genome of the heterologous host.

Several site-specific recombinases have been used as tools for engineering *S. meliloti*. The tyrosine recombinase family includes integrases from coliphage λ (also known as λ Integrase),[37] Cre from bacteriophage P1,[38] and Flp from *S. cerevisiae*,[39] which have been used to engineer *S. meliloti*. The serine recombinases family of *Streptomyces* temperate phage (Φ)C31 integrase has also been used to engineer *S. meliloti* (the details are shown in Table 1).[40]

4.1. Use λ Integrase as a Tool for Engineering *S. meliloti*. λ integrase is a typical member of the tyrosine recombinase family. It mediates integration of the phage λ genome into the bacterial chromosome together with the integration host factor IHF. In addition, integrase is involved in the excision of the phage λ genome out of the bacterial chromosome, in collaboration with Fis and Xis as well as IHF.[41] λ integrase catalyzes a single DNA crossover between specific "attachment" sites, *attB* on the bacterial chromosome and *attP* on the circularized phage genome, and creates an integrated λ prophage flanked by two new attachment sites, *attL* and *attR*.

A powerful gene cloning methodology for *S. meliloti* had been constructed by using a BP Clonase enzyme cocktail containing λ integrase and *E. coli* IHF.[37] This λ integrase recombination method combined a PCR product of the target gene from *S. meliloti* flanking *attB* sites into *attP* sites in a plasmid. The toxic *ccdB* gene, which served as a counter-selectable marker, was situated between two *attP* sites.

4.2. Use Flp/*FRT* and Cre/*loxP* Systems As Tools for Engineering *S. meliloti*. In contrast to λ integrase, numerous members of the tyrosine recombinase family are capable of catalyzing reversible site-specific recombination reactions between two identical sites that are approximately 35 bp long without the necessity of accessory proteins.[36] The Flp/*FRT* system and Cre/*loxP* system are two typical representatives of "simple" recombination systems, which function in a wide variety of different organisms.[28,42,43]

In both systems, the site-specific recombinase should be expressed from an inducible promoter on one plasmid that is replicative in *S. meliloti* and contains a selective marker. Site-specific recombinases are extremely valuable tools in genetic engineering, as they are able to precisely shear and recombine DNA at specific locations in the genome. However, the expression of site-specific recombinases, if uncontrolled, may cause unwanted alterations in the genome in cells or periods where recombination is not desired, which may interfere with the results of the study or even lead to embryonic lethality in experimental animals.[44] The use of inducible promoters allows the researcher to turn on or off specific recombinase activity at specific times and/or locations as needed, which is important for avoiding genetic recombination in nontarget cells or in inappropriate periods, and also reduces background activity of specific recombinases (i.e., nonspecific expression of specific recombinases when recombination is not desired), which can help to minimize false-positive results and contribute to improved experimental accuracy and reliability.[44] In conclusion, the use of inducible promoters to control the expression of specific recombinases not only achieves precise control of the gene editing process but also helps to avoid the occurrence of nonspecific recombination, thus playing an important role in complex gene function studies. At least two recombinase recognition target sequences should be designed on two suicide vectors, respectively. The vectors each contain a selective marker that is distinct from the other markers and different from the selective marker on the recombinase expression vector. Additionally, they include a homologous sequence that was used to integrate the recognition target into the genome of *S. meliloti*.[24] Following the formation of a deletion, one *FRT* (5′-GAAGTTCCTATACTTTCTA-

https://doi.org/10.1021/acssynbio.4c00348
ACS Synth. Biol. 2024, 13, 3497−3506

Table 1. Non-CRISPR/Cas Systems Mediated Genetic Engineering Strategies

Tn5 transposon							
type	consistent	substrate	insertion site	homologous arm length	template	efficiency	ref
Tn5 Transposon	*IS50R, IE, OE*	dsDNA	Multiple recognition sites	None	plasmid	up to 100%	27, 49

λ Red recombination							
type	consistent	substrate	insertion site	homologous arm length	template	efficiency	ref
λ Red recombination	*redα*, *redβ*, and *redγ* recombinase,homologous arm	dsDNA	specific	∼500bp	linear targeting DNA	from 35 to 100%	30, 32

Site-specific recombination						
type	representative member	recombination mechanism	recognition site	template	efficiency	ref
Tyrosine-type recombinase	λ Integrase	Formation and resolution of Holliday junction	Nonidentical *attP* and *attB*	plasmid	66.3%	33, 36, 37, 41, 50
	Flp/FRT and Cre/loxP system	Formation and resolution of Holliday junction	Identical *loxP* (Cre) (5′-ATAACTTCGTATAGCATACATTATACGAAGTTAT) *FRT* (Flp) (5′-GAAGTTCTATACTTTCTAGATAGA ATTTC)	plasmid	up to 100%	24, 33, 36, 39, 41,46
Serine-type recombinase	ΦC31 integrase	180° Rotation and rejoining of cleaved DNAs	Nonidentical *attP(5′-CCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGG)* and *attB(5′-GGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTA)*	plasmid	up to 100%	33, 36, 40, 41, 48

GAGAATAGGAACTTC) or *loxP* (5′-ATAACTT-CGTATAGCATACATTATACGAAGTTAT) site remains in the genome, which can be used for a subsequent round of recombination to enlarge the deletion.[37,38] However, the Flp/*FRT* system cannot be used to delete two independent regions in the genome of *S. meliloti*, as the region between the two deletions will be removed by expressing the Flp once again.[39,45] The Cre/*loxP* system using mutant *lox* sites can construct two or more independent deletions within *S. meliloti*, as the *lox* sites left after recombination are poor Cre substrates.[46]

4.3. Use Large Serine Recombinase As Tool for Engineering *S. meliloti*. The typical serine recombinases are resolvase/invertases, which are small proteins with only 180 amino acids.[36] The ΦC31 integrase belongs to the large serine recombinase family because it contains a catalytic domain of about 140 amino acids and a C-terminal domain of 300−500 amino acids, which is much larger than that in the typical serine recombinases.[35] The ΦC31 integrase combines the simplicity of Cre/*loxP* and the directionality of λ integrase without the need for an accessory protein (such as Xis or recombination directionality factor, RDF).[41,47]

The ΦC31 integrase mediates unidirectional recombination between two specific sites: *attB* (34 bp, 5′-GTGCCAG-GGCGTGCCCTTGGGCTCCCCGGGCGCG) and *attP* (39 bp, 5′-CCCCAACTGGGGTAACCTTTGAGTTCTCT-CAGTTGGGG).[48] The *attL* or *attR* sites cannot be recognized by ΦC31 recombinase and will be left in the genome after recombination. Therefore, this system can be used to construct two or more independent deletions in the same strain.

Similar to Cre and Flp, the ΦC31 integrase should also be expressed under an inducible promoter on a replicative plasmid in *S. meliloti*. The recognition target sequences, *attB* sequence and *attP* sequence, should be designed on two suicide vectors, respectively. Heil et al. used the ΦC31 integrase system to mediate cassette exchange.[40] In this method, a "landing pad" (LP) sequence contains a spectinomycin-resistance gene and the *β*-glucuronidase gene from *E. coli* flanked by *attP* sites were integrated into the genome of *S. meliloti*, then a single *attB* flanked DNA cassette could be integrated into the LP locus.

In conclusion, all the site-specific recombination systems can be employed to construct a basic deletion mutant. However, the recombinase needs to be carefully selected for different downstream applications. A site-specific recombination system that leaves a site in the genome that can be recognized by recombinase could be employed in a second round of recombination to enlarge the deletion. While the system that leaves a site in the genome that cannot be recognized by recombinase could be used for the construction of two or more independent deletions in the same strain. Moreover, the vectors used to integrate the recombinase recognition sites into the genome should be carefully designed, as different designs will yield disparate outcomes, including varying amounts of selective marks retained in the genome after recombination or result in the region of interest being reversed rather than deleted (Figure 3).

