Biotechnology Journal
www.biotechnology-journal.com

CRISPR with a Happy Ending: Non-Templated DNA Repair for Prokaryotic Genome Engineering

*Max Finger-Bou, Enrico Orsi, John van der Oost, and Raymond H. J. Staals**

The exploration of microbial metabolism is expected to support the development of a sustainable economy and tackle several problems related to the burdens of human consumption. Microorganisms have the potential to catalyze processes that are currently unavailable, unsustainable and/or inefficient. Their metabolism can be optimized and further expanded using tools like the clustered regularly interspaced short palindromic repeats and their associated proteins (CRISPR-Cas) systems. These tools have revolutionized the field of biotechnology, as they greatly streamline the genetic engineering of organisms from all domains of life. CRISPR-Cas and other nucleases mediate double-strand DNA breaks, which must be repaired to prevent cell death. In prokaryotes, these breaks can be repaired through either homologous recombination, when a DNA repair template is available, or through template-independent end joining, of which two major pathways are known. These end joining pathways depend on different sets of proteins and mediate DNA repair with different outcomes. Understanding these DNA repair pathways can be advantageous to steer the results of genome engineering experiments. In this review, we discuss different strategies for the genetic engineering of prokaryotes through either non-homologous end joining (NHEJ) or alternative end joining (AEJ), both of which are independent of exogenous DNA repair templates.

1. Introduction

Climate change, growing world population, and scarcity of resources are issues gaining increasing attention from society and from the research community. Consequently, developing strategies for decoupling economic growth from the emission of greenhouse gases has become a pressing issue.[1] Biotechnology, at the core of the emerging concept of bio-economy, aims to facilitate the replacement of petroleum-based chemical synthesis by biocatalysis in which microbial cell factories convert renewable feedstocks into a wide range of products.[2–4] These sustainable products can be high value molecules such as pharmacologically active compounds,[5] but also cheaper commodity chemicals[6] and biofuels.[7] While harnessing microbial production for the pharmaceutical and nutraceutical sectors is already a reality,[8–10] the replacement of many petroleum-based commodity chemicals with their greener counterparts is still to be realized. The available microbial engineering tools, as well as our understanding about their precise molecular workings, are still often the bottleneck for the optimization of microbial cell factories. In cases where homologous recombination is inefficient, template-independent DNA repair represents an attractive alternative for prokaryotic genome editing. In this mini-review, we provide a concise summary of the two known prokaryotic template-independent end joining pathways, and we elaborate on different strategies to employ clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (CRISPR-Cas) systems and other nucleases in combination with these native or heterologously expressed DNA repair pathways. We hope our work will encourage researchers to explore the emerging field of non-templated prokaryotic engineering.

M. Finger-Bou, Prof. J. van der Oost, Dr. R. H. J. Staals
Laboratory of Microbiology
Wageningen University and Research
Wageningen 6708 WE,, The Netherlands
E-mail: raymond.staals@wur.nl

E. Orsi
Bioprocess Engineering
Wageningen University and Research
Wageningen 6708 PB The Netherlands

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/biot.201900404

DOI: 10.1002/biot.201900404

2. CRISPR-Cas Systems, User-Friendly Tools for Microbial Engineering

Although several strategies have been applied for microbial genome engineering with moderate success before the rise of the CRISPR-Cas tools, there are certain drawbacks associated with their use. Allelic exchange, based on the introduction of positive and/or negative markers through homologous recombination, is often reported to carry high levels of false positive mutants, particularly in the case of negative selection

markers.[11,12] An elegant alternative to introduce point mutations as well as big deletions and insertions is recombineering, which typically exploits bacteriophage proteins to mediate genome manipulations.[13] While these enzymes increase the efficiency of in vivo recombination, the heterologous expression of bacteriophage proteins required for recombineering can be cumbersome. Moreover, this approach necessitates the generation of a recombination template for every gene to be mutated,[14] which is a bottleneck in large mutagenesis experiments. Whereas selection can be carried through positive and/or negative markers, scar-less mutations require multiple rounds of crossover recombination, which often complicates experiments as it involves the screening of many colonies and it often entails high rates of false positive mutants.[15]

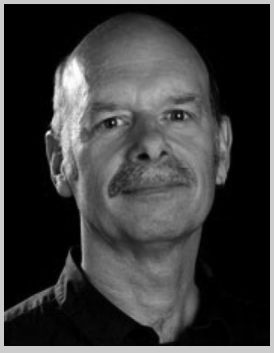
Additionally, numerous efforts have been focused at engineering sequence-specific endonucleases. In particular, zinc finger nucleases (ZFNs)[16] and transcription activator-like effector nucleases (TALENs)[17] have been extensively exploited for genome editing. These synthetic protein-complexes recognize specific nucleic acid sequences by protein-DNA interactions. Reprogramming their target specificity involves time-consuming protein engineering, which is inconvenient when multiple sequences need to be targeted. Alternatively, homing mega-nucleases, like I-SceI, can be repurposed to drive targeting of their recognition sites, but this requires the presence or introduction of a restriction site sequence in the DNA to be targeted,[18] thereby limiting its convenience.

