Essential Laboratory Techniques: Plasmid Transformation

Expert Insights

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Imprint

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Contents

Editorial

Plasmid transformation is a fundamental technique in life science research that allows for the expression of exogenous DNA in host cells, typically bacterium. To accomplish this, a process of horizontal gene transfer occurs where bacteria take up exogenous DNA, which then express the genetic material. This technique is the underpinning of fields such as molecular biology and protein science, and can be used in the ~~manufacture~~ of pharmaceuticals such as insulin. While this technique does not require a living donor cell (only persistent DNA), not all bacteria are capable of taking up exogenous DNA or do it at a sufficient rate to be tractable in the laboratory. Using chemicals or electrical pulses, however, one can acquire artificially competent bacterial cells. There are a number of different factors that can affect the transformation efficiency, including the DNA used, the choice of microbial media, the type of agar plate, the particular protocol, and the length of time between the transformation reaction and plating. A thorough understanding of this technique, from the reagents to the instruments and precise protocol used, is imperative when trying to generate artificially competent cells and optimize the transformation efficiency. Due to the importance of this fundamental technique for life science research, this booklet provides a thorough overview of plasmid transformation.

First, Riggs *et al*. (2018) in *Current Protocols: Essential Laboratory Techniques* provides an overview of plasmid expression vectors and their corresponding elements, in addition to information on types of host organisms (to guide the selection of the best vector/host combination for a given target protein). Additionally, Riggs et al. details other features that contribute to protein expression, maintenance, or purification. Next, Renzette *et al*. (2011) in *Current Protocols: Microbiology* details two protocols to make chemically competent or electrocompetent *E. coli* cells, discussing important parameters and considerations. Following these protocols, Cheng *et al*. (2022) in Journal of Applied Microbiology describes a rapid transformation method for the anaerobic cellulolytic bacterium *Ruminiclostridium cellulolyticum*. By functionally expressing the gene encoding for CceI methyltransferase, resulting in an *in vivo* methylation system, the transformation efficiency of *R. cellulolyticum* was improved. Next, Seidman *et al*. (2001) in *Current Protocols: Molecular Biology* presents protocols for introducing plasmid DNA into cells via calcium chloride or electroporation. Xu et al. (2020) in *Environmental Microbiology* reviews the type I CRISPR-Cas systems for prokaryotic genome editing. Finally, this booklet is rounded out by a white paper from IKA Werke. Haupt and Wiggenhauser (2022) discuss important considerations for essential laboratory equipment when performing plasmid transformations in *E. coli*.

The studies, protocols, and reviews discussed here focus on the theory, technique and applications of plasmid transformation. Through these article summaries and expert insights, we hope to educate researchers on important considerations and strategies to enhance the efficiency for successful plasmid DNA transformation.

Emily E. Frieben, PhD
Associate Editor, Wiley Interdisciplinary Reviews

Overview of Protein Expression Vectors for *E. coli*

Paul D. Riggs

This article describes the elements of plasmid expression vectors: the promoter, host organism, other expression features, plasmid origin of replication and additional features such as sequences and tags.

Elements of an expression vector

Promoters

The promoter is the defining element of any expression vector since it drives gene transcriptions (i.e. DNA⟶RNA). Moreover, the promoter is typically paired with a ribosome binding site (RBS) that promotes subsequent translation (i.e. RNA⟶protein). Most expression vectors utilize promoters based on historical performance and/or availability, but many alternative promoters can have significant advantages in particular situations. The Lac and T7 promoters are perhaps the most widely utilized promoters; however, other promoters are also frequently employed.

Lac promoter

The Lac promoter and its derivative were the first to control recombinant protein expression. These promoters can be induced with isopropyl ß-D-1-thiogalactopyranoside (IPTG), and mutant (lacUV5) and hybrid (Ptac) Lac promoters have been discovered and constructed. The lacUV5 promoter is about 2× stronger than the wild-type (wt) lac promoter, and Ptac is about 10× stronger than the lacUV5 promoter. These promoters utilize the normal *Escherichia* coli RNA polymerase and can be moved into any *E. coli* strain for expression. Under conditions where the high-level expression may not give the best yield (e.g. aggregation-prone proteins), a weaker promoter may be desirable.

T7 promoter

The most widely used promoter for protein production is the T7 gene 10 promoter. It encodes for the major capsid protein of the phage, and its promoter and RBS provide strong transcription and translation signals for robust protein production. Since the phage T7 encodes for an RNA polymerase that recognizes its own promoters, and the *E. coli* RNA polymerase does not recognize those promoters, it is possible to subclone the gene of interest into a T7 vector (PT7 vector) and transform a host that lacks the T7 RNA polymerase, with little or no protein expression. The plasmid can then be isolated and transformed into a host with an inducible T7 RNA polymerase, thus separating the subcloning step from the production step. This property is useful for proteins that may be toxic to *E. coli*. The strong, reliable expression has made T7 promoter vectors the most popular first choice for *E. coli* protein production. Many PT7 vector variations are commercially and non-commercially available.

Other phage promoters

Promoters from phages other than T7 have gained attention due to their strong activity. For example, the phage lambda (PL) promoter is transcribed by the *E. coli* RNA polymerase and is controlled by the cI phage repressor. A commercially available system for inducing expression with PL vectors employs the pLex system (Invitrogen/ThermoFisher), which can be induced with the addition of tryptophan. Moreover, pQE vectors from Qiagen use the T5 promoter, with dual adjacent lac repressor binding sites, to make it IPTG inducible. The phage T5 promoter is transcribed by the *E. coli* RNA polymerase. Still, many pQE plasmids do not have the lacIq repressor gene, so the compatible plasmid pRep4 carrying lacI or a host with lacIq is required.

Other common promoters

The pBAD series of vectors use the arabinose promoter, which in *E. coli* transcribes genes to utilize arabinose. The induction of the arabinose promoter can produce elevated levels of protein expression, though not as high as the T7, tac, PL or T5 promoters. Many pBAD vectors are based on the pBR322-compatible p15A plasmid origin, making these vectors popular for co-expressing chaperones, secondary subunits or accessory factors, and a protein of interest in another expression vector.

Expression vectors based on the rhamnose promoter are known for their tunability. In *E. coli*, the rhamnose promoter transcribes the genes for rhamnose utilization and is under catabolite repression. At intermediate rhamnose concentrations, the vector stops expressing the protein

of interest when the rhamnose runs out, giving an intermediate product level due to accumulation rather than an intermediate rate of expression.