5. CRISPR-MEDIATED GENETIC ENGINEERING STRATEGIES

In the past decade, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein systems have been widely used as genome engineering

https://doi.org/10.1021/acssynbio.4c00348
ACS Synth. Biol. 2024, 13, 3497−3506

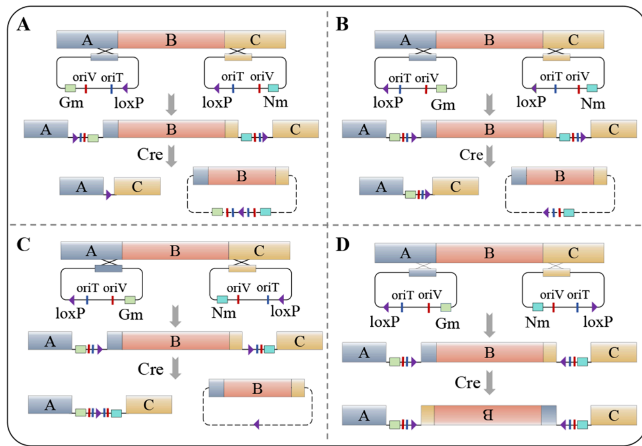

Figure 3. Influence of integration vector design on recombination end-product is examined using the Cre/*loxP* system as an illustrative example. According to the different organization of the integration vectors, the final deletion may be marked by either (A) zero selective markers, (B) one selective marker, or (C) two selective markers. (D), the FRT sites are introduced into the genome in the opposite direction, resulting in an inversion rather than a deletion.

tools in microorganisms.[51] The CRISPR/Cas systems can be classified into two primary categories (class I and class II) that are further subdivided into six distinct types (types I–VI) based on the differences in the structure and function of the Cas protein.[51]

The CRISPR/Cas9 system is regarded as the most powerful genome editing toolkit.[52] The Cas9 is a signature gene of the type II system. The Cas9 protein from *Streptococcus pyogenes* (SpyCas9) is 1368 amino acids long and consists of six components: RECI, RECII, Arginine-rich bridge helix, PAM Interacting, HNH, and RuvC. RECI is the Recognition lobe, which is mainly responsible for binding gRNA. Arginine-rich bridge helix initiates cleavage activity upon binding to the target sequence. PAM Interacting interacts with specific PAMs, which are responsible for binding to the target sequence.[53] HNH and RuvC are nuclease domains used to cleave the target sequence. In the absence of gRNA, the Cas9 protein does not have cleavage activity. gRNA has a 20–24 nucleotide sequence complementary to the target sequence at its 5′ end. After the gRNA and Cas9 nuclease form a complex, Cas9 recognizes the PAM sequence (the SpyCas9 is 5′-NGG-3′), and if there is a complementary sequence to the 5′ end of the gRNA near the PAM sequence, the gRNA/Cas9 complex cleaves the target DNA.[54] Cas9 then uses its HNH and RuvC nuclease domains to cleave the dsDNA at 3 bp upstream of the PAM to generate double-stranded breaks (DSBs). The HNH domain cleaves the strand of DNA that is complementary to the target sequence, while the RuvC domain cleaves the other strand.[55] These DSBs can be repaired by homology-directed repair (HDR) or by nonhomologous end joining (NHEJ) (Figure 4A).[56,57] The CRISPR/Cas9 system coupled with 500 bp homology arms

