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Advances and challenges in CRISPR/Cas9 microbial genome editing: a review

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Abstract

CRISPR/Cas9 has revolutionized genome editing, providing a powerful and precise tool for modifying genetic material across various biological systems. This review explores its mechanisms, applications, and future directions, highlighting its impact on human health, microbial engineering, and protist research. CRISPR-based genome editing has enabled breakthroughs in treating genetic disorders and microbiome modulation, with promising applications in oral health for bacterial regulation and antibiotic resistance management. Beyond medical applications, CRISPR technology has facilitated genetic manipulation in protists, advancing research on parasite biology, host-pathogen interactions, and industrial biotechnology. Despite its vast potential, CRISPR/Cas9 presents challenges, including off-target effects, immunogenicity, and technical barriers in genome delivery and repair mechanisms. Addressing these limitations requires the development of high-fidelity Cas variants, improved delivery methods, and advanced detection tools. As research progresses, CRISPR technology is expected to play a transformative role in medicine, agriculture, and biotechnology. With continuous advancements, CRISPR/Cas9 remains at the forefront of genetic engineering, offering novel solutions for scientific and medical challenges.

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Introduction

Genome editing technologies have rapidly advanced, offering unprecedented precision in genetic modifications. The foundation of genetic engineering was laid in 1974 when Jaenisch and Mintz demonstrated the stable integration of viral DNA into the mouse genome, marking a significant milestone in molecular biology (Jones 2011; Jaenisch and Mintz 1974). A transformative breakthrough came in 2012 when Emmanuelle Charpentier and Jennifer Doudna successfully

reconstituted the clustered regularly interspaced short palindromic re-peats and the associated Cas 9 proteins (CRISPR/Cas9) system in vitro, demonstrating its ability to target specific DNA sequences with programmable precision. This discovery revolutionized genome editing, establishing CRISPR/Cas9 as a versatile and efficient tool for genetic modifications across diverse species. Due to its ease of adaptability in biomedical laboratories, Charpentier and Doudna

were awarded the Nobel Prize in Chemistry in 2020 (Okzan 2021; Jinek *et al.* 2012).

The discovery of CRISPR and the associated Cas proteins in prokaryotic and eukaryotic genomes represents one of the most groundbreaking advancements in modern biology (Shmakova *et al.* 2022). This technology utilizes the Cas9 nuclease, guided by a synthetic RNA (gRNA), to precisely target and cleave specific genomic sequences, enabling the removal, modification, or insertion of genetic material *in vivo*. CRISPR-based genome editing holds immense potential for biological research, but also for clinical and biotechnological applications. Its simplicity, cost-effectiveness, and high precision have revolutionized biotechnology and medicine, with applications spanning disease management, drug development, and beyond. This review explores the CRISPR/Cas9 system, highlighting its transformative role in medicine, microbial engineering, and biotechnology.

CRISPR/Cas9 system and genome editing mechanism

The CRISPR/Cas9 system represents a groundbreaking advancement in gene editing, enabling precise modifications to the genomes of living organisms. CRISPR/Cas9 is naturally present in approximately 5–7% of prokaryotic bacteria, particularly in the Proteobacteria and Firmicutes phyla, and can be used for genome editing in virtually all living organisms, including humans (Wang *et al.* 2016; Makarova *et al.* 2011).

Based on the structure and function of Cas proteins, the CRISPR/Cas system is classified into Class I (Types I, III, and IV) and Class II (Types II, V, and VI). Type I, II, and V systems recognize and cleave DNA, while Type VI targets RNA, and Type III can modify both DNA and RNA. The mechanism of Type IV remains largely unknown (Hryhorowicz *et al.* 2023; Liu *et al.* 2020). The characteristics of various types of CRISPR/Cas system are summarized in Table 1. In genetic engineering, the Type II CRISPR/Cas9 system from *Streptococcus pyogenes* (SpCas9) is the most widely studied and utilized due to its high efficiency and precision. Its mechanism involves three key steps: recognition, cleavage, and repair (Mengstie and Wondimu 2021; Le Rhun *et al.* 2019; Zhou *et al.* 2018).

The Type II Cas9 enzyme consists of two main components: the guide RNA (gRNA) and the CRISPR-associated protein 9 (Cas9) (Zhang and Li 2021). The gRNA is a duplex structure composed of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). For practical applications in genetic engineering, this duplex can be replaced by a synthetic single-guide RNA (sgRNA), a chimeric RNA that simplifies the CRISPR/Cas9 system (Bhattacharjee *et al.* 2022; Martinez-Lage *et al.* 2018).

A short DNA sequence known as the "Protospacer-Adjacent Motif" (PAM) is essential for CRISPR function and follows the sgRNA's unique sequence, which is recognized by Cas9 (Martinez-Lage *et al.* 2018). The PAM sequence plays a crucial role in target recognition, ensuring that Cas9 distinguishes foreign DNA from the host's own genome, thereby preventing self-cleavage (Zhu 2022).

Upon expression in the cell, the Cas9 nuclease and sgRNA form a ribonucleoprotein (RNP) complex, which the sgRNA directs to the target DNA sequence through Watson-Crick base pairing (Lino *et al.* 2018). Cas9 then induces a double-strand break (DSB) by precisely cleaving both DNA strands, generating blunt ends. In eukaryotic cells, DSBs activate cellular repair mechanisms: homology-directed repair (HDR) or non-homologous end joining (NHEJ) (Nambiar *et al.* 2022).

HDR is a highly precise repair pathway that requires a homologous DNA template to guide error-free repair. In contrast, NHEJ, the predominant repair mechanism, directly joins the broken DNA ends without a homologous template. NHEJ is generally active throughout the cell cycle, including mitosis and is considered the predominant repair pathway for double-strand breaks (DSBs) in mammalian cells (Yang *et al.* 2020). However, during mitosis, certain regulatory mechanisms – such as phosphorylation of repair factors and exclusion of proteins like 53BP1 from chromatin, can suppress or modify NHEJ activity to prevent erroneous repair (Scully *et al.* 2019).

Table 1: General characteristics of CRISPR/Cas systems according to types (Liu *et al.* 2022).

Type	Class	Signature protein	Effector	PAM sequence	Target substrate	Cleavage product
I	1	Cas3 (or Cas3')	Cascade	3-nt	DNA	SSBs
II	2	Cas9	crRNA and tracrRNA (sgRNA)	5'-NGG-3'	DNA	DSB (flat end)
III	1	Csm (III-A)/ Cmr (III-B)	Cascade	without PAM	DNA/RNA	SSBs at every 6-nt
IV	1	unknown	unknown	unknown	unknown	unknown
V	2	Cas12a	crRNA	5'-YTN-3', 5'- TTTN-3'	DNA	DSB (sticky end with 5 nucleotides protruding)
VI	2	Cas13	Csm/Cmr	without PAM	RNA	RNA cleavage

Applications of CRISPR/Cas system

Beyond its fundamental role in genome editing, the CRISPR/Cas9 system has vast potential across various fields, ranging from human health to microbial engineering, and biotechnology. Its applications extend beyond therapeutic gene editing to include the manipulation of microbial genomes, disease prevention, advancements in industrial, and agricultural biotechnology (Chen *et al.* 2025; Jakočiūnas *et al.* 2016; Schumann *et al.* 2015; Zhang *et al.* 2015). Numerous studies have explored the role of CRISPR/Cas9 in human health, particularly in treating genetic disorders and cancer (Stefanoudakis *et al.* 2023; Kan and Doudna 2022; Katti *et al.* 2022; Wu *et al.* 2020). However, in this review, we focus on its applications in microbial genome editing, emphasizing both its potential in human health, and its broader use in biotechnology. A particularly promising area of research is the role of CRISPR/Cas9 in modulating the oral microbiome and combating oral diseases. By targeting bacterial physiology, CRISPR/Cas9 may provide innovative strategies for regulating microbial populations, improving oral health, and addressing conditions such as periodontitis and oral cancer. The following sections explore its role in oral microbiome regulation, microbial genome editing, and other transformative applications in biotechnology and medicine.

Besides these classical repair pathways, alternative error-prone end-joining mechanisms, such as microhomology-mediated end joining (MMEJ),

also exist. MMEJ is a highly mutagenic process that repairs DSBs by annealing short microhomologous sequences (2–20 bp) flanking the break site (Seol *et al.* 2018). Following DSB formation, additional gene modifications can be performed depending on the specific application, including disease treatment and functional genomic studies. Figure 1 provides a schematic representation of the CRISPR/Cas9 mechanism, highlighting its key components and molecular processes involved in target recognition, cleavage, and genome editing. Figure 1A depicts the natural function of the CRISPR/Cas system in bacterial defines against viral infections. When a bacteriophage injects its DNA into a bacterial cell, the CRISPR system incorporates a fragment of the viral DNA (spacer) into its CRISPR array, creating a genetic memory. This sequence is transcribed into precursor CRISPR RNA (pre-crRNA), which is processed by tracrRNA and RNase III into mature crRNA. The crRNA, in complex with Cas9, guides the protein to a matching viral DNA sequence, leading to targeted cleavage and destruction of the viral genome through a double-stranded break.

The Cas9 protein (Figure 1B), guided by a synthetic single-guide RNA (sgRNA), binds to a target DNA sequence adjacent to a protospacer adjacent motif (PAM), inducing a site-specific double-stranded break (DSB). The cell repairs this break via two pathways: (i) Non-homologous end joining (NHEJ). An error-prone repair mechanism that introduces small insertions or deletions (indels), potentially leading to gene knockout.

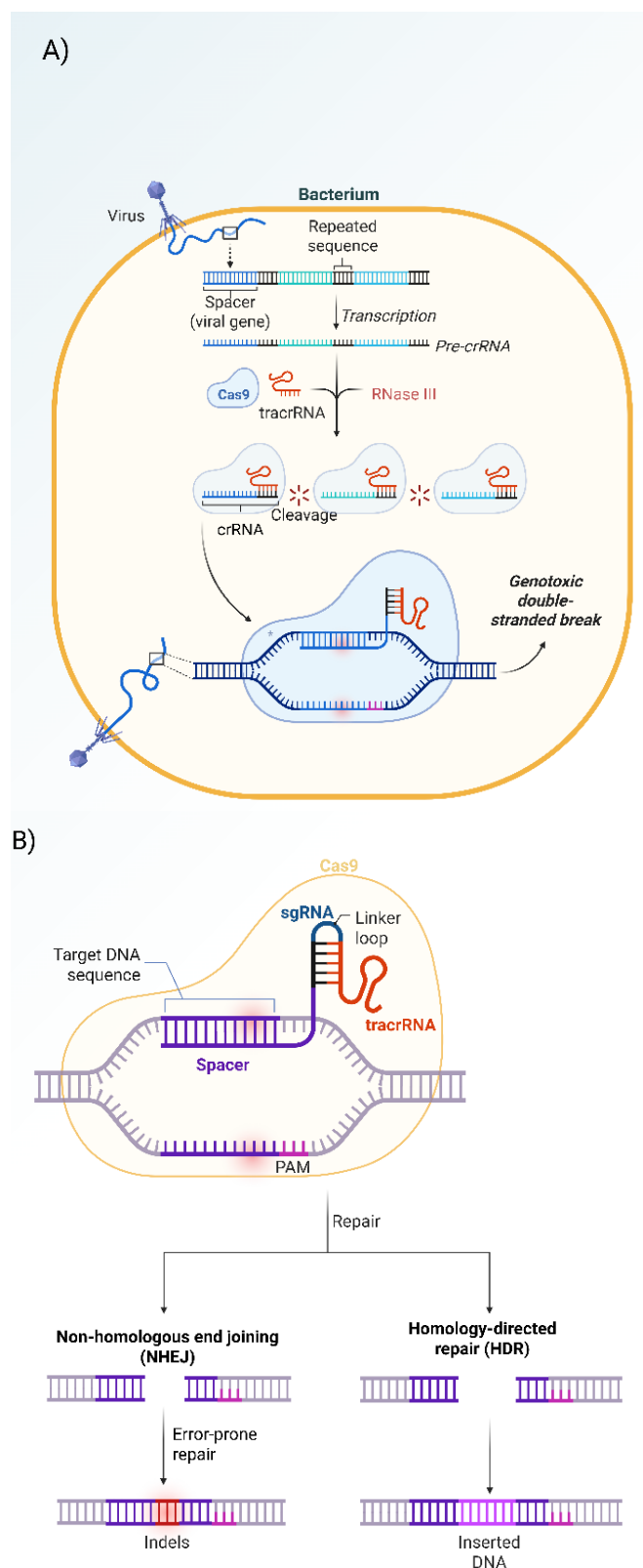


Figure 1: Mechanism of CRISPR/Cas9 genome editing (A) CRISPR/Cas as a bacterial immune system; (B) CRISPR/Cas9 in genome editing (BioRender 2020).

(ii) Homology-directed repair (HDR): A precise repair mechanism incorporating a donor DNA template, allowing for targeted gene correction or sequence insertion

The CRISPR/Cas system and oral bacteria

CRISPR-associated genes in oral bacteria and archaea play a crucial role in adaptive immunity and bacterial physiology, influencing various cellular processes (Gong *et al.* 2020; Makarova *et al.* 2011). The CRISPR/Cas system in oral bacteria is involved in regulating bacterial growth, DNA repair, virulence, metabolism, biofilm formation, interspecies interactions, and overall microbial survival (Chopra *et al.* 2025).

Recent studies have identified multiple CRISPR/Cas system types (I, II, III, V, and VI) within the human oral microbiome, while Type IV has not been detected (Gong *et al.* 2018; Makarova *et al.* 2011). Among these, Types I and II are the most prevalent, suggesting that oral bacteria primarily rely on these systems for genetic defense and potentially other functional roles. Notably, certain oral pathogenic bacteria, such as *Porphyromonas gingivalis*, harbor a Type III CRISPR/Cas system, which primarily targets mRNA and other RNAs, indicating its involvement in post-transcriptional gene regulation (Gong *et al.* 2020). *P. gingivalis* is an anaerobic, Gram-negative pathogen of oral cavity that plays a major role in periodontitis and has been implicated in systemic diseases such as rheumatoid arthritis, atherosclerosis, cardiovascular diseases, Alzheimer's disease, pneumonia, and pregnancy complications (Li *et al.* 2022).

A deeper understanding of CRISPR/Cas systems in oral bacteria is essential for elucidating their role in maintaining microbiome stability, modulating pathogenicity, and influencing disease progression (Chopra *et al.* 2025). The CRISPR/Cas system plays a critical role in maintaining microbial homeostasis by limiting horizontal gene transfer and providing adaptive immunity against bacteriophages and foreign genetic elements (Watson *et al.* 2021). Disruptions in this system can contribute to microbial dysbiosis, leading to bacterial plaque formation, inflammation, and the

onset of oral diseases such as dental caries and periodontitis (Giordano-Kelhoffer *et al.* 2022; Lemos *et al.* 2019; Yu *et al.* 2019). Oral microbiome dysbiosis, characterized by imbalances in bacterial composition and metabolic activity, can facilitate the overgrowth of cariogenic species (e.g. *Streptococcus mutans*) or pathogenic bacteria (e.g. *P. gingivalis*).

S. mutans, a Gram-positive bacterium, is a primary etiological agent of dental caries due to its ability to synthesize an extracellular polysaccharide (EPS) matrix, which promotes biofilm formation and enhances bacterial adherence to tooth enamel and microbial communities (Gong *et al.* 2020; Lemos *et al.* 2019; Gong *et al.* 2018). The CRISPR/Cas system is naturally occurring in *S. mutans* as a defense mechanism against foreign genetic elements. On the other hand, it could be used as a potentially non-toxic and targeted approach for dental caries prevention (Gong *et al.* 2018).

A gene-editing approach using self-targeting CRISPR arrays combined with editing templates was developed by Gong *et al.* (2018) to target virulence genes encoding glycosyltransferases (*gtfs*) in *S. mutans*. This strategy effectively reduced EPS synthesis and biofilm formation. Gene *gtfB* and *gtfC* are essential for the sucrose-dependent colonization of hard surfaces by *S. mutans*, further for EPS production and biofilm architecture and they play a crucial role in the pathogenesis of dental caries. Therefore, these genes have been identified as promising targets for preventing dental caries and their simultaneous knock out leads to bio-film reduction (Koo *et al.* 2010).

Beyond biofilm regulation, CRISPR/Cas, especially loci CRISPR1 and CRISPR2 in *S. mutans* may influence antibiotic resistance. Studies analyzing these loci in dental plaque biofilms of healthy and periodontitis patients, showed that healthy individuals had more similar CRISPR components suggesting that an intact CRISPR system contributes to microbial balance by defending against phage invasion and limiting the spread of antibiotic resistance genes. Consequently, modulating CRISPR/Cas activity could serve as a novel strategy for microbiome engineering and disease control (Chavez-Granados *et al.* 2022; Yu *et al.* 2019).

Recent studies have highlighted the crucial role of the CRISPR/Cas system in modulating *P. gingivalis* virulence (Chopra *et al.* 2025; Chavez-Granados *et al.* 2022). The Cas3 protein, an essential nuclease in the Class 1 Type I CRISPR/Cas system, has been shown to influence the pathogenicity of *P. gingivalis*. The *cas3* gene is involved in the regulation of cytokine production, particularly actin and ankyrin, which are critical for cytoskeletal organization, as well as microRNA (miRNA) expression, both of which contribute to inflammatory responses in periodontitis (Chopra *et al.* 2025). Experimental knock out of *cas3* has been associated with increased virulence, as evidenced by heightened pathogenicity and increased mortality in *in vitro* infection models. These findings suggest that Cas3 may act as a regulatory element, modulating the expression of virulence-associated genes and playing a key role in the pathogenesis of *P. gingivalis* (Chavez-Granados *et al.* 2022; Solbiati *et al.* 2020).

Challenges, limitations, and future directions of CRISPR/Cas technology in oral microbiome engineering

Despite the promise of CRISPR/Cas technology in modifying the oral microbiome, several challenges must be addressed to ensure its safe and effective application (Chopra *et al.* 2025). One of the primary concerns is CRISPR-associated cytotoxicity, which can reduce the viability of epithelial and gingival cells following gene-editing interventions. This toxicity poses a significant challenge, particularly in delicate tissues such as the oral mucosa, potentially limiting the therapeutic potential of CRISPR-based treatments (Chopra *et al.* 2025; Azeez *et al.* 2024).

Furthermore, genetic manipulation of anaerobic oral bacteria, such as *P. gingivalis*, remains technically challenging due to its anaerobic growth requirements, which complicate conventional transformation techniques. Effective CRISPR-based editing in such bacteria necessitates optimized delivery methods and selection markers. Additionally, *P. gingivalis* harbors an endogenous CRISPR/Cas system that may interfere with the introduction of exogenous gene-editing tools,

requiring careful design to circumvent these limitations (Chopra *et al.* 2025; Gong *et al.* 2018). Another significant challenge is the immunogenicity of CRISPR components. Since Cas9 is derived from bacterial species, the human immune system may recognize it as a foreign antigen, potentially triggering immune responses such as inflammation or rejection of genetically modified cells. This response could limit the safety and effectiveness of CRISPR-based therapies (Azeez *et al.* 2024).

Additionally, off-target effects remain a critical concern in CRISPR genome editing. Unintended genetic modifications can lead to unpredictable gene disruptions, permanent alterations, and potential health risks (Chehelgerdi *et al.* 2024). These off-target mutations may result in genomic instability, disruption of essential gene functions, epigenetic modifications, or even carcinogenesis, raising significant safety concerns regarding the long-term application of CRISPR technology (Chopra *et al.* 2025; Chehelgerdi *et al.* 2024). Therefore, precise detection and regulation of these unintended alterations are essential to minimize potential adverse effects.

Moreover, CRISPR-mediated gene editing has been associated with large, unintended deletions, which may contribute to pathological consequences. These large-scale deletions can lead to irreversible genetic damage, complicating the controlled application of CRISPR in therapeutic settings (Kosicki *et al.* 2018). The unpredictability of extensive genomic alterations presents a major obstacle in achieving targeted and safe gene modifications, particularly in clinical applications requiring high precision (Chopra *et al.* 2025). To overcome these challenges, future advancements must focus on high-fidelity CRISPR variants, enhanced delivery methods, and improved detection techniques for off-target effects. Continued research is essential to ensure the safe and effective application of CRISPR technology in oral microbiome engineering and broader biomedical fields.

The use of CRISPR in human-associated microbiomes holds immense potential, yet safety and ethical considerations must be carefully addressed before clinical translation (Ansori *et al.* 2023; Gong *et al.* 2020). Future research should

prioritize optimizing CRISPR delivery methods, including bacteriophage-based vectors and nanoparticle-mediated approaches, to enhance editing efficiency in oral bacteria (Ansori *et al.* 2023). While CRISPR has emerged as a leading tool for microbiota modification, its specific applications in the human oral microbiome remain largely unexplored (Chopra *et al.* 2025; Abavisani *et al.* 2024). Harnessing CRISPR technology could provide valuable insights into host-microbiota interactions and aid in the identification of key biological pathways involved in oral and systemic health. While preclinical studies suggest that CRISPR-based approaches may be effective for microbiome-related disorders, addressing ethical and regulatory challenges is crucial to ensure compliance with established safety guidelines (Abavisani *et al.* 2024).

Additionally, expanding the application of CRISPR interference (CRISPRi) for epigenetic modifications and host-microbe interaction studies could deepen our understanding of the oral microbiome's role in both oral and systemic diseases (Chopra *et al.* 2025; Ghavami and Pandi 2021). CRISPRi is an RNA-guided technology based on the CRISPR system type II, that enables highly specific transcriptional silencing in both prokaryotic and eukaryotic cells. This method employs a catalytically inactive Cas9 (dCas9), which binds to DNA without cleaving it, thereby leading to repression of gene expression without inducing double-strand break. CRISPRi bypasses the need for homologous recombination, simplifying genetic manipulation process (Ghavami and Pandi 2021). In a study by Zhou *et al.* (2024), they studied gene inactivation and functionality in oral pathogenic bacteria *Fusobacterium nucleatum* using CRISPRi. *F. nucleatum* is an anaerobic bacterium associated with periodontitis, systemic inflammatory diseases, and rheumatoid arthritis. In general, fusobacterial strains are characterized with difficulties to perform genetic manipulations. Using CRISPRi the authors suppressed essential gene *bamA*, leading to disruption of membrane integrity and inhibition of bacterial growth (Zhou *et al.* 2024). By leveraging CRISPRi for gene silencing and functional genomic studies, researchers can gain novel insights into microbial contributions to disease progression and explore

therapeutic strategies beyond direct genetic modifications (Chopra *et al.* 2025; Ghavami and Pandi 2021).

As CRISPR technology continues to evolve, its integration into oral microbiome research and therapeutic applications will require multidisciplinary collaboration, encompassing microbiology, genetic engineering, immunology, and bioethics. Addressing current limitations while exploring innovative applications will be essential for fully realizing the potential of CRISPR in oral and systemic health.

While CRISPR/Cas technology has demonstrated significant potential in modifying the human-associated microbiome, its applications extend far beyond human health. CRISPR-based genome editing is increasingly being utilized in diverse microorganisms, including protists, where it has revolutionized the study of molecular genetics, host-pathogen interactions, and biotechnology. The ability to manipulate protist genomes with precision has provided new insights into their biology, disease mechanisms, and potential applications in industrial and environmental biotechnology. The following section explores how CRISPR/Cas9 has been adapted for genome editing in protists, highlighting key advancements, challenges, and future directions in this rapidly evolving field.

Genome Editing in Protists using CRISPR/Cas9

While CRISPR/Cas9 has been widely explored for its applications in human health, its impact extends far beyond clinical applications. CRISPR-based genome editing has also revolutionized the study of eukaryotic microorganisms, providing powerful tools for fundamental research and biotechnological advancements. In protists, CRISPR/Cas9 has enabled precise genetic modifications, facilitating the study of gene function, host-pathogen interactions, and metabolic pathways (Horáčková *et al.* 2022; Grzybek *et al.* 2018; Lander *et al.* 2016a). The first successful application of CRISPR/Cas9 in protists was reported in 2014, demonstrating its potential for functional genomics in single-celled eukaryotes (Ghorbal *et al.* 2014). Since then, numerous studies

have employed CRISPR/Cas9 to manipulate genes in both pathogenic and non-pathogenic protists, leading to significant advancements in understanding their biology and identifying potential therapeutic targets (Horáčková *et al.* 2022; Gomaa *et al.* 2022; Faktorová *et al.* 2020; Lander *et al.* 2016a).

CRISPR/Cas9 applications in pathogenic protists

One of the earliest and most impactful applications of CRISPR/Cas9 in protists focused on *Plasmodium falciparum*, the causative agent of malaria. In a landmark study, researchers first validated the system by targeting an *EGFP* reporter gene, demonstrating that CRISPR/Cas9 can introduce precise double-strand breaks that are repaired by homologous recombination. They then extended the approach to modify an endogenous gene specifically, the gene encoding knob-associated histidine-rich protein (KAHRP). KAHRP is critical for the formation of knobs on the surface of infected red blood cells, which mediate cytoadherence and are key to malaria pathogenesis. To disrupt KAHRP, a single guide RNA (sgRNA) was designed to target its coding region and was co-transfected with a donor DNA template that carried homology arms flanking a drug-selectable marker (HDHFR). Successful integration of this cassette via homologous recombination resulted in the loss of KAHRP expression, providing crucial insights into host-pathogen interactions and red blood cell remodelling (Lander *et al.* 2016a; Ghorbal *et al.* 2014; Wagner *et al.* 2014).

Similarly, *Toxoplasma gondii* has been extensively studied using CRISPR/Cas9, where gene knockouts have helped to elucidate the role of specific proteins in parasite virulence and immune evasion. In this study, the authors first selectively inactivated the UPRT gene because its disruption leads to fluorodeoxyribose (FUDR) resistance. This characterization not only provides a direct and quantitative marker to monitor the efficacy of gene disruption but also serves as a practical platform for subsequent experiments. They then focused on the modification of the ROP18 gene, which encodes a secreted serine/threonine kinase identified as a key

determinant of acute virulence. Disruption of *ROP18*, particularly in the *T. gondii* type I natural isolate GT1, revealed that this gene plays a much more critical role in the pathogenicity of natural strains than previously thought. Complementation experiments, in which the lost function of *ROP18* was restored by reintroducing the gene into a neutral genomic locus, further confirmed its essential role in modulating virulence (Shen *et al.* 2014).

This system has also been adapted for *Cryptosporidium parvum*, another apicomplexan parasite, to investigate genes essential for intracellular survival. In a recent study, researchers established a robust transfection protocol for *C. parvum* sporozoites and developed an in vivo mouse model to select for stable transgenic parasites. They engineered reporter strains by integrating a highly sensitive nanoluciferase (*Nluc*) gene alongside a drug-selectable marker, enabling precise monitoring of parasite infection both in vitro and in vivo. Notably, the study targeted the thymidine kinase (*TK*) gene—a gene acquired via horizontal gene transfer that is implicated in nucleotide metabolism and antifolate resistance. Disruption of *TK* via homologous recombination led to complete loss of its expression and increased susceptibility to the anti-folate drug trimethoprim, thereby underscoring its critical role in intracellular survival (Vinayak *et al.* 2015). These studies demonstrated that CRISPR/Cas9 is an effective tool for protist genome editing, despite the unique challenges posed by their complex cellular and genomic structures (Faktorová *et al.* 2020).

The adoption of CRISPR/Cas9 in kinetoplastid parasites, such as *Leishmania* spp. and *Trypanosoma cruzi*, marked another milestone in protist research. In *L. donovani*, gene knockouts of the *LdMT* gene, which encodes a miltefosine transporter have been achieved without requiring selection markers, streamlining genetic modifications and accelerating functional studies. In this approach, the CRISPR/Cas9 system was used to create a double-strand break at the *LdMT* locus, and an oligonucleotide donor carrying the stop codons was introduced using homology-directed repair. This method circumvents the need to insert exogenous selectable markers (such as antibiotic resistance genes), resulting in a cleaner

genetic modification process that facilitates rapid functional analysis of gene roles, including drug resistance mechanisms (Lander *et al.* 2016a; Zhang and Matlaszewski 2015).

Similarly, in *T. cruzi*, researchers targeted genes involved in calcium metabolism and acidocalcisome function by employing CRISPR/Cas9-mediated endogenous tagging. In this approach, they specifically tagged the inositol-1,4,5-trisphosphate receptor (*TcIP3R*) at its native locus without overexpression, thereby ensuring that its detected localization accurately reflected its true distribution within the cell. This precise tagging revealed that *TcIP3R* localizes to acidocalcisomes rather than the endoplasmic reticulum—a finding that contrasts with earlier results obtained by overexpression methods. As a result, the study provided novel insights into the role of *TcIP3R* in regulating calcium signalling, which is critical for parasite survival and virulence, and highlighted its potential as a therapeutic target (Lander *et al.* 2016a; Lander *et al.* 2016b). These findings highlight the versatility of CRISPR/Cas9 in editing complex protozoan genomes and identifying new potential drug targets (Faktorová *et al.* 2020).

CRISPR/Cas9 in non-pathogenic and biotechnologically relevant protists

Beyond pathogenic protists, CRISPR/Cas systems have also been applied to model organisms such as *Euglena gracilis*, a photosynthetic flagellate widely studied for its unique metabolic pathways and potential industrial applications. Recent studies have successfully introduced gene modifications in *E. gracilis* using CRISPR-based methods. For example, Nomura *et al.* (2024) used a Cas12a ribonucleoprotein (RNP) system to target two key genes: *EgGSL2*, a glucan synthase-like gene implicated in paramylon synthesis, and *EgcrtB*, a phytoene synthase gene essential for carotenoid biosynthesis. By disrupting these genes, they achieved high mutagenesis rates of approximately 77–95% and demonstrated efficient knock-in and base editing using single-stranded DNA donor templates. Their aim was to modulate metabolic pathways associated with polysaccharide and pigment production, thereby enhancing the

organism's potential for biofuel production and industrial biotechnology (Nomura *et al.* 2024; Nomura *et al.* 2020; Nomura *et al.* 2019). Similarly, in a separate study aimed at improving harvesting efficiency, researchers generated non-motile *E. gracilis* mutants by targeting the Bardet–Biedl syndrome genes (*EgBBS7* and *EgBBS8*) using CRISPR/Cas9. The objective in this study was to disrupt flagellar function, resulting in enhanced gravