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Molecular methods for high-throughput, multiplexed, and automated genome editing in prokaryotes and eukaryotes

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Novel approaches to genome engineering are crucial to rapidly advance the capabilities of strain engineering and synthetic biology. With ongoing developments in DNA editing techniques, researchers have begun to engineer organisms at higher throughput and can now perform multiple genome modifications simultaneously. As laboratory automation becomes more accessible, workflows are being transferred to robot-assisted platforms, enabling large-scale and highly parallelized genome editing campaigns. These platforms play a key role in fully utilizing the potential of modern molecular biology tools. Here, we review recent developments in technologies for high-throughput, multiplexed, and automated strain engineering in prokaryotic and eukaryotic organisms.

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Introduction

Recent advances in genome engineering techniques have enabled scientists to rapidly construct genetically modified organisms. Consequently, we have now reached a point where methodological progress is surpassing the capabilities of traditional manual workflows. To keep pace with these developments, the automation of genome editing is becoming increasingly important. As a result, embedding genome editing into the widely applied design–build–test–learn (DBTL) cycle through robot-assisted approaches allows researchers to scale up the construction of genetic variants efficiently.

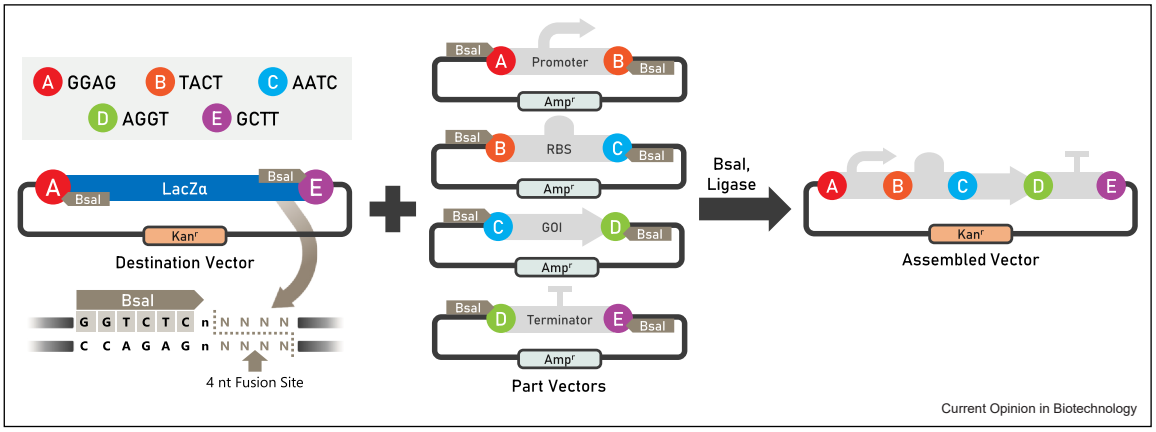
Integrating molecular tools for genome editing with robotic platforms requires standardized, modular workflows tailored to automation. Such workflows enhance reproducibility and enable both miniaturization and parallelization for high-throughput strain construction. Biofoundries represent comprehensive end-to-end platforms capable of fully integrating automated DBTL cycles to generate thousands of strain variants within weeks [1,2]. They combine molecular biology, microbiology, bioinformatics, precise liquid handling, and analytical processes into a unified platform with minimal human intervention. Thus, they have the potential to address the critical need for automation in genome engineering beyond plasmid-based systems and enable genome-scale modifications while maintaining both throughput and flexibility.

In this review, we present methods and tools for advanced genome editing across a range of organisms of biotechnological interest. Because fully automated biofoundries are still rare, highly specialized, and require substantial initial investment, we also highlight technologies that enable high-throughput and multiplexed genome editing with manual or partially automated workflows.