The discovery of an adaptive immunity system in prokaryotes,[19–22] namely CRISPRs and Cas, has shaken the grounds of genome editing. Unlike the previously engineered endonucleases, the different Cas effector proteins are guided to the target nucleic acids by small RNA molecules.[22] As such, CRISPR-Cas systems are much easier to reprogram towards targeting other DNA sequences than previous genome engineering technologies.[15]

Given its advantages over other traditional mutagenesis methods, the RNA-guided DNA endonuclease activity of CRISPR-Cas systems has rapidly become the standard tool for modern genome editing, initially by allowing for easy counter-selection after homologous recombination experiments.[23] New variants are still being discovered and added to the prokaryotic CRISPR toolbox,[24] such as RNA-guided RNA endonucleases,[25] thermophilic Cas proteins variants [26,27] or RNA-guided DNA integrases.[28,29] Many exciting synthetic variants have been engineered to allow for novel functions such as transcriptional regulation,[30,31] DNA nicking,[32] in vivo base editing,[33] novel fusions of different nucleases,[34] and protein chimeras that edit by reverse transcription of a prime editing guide RNA.[35]

Despite the emergence of novel functionalities in the CRISPR-Cas toolkit, the main feature of these and other nucleases is the generation of target-specific double-strand DNA breaks to initiate a certain edit at the target site. This type of DNA break is lethal if left unrepaired; DNA repair mechanisms are therefore key for cell survival, but also for the introduction of the desired mutations.

Max Finger-Bou received his BSc degree in Biotechnology in 2015, at the University of Barcelona, Spain. He pursued his MSc in Cellular and Molecular Biotechnology at Wageningen University, the Netherlands, with a thesis on developing thermophilic CRISPR-Cas genome editing tools in van der Oost's group. For his MSc internship, he worked on the and genetic engineering of synthetic carbon fixation pathways in Bar-Even's group at the Max Planck Institute of Molecular Plant Physiology, Germany. He now pursues his PhD at Wageningen University, working on synthetic biology and DNA repair under the supervision of John van der Oost, Raymond Staals and Nico Claassens.



John van der Oost completed his PhD at the Vrije Universiteit in Amsterdam. After postdoc positions in Helsinki, Heidelberg and Amsterdam, he became group leader of the Bacterial Genetics group in the Laboratory of Microbiology at Wageningen University. In 2005, John was appointed Professor, in 2013 he was elected as EMBO member, in 2017 as member of the Royal Dutch Society for Arts and Sciences (KNAW). In 2018, he received the Dutch Spinoza Prize for his fundamental research on the prokaryotic immunity CRISPR-Cas systems.

Raymond H.J. Staals conducted his PhD studies (on the human RNA exosome) at the Radboud University Nijmegen, the Netherlands, in the department of Biomolecular Chemistry. He then moved to the Wageningen University, where he worked for three years on type III and other CRISPR-Cas systems in John van der Oost's group. Raymond continued his postdoctoral studies on CRISPR-Cas adaptation by joining Peter Fineran's lab at Otago University, New Zealand. In 2017, he returned to Wageningen University to start his own group.

3. Non-Templated DNA Repair in Prokaryotes

When a copy of the broken DNA is available, prokaryotes can repair double-strand DNA breaks with accuracy.[36] For most prokaryotes, a repair template is only available during the logarithmic phase of growth, when more than one copy of the chromosome is present in the cell during replication.[37] In bacteria, chromosomal breaks can be recognized by multi-subunit helicase-nuclease complexes which process DNA ends and drive homologous recombination.[38] For instance, the well-studied RecBCD complex recognizes and processes DNA ends, and loads multiple RecA proteins onto resected, single stranded DNA, which facilitates homologous recombination,[38] leading to the accurate repair of the chromosomal DNA break.

Naturally, when no repair template is available, cells rely on their intrinsic ability to join DNA ends. Understanding the mechanisms that drive these alternative repair pathways is key to embracing the full potential of template-independent genome editing.[39] Microbes rely on different sets of enzymes which protect, process, and ligate DNA ends.[40,41] To date, two natural pathways have been described in bacteria: non-homologous end joining (NHEJ)[42,43] (**Figure 1**A) and alternative end joining (AEJ; also referred to as microhomology-mediated repair, MMEJ)[44,45] (Figure 1B).

NHEJ is thought to be active in all eukaryotes[46] a sub-set of bacteria[47] and archaea.[48] Very well studied in humans, NHEJ involves more than ten proteins, but the core consists of the heterodimer Ku70/Ku80 and the ligase IV, as extensively reviewed elsewhere.[46,49] In contrast, the mechanisms governing DNA repair in absence of a repair template have remained more elusive in prokaryotes. Being firstly predicted through in silico analyses, the prokaryotic NHEJ machinery was suggested to be considerably simpler than its eukaryotic counterpart, with only two proteins predicted to intervene, Ku and LigD.[50,51] While NHEJ is more common in bacteria, it is considered rare in archaea, as not many species have the Ku protein;[48] so far, the full, canonical NHEJ system has only been described in the archaeal species *Archaeoglobus fulgidus*[50] and *Methanocella paludicula*.[52]

Just like their eukaryotic homologs, prokaryotic Ku proteins form a ring-like structure that encloses broken DNA ends, protects them from the activity of cellular exonucleases and recruits LigD.[40] LigD, in turn, is a multidomain protein with nuclease, polymerase and (ATP-dependent) ligase activities, organization of which varies between species, and it can also be present as a holoenzyme made of subunits.[47,53] Upon its recruitment by Ku, LigD processes the DNA ends with its nuclease and polymerase activities and ligates them in an ATP-dependent manner [54,55] (Figure 1A). While the proteins are known to be essential during stationary phase in irradiated cultures and in spores during desiccation, they can be knocked out without apparent detrimental effects to cellular fitness under normal growth conditions.[37,56]