Other promoters

Other expression vectors that use the tet promoter are inducible with anhydrotetracycline and have occasionally been used for expression in *E. coli*, although they are more popular for mammalian expression. The XylS/Pm promoter is inducible with toluic acid and related compounds and has been used successfully to express some secreted proteins that were poorly expressed using PT7 and lac UV5 vectors. Another set of *E. coli* protein expression vectors was constructed based on the lux quorum-sensing regulon from *Vibrio fischeri*. In this system, bacterial cells secrete a small molecule (e.g. homoserine lactone) that builds up as the population grows, and when cellular receptors for the molecule signal that the population density is high enough, they activate a transcription factor to express certain genes that are advantageous at high cell densities. The lux quorum sensing system allows autoinduction of the protein of interest as the cells approach the stationary phase.

The pDAWN and pDUSK expression vectors use the FixK2 promoter and the YF1 and FixJ genes from *Bradyrhizobium japonicum* to give blue light-controlled expression in *E. coli*. Furthermore, promoters induced by propionate (pPro vectors) and the CAP-cAMP complex (e.g. cstA and cspD promoters) have been developed. These alternative expression systems are mostly used in synthetic biology applications, where optimal protein expression yields are less important than the controlled expression.

Host organisms

Choosing a suitable host for an expression vector is an important part of designing a successful system.

Hosts for T7 promoter vectors: basic strains

The original *E. coli* strain constructed with T7 promoter vectors was BL21(λDE3). It is an *E. coli* B strain that has several properties for producing most heterologous proteins. The combination of high protein production and Lon protease deficiency makes *E. coli* B the first choice for most proteins. Since BL21(λDE3) carries the T7 RNA polymerase on a λDE3 prophage under the control of the lac UV5 promoter, it can be induced with IPTG. One problem researchers encounter with T7 promoter vectors is that the uninduced expression level can sometimes be toxic to *E. coli*.

Another strain similar to BL21(λDE3) is T7 Express (New England Biolabs). It is also an *E. coli* B strain with the same high yield and protease-deficient properties. The major difference from BL21(λDE3) is that the T7 RNA polymerase is integrated into the chromosome at the lac locus under the control of the wt lac promoter instead of on a prophage under the lacUV5 promoter. The induced level of T7 RNA polymerase is slightly lower than in BL21(λDE3), but so are the uninduced expression levels.

Hosts for T7 promoter vectors: tunable expression

There are situations where the highest protein expression is not desirable. In these cases, using intermediate concentrations of the inducer IPTG to get intermediate levels of T7 RNA polymerase is a viable strategy. The Tuner *E. coli* strain (Novagen) is BL21(λDE3) lacY, permitting a more graded expression level in response to intermediate levels of IPTG. The LEMO21(DE3) strain (New England Biolabs) also includes a plasmid that expresses the lysY gene, which indirectly modulates T7 RNA polymerase activity.

Hosts for expression vectors: rare codons

Proteins from foreign organisms are often encoded by genes that use codons that are rarely used in *E. coli.*, leading to low protein yields. While there are several solutions to dealing with rare codons, overexpressing their tRNAs on a plasmid compatible with the expression vector is a viable option. The Rosetta strains (Novagen) contain a plasmid that encodes six (pRARE) or seven (pRARE2) rare codon tRNAs.

Hosts for expression vectors: disulphide-bonded proteins

Proteins with disulphide bonds are generally secreted through the cytoplasmic membrane in prokaryotes and trafficked through the endoplasmic reticulum in eukaryotes. There are two options for expressing disulphide-bonded proteins in E coli: (1) including a signal sequence to export the polypeptide into the periplasm, or (2) using an engineered *E. coli* strain that can form disulphide bonds in the cytoplasm. The Origami strain and its derivatives (Novagen) and SHuffle and its derivatives (New England Biolabs) attenuate the cell's reducing power and improve disulphide bond formation.

Other expression features

Selectable markers

The second element of expression plasmids is the selectable marker, most often an antibiotic resistance marker. Historically, ampicillin resistance is the most utilized; however, kanamycin and chloramphenicol resistance are two other commonly used selectable markers in protein expression plasmids. Other selectable markers are genes that code for resistance to tetracycline, spectinomycin, streptomycin and erythromycin, which bind to the ribosome and inhibit protein synthesis.

Plasmid origin of replication

The origin of replication of an expression vector can have a strong influence on induced protein expression levels, the induction ratio and ease of use. The most common one is the pMB1 origin found in pBR322 and derivatives thereof. The pUC plasmid origin has a higher copy number than derivatives of pMB1. However, the higher copy number of pUC vectors produces lower induction ratios. The p15a origin is compatible with pMB1 and can be maintained together in the same cell, which is useful when co-expressing two proteins. The pSC101 replication origin is also compatible with pMB1 but has a low copy number, making it difficult to isolate plasmid DNA. The pET-coco vectors lower the uninduced level of protein expressed from the T7 promoter in the vector, which attenuates gene expression due to the low gene dosage. Upon induction with arabinose, the *oriV* origin increases the plasmid copy number to 20-50/cell, and IPTG induction of T7 RNA polymerase then overexpresses the gene of interest.

Additional features

Expression vectors often contain additional features that either contribute to their maintenance in the cell, enhance the expression of classes of proteins, and/or aid in the purification of the protein of interest. For example, vectors often include transcription terminators, sometimes upstream of the promoter, to prevent unwanted expression before induction and often downstream from the expressed gene to prevent transcriptional interference with antibiotic resistance or replication. Several other features are available that can increase the utility of the expression vector.

Signal sequences

Some vectors have signal sequences that initiate the export of the protein to the

periplasmic space. Some systems, like the *E. coli* type V secretion system, can secrete proteins into the media. Another system uses the Pet protein, a serine protease autotransporter protein that can release the protein of interest following outer membrane insertion. Secretion into the medium can streamline purification, avoid toxic effects and make continuous production possible.

Affinity tags

Affinity tags are proteins encoded on an expression vector designed to be translationally fused to the protein of interest to aid in purification. Examples include the His-tag, glutathione-S-transferase (GST), maltose-binding protein (MBP), chitin-binding domain (CBD), the Snap-tag, the Flag-tag, calmodulin-binding peptides, the Strep II-tag and biotin acceptor peptide (BAP).

Solubility tags

Solubility tags are proteins or peptide sequences that assist the protein of interest in folding into its native, soluble conformation. Previous studies demonstrated that MBP, N-utilization substance protein A (NusA) and thioredoxin (Trx) enhance the solubility of recombinant proteins. Additionally, small ubiquitin-related modifier (SUMO) has been shown to modify protein function and improve solubility. Other small protein and peptide tags, such as the SET-tag, the FH8-tag, the Skp-tag and the T7PK-tag, can also enhance solubility.