DNA synthesis and plasmid construction

While this review focuses on the latest genome editing technologies, reliable and modular DNA assembly techniques are indispensable for high-throughput, parallelized strain construction. The development of Golden Gate Assembly (GGA) — a method for the simultaneous and directional assembly of multiple DNA fragments using type IIS restriction enzymes and T4 DNA ligase [3] — has greatly advanced synthetic biology. GGA and derivative cloning frameworks, such as Modular Cloning (MoClo) [4], enable researchers to hierarchically assemble modular DNA parts and have paved the way for standardized, high-throughput plasmid construction (Figure 1). Since then, numerous GGA-based cloning frameworks have been established for a wide range of organisms, including bacteria [5–7], yeast [8] and fungi [9], plants [10], and mammalian cells [11]. More recently, the high fidelity of GGA has been leveraged to construct hundreds of codon-harmonized genes from oligonucleotide pools, reducing the cost of gene synthesis by more than threefold [12]. These advances inevitably require the handling of large datasets to plan and

Figure 1



Modular Cloning (MoClo) principle based on Golden Gate Assembly with a standardized cloning syntax. For example, the standardized 4-nt fusion sites (A to E) are used for the directional assembly of an expression vector, starting from a destination vector and four individual genetic components that constitute a transcriptional unit in a single reaction using *Bsa*I and ligase enzymes. The development of MoClo cloning frameworks has enabled standardized, high-throughput plasmid construction applied in various genome editing technologies. RBS, ribosome binding site; GOI, gene of interest; Kan^r, kanamycin resistance; Amp^r, ampicillin resistance.

construct DNA modules. As a result, research groups have begun developing platforms that assist plasmid construction through both digital tools and wet lab automation [13–15].

Other assembly methods have also been developed to provide modular DNA constructs for higher throughput. For example, PlasmidMaker combines a user-friendly design interface with a fully integrated robotic system for DNA assembly using *Pyrococcus furiosus* Argonaute (*Pf*Ago)-based artificial restriction enzymes [15]. The potential of PlasmidMaker was demonstrated by constructing over 100 plasmids of varying size and complexity for different species. Another strategy to accelerate plasmid DNA supply for genomic engineering is phage enzyme-assisted *in vivo* DNA assembly [16]. This study verified successful *in vivo* assembly through the expression of phage-derived DNA exonucleases and ligases, not only in *E. coli* but also in the Gram-negative bacteria *Ralstonia eutropha* and *Pseudomonas putida*, the Gram-positive bacterium *Lactobacillus plantarum*, and the eukaryotic yeast *Yarrowia lipolytica*. Further development of *in vivo* assembly methods may enable direct DNA assembly in diverse organisms, thereby bypassing cloning hosts completely. This could reduce the cost and time for genome engineering, particularly when coupled with other tools such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated machineries, as recently reported for filamentous fungi [17,18].

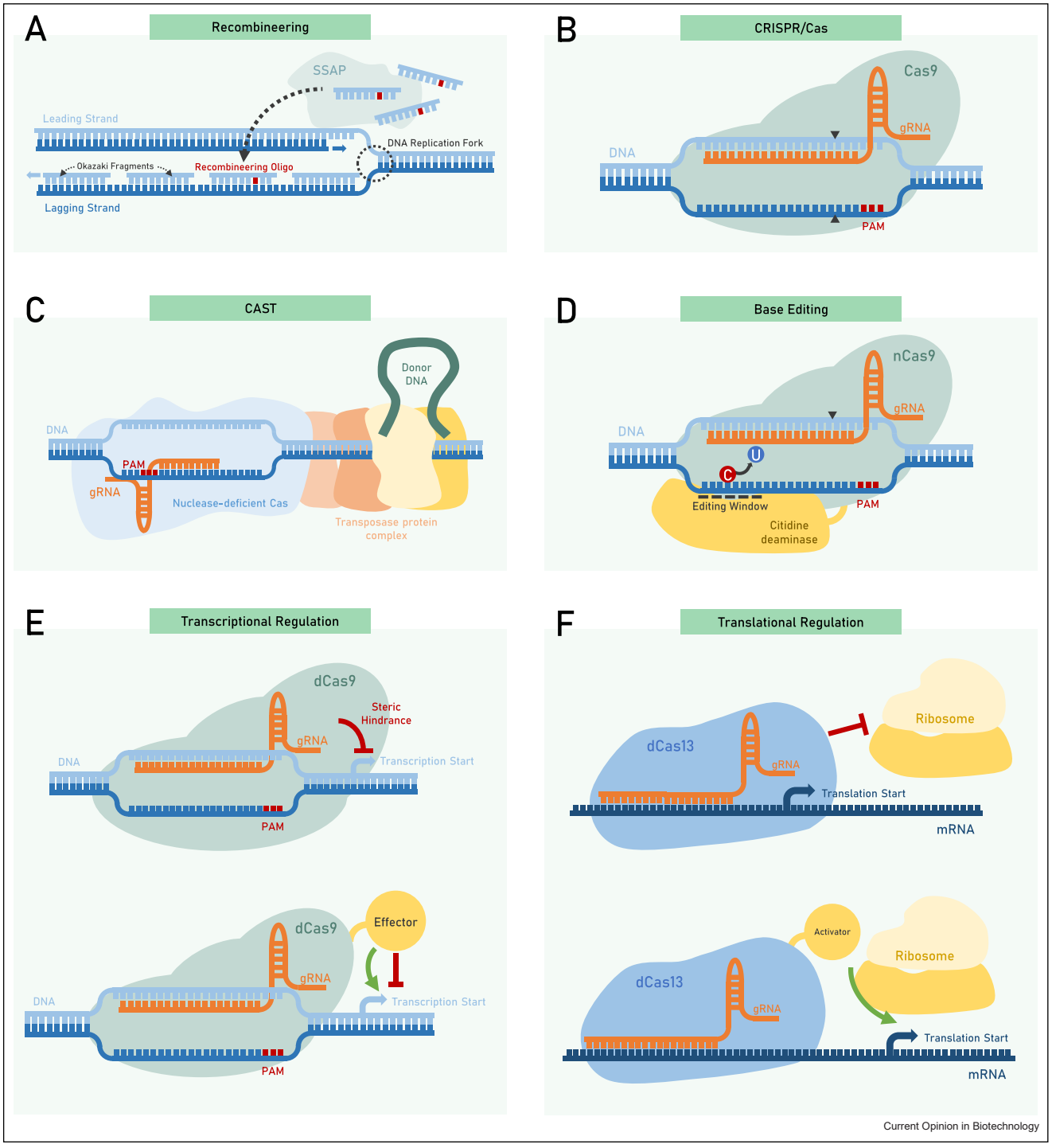
Genome editing technologies

In recent years, technologies for engineering prokaryotes and eukaryotes have demonstrated significant progress, resulting in a substantial volume of scientific publications. Therefore, we focus on methodological strategies suitable for high-throughput, multiplexed, and automated genome editing (Figure 2).

Recombineering

Recombineering was developed for DNA editing in bacteria more than two decades ago and is still being actively refined to improve recombination efficiency, DNA cargo capacity, and multiplexing [19]. It typically employs phage proteins such as RecE/T from the Rac prophage or Redα/β from the λ phage to promote homologous recombination during DNA replication, using single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) templates that carry the desired mutation(s) (Figure 2a). This approach is inherently suitable for high-throughput experiments because recombination templates are typically supplied as synthetic oligonucleotides (for point mutations) or linear polymerase chain reaction (PCR) products (for larger DNA modifications), which are co-transformed with the plasmid-borne recombination machinery (Table 1). This eliminates the need for plasmid construction, thereby simplifying mutational design and accelerating the generation of genetic variants. Recent studies have demonstrated the potential of recombineering for high-throughput and multiplexed genome editing, enabling modifications ranging from single-nucleotide substitutions to large deletions and insertions in various bacteria [20–24]. A notable recent application of recombineering is the generation of combinatorial plasmid libraries through a multiplex, one-step, single-stranded DNA annealing protein (SSAP)-mediated plasmid diversification protocol (MOSAIC) [25]. In this method, mutant ssDNA oligonucleotides and the plasmid to be diversified are co-transformed into *E. coli* carrying a recombineering plasmid that expresses RecT from *Collinsella stercoris* and a dominant negative *E. coli* MutL variant for the transient repression of

Figure 2



Schematic overview of genome engineering and gene regulation strategies. **(a)** Recombineering enables homologous recombination with short ssDNA template oligonucleotides using phage-derived recombinases. **(b)** CRISPR/Cas systems facilitate targeted DNA cleavage directed by guide RNAs (gRNA), followed by repair using dsDNA templates. **(c)** CRISPR-associated transposases (CASTs) mediate RNA-guided DNA integration without the need for double-strand breaks and homologous repair templates. **(d)** Base editing enables single-nucleotide conversions without inducing double-strand breaks by fusing cytidine or adenine deaminases to catalytically dead Cas proteins (dCas) or nickase Cas proteins (nCas). **(e)** Transcriptional regulation can be achieved through CRISPR interference (CRISPRi, top) or activation (CRISPRa, bottom) via steric hindrance, using catalytically dead Cas proteins fused to effector proteins (activators or repressors). **(f)** Translational regulation is mediated by nuclease-deficient Cas proteins (e.g. dCas13) that either bind target mRNA to reduce ribosome binding efficiency by steric hindrance (top) or fused to activator proteins to increase translation efficiency (bottom). PAM, protospacer adjacent motif; SSAP, single-stranded DNA annealing protein.

Table 1

Advantages and disadvantages of current genome editing and gene regulation methods.

Method	Advantages	Disadvantages
Recombineering	• High precision • Scarless edits • Short linear and homologous DNA as a repair template • Multiplexing possible	• Requires phage/host recombination proteins • Off-target effects observed
CRISPR/Cas	• Programmable • High target specificity • Molecular tools for a broad range of hosts available • Multiplexing possible	• Requires nearby PAM • Mutation of the PAM site is necessary • DSBs trigger DNA damage responses and lead to lower cell survival rates • Off-target effects observed
CRISPR-associated transposases (CASTs)	• RNA-guided DNA integrations without DSBs or homology arms • Integration of large DNA cargos (multi-kb)	• Complex on the molecular level (multi-protein machineries) • Not scarless (transposon end sequences remain) • Target immunity reduces editing efficiency
Base Editing	• High editing efficiencies • High cell survival rates • Multiplexing possible	• Editing window and PAM constraints • Limited to specific base transitions • Off-target mutations (Bystander edits)
Gene Expression Regulation	• Tuneable regulation • Simultaneous up- and downregulation of different genes possible • No DSBs introduced • Applicable for functional genomics • Multiplexing possible	• No permanent edits • Incomplete repression/activation • Genomic context-dependent • dCas-toxicity at high expression levels • Off-target effects observed
Evolution-coupled techniques	• Unbiased discovery of beneficial mutations • Scalable • Effective for complex and polygenic traits	• Time-consuming (weeks to months) • High phenotyping, sequencing, and reverse engineering effort • Need for selection pressure

mismatch repair. Recombineering occurs during cell growth and is subsequently validated by plasmid sequencing. This method could serve as an alternative to GGA for generating diversified genetic parts and could synergize with high-throughput genome editing approaches.

As CRISPR/Cas methods (Figure 2b) are widely employed to engineer prokaryotic and eukaryotic systems, recent efforts have also started to combine recombineering with CRISPR/Cas9, enabling two-step recombination in a single transformation event in *E. coli*, leading to the development of GIDGE (Guide sequence-independent and donor DNA mediated genomic editing) [21]. This method employs selectable markers during the first genomic integration event, followed by their excision through the induction of a Cas9-mediated double-strand break (DSB) to promote a second recombination event. With this set-up, the authors performed high-throughput engineering of single-gene knockout mutants in a 96-well plate format, which can readily be adapted to robot-assisted platforms that commonly operate in this format.

Because several other CRISPR-based technologies have been developed in recent years, various CRISPR-associated methods for rapid strain engineering are presented in the following sections. These include CRISPR/Cas-based editing (Figure 2b), CRISPR-associated transposases (CASTs) (Figure 2c), base editing (Figure 2d), and gene expression regulation with catalytically dead Cas proteins (dCas) (Figure 2e,f). Although the regulation of gene expression is, by definition, not genome editing, we consider it an important tool for strain engineering as a whole.

CRISPR/Cas genome engineering

Owing to the DSB-inducing nature of commonly used Cas proteins, performing CRISPR/Cas-based multiplex editing is not trivial, as it imposes considerable stress on the targeted cells (Figure 2b). Despite these challenges, previous studies have successfully achieved multiplexed editing in bacteria [26], yeast [27], plants [28,29], and mammalian cells [30]. Since higher degrees of multiplexing become progressively more challenging and inefficient, we believe that classical CRISPR/Cas-based editing approaches could benefit from parallelization in automated laboratory platforms. This would compensate for low editing efficiencies by increasing the overall throughput. Alternatively, high-throughput editing in *E. coli* using the CREATE (CRISPR-Enabled Trackable Genome Engineering) method could be achieved by generating large mutational libraries for point mutations in regulatory genes with barcode-enabled mutational

tracking to screen for beneficial mutations [31]. When working with organisms that allow for *in vivo* DNA assembly, genome engineering can be accelerated by coupling *in vivo* assembly with targeted genomic integration, orchestrated by Cas-proteins. Using this approach, a recent study achieved genomic integration of a 37 kb gene cluster in *Trichoderma reesei* with Cas9 (20% integration efficiency), which was preassembled *in vivo* from four fragments before integration (70% assembly efficiency) [18]. In this case, *in vivo* assembly reduces cloning time, while the ability to integrate large DNA sequences decreases the number of editing cycles required to modify the target organism.

CRISPR-associated transposases

CRISPR-associated transposases (CASTs) are programmable mobile genetic elements that enable targeted genomic integration of large genetic cargo by combining nuclease-deficient CRISPR/Cas systems with transposons (Figure 2c) [32]. Although the integration cascade involves multiple proteins and is relatively complex, CASTs neither induce DSBs nor require homology arms as templates, which reduces cellular stress and simplifies the design of genetic tools. Although still a relatively new method, CASTs have already been applied for the modification of various bacteria [33–35], plant cells [36], and human cell lines [37]. In bacteria, CAST-based methods have successfully enabled the insertion of large operons and biosynthetic pathways (< 10 kb) into host genomes and supported multiplex genomic integration of a GFP reporter via expression of a crRNA array [33,38].

Base editing

Genome editing without inducing DSBs is also possible with so-called base editors (BEs) (Figure 2d). As the name implies, BEs facilitate single-nucleotide substitutions, guided by either a dCas or a Cas nickase (nCas) that cuts only one strand and is fused to an adenine or cytosine base editor (ABE and CBE, respectively). Although BEs depend on proximal protospacer adjacent motifs (PAMs) because of their limited editing window and may cause off-target effects, they have nevertheless proven effective for genome-wide essentiality screens in bacteria [39]. Moreover, BE-derived methods are currently employed for high-throughput and high-level multiplex editing, enabling the simultaneous modification of up to ten genes in bacteria [40–43]. The suitability of base editing for automation was demonstrated in a study on *Corynebacterium glutamicum* [2]. In this context, the authors automated plasmid construction and *in vivo* editing, requiring only a few offline steps. As a demonstrator, 94 putative nonessential transcription factors were inactivated by introducing premature stop codons, achieving editing efficiencies above 50%. With multiple research groups reporting successful applications of various BEs, base editing represents a valuable addition to the genome editing repertoire.

Cas-mediated gene expression regulation

Cas-mediated regulation of gene expression can be achieved either via the editing methods described above or through nuclease-deficient Cas variants fused to activators (CRISPRa) and repressors (CRISPRi, i for interference or inhibition) (Figure 2e). Although CRISPRa/i is not genome editing per se, it is well suited for multiplexed targeting, which is particularly valuable for large-scale and automated genomic screens. Some of the recent developments include multifunctional genome-wide screens [44,45], as well as improved multiplexing in plants [46] and mammalian cells [47]. Other studies achieved simultaneous gene activation and inactivation by employing orthogonal Cas proteins in *S. cerevisiae*, or parallel gene disruption and repression by using full-length and truncated guide RNAs (gRNAs) in *E. coli*, respectively [48,49]. When combined with biosensor-assisted strain engineering, CRISPRa/i techniques have enabled large-scale and multiplexed genome engineering, as well as high-throughput phenotyping, addressing a major bottleneck in strain engineering [50].

More recently, the discovery of the RNA-targeting Cas13 protein family permitted translational repression (dCas13), independent of a protospacer flanking site in both prokaryotes [51–54] and eukaryotes [55,56]. Similar to other Cas systems, dCas13 can be fused to activators — in this case, translation initiation activators — to enhance target protein production for strain engineering (Figure 2f) [57]. These studies demonstrate the potential of advanced gene expression regulation for strain engineering and expand the repertoire for modern genome engineering tools.

Microfluidics and evolution-coupled techniques

The transfer of genome engineering technologies to robot-assisted platforms typically requires the miniaturization of laboratory workflows into microtiter plate (MTP) formats. Although still uncommon, further miniaturization to the microfluidic scale would support even higher throughput. Multiple research groups have demonstrated that the methods discussed above can be implemented in microfluidic devices. A first step toward the miniaturization of genome editing was achieved through plasmid construction via GGA and subsequent heat shock transformation into the commonly used cloning host *E. coli* on a nanoliter scale [58]. Automated, parallel editing of up to 100 *E. coli* strains in a microfluidic device was achieved using a combination of CRISPR/Cas9 and λ red recombineering [59]. Additionally, microfluidic chips can serve as screening platforms when CRISPRi is employed, as shown in a genome-wide screening in *C. glutamicum* [60]. Alternatively, such devices can be employed as microdroplet culture systems, for instance, for automated and parallelized microbial adaptive laboratory evolution (ALE), which remains a widely used approach for identifying novel engineering targets. Beyond microfluidics, automated lab-scale ALE

systems are currently under development to increase parallelization while reducing manual labor [61–64].

Integrated workflow automation

Some of the methods presented above have already been converted into (partially) automated workflows. Additionally, case studies describing integrated automated workflows in biofoundries are now available. An increasing number of biofoundries are currently being established to automate DBTL cycles, and many of these are joining the Global Biofoundry Alliance to coordinate and collaborate more effectively [65]. Recent advances in automated platforms for genome engineering with minimal human intervention have become accessible across a range of organisms. Most recently, a group of scientists successfully integrated protein language models with a biofoundry for automated protein evolution (PLMeAE) in successive DBTL cycles [66]. The study used the *p*-cyanophenylalanine tRNA synthetase of *Methanocaldococcus jannaschii* as a model enzyme for four rounds of evolution in 10 days, resulting in a 2.4-fold increase in enzyme activity, depicting an alternative approach to classical genome editing. CRISPR/Cas-based genome editing in bacteria was recently implemented on the AutoBioTech platform, a biofoundry consisting of 14 devices for automated strain construction without human interaction at Forschungszentrum Jülich, Germany [67]. The processes include GGA-based plasmid construction, transformation of Gram-positive and Gram-negative bacteria via heat shock, electroporation, or conjugation, and Cas9-assisted genome editing in *E. coli*. Similarly, automated high-throughput genome engineering using CRISPR/Cas9 was demonstrated in *Pichia pastoris* on the iBioFoundry platform [68]. The FAST-PB (fast, automated, scalable, high-throughput pipeline for plant bioengineering) platform utilized a CRISPRa approach for large-scale profiling to improve lipid production in plant cells [69]. Editing of mammalian cell lines in biofoundries has been achieved with BEs [1] and Cas9 [70]. These case studies demonstrate the broad host range and methodological versatility already attainable through biofoundry-guided engineering. Beyond their applied value, the increasing robustness and accessibility of automated technologies render biofoundries a key asset for accelerating our understanding of biological systems.

Conclusions

Modern genome editing methods that allow for automatable, high-throughput, and multiplexed workflows are essential to meet the growing demand for tailor-made organisms in biotechnological and pharmaceutical applications. Since laboratory automation requires substantial financial investment, we argue that the development of methods enabling higher throughput or multiplexed editing at the laboratory scale is equally important. These advancements form the foundation of modern biofoundries, in which the manual strain construction workflows are transferred to fully automated — and in the future fully autonomous and AI-driven — DBTL cycles.

CRediT authorship contribution statement

DK: Conceptualization, Visualization, Writing – original draft. **JM:** Conceptualization, Project administration, Supervision, Visualization, Writing – review & editing.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT in order to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare no conflict of interest.

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- •• of outstanding interest

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