Despite Ku and LigD being thought to be the only proteins able to fix double-strand DNA breaks and prevent cellular death,[41] a Ku- and LigD-independent end joining pathway has been described in *Escherichia coli*[57] Named alternative end-joining (AEJ), it was proven to mediate plasmid and genomic DNA recircularization in vivo without neither Ku nor LigD. A characteristic feature of AEJ is the large reliance on microhomologies (1–9 nucleotides), which are exposed due to the action of the RecBCD complex and enable DNA end annealing and ligation by the NAD-dependent DNA ligase A (LigA)[57] (Figure 1B).

4. Strategies for Non-Templated Prokaryotic Genome Engineering

In **Table 1**, a summary is given of the different strategies successfully applied for non-templated prokaryotic engineering, including the edited species, the used endonucleases, the origin of the DNA repair mechanisms employed and an outline on the observed mutations and the presence of microhomologies.

Both native and heterologous DNA repair systems have been successfully employed for template-independent genome engineering of archaea and several bacteria. Although conventional engineering methods are still being used (e.g., I-SceI[57–60]), the class 2 type II CRISPR-Cas9 system from *Streptococcus pyogenes* is nowadays the most frequently used tool for the prokaryotic genome engineering.[36,61–66] Additionally, other CRISPR nucleases are gaining popularity, such as Cas12a from *Francisella novicida*,[67–69] which has different properties than Cas9,[70] and other newly engineered CRISPR-Cas variants.[71] Class 1 CRISPR systems have also been used[72,73] but are far less usual.

Ku and LigD have been, for long, the go-to proteins to transplant template-independent DNA repair pathways. However, the use of different ligases or Ku-like proteins is surging. Gam proteins from bacteriophage μ[59] and λ[65] have been successfully demonstrated to protect linear DNA ends from degradation by cellular nucleases. Interestingly, μGam has been proven to promote binding of LigA to DNA ends,[59] and the ligase from bacteriophage T4 has been used as a single component NHEJ protein, increasing the surviving rates of Cas9-targeted bacteria.[65] Non-canonical end joining proteins prove therefore a useful addition to the molecular toolbox of non-templated genome engineering.

Altogether, the outcomes of template-independent genome editing with CRISPR-Cas systems or other endonucleases can be roughly grouped in three categories: gene inactivation, gene insertion and genome minimization.

4.1. Gene Inactivation

The most straightforward approach to take advantage of the error-prone nature of NHEJ is to generate small indels in protein-encoding genes, causing frameshifts or nonsense mutations, which disrupt the function of genes. An efficient NHEJ pathway in combination with CRISPR-Cas targeting can provide an excellent platform to perform high-throughput functional genomics in different organisms without the need of templates for homologous recombination.[74]

4.2. DNA Insertion

It was demonstrated in *E. coli* that antibiotic resistance cassettes can be acquired through AEJ, independently of Ku and LigD,[57]