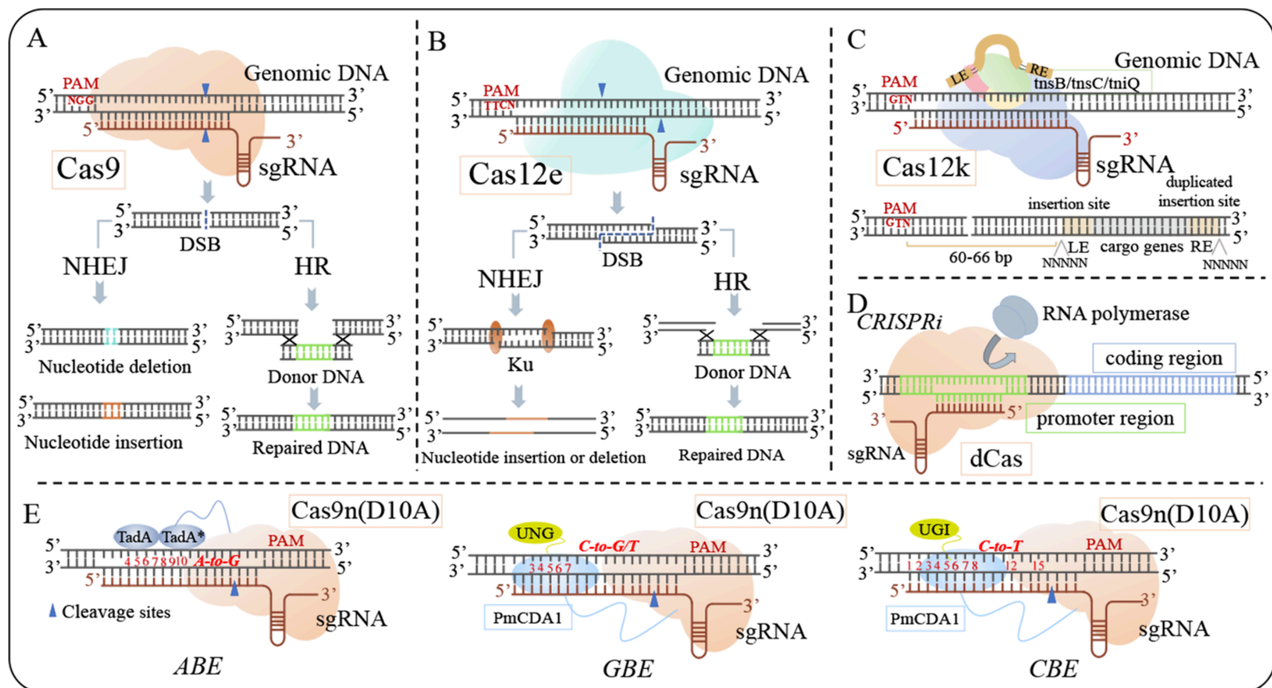

Figure 4. Schematic of the mechanisms of various types of CRISPR systems. (A) The Type II Cas9. The targeting effect of the sgRNA directs the Cas9 protein to cleave both the complementary and noncomplementary strands in the presence of the PAM sequence (5′-NGG-3′), forming a blunt-ended nick. (B) The CRISPR/Cas12e system is a method of cleaving DNA, resulting in DNA double-strand breaks (DSBs). These can be repaired by two main mechanisms: homologous recombination (HR) or nonhomologous end joining (NHEJ). HR is employed for precision editing, while NHEJ is used for nonprecision editing. (C) The CRISPR-associated transposase (CAST) system. In the presence of a PAM sequence (5′-GTN-3′), the targeting effect of sgRNA was used to achieve gene integration by unidirectional insertion of a donor gene 60–66 bp downstream. (D) The CRISPR-mediated transcription repression (CRISPRi). dCas targets promoter regions or coding regions of structural genes, competing with RNA polymerase for promoter binding or preventing polymerase migration to inhibit gene transcription. (E) The Adenine Base Editor (ABE), Glycosylase Base Editor (GBE), and Cytidine Base Editor (CBE) systems catalyze the efficient conversion of A to G, C to G/T, and C to T in *S. meliloti*, respectively.

https://doi.org/10.1021/acssynbio.4c00348
ACS Synth. Biol. 2024, 13, 3497–3506

has been tried to be used in *S. meliloti* 320; however, few transformants could be obtained under noninduced conditions.[58] The excessive cutting activity and toxicity of Cas9 and the undetectable recombination ability severely limit the application of the CRISPR/Cas9 system in *S. meliloti* 320.