Other small peptide tags

Epitope tags

Epitope tags are peptide sequences fused to proteins that allow immunodetection following protein expression. The four most popular epitope tags are the hemagglutinin-tag (HA-tag), the myc-tag (derived from the c-myc gene product), the Flag-tag and the His-tag. The HA-tag, c-myc-tag and FLAG-tag are primarily detection tags, while the His-tag is a better affinity tag than an epitope tag. Other epitope tags on pET vectors from Novagen include the T7 tag, the S-tag, the HSV-tag, and the Strep II-tag. NusA, CBD, MBP, GST and SUMO could also be used as epitope tags since antibodies against these proteins/peptides are available.

Protease sites

Most expression vectors that express affinity or solubility tags are available with specific protease sites that allow separation of the tag from the protein of interest after purification. Factor Xa, enterokinase or thrombin recognition sites have been shown to remove protein tags effectively; however, both enzymes also can cleave secondary sites. The highly specific tobacco etch virus (TEV) cysteine protease removes tags but leaves an extra amino acid at the N-terminus. Moreover, the rhinovirus 3C protease is quite specific, and a GST-tagged version of it, called PreScission protease, is available (GE Life Sciences).

Conclusion

The choice of expression vector and host can profoundly affect the success of producing a recombinant protein in *E. coli*. The information in this article can be used to select the best vector/host combination for a particular protein and determine whether other elements could facilitate protein production and purification.

Digest of
Riggs, PD. Overview of protein expression vectors for E. coli. Current Protocols Essential Laboratory Techniques, 17, e23. DOI: 10.1002/cpet.23.

Generation of Transformation Competent *E. coli*

Nicholas Renzette

This unit describes two protocols to make competent E. coli cells. Basic Protocol 1 describes the preparation of chemically competent cells, and Basic Protocol 2 describes the preparation of electrocompetent cells. The competent cells made using either protocol are suitable for transformation with exogenous linear or double-stranded DNA. Electrotransformation is more efficient than chemical transformation, but the latter does not require an electroporator, which is not always available.

The rubidium chloride method for preparing chemically competent cells is more laborious than the calcium chloride method but tends to produce higher transformation efficiencies, especially with larger plasmids. Thus, competent cells prepared with this method can be used in various applications.

Preparation of chemically competent cells

The rubidium chloride method for making chemically competent *E. coli* cells involves a two-step process of centrifugation and resuspension in buffers with decreasing concentrations of rubidium chloride. This protocol yields approximately 12 100-µl aliquots of competent cells.

Protocol

1. Inoculate 2 ml of LB broth with a single colony of the appropriate E. coli strain. Grow the culture overnight at 37°C. For most strains, antibiotics should not be added to the LB broth. However, if the strain harbors a pre-existing resistance cassette, media with the appropriate antibiotic(s) can be used.
2. Dilute 20 µl of the overnight culture into 10 ml of LB broth. Grow at 37°C with shaking to an OD600 = 0.3 (early log phase). The volume of the culture can be adjusted depending on the amount of competent cells desired. Scale buffer quantities appropriately.
3. Transfer the culture into 50-ml centrifuge tubes and incubate in an ice-water bath for 20 min at 4°C. Note: While the culture is on ice, prechill the centrifuge by running it for 10 min at 2000× g at 4°C.
4. Centrifuge the cells for 10 min at 2000× g at 4°C.
5. Decant the supernatant. Resuspend the cell pellet in 3.3 ml of TB-I and gently swirl the tube in an ice-water bath to resuspend the cells. Gentle, repeated pipetting of the cells can also be performed if necessary.
6. Incubate the cell resuspension in an ice-water bath for 2 hr. After 100 minutes, prechill the centrifuge as described in Step #3.
7. Centrifuge the cells for 10 min at 2000× g at 4°C.
8. Decant the supernatant. Resuspend the cells in 1.1 ml of TB-II.
9. Aliquot 100-µl of the cell suspension into rechilled 1.5-ml microcentrifuge tubes. The cells can be used immediately or stored at –80°C.

Electrocompetent cells

Protocol

1. Inoculate 2 ml of LB broth with a single colony of the appropriate E. coli strain. Grow the culture overnight at 37°C.
2. Dilute 200 µl of overnight culture into 100 ml LB broth. Grow at 37°C with shaking to an OD600 = 0.3 (early log phase). Note: While the culture is growing, prechill the centrifuge by running it for 10 min at 2000× g at 4°C. The volume of the culture can be adjusted depending on the amount of competent cells desired. Adjust the volumes of 10% glycerol accordingly in later steps.
3. Transfer the culture into 250-ml centrifuge tubes. Centrifuge the cells for 10 min at 2000× g at 4°C.
4. Remove the supernatant. Gently resuspend the pellet in 1× vol of 10% glycerol. This volume depends on the culture volume used in Step #2.
5. Centrifuge the cells for 10 min at 2000× g at 4°C.

6. Remove the supernatant. Gently resuspend the pellet in 1× vol of 10% glycerol.
7. Centrifuge the cells for 10 min at 2000× g at 4°C.
8. Decant the supernatant. Gently resuspend the pellet in 1 ml of 10% glycerol. Note: Remove as much of the supernatant as possible in this step and after the next centrifugation to remove residual ions.
9. Centrifuge the cells for 10 min at 2000× g at 4°C.
10. Remove the supernatant. Resuspend the pellet in 500 µl of 10% glycerol.
11. Aliquot 50-µl of the cell suspension into re-chilled 1.5-ml microcentrifuge tubes. The cells can be stored at -80°C.

Note: chemically competent and electrocompetent cells can be stored indefinitely at -80°C under stable temperature conditions. There will be a minor loss of transformation efficiency with time, but this is inconsequential for short storage periods (e.g. <2 years).

Reagents and solutions

Use deionized, distilled water in all recipes and protocol steps.