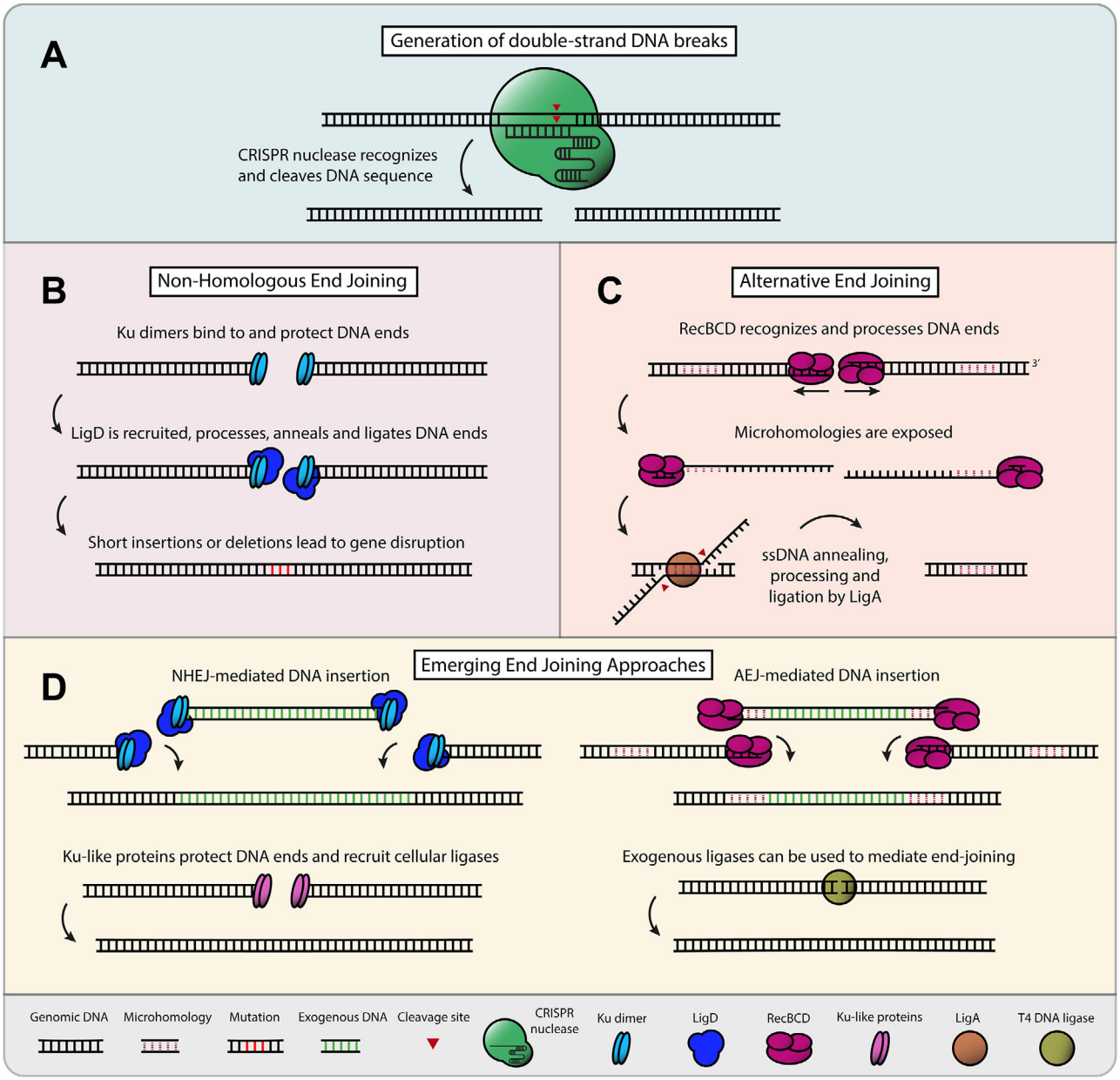


Figure 1. Schematic representation of a genome engineering experiment where the genome is targeted by a nuclease and different DNA repair pathways can mend the double-strand DNA break. A) A CRISPR nuclease recognizes and cleaves a target sequence, generating a double-strand DNA break (not to scale). B) In non-homologous end joining, Ku dimers bind to DNA ends and protect them against the effect of cellular nucleases, and they recruit LigD (or multiple subunits) which then processes, anneals and ligates DNA ends, often generating short insertions or deletions which induce frameshift mutations, leading to gene disruption. C) In alternative end joining (also named microhomology mediated repair), RecBCD (or other complexes) recognize and resect DNA ends, when short stretches of microhomology (1–9 nt) are exposed, which then allow the annealing of the processed ssDNA. The protruding ssDNA ends are digested by cellular nucleases and the junctions are sealed by cellular DNA ligase A. D) Novel approaches are emerging where both end joining mechanisms are used to insert exogenous DNA in the genome, Ku-like proteins like Gam from phages μ and λ, and other ligases like phage T4 DNA ligase are also being used to mediate synthetic end joining.

allegedly relying on the action of the essential LigA and other cellular components. Additionally, it was recently proven that Ku and LigD can also mediate acquisition of DNA through classical NHEJ.[60] Further characterization of the different DNA repair mechanisms will likely be needed to make DNA insertion a reliable feature of engineering through end-joining pathways.

4.3. Genome Minimization

Whereas the use of CRISPR technologies has been mostly targeted at small scale genome engineering, DNA repair pathways independent of exogenous template offer the possibility of minimizing bacterial genomes with ease, opening up opportunities for both fundamental and applied biotechnological research.[75]

Table 1. Summary of the published studies to date where CRISPR and other endonucleases are employed for prokaryotic genome editing in combination with either native or heterologously expressed DNA repair systems.