Cas12e from *Deltaproteobacteria* is one of the effector proteins of the type V family (type V-E) and consists of only 986 amino acids, which has the advantage of a small molecular weight compared with Cas9, making it more efficient for editing. Cas12e, unlike Cas9, has only one nuclease domain, RuvC, which is used to cleave double-stranded DNA. The structure of the DpbCas12e-sgRNA (single guide RNA)-DNA complex reveals that it contains a nontarget-strand binding (NTSB) domain and a target-strand loading (TSL) domain.[59] When the nontarget DNA strand is cleaved by the RuvC domain, the complex undergoes a conformational change so that the target DNA strand is also cleaved by the RuvC domain. DpbCasX binds to sgRNA and cleaves dsDNA through the 5′-TTCN-3′ protospacer adjacent motif (PAM) and a 20 nucleotide sequence at the 5′ end of the sgRNA that is complementary to the target sequence. DpbCasX cleaves the DNA at 12−14 nucleotides after the PAM on the nontarget strand and at 22−25 nucleotides after the PAM on the target strand, yielding 5′-overhangs that are approximately 10 nucleotides in length (Figure 4B).[59] Reducing the sgRNA sequence from 20 nt to 16 nt results in the cleavage of Cas12e closer to the PAM on the nontarget DNA strand, resulting in longer 5′-overhangs.[60]

A CRISPR/Cas12e-based genome engineering toolkit containing Cas12e, single guide RNA, and 500 bp homology arms was constructed in *S. meliloti* 320. This toolkit was designed to address the low homologous recombination efficiency of *S. meliloti* 320 by adjusting the Cas12e cutting activity.[61] The CRISPR/Cas12eGET system generates DNA double-strand breaks (DSBs) for precision editing by homologous recombination (HR) or for nonprecision editing by repair through nonhomologous end-joining (NHEJ). Ku proteins, in the form of either homodimers or heterodimers, bind to DNA ends, initiating NHEJ and preventing end resection. Therefore, knockdown of Ku protein reduces the competition between NHEJ and HR for repair, tipping the balance in favor of HR.[61]

The discovery of the Cas12k-based Tn7-like transposon was due to the fact that the type V CRISPR/Cas system encodes effector proteins that are very similar to the TnpB nuclease. CRISPR-associated transposase from cyanobacteria *Scytonema hofmanni* (ShCAST) contains Tn7-like transposase subunits and a CRISPR array and type V−K CRISPR effector (Cas12k).[62] Tn7-like transposase subunits contain characteristic terminal structures (LE and RE) and the related transposase genes *tnsB*, *tnsC*, and *tniQ*. Upon binding to the sgRNA, Cas12k recruits the associated transposon and cargo genes to the target site via 5′-GTN-3′ PAMs and a 23 nucleotide sequence complementary to the target sequence at the 5′ end of the sgRNA, inserting transposon LE and RE sequences and cargo genes downstream of the PAM in a unidirectional manner at 60−66 bp and resulting in the duplication of 5-bp insertion sites (Figure 4C).[58,62] ShCAST for DNA insertion had been developed in *S. meliloti* 320.[58] This CAST system consists of Cas12k and sgRNA for the CRISPR machinery, TnsB-TnsC-TniQ for the transposition machinery, and a cargo DNA flanked by transposon left end (LE) and right end (RE) elements. This system has been demonstrated to integrate genomic fragments up to 10 kb in size in *S. meliloti* 320 with up to 100% efficiency, and gene integration occurred mainly at 62−65 base pairs downstream of the PAM sequences. However, it has been observed that this process leaves scars at the targeted site, and the off-target rate is 17.48% in the whole genome.

The Cas12k protein within the CAST system possesses a naturally inactivated endonuclease domain. Upon binding of the Cas12k-sgRNA complex to the target gene, it will block the transcriptional elongation of the gene and down-regulate the gene expression (Figure 4D). Cas12k and dCas9-mediated CRISPRi systems were constructed and compared in *S. meliloti* 320.[58] Both systems could down-regulate the gene expression level. The maximum knock-down efficiency of the Cas12k-mediated CRISPRi system reached 90%, which was slightly higher than that of the dCas9-mediated CRISPRi system. Furthermore, the dCas9-mediated CRISPRi system imposed a greater growth burden on the host cell than the Cas12k-mediated CRISPRi system. Compared with dCas9, Cas12k comprises only 639 amino acids and does not necessitate the mutation of the Cas protein.