Glycerol, 10% (v/v)

Dilute 1 vol glycerol in 9 vol ddH2O
Filter sterilize
Store for up to 1 year at 4°C

Luria-Bertani broth (LB broth)

10 g tryptone
5 g yeast extract
10 g NaCl
Bring volume to 1000 ml with ddH20
Sterilize immediately by autoclaving
Store for up to 6 months at room temperature

Transformation buffer I (TB-I)

30 ml 1 M potassium acetate (60 mM final)
40 ml 1 M MnCl2 (80 mM final)
50 ml 1 M RbCl (100 mM final)
5 ml 1 M CaCl2 (10 mM final)
75 ml 15% (v/v) glycerol
Adjust volume to 500 ml with ddH2O
Filter sterilize
Store for up to 1 year at 4°C

Transformation buffer II (TB-II)

5 ml 1M MOPS, pH 7.0 (10 mM final)
5 ml 1 M RbCl (10mM final)
50 ml 1 M CaCl2 (100 mM final)
75 ml 15% (v/v) glycerol
Adjust volume to 500 ml with ddH2O
Filter sterilize
Store for up to 1 year at 4°C

Commentary

Background information

The transformation efficiency and stability of the exogenous nucleic acid after transformation depend on the choice of *E. coli* strain for making competent cells. The protocol described here will make all *E. coli* strains competent for nucleic acid uptake. DH5α is a widely used strain for standard plasmid cloning due to high transformation efficiencies and blue-white screening. XL-1 Blue (and related XL strains) have a similar genotype to DH5α and permit blue-white screening. DH10B is a suitable strain for transforming methylated DNA from eukaryotic cells. Additionally, the BL21(DE3) strain and derivatives contain a prophage (λDE3) encoding an IPTG-inducible T7 RNA polymerase, which is useful for overexpressing proteins from exogenous plasmids.

Anticipated results

For chemically competent cells, transformation efficiencies should be 10^7 10^8 CFU/µg of small plasmid DNA (such as pUC18 or pUC19), and for electrocompetent cells, efficiencies of $\geq 10^{10}$ CFU/µg of small plasmid DNA. When transforming larger DNA molecules or exogenous DNA expressing products that reduce the viability of the recipient strain, attenuated efficiencies can occur.

Critical parameters

Regardless of the method, the temperature of the cells must be maintained at 4°C after the initial cooling (i.e. Step #3 in both protocols).

Time considerations

Wild-type *E. coli* in LB broth take ~2 hr to reach early log phase. However, different media, such as glucose minimal medium or altered strain genetics, can increase this time. Thus, it is important to monitor the culture's OD regularly. After reaching early log phase, it takes about 3 hr to prepare chemically competent cells. The preparation time of electrocompetent cells is around 1 hr.

Digest of
Renzette, N. Generation of transformation competent E. coli. Current Protocols in Microbiology, A.3L.1, August 2011. DOI: 10.1002/9780471729259.mca03ls22.

Development of an *in vivo* methylation system for transformation of *Ruminiclostridium cellulolyticum*

Ying Cheng, Yuanyuan Jiang, Zhenxing Ren *et al*.

This study aims to develop a rapid transformation method for R*uminiclostridium cellulolyticum*. This anaerobic cellulolytic bacterium secretes abundant multi-enzymatic complexes called cellulosomes that can degrade plant cell components. This bacterium is a promising host for producing renewable green chemicals from cellulose, including lignocellulose biofuel production.

Previous studies showed that in *R. cellulolyticum*, the CceI restriction system protects against foreign DNA, thus, limiting its utility for genetic engineering tools. This study functionally expressed the gene encoding CceI methyltransferase (M.CceI) of *R. cellulolyticum* H10 in E. coli, resulting in an in vivo methylation system for improving the transformation efficiency of *R. cellulolyticum*.

Materials and Methods

The bacterial strains used included *E. coli* DH5α, JM109, Top10 and HSTT08 and wild-type *R. cellulolyticum*. The plasmids used included pMTC6 (shuttle vector with a fluorescent protein gene), pET28a-M.CceI, pGEX-M.CceI pSY6 and pSY6-CipUTR. The culture media were supplemented with ampicillin, kanamycin or erythromycin when required. Crude protein extracts were analyzed by agarose gel electrophoresis to characterize the restriction and methylation specificity.

Standard molecular biology techniques were utilized for plasmid construction. Briefly, M.CceI was amplified by PCR, yielding a 1251 bp fragment (**Fig. 1**). A PthI promoter was fused to M.CceI by single overlap extension (SOE). The expression cassette was restriction digested and cloned into pET28a, generating pET28a-M.CceI. For targeted disruption of the 5'UTR of the cip-cel operon, the targeting region identified by SOE PCR was restriction digested and cloned into the Clostron plasmid pSY6, generating pSY6-CipUTR.

For M.CceI protein overexpression, an M.CceI fragment was restriction digested and cloned into pGEX-6P-1, generating pGEX-M.CceI. HST08 cells containing pGEX-M.CceI were grown in LB medium containing ampicillin. Overexpression of the M.CceI fused to GST was induced by adding IPTG and incubating the cultures at 16°C until OD600 = 0.6. Cells were harvested by centrifugation and lysed by sonication. The soluble protein fraction was applied to a GST-NTA column and eluted with reduced glutathione.

Before electrotransforming *R. cellulolyticum*, the plasmid DNAs were methylated in vitro by commercially available M.MspI or in vivo by recombinant M.CceI. Electrotransformation was performed as described by others, and transformation efficiency was evaluated by counting the colonies grown on erythromycin-containing agar plates.

The 5'-UTR of the cip-cel operon in *R. cellulolyticum* was disrupted by using pSY6-CipUTR. The disruption was confirmed by colony PCR yielding a 1400 kb product using a specific primer set. For plasmid curing, the mutant harboring pSY6-CipUTR was successively inoculated and cultivated in liquid or solid GS-2 medium without erythromycin several times until erythromycin resistance was lost.

The abundance of the cipC transcript via quantitative reverse transcription-PCR (qRT-PCR) was measured to analyze the transcription of the cip-cel operon in the mutant. The qRT-PCR data were normalized to the abundance of Ccel_0312 transcript encoding beta-subunit of DNA-directed RNA polymerase.

Results

The approach outlined in this manuscript demonstrates that with the pMTC6 *E. coli-Clostridium* shuttle plasmid, this approach can result in a transformation efficiency of 2.6 × 103±0.23 × 103

Figure 1

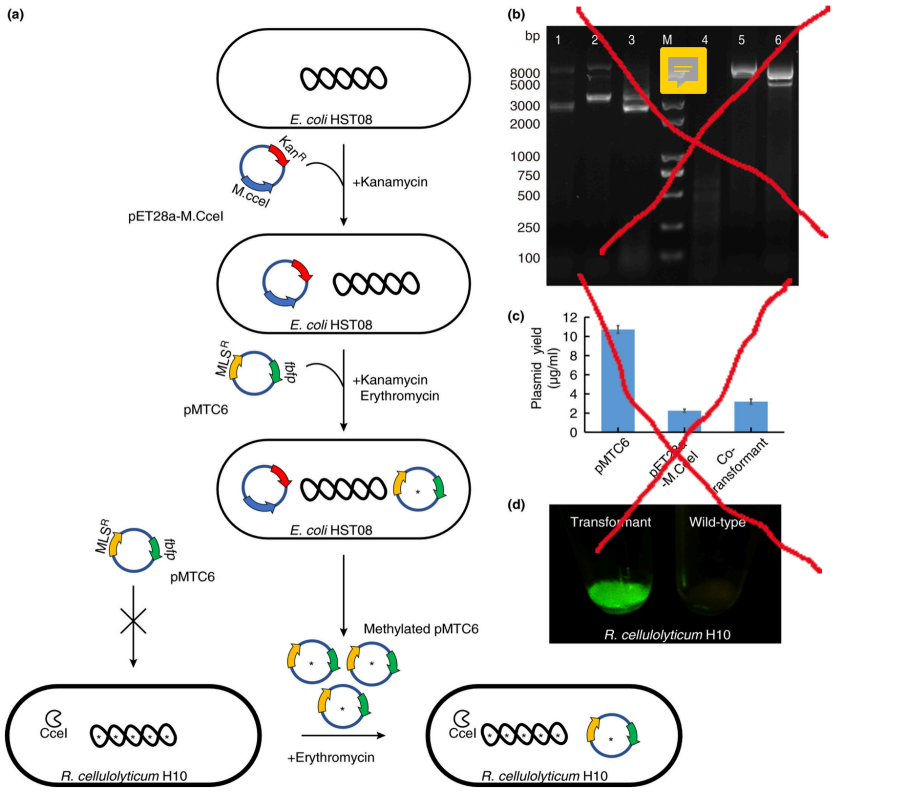

Fig. 1: *Schematic of in vivo methylation and transformation of pMTC6 into Ruminiclostridium cellulolyticum H10.*

CFU/μg plasmid DNA in R. *cellulolyticum* H10 cells. This system could confer the M.CceI-specific DNA methylation pattern to its resident plasmid, making it resistant to the CceI restriction and facilitating transfer into *R. cellulolyticum*.

Discussion

In this study, we generated an in vivo methylation system of *R. cellulolyticum*, allowing interspecies DNA transfer and improving transformation efficiency. It is plausible that metabolically engineered *R. cellulolyticum* could augment cellulose-based biofuel production.

Digest of
Cheng Y, Jiang Y, Ren Z. et al. Development of an in vivo methylation system for transformation of Ruminiclostridium cellulolyticum. Journal of Applied Microbiology 2022 Mar;132(3):1926-1935. DOI: 10.1111/jam.15367.

Introduction of Plasmid DNA into Cells

Christine E. Seidman, Kevin Struhl, Jen Sheen, Timm Jessen.

Basic Protocol 1 — Transformation using calcium chloride

Prepare competent cells

1. Inoculate 50 ml LB medium with a single colony of *E. coli* cells. Grow overnight at 37°C with moderate shaking.
2. Dilute 4 ml of the culture in 400 ml LB medium in a sterile 2-liter flask. Grow at 37°C, with shaking, to an OD590 = 0.375 (early- or mid-log phase).
3. Aliquot the culture into eight 50-ml prechilled, sterile polypropylene tubes and chill on ice for 5-10 min. Cells should be kept cold for all subsequent steps.
4. Centrifuge the cells for 7 min at 1600× g at 4°C.
5. Decant the supernatant and gently resuspend each pellet in 10 ml ice-cold CaCl2 solution.
6. Centrifuge the cells for 5 min at 1100× g at 4°C. Discard the supernatant and resuspend each pellet in 10 ml ice-cold CaCl2 solution. Incubate the resuspended cells on ice for 30 min.
7. Centrifuge the cells for 5 min at 1100× g at 4°C. Discard the supernatant and resuspend each pellet in 2 ml ice-cold CaCl2 solution.
8. Aliquot 250-µl aliquots of the cell suspension into prechilled, sterile polypropylene tubes. Freeze immediately at −70°C.

Assess the competency of cells

9. Transform 100 µl of competent cells with 10 ng of pBR322 following steps #11-16 below. Plate 1, 10, and 25 µl of the transformation culture on LBAMP plates and incubate at 37°C overnight.
10. Calculate the number of transformant colonies per aliquot volume (µl) × 105: This is equal to the number of transformants per microgram of DNA.

Transform competent cells

11. Aliquot 10 ng of DNA (10-25 µl) into a sterile 15-ml round-bottom test tube and place on ice.
12. Rapidly thaw the competent cells and dispense 100 µl immediately into the DNA-containing test tubes. Gently swirl tubes and place them in ice for 10 min.
13. Heat shock the cells by placing tubes into a 42°C or 37°C water bath for 2 or 5 min, respectively.
14. Add 1 ml LB medium to each tube. Incubate 1 hr at 37°C on a roller drum.
15. Plate aliquots of transformation culture on appropriate plates.
16. Allow plates to dry and incubate at 37°C for 12-16 hr.

Alternate Protocol 1 — One-step preparation and transformation of competent cells

Protocol

1. Dilute a fresh overnight culture of bacteria 1:100 into LB medium and grow at 37°C until OD600 = 0.3-0.4.
2. Add an equal volume of ice-cold 2× TSS to the cell suspension and gently mix on ice. To use frozen cells for transformation, thaw them slowly and then use them immediately. Pelleting the cells by centrifugation at 1000× g for 10 min, 4°C may increase transformation frequency. Discard the supernatant and resuspend the cell pellet at one-tenth of the original volume in 1× TSS. Proceed with transformation as in step #3.
3. Add 100 µl competent cells and 1-5 µl DNA (0.1-100 ng) to an ice-cold polypropylene or glass tube. Incubate at 4°C for 5-60 min.
4. Add 0.9 ml LB medium containing 20 mM glucose and incubate at 37°C for 30-60 min with mild shaking to allow expression of the antibiotic resistance gene. Select transformants on appropriate plates.

Basic Protocol 2 — High-efficiency transformation by electroporation

Prepare the cells

1. Inoculate a single colony of *E. coli* cells into 5 ml LB medium and grow at 37°C 5 hr to overnight with moderate shaking.
2. Inoculate 2.5 ml of the culture into 500 ml LB medium in a sterile 2-liter flask. Grow at 37°C, with shaking, to an OD_{600} = 0.5-0.7.
3. Chill cells in an ice-water bath for 10-15 min and transfer them to a prechilled 1-liter centrifuge bottle. Cells should be kept at 2°C for all subsequent steps.
4. Centrifuge cells 20 min at 4200 rpm in Beckman J-6M rotor, 2°C.
5. Decant the supernatant and resuspend the pellet in 5 ml ice-cold water. Add 500 ml ice-cold water and mix well. Centrifuge cells as in step #4.
6. Decant supernatant immediately and resuspend the pellet by swirling in the remaining liquid. The pellet can be made tighter by substituting ice-cold sterile HEPES (1 mM, pH 7.0) for the ice-cold water in step #5.
7. 7. Add another 500 ml ice-cold water, mix well, and centrifuge again as in step #4.
8. Decant supernatant immediately and resuspend the pellet by swirling in the remaining liquid.

9a. If fresh cells are to be used for electroporation, place

suspension in a prechilled, narrow-bottom, 50-ml polypropylene tube and centrifuge at 4200 rpm for 10 min at 2°C. Estimate the pellet volume (usually ~500 µl from a 500-ml culture) and add an equal volume of ice-cold water to resuspend cells (on ice). Aliquot 50 to 300 µl cells into prechilled microcentrifuge tubes.

9b. If frozen cells are to be used for electroporation, add 40 ml ice-cold 10% glycerol to the cells and mix well. Centrifuge cells as described in step #9a. Estimate the pellet volume and add an equal volume of ice-cold 10% glycerol to resuspend cells (on ice). Place 50- to 300 µl aliquots of cells into prechilled microcentrifuge tubes and freeze on dry ice. Store at –80°C.

Transform the cells

10. Set the electroporation apparatus to 2.5 kV, 25 µF. Set the pulse controller to 200 or 400 ohms.
11. Add 5 pg to 0.5 µg plasmid DNA/µl to tubes containing fresh or thawed cells(on ice). Mix by tapping the tube or by swirling the cells with the pipettor.
12. Transfer the DNA and cells into a cuvette that has been chilled for 5 min on ice, shake slightly to settle the cells to the bottom, and wipe the ice and water from the cuvette with a Kimwipe.
13. Place the cuvette into the sample chamber.
14. Apply the pulse.
15. Remove the cuvette. Immediately add 1 ml SOC medium and transfer to a sterile culture tube with a Pasteur pipet. Incubate for 30-60 min with moderate shaking at 37°C.
16. Plate aliquots of the transformation culture on LB plates containing antibiotics.

Alternate Protocol 2 — Direct electrophoretic transfer of plasmid DNA from yeast into *E. coli* Protocol

1. Prepare electrocompetent KC8 cells (see Basic Protocol 2, steps 1 to 9a), resuspending the final cell pellet in ice-cold water to obtain an OD_{600} = 100. Fresh KC8 cells work better in this electroporation method than frozen ones.
2. Aliquot 65-µl of the electrocompetent *E. coli* KC8 cells into ice-cold microcentrifuge tubes.
3. Scrape off ~10 µl of yeast from a streak colony of EGY48 harboring the respective "prey" plasmid derivative of pJG4-5 and grown on Gal/Raff/Xgal/CM plates. Resuspend the yeast cells in the KC8 suspension by swirling the stick used for scraping off the cells. Keep the microcentrifuge tube on ice as much as possible. Do not vortex.
4. Set the electroporation apparatus to 1.5 kV, 25 µF, and the pulse controller to 100 ohms. Transfer the cell suspension into a 0.2-cm cuvette that has been chilled for 5 min on ice, shake slightly to settle the cells to the bottom, and wipe the ice and water from the cuvette with a Kimwipe. Pasteur pipettes will facilitate placing the cell suspension at the bottom of the cuvette. Avoid any air bubbles.
5. Place the cuvette in the sample chamber of the apparatus and pulse. Take the cuvette out and place it on ice for ≥45 sec. The expected time constant for the first pulse is 2.2 – 2.4 msec.
6. Set the electroporation apparatus to 2.5 kV, 25 µF, and the pulse controller to 200 ohms. Wipe the cuvette, place it in the sample chamber, and pulse. The expected time constant for the second pulse is 4.2 – 4.8 msec.
7. Remove the cuvette, immediately add 1 ml LB medium, and transfer the suspension into a microcentrifuge tube. Incubate for 45 min at room temperature.
8. Spread 150 µl of the suspension evenly onto M9 minimal medium plates containing 100 µg/ml ampicillin, leucine, histidine, and uracil. Incubate ≥24 hr at 37°C.
9. Pick a single KC8 colony and inoculate 1.5-5 ml M9 minimal medium (Leu^+, His^+, Ura^+, 100 µg/ml Amp) or LB (100 µg/ml Amp) and grow at 37°C. Harvest at an appropriate OD.

Commentary

Basic Protocol 1 described here provides good transformation efficiencies, permits long-term storage of competent cells, and is relatively uncomplicated.

Alternate Protocol 2 presents an application of electrophoretic transformation whereby a "shuttle vector" can be directly transferred between two species. Shuttle vectors have become increasingly popular in recent years, with those that facilitate the transfer of plasmid DNA between yeast and *E. coli* being particularly useful. Moreover, Alternate Protocol 2 bypasses the need for plasmid isolation and offers a one-step method to obtain the same result.

Critical parameters

Calcium transformation

In Basic Protocol 1, the preparation of competent cells with a high transformation efficiency is thought to depend on (1) harvesting bacterial cultures in the logarithmic phase of growth, (2) keeping cells on ice throughout the procedure, and (3) prolonged CaCl2 exposure.

Transformation by electroporation

The best results are obtained when cells are harvested at an OD_{600} = 0.5-0.6. In general, successful electroporation of *E. coli* requires long, strong pulses. The SOC medium must be added immediately after electroporation. In the described procedure, the number of transformants increases linearly with input DNA over a very wide range (from 5 pg to 500 ng) without affecting the transformation efficiency significantly.

Anticipated results

Calcium and one-step transformation In Basic Protocol 1, transformation efficiencies of 106-108 should be obtained with *E. coli* MC1061 or DH1. In Alternate Protocol 1, transformation frequencies should range from 10^6 – 10^7 colonies/µg DNA.

Transformation by electroporation

Using Basic Protocol 2, efficiencies of 10^9 – 10^{10} transformants/µg have been obtained with super pure pUC19 DNA, homemade pUC18 DNA and cDNA libraries in MC1061/P3. Using Alternate Protocol 2, 50-200 KC8 colonies were obtained per plate when 150 µl of the 1 ml LB suspension was employed.

Digest of
Christine E. Seidman, Kevin Struhl, Jen Sheen, Timm Jessen. Introduction of plasmid DNA into cells. Current Protocols in Molecular Biology, Supplement 37. DOI: 10.1002/0471142727.mb0108s37

Harnessing the type I CRISPR-Cas systems for genome editing in prokaryotes

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CRISPR-Cas systems constitute the adaptive immune system of prokaryotes and are found in nearly half of bacterial genomes and the majority of archaeal genomes. There are two general classes. Class 1 systems employ a multi-subunit effector complex to interfere with DNA/RNA and include type I, type III, and rare type IV systems with the signature proteins Cas3, Cas10, and DinG, respectively. On the other hand, class 2 systems are distinguished by one single effector module, and further divided into type II, type V and type VI with signature nucleases Cas9, Cas12, and Cas13, respectively.

Class 2 CRISPR-Cas systems have been reprogrammed extensively for genome editing and various genetic applications. However, their applications in prokaryotes are limited since the heterologous Cas9-based method relies on the efficient delivery of two plasmids and optimal expression of the two modules, making it difficult to control DNA homeostasis in microbial cells. Moreover, it has been reported that overexpressing large, multi-domain nucleases (e.g. Cas9 or Cas12a) in certain genotypes is cytotoxic. Thus, CRISPR-Cas systems belonging to other classes and types and implementing additional reprogramming strategies with application potentials that are not accessible with the heterologous Cas9 systems must be explored.

Single-effector class 2 CRISPR-Cas systems constitute only 10% of all CRISPR-Cas systems identified. The remaining 90% belongs to the multimeric class 1 system (Makarova et al., 2017), with the type I CRISPR-Cas system being the most common type in bacteria and archaea. In recent years, repurposing these widespread, endogenously encoded CRISPR-Cas systems for 'built-in' genome editing is emerging as a promising genetic manipulation strategy in prokaryotes. This approach represents the only available genetic tool for functional genomics investigations in certain genetically recalcitrant organisms or strains. This progress review describes the general workflow of CRISPR-based genetic toolkits and summarizes their establishment in many prokaryotes by harnessing the most widespread, diverse type I CRISPR-Cas systems in their genomes. The review also discusses factors affecting this editing platform's success and efficiency and troubleshooting.

CRISPR-Cas immunity

CRISPR-Cas systems belonging to the different classes and types share a similar adaptive immune process but differ in terms of the Cas proteins involved, the architecture of the crRNA and the fashion of DNA interference. The process involves three stages: (1) adaptation, (2) crRNA processing, and (3) interference. During the adaptation stage, a DNA fragment from an invading foreign genetic element is acquired and incorporated into the CRISPR array by the conserved Cas1 and Cas2 proteins to generate a new spacer. During the crRNA processing stage, the CRISPR array is transcribed into a long pre-crRNA driven by the leader element. Then, the pre-crRNA is processed into mature crRNAs by an RNase of the Cas6e family in the type I-E (Cas5 in the type I-C) or RNase III and Cas9 in the type II-A system. During the interference stage, PAM sequences are scanned for potential targets by a multiprotein Cascade (type I-E) or Cas9 bound by the crRNA (type II-A). Complementary base pairing between the crRNA and the target strand forms a triple-stranded R-loop structure which recruits or drives the Cas effectors to break the DNA target, resulting in single-strand DNA degradation (type I-E) or double-strand DNA break (DSB) (type II-A).

Figure 1

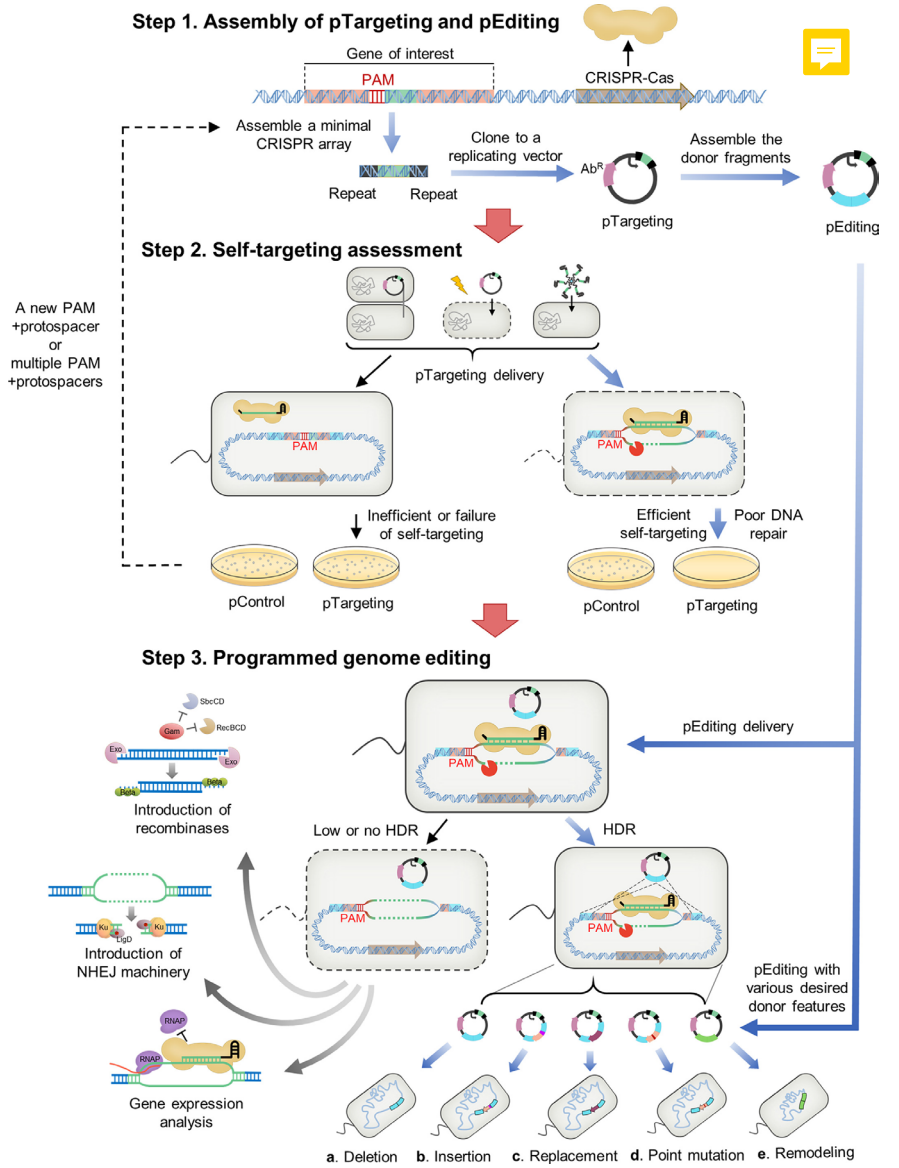

Fig. 1: *Figure legend Fig. 1: Principles and workflow for harnessing the native type I CRISPR-Cas system for genome editing. In the first step, a target sequence containing a PAM and a protospacer within the gene of interest is selected and a targeting plasmid (pTargeting) is assembled to validate the self-targeting activity. In the second step, pTargeting is introduced into the cell. In the presence of a functional native CRISPR-Cas system, a DNA interference Cascade is formed and cause self-targeting of the host genome, resulting in cell death and consequently failure of colony recovery. If inefficient or failure of self-targeting occurs, a new target or multiple targets can be selected for self-targeting assay. In the last step, an editing plasmid (pEditing) is generated by assembling donor fragments into pTargeting and then delivered into the host cell to achieve programmed genome editing through HR-mediated DSB repair. Introduction of exogenous recombinases or NHEJ machinery can potentially increase the editing efficiency if endogenous HDR is insufficient. CRISPR-based gene regulation (eg. CRISPRi) provides as an alternative strategy for functional genomics if the host cell has inefficient capacity for DSB repair.*

General workflow of repurposing the native type I CRISPR-Cas systems for genome editing

In the first step, a target sequence containing a PAM and a protospacer within the gene of interest is selected, and a targeting plasmid (pTargeting) is assembled to validate the self-targeting activity (**Fig. 1**). In the second step, pTargeting is introduced into the cell. In the presence of a functional native CRISPR-Cas system, a DNA interference Cascade is formed and causes self-targeting of the host genome, resulting in cell death and the inability to recover the colony. If inefficient or failure of self-targeting occurs, a new target or multiple targets can be selected for self-targeting assay. In the last step, an editing plasmid (pEditing) is generated by assembling donor fragments into pTargeting and then delivering them to the host cell to edit the genome editing via HR-mediated DSB repair. The introduction of exogenous recombinases or NHEJ machinery can increase the editing efficiency if endogenous HDR is insufficient. Furthermore, CRISPR-based gene regulation (e.g. CRISPRi) provides an alternative strategy for functional genomics if the host cell has an inefficient DSB repair capacity.

Factors affecting the efficiency of CRISPR-based genome editing and corresponding solutions

Editing plasmid and its delivery

Although the native CRISPR-based genome-editing system is simpler than the

Cas9-based editing platform and can be conveniently established once the PAM, spacer and repeats of the native CRISPR-Cas system are determined, the platform still relies on the introduction of a minimum of one plasmid to deliver the programmed mini-CRISPR and donor template. A replicating plasmid expresses the mini-CRISPR and simultaneously provides the donor sequence for homologous recombination in the strain of interest.

The plasmid delivery method is also important for the success of genome editing. Transformation is the commonly employed method in bacteria for plasmid delivery. However, conjugation, a DNA transfer process that occurs in the natural environment through direct contact of a donor cell to a recipient cell with broad host ranges, has also been reported. One conjugation system called XPORT efficiently transferred DNA in 35 different Gram-positive genotypes and directly transformed undomesticated soil bacteria. Moreover, nanoparticle-conjugated crRNA and donor DNA can be directly delivered into prokaryotic cells to bypass the requirement of species-compatible plasmids and overcome poor transformation efficiency.

Capacity of DNA repair

Many prokaryotes have poor frequencies of HR to cope with the highly efficient CRISPR-Cas-induced DNA breakage. This problem can often be solved by adding recombination systems which can augment the genome-editing efficiency in combination with CRISPR-Cas systems. Alternatively, controlling mini-CRISPR expression in pEditing by including an inducible promoter also helps with the rate of DNA breakage and HR-directed DNA repair.

The length of the donor template also affects the HR process. Longer donors generally result in a higher recombination frequency than shorter ones. Although NHEJ is not common in prokaryotes, recently, studies have demonstrated the successful application of NHEJ in *E. coli* and Mycobacterium tuberculosis for deleting large chromosome fragments in combination with CRISPR-Cas cleavage. Lastly, CRISPR interference (CRISPRi) can be considered an alternative strategy to investigate gene functions via transcriptional repression if the recombination capacity cannot achieve the desired genome editing.

False positive colonies

Sometimes false positive colonies with un-edited genotypes are recovered with high frequency. This phenomenon can be avoided by employing a dual selection system in the editing plasmid. The emergence of false positive colonies may also be caused by mutations in the plasmid-encoded spacers or chromosome-encoded Cas machinery. This deficiency can be detected by including a self-targeting plasmid (without the repair donor) as a control in the editing reaction setup and verified by sequencing the spacer sequence and cas genes.

Occurrence of anti-CRISPR elements and R-M systems

Prophages frequently carry anti-CRISPR genes that suppress the CRISPR-Cas immune system of bacterial hosts, limiting the exploitation of native CRISPR-Cas systems for genome editing. A group of conserved genes named *aca* was found to be frequently adjacent to the anti-CRISPR genes. Aca proteins are autoregulators that repress the transcription of anti-CRISPR genes, and their overexpression acts as a novel 'anti-anti-CRISPR' strategy. The co-occurrence of other DNA targeting modules, such as the R-M system, was also found to perturb the efficiency of I-F CRISPR-based genome editing; thus, eliminating or inactivating DNA targeting modules in these strains is necessary to improve editing efficiency.

Future perspectives

Transformation of an 'all-in-one' plasmid containing a miniCRISPR and a repair donor could generate mutations in a prokaryotic host once its native CRISPR-Cas system is characterized. Harnessing the native CRISPR-Cas system represents a promising and sometimes the only strategy for editing the genomes of genetically recalcitrant species.

These systems provide valuable tools for functional genomics of widely isolated, non-model strains and promote the dissecting and engineering of clinical, environmental and industrial microbial species. However, half of the bacteria are CRISPR-free, and the native CRISPR-Cas systems in certain strains degenerate without interference activity, thus, reducing the utility of native CRISPR-Cas systems. Developing transferable type I CRISPR-Cas systems could facilitate DNA introduction into different hosts. The remarkable diversity of CRISPR-Cas systems in prokaryotes offers tremendous opportunities for various applications in addition to genome editing.

Digest of
Xu Z, Li Y, Li M et al. Harnessing the type I CRISPR-Cas systems for genome editing in prokaryotes. Environmental Microbiology (2021), 23(2): 542–558. DOI: 10.1111/1462-2920.15116.