Edited species	Endonuclease	DNA repair	Observed mutations	(Micro)homologies	Reference
Mycobacterium smegmatis	I-SceI	Native	Up to 221 bp	Yes	[58]
Escherichia coli	I-SceI, 2 sites	Native	Up to 12.3 kb deletions	Yes	[57]
		Native	Insertion of about 1kb	Yes	
Pectobacterium atrosepticum	Native (Type I-F CRISPR)	Native	40.2 kb deletion	Yes	[72]
Sterptomyces coelicolor	SpyCas9, 1 gRNA	Native	Small insertions (1/4 colonies) Up to 37 kb deletions (3/4 colonies)	Not reported	[61]
		ScaLigD	Small indels (16/16 colonies)	Not reported	
E. coli	SpyCas9 (nickase), 1 gRNA	Native	1 kb deletion	Yes	[66]
	SpyCas9 (nickase), 2 gRNA	Native	36–97 kb deletions	Yes	
E. coli	SpyCas9, 1 gRNA	Native	Up to 27.8 kb deletions	Yes	[36]
		MtuKuLigD	9–298 bp deletions	Yes	
		μGam	Not reported	Not reported	
E. coli	SpyCas9, 1 gRNA	MtuKuLigD	10–267 bp deletions	Yes	[62]
	SpyCas9, 2 gRNA	MtuKuLigD	Up to 17 kb deletions	Not reported	
E. coli	SpyCas9, 1 gRNA	BsuKuLigD	13–172 bp deletions	Yes	[63]
	SpyCas9, 1 gRNA	MtuKuLigD	10–26 bp deletions	Yes	
	SpyCas9, 1 gRNA	MsmKuLigD	13–37 bp deletions	Yes	
	SpyCas9, 2 gRNA	MsmKuLigD	Up to 123 kb deletions	Yes	
Methanosarcina acetivorans	SpyCas9, 1 gRNA	MpaKuLigD (*: subunits expressed instead of holoenzyme)	75 bp–2.7 kb deletions	Yes	[64]
	SpyCas9, 2 gRNA	MpaKuLigD* (*: subunits expressed instead of holoenzyme)	1.3 kb deletions	Yes	
Mycobacterium smegmatis	FnCpf1, 1 gRNA	Native (MsmKuLigD)	Up to 1.5 kb deletions	Not reported	[67]
S. coelicolor	FnCpf1, 1 gRNA	MsmKuLigD	409–1624 bp deletions	Not reported	[69]
	FnCpf1, 2 gRNA	MsmKuLigD	Up to 28 kb deletions	Not reported	
E. coli	I-SceI	μGam, EcoLigA		Yes	[59]
Sinorhizobium meliloti	I-SceI	Native (SmeKuLigD)	Plasmid deletions of up to 994bp	Not reported	[60]
		Native (SmeKuLigD)	Insertion of 1.3 kb resistance cassette	Not reported	
		Native (SmeKu3-4)	Up to 343 bp deletion in chromosome	Not reported	
E. coli	SpyCas9, 1 gRNA	MtuKuLigD	Not reported	Not reported	[65]
	SpyCas9, 1 gRNA	T4 ligase	Up to 35 kb deletions	Yes	
	SpyCas9, 1 gRNA	T4 ligase, λGam	Up to 35 kb deletions	Not reported	
E. coli	xCas9-3.7, 1 gRNA	Native	Up to 1.7 kb deletions	Yes	[71]
	xCas9-3.7, 2 gRNA	Native	Up to 83 kb deletions	Yes	
Pseudomonas aeruginosa	Native, CRISPR type I-C, 1 gRNA	Native	7–424 kb deletions	Yes	[73]
Pseudomonas syringae	Pae CRISPR type I-C, 1 gRNA	Native	55–101 kb deletions	Yes	
E. coli		Pae CRISPR type I-C	Native	17–106 kb deletions	Yes

Spy, *Streptococcus pyogenes*; Fn, *Francisella novicida*; Sca, *Streptomyces carneus*; Mtu, *Mycobacterium tuberculosis*; Msm, *Mycobacterium smegmatis*; Bsu, *Bacillus subtilis*; Mpa, *Methanocella paludicola*; Sda, *Streptomyces daghestanicus*; Pat, *Pectobacterium atrosepticum*; Ppu, *Pseudomonas putida*; Eco, *Escherichia coli*; gRNA, *guide RNA*.

Both NHEJ and AEJ have been successfully used to generate large deletions in prokaryotes (Table 1). In these cases, the extent of the introduced genomic deletion seems determined by the presence of essential genes in proximity of the targeted locus,[57] highlighting the role of DNA repair in mediating large-scale genome rearrangements. Notably, the relatively simple CRISPR type I-C system was recently shown to mediate deletions of up to 424 kb in *Pseudomonas aeruginosa* using only one guide, allegedly facilitated by AEJ.[73]

5. Native Versus Heterologous DNA End Joining for Prokaryotic Engineering

Whether it is HDR, AEJ, NHEJ, or a combination thereof, all microorganisms have at least one of these DNA repair pathways. It is therefore possible, theoretically, to apply CRISPR-Cas tools without the heterologous expression of any exogenous DNA repair pathway. In practice, however, whether native DNA repair systems are adequate to genetically engineer a microorganism

depends on two aspects: the type of native DNA repair system and the specific genetic engineering goal.

The spectra of mutations mediated by the different types of DNA repair differ substantially from NHEJ to AEJ. While repair mediated by NHEJ is typically associated with short, unpredictable nucleotide insertions, or deletions,[47] repair by AEJ can generate deletions from dozens of nucleotides[57] to hundreds of kb[73] and is driven by microhomology. Microhomologies can be computationally predicted,[76] which can be instrumental in establishing AEJ as a valuable tool for prokaryotic genome engineering.

Depending on the specific genetic engineering goal, a DNA repair pathway might be more favorable than the other. Small mutations introduced by NHEJ can be very useful to disrupt the function of several genes in iterative cycles or multiplex genome editing,[77] as well as to carry functional genomics studies.[74] On the other hand, large genomic deletions driven by AEJ can facilitate the genome minimization of prokaryotes and help unravel the poorly understood secrets of the noncoding genome.[78]

6. Future Directions

A few challenges are to be faced when it comes to the development of the field. It has been shown that interactions occur between prokaryotic DNA repair pathways and certain CRISPR-Cas systems,[79] further computational analysis suggesting that there might exist incompatibility between some DNA repair mechanisms and certain biochemical properties of CRISPR immunity.[80] Additionally, it is not easy to currently distinguish between the outcomes of repair by NHEJ and AEJ without intensive sequence analysis, which prevents some studies from having clear conclusions about the DNA repair pathways involved in specific experiments. Further study and elucidation of these phenomena in combination with computational tools to predict outcomes of DNA repair will likely facilitate the establishment of better rules and principles for non-templated prokaryotic genome engineering. Despite these caveats, novel combinations of CRISPR-Cas nucleases and DNA processing/repair enzymes are expected to flourish in the near future, providing the field of genome engineering with unprecedented power.