Cytosine base editors (CBE) were developed by fusion expression of nuclease-dead Cas9 (dCas9), which is fully inactivated by endonuclease, or nCas9, which creates single-strand breaks (nicks) in target DNA through mutations in either the RuvC or HNH nuclease domains of Cas9, with cytosine deaminase.[63] CBE is directed to the target site by dCas9 or nCas9, and cytosine deaminase converts cytosine (C) to uracil (U), which is then converted to thymine (T) during DNA replication and repair, thus realizing the free conversion of C·G-T·A base pairs. Similarly, the fusion of adenine deaminase developed adenine base editor (ABE).[64] Adenine deaminase will convert purine (adenine, A) to hypoxanthine (inosine, I); I is recognized by the polymerase as guanine (guanin, G) to achieve A·T-G·C base pair conversion. A new glycosylase base editor (GBE) was developed using the cytosine deaminase-nCas9-Ung protein complex.[65] GBE brings uracil-N-glycosylase (UNG) to the uracil base formed by cytosine deaminase. The uracil-N-glycosyltransferase (UNG) is induced to bring the uracil base to the uracil site, where the uracil is removed, creating an apurinic/apyrimidinic-free site. The DNA damage site then induces the initiation of DNA repair, which can induce base reversal between pyrimidine and purine. These techniques allow efficient substitution of specific genomic bases without the need to create DNA double-strand breaks (Figure 4E). Single plasmid CRISPR/nCas9^{D10A}-mediated base editing tools, including the adenine base editor (ABE) system, cytidine base editor (CBE), and guanine base editor (GBE), have been established in *S. meliloti*.[66,67] The editing efficiency of these base editors is affected by the strength of promoters for gRNA and fusion proteins that contained Cas9 nickase and other auxiliary enzymes. The optimal promoter for gRNA is the pRpmJ promoter. The fusion proteins of CBE and GBE were both driven by P_{HemA}, while TadA−TadA*-nCas9, the fusion protein of ABE, could be driven by P_{HemA}, P_{Neo}, or P_{Tau}. All of these factors exhibited nearly 100% editing efficiency.[66] In the case of CBE used in *S. meliloti*, the editing efficiency of PmCDA1-CBE is slightly higher than that of rAPOBEC1-CBE, and the editing window is slightly wider. GBE preferred to produce C to G with efficiencies ranging from 30% to 80% in *S. meliloti*, which was previously observed to predominantly convert C to A in *E. coli*.[65] CBE and ABE have been

Table 2. CRISPR/Cas Systems-Mediated Genetic Engineering Strategies

CRISPR/Cas system									
type	subtype	PAM	Substrate	Length	Consistent	The type of donor	Homologous arm length	Efficiency	ref.
Cas12e(CasX)	Type V-E	5′-TTCN	dsDNA	986 amino acids	Cas12e;sgRNA	plasmid	500 bp	50%−60%	59, 61
Cas12k	Type V-K	5′-GTN	dsDNA and ssDNA	639 amino acids	Cas12k;sgRNA;TniQ/TnsB/C transposable enzyme	plasmid	None	100% (fragment length <10kb)	58, 62

CRISPR-mediated base editing tools							
type	Cas based system	type of base transitions	editing window	editing site	the promoter of the fusion protein	efficiency	ref
Containing adenosine base editors (ABEs)	CRISPR/nCas9-mediated	A to G	7 bases	positions 4−10, with PAM at positions 21−23	P_{HemA}, P_{Neo}, or P_{Tau}	60−100%	66, 67
Cytidine base editors (CBEs)	CRISPR/nCas9-mediated	C to T	10 bases	positions 1−8, 12, and 15, with PAM at positions 21−23	P_{HemA}	75−100%	66, 67
Glycosylase base editors (GBEs)	CRISPR/nCas9-mediated	C to G/T	5 bases	positions 3−7, with PAM at positions 21−23	P_{HemA}	C to G 30−80%; C to T 20−70%	65−67

demonstrated to be capable of simultaneously editing multiple genes with an efficiency of 50−90% (Table 2).

6. CONCLUSION

S. meliloti is a significant soil bacterium with the potential to be used for the production of high yields of nutritional chemicals, such as vitamin B_{12}.[7] Many kinds of genetic engineering strategies have been used to investigate the genetic modification of *S. meliloti*. The Tn5-based transposon method allows for the analysis of tens to hundreds of genes simultaneously, which could rapidly narrow down the search for the genes of a particular phenotype. Site-specific recombination systems are highly efficient in deleting small or large gene fragments. Whether the specific sites left after recombination can be recognized by the recombinase determines whether a second or more rounds of operations could be performed to result in expanding the deletion or constructing independent deletions. The above two methods can be applied in combination. The Cre/*loxP* system and Tnp from Tn5 were combined to analyze conditional genetic.[38]

Homologous recombination methods could be employed to engineer *S. meliloti* strain without leaving scars on the genome. However, methods based on endogenous recombinase are generally inefficient due to the weak homologous recombination ability of *S. meliloti*, while the methods based on exogenous recombinase may only work in some strains. The emergence of the CRISPR/Cas system provides a more convenient and efficient genetic manipulation tool for *S. meliloti*. The introduction of CRISPR/Cas12e systems has led to an increase in screening pressure, resulting in enhanced homologous recombination efficiency. The cutting activity and toxicity of the CRISPR/Cas12e system match the homologous recombination ability of *S. meliloti* 320. Base editor systems could simultaneously edit multiple genes without forming double-strand breaks (DSB), and dCas9 itself can be used to suppress gene transcription. The CRISPR-associated transposase system can be used to integrate large fragments into the genome and combines the high efficiency of transposase with the targeting of the CRISPR/Cas12k system. The CRISPR/Cas12k system can be used to suppress gene transcription, as Cas12k has a naturally inactivated endonuclease domain.

The implementation of diverse genetic engineering strategies has facilitated remarkable applications in *S. meliloti*. However, the development of diverse genome editing tools with complementary strategies is essential. The future discovery and characterization of novel genetic engineering strategies could further enhance the toolkit for *S. meliloti* and other Rhizobium species, thereby advancing its applications.

AUTHOR INFORMATION

Corresponding Authors

Huina Dong − *Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; University of Chinese Academy of Sciences, Beijing 100049, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*; Email: dong_hn@tib.cas.cn

Zhaoxia Jin − *School of Biological Engineering, Dalian Polytechnic University, Dalian 116034, China*; Email: jinzx2018@163.com

Dawei Zhang − *Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; University of Chinese Academy of Sciences, Beijing 100049, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*; orcid.org/0000-0002-5857-394X; Email: zhang_dw@tib.cas.cn

Authors

Xuan Liu − *School of Biological Engineering, Dalian Polytechnic University, Dalian 116034, China; Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*

Huiying Wang − *Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*

https://doi.org/10.1021/acssynbio.4c00348
ACS Synth. Biol. 2024, 13, 3497−3506

Xinyi Ren − *Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*

Xia Yang − *Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*

Tingting Li − *School of Biological Engineering, Dalian Polytechnic University, Dalian 116034, China; Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*

Gang Fu − *Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; University of Chinese Academy of Sciences, Beijing 100049, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*

Miaomiao Xia − *Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*

Huan Fang − *Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; University of Chinese Academy of Sciences, Beijing 100049, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China;* orcid.org/0000-0003-0317-5394

Guangqing Du − *Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acssynbio.4c00348

Author Contributions

[#]L.X., D.H., and W.H. contributed equally to this work. L.X., D.H., and W.H. wrote and edited the manuscript. R.X., Y.X., L.T., F.G., X.M., F.H., D.G. provided feedback on the manuscript. J.Z. and Z.D. provided funding support and coordinated project management.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Key R&D Program of China (2022YFC2106100), the National Science Fund for Distinguished Young Scholars (22325807), the National Natural Science Foundation of China (22178372, 32300069, 42177112), the Science Research Project of Liaoning Provincial Education Department (LJKMZ20220882), and the Tianjin Synthetic Biotechnology Innovation Capacity Improvement Project (TSBICIP-KJGG-011 and TSBICIP-CXRC-055).

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https://doi.org/10.1021/acssynbio.4c00348
ACS Synth. Biol. 2024, 13, 3497−3506