Acknowledgements

The Netherlands Organization for Scientific Research (NWO) is acknowledged for financial support through a Gravitation grant (BaSyC, 024.003.019) to M.F.B and J.v.d.O, and a VENI grant (016.Veni.171.047) to R.H.S. E.O. acknowledges the public-private NWO-Green Foundation for sustainable production and supply chains in agriculture and horticulture for their financial support (870.15.130, 2015/05279/ALW).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

AEJ, CRISPR-Cas, DNA repair, genome editing, NHEJ

Received: January 14, 2020
Revised: May 4, 2020
Published online: June 29, 2020

[1] M. S. G. Lopes, *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 813.
[2] A. Aguilar, T. Twardowski, R. Wohlgemuth, *Biotechnol. J.* **2019**, *14*, 1800638.
[3] A. Tylecote, *Res. Policy* **2019**, *48*, 858.
[4] R. A. Sheldon, *Green Chem.* **2014**, *16*, 950.
[5] C. J. Paddon, P. J. Westfall, D. J. Pitera, K. Benjamin, K. Fisher, D. McPhee, M. D. Leavell, A. Tai, A. Main, D. Eng, D. R. Polichuk, K. H. Teoh, D. W. Reed, T. Treynor, J. Lenihan, M. Fleck, S. Bajad, G. Dang, D. Dengrove, D. Diola, G. Dorin, K. W. Ellens, S. Fickes, J. Galazzo, S. P. Gaucher, T. Geistlinger, R. Henry, M. Hepp, T. Horning, T. Iqbal, et al., *Nature* **2013**, *496*, 528.
[6] H. Yim, R. Haselbeck, W. Niu, C. Pujol-Baxley, A. Burgard, J. Boldt, J. Khandurina, J. D. Trawick, R. E. Osterhout, R. Stephen, J. Estadilla, S. Teisan, H. B. Schreyer, S. Andrae, T. H. Yang, S. Y. Lee, M. J. Burk, S. Van Dien, *Nat. Chem. Biol.* **2011**, *7*, 445.
[7] P. P. Peralta-Yahya, F. Zhang, S. B. Del Cardayre, J. D. Keasling, *Nature* **2012**, *488*, 320.
[8] D. J. Pollard, J. M. Woodley, *Trends Biotechnol.* **2007**, *25*, 66.
[9] F. M. Schempp, L. Drummond, M. Buchhaupt, J. Schrader, *J. Agric. Food Chem.* **2018**, *66*, 2247.
[10] M. Sauer, D. Mattanovich, *J. Chem. Technol. Biotechnol.* **2012**, *87*, 445.
[11] J. Hashimoto, B. Stevenson, T. M. Schmidt, *J. Bacteriol.* **2003**, *185*, 966.
[12] J. S. Ludu, E. B. Nix, B. N. Duplantis, O. M. De Bruin, L. A. Gallagher, L. M. Hawley, F. E. Nano, *FEMS Microbiol. Lett.* **2008**, *278*, 86.
[13] S. K. Sharan, L. C. Thomason, S. G. Kuznetsov, D. L. Court, *Nat. Protoc.* **2009**, *4*, 206.
[14] T. Baba, T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, H. Mori, *Mol. Syst. Biol.* **2006**, *2*, 2006.0008.
[15] W. Jiang, D. Bikard, D. Cox, F. Zhang, L. A. Marraffini, *Nat. Biotechnol.* **2013**, *31*, 233.
[16] F. D. Urnov, E. J. Rebar, M. C. Holmes, H. S. Zhang, P. D. Gregory, *Nat. Rev. Genet.* **2010**, *11*, 636.
[17] D. F. Voytas, A. J. Bogdanove, *Science* **2011**, *333*, 1843.
[18] B. L. Stoddard, *Q. Rev. Biophys.* **2005**, *38*, 49.
[19] R. Barrangou, C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D. A. Romero, P. Horvath, *Science* **2007**, *315*, 1709.
[20] P. Horvath, R. Barrangou, *Science* **2010**, *327*, 167.
[21] J. E. Garneau, M.-È. Dupuis, M. Villion, D. A. Romero, R. Barrangou, P. Boyaval, C. Fremaux, P. Horvath, A. H. Magadán, S. Moineau, *Nature* **2010**, *468*, 67.
[22] S. J. J. Brouns, M. M. Jore, M. Lundgren, E. R. Westra, R. J. H. Slijkhuis, A. P. L. Snijders, M. J. Dickman, K. S. Makarova, E. V. Koonin, J. van der Oost, *Science* **2008**, *321*, 960.
[23] I. Mougiakos, E. F. Bosma, J. Ganguly, J. van der Oost, R. van Kranenburg, *Curr. Opin. Biotechnol.* **2018**, *50*, 146.
[24] K. S. Makarova, Y. I. Wolf, J. Iranzo, S. A. Shmakov, O. S. Alkhnbashi, S. J. J. Brouns, E. Charpentier, D. Cheng, D. H. Haft, P. Horvath, S. Moineau, F. J. M. Mojica, D. Scott, S. A. Shah, V. Siksnys, M. P. Terns, Č. Venclovas, M. F. White, A. F. Yakunin, W. Yan, F. Zhang, R. A. Garrett, R. Backofen, J. van der Oost, R. Barrangou, E. V. Koonin, *Nat. Rev. Microbiol.* **2020**, *18*, 67.
[25] O. O. Abudayyeh, J. S. Gootenberg, S. Konermann, J. Joung, I. M. Slaymaker, D. B. T. Cox, S. Shmakov, K. S. Makarova, E. Semenova, L. Minakhin, K. Severinov, A. Regev, E. S. Lander, E. V. Koonin, F. Zhang, *Science* **2016**, *353*, aaf5573.

[26] I. Mougiakos, P. Mohanraju, E. F. Bosma, V. Vrouwe, M. F. Bou, M. I. S. Naduthodi, A. Gussak, R. B. L. Brinkman, R. van Kranenburg, J. van der Oost, *Nat. Commun.* **2017**, *8*, 1647.
[27] L. B. Harrington, D. Paez-Espino, B. T. Staahl, J. S. Chen, E. Ma, N. C. Kyrpides, J. A. Doudna, *Nat. Commun.* **2017**, *8*, 1424.
[28] S. E. Klompe, P. L. H. Vo, T. S. Halpin-Healy, S. H. Sternberg, *Nature* **2019**, *571*, 219.
[29] J. Strecker, A. Ladha, Z. Gardner, J. L. Schmid-Burgk, K. S. Makarova, E. V. Koonin, F. Zhang, *Science* **2019**, *365*, 48.
[30] D. Bikard, W. Jiang, P. Samai, A. Hochschild, F. Zhang, L. A. Marraffini, *Nucleic Acids Res.* **2013**, *41*, 7429.
[31] L. A. Gilbert, M. H. Larson, L. Morsut, Z. Liu, G. A. Brar, S. E. Torres, N. Stern-Ginossar, O. Brandman, E. H. Whitehead, J. A. Doudna, W. A. Lim, J. S. Weissman, L. S. Qi, *Cell* **2013**, *154*, 442.
[32] B. Shen, W. Zhang, J. Zhang, J. Zhou, J. Wang, L. Chen, L. Wang, A. Hodgkins, V. Iyer, X. Huang, W. C. Skarnes, *Nat. Methods* **2014**, *11*, 399.
[33] N. M. Gaudelli, A. C. Komor, H. A. Rees, M. S. Packer, A. H. Badran, D. I. Bryson, D. R. Liu, *Nature* **2017**, *551*, 464.
[34] P. Cameron, M. M. Coons, S. E. Klompe, A. M. Lied, S. C. Smith, B. Vidal, P. D. Donohoue, T. Rotstein, B. W. Kohrs, D. B. Nyer, R. Kennedy, L. M. Banh, C. Williams, M. S. Toh, M. J. Irby, L. S. Edwards, C.-H. Lin, A. L. G. Owen, T. Künne, J. van der Oost, S. J. J. Brouns, E. M. Slorach, C. K. Fuller, S. Gradia, S. B. Kanner, A. P. May, S. H. Sternberg, *Nat. Biotechnol.* **2019**, *37*, 1471.
[35] A. V. Anzalone, P. B. Randolph, J. R. Davis, A. A. Sousa, L. W. Koblan, J. M. Levy, P. J. Chen, C. Wilson, G. A. Newby, A. Raguram, D. R. Liu, *Nature* **2019**, *576*, 149.
[36] L. Cui, D. Bikard, *Nucleic Acids Res.* **2016**, *44*, 4243.
[37] R. S. Pitcher, A. J. Green, A. Brzostek, M. Korycka-Machala, J. Dziadek, A. J. Doherty, *DNA Repair* **2007**, *6*, 1271.
[38] D. B. Wigley, *Nat. Rev. Microbiol.* **2013**, *11*, 9.
[39] W. Y. Wu, J. H. G. Lebbink, R. Kanaar, N. Geijsen, J. van der Oost, *Nat. Chem. Biol.* **2018**, *14*, 642.
[40] R. Bowater, A. J. Doherty, *PLoS Genet.* **2006**, *2*, 93.
[41] N. C. Brissett, A. J. Doherty, *Biochem. Soc. Trans.* **2009**, *37*, 539.
[42] M. Della, P. L. Palmbos, H. M. Tseng, L. M. Tonkin, J. M. Daley, L. M. Topper, R. S. Pitcher, A. E. Tomkinson, T. E. Wilson, A. J. Doherty, *Science* **2004**, *306*, 683.
[43] R. S. Pitcher, T. E. Wilson, A. J. Doherty. *New Insights into NHEJ Repair Processes in Prokaryotes*, Taylor and Francis, London **2005**.
[44] J. Aniukwu, M. S. Glickman, S. Shuman, *Genes Dev.* **2008**, *22*, 512.
[45] C. Bertrand, A. Thibessard, C. Bruand, F. Lecointe, P. Leblond, *Mol. Microbiol.* **2019**, *111*, 1139.
[46] M. R. Lieber, *Annu. Rev. Biochem.* **2010**, *79*, 181.
[47] S. Shuman, M. S. Glickman, *Nat. Rev. Microbiol.* **2007**, *5*, 852.
[48] M. F. White, T. Allers, *FEMS Microbiol. Rev.* **2018**, *42*, 514.
[49] H. H. Y. Chang, N. R. Pannunzio, N. Adachi, M. R. Lieber, *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 495.
[50] L. Aravind, E. V. Koonin, *Genome Res.* **2001**, *11*, 1365.
[51] G. R. Weller, B. Kysela, R. Roy, L. M. Tonkin, E. Scanlan, M. Della, S. K. Devine, J. P. Day, A. Wilkinson, F. d'Adda di Fagagna, K. M. Devine, R. P. Bowater, P. A. Jeggo, S. P. Jackson, A. J. Doherty, *Science* **2002**, *297*, 1686.
[52] E. J. Bartlett, N. C. Brissett, A. J. Doherty, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, E1984.
[53] R. S. Pitcher, N. C. Brissett, A. J. Doherty, *Annu. Rev. Microbiol.* **2007**, *61*, 259.
[54] H. Zhu, S. Shuman, *J. Biol. Chem.* **2005**, *280*, 418.
[55] P. A. Nair, P. Smith, S. Shuman, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 12822.
[56] R. Moeller, M. Raguse, G. Reitz, R. Okayasu, Z. Li, S. Klein, P. Setlow, W. L. Nicholson, *Appl. Environ. Microbiol.* **2014**, *80*, 104.
[57] R. Chayot, B. Montagne, D. Mazel, M. Ricchetti, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 2141.
[58] N. C. Stephanou, F. Gao, P. Bongiorno, S. Ehrt, D. Schnappinger, S. Shuman, M. S. Glickman, *J. Bacteriol.* **2007**, *189*, 5237.
[59] S. Bhattacharyya, M. M. Soniat, D. Walker, S. Jang, I. J. Finkelstein, R. M. Harshey, *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E11614.
[60] P. Dupuy, L. Sauviac, C. Bruand, *Nucleic Acids Res.* **2019**, *47*, 1335.
[61] Y. Tong, P. Charusanti, L. Zhang, T. Weber, S. Y. Lee, *ACS Synth. Biol.* **2015**, *4*, 1020.
[62] T. Su, F. Liu, P. Gu, H. Jin, Y. Chang, Q. Wang, Q. Liang, Q. Qi, *Sci. Rep.* **2016**, *6*, 37895.
[63] X. Zheng, S. Y. Li, G. P. Zhao, J. Wang, *Biochem. Biophys. Res. Commun.* **2017**, *485*, 768.
[64] D. D. Nayak, W. W. Metcalf, *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 2976.
[65] T. Su, F. Liu, Y. Chang, Q. Guo, J. Wang, Q. Wang, Q. Qi, *Synth. Syst. Biotechnol.* **2019**, *4*, 107.
[66] K. Standage-Beier, Q. Zhang, X. Wang, *ACS Synth. Biol.* **2015**, *4*, 1217.
[67] B. Sun, J. Yang, S. Yang, R. D. Ye, D. Chen, Y. Jiang, *Biotechnol. J.* **2018**, *13*, 1700588.
[68] Z.-H. Li, M. Liu, X.-M. Lyu, F.-Q. Wang, D.-Z. Wei, *J. Basic Microbiol.* **2018**, *58*, 1100.
[69] L. Li, K. Wei, G. Zheng, X. Liu, S. Chen, W. Jiang, Y. Lu, *Appl. Environ. Microbiol.* **2018**, *84*, e00827.
[70] T. Swartjes, R. H. J. Staals, J. van der Oost, *Biochem. Soc. Trans.* **2020**, *48*, 207.
[71] C. Huang, T. Ding, J. Wang, X. Wang, J. Wang, L. Zhu, C. Bi, X. Zhang, X. Ma, Y.-X. Huo, *Appl. Microbiol. Biotechnol.* **2019**, *103*, 20.
[72] R. B. Vercoe, J. T. Chang, R. L. Dy, C. Taylor, T. Gristwood, J. S. Clulow, C. Richter, R. Przybilski, A. R. Pitman, P. C. Fineran, *PLoS Genet.* **2013**, *9*, e1003454.
[73] B. Csörgő, L. M. León, I. J. Chau-Ly, A. Vasquez-Rifo, J. D. Berry, C. Mahendra, E. D. Crawford, J. D. Lewis, J. Bondy-Denomy, *bioRxiv* **2019**, 860999.
[74] K. Ford, D. McDonald, P. Mali, *J. Mol. Biol.* **2019**, *431*, 48.
[75] E. Martínez-García, V. de Lorenzo, *Curr. Opin. Biotechnol.* **2016**, *42*, 216.
[76] S. Bae, J. Kweon, H. S. Kim, J. S. Kim, *Nat. Methods* **2014**, *11*, 705.
[77] B. Adiego-Pérez, P. Randazzo, J. M. Daran, R. Verwaal, J. A. Roubos, P. Daran-Lapujade, J. van der Oost, *FEMS Microbiol. Lett.* **2019**, *366*, fnz086.
[78] A. Montalbano, M. C. Canver, N. E. Sanjana, *Mol. Cell* **2017**, *68*, 44.
[79] A. Bernheim, A. Calvo-Villamañán, C. Basier, L. Cui, E. P. C. Rocha, M. Touchon, D. Bikard, *Nat. Commun.* **2017**, *8*, 2094.
[80] A. Bernheim, D. Bikard, M. Touchon, E. P. C. Rocha, *Philos. Trans. R. Soc., B* **2019**, *374*, 20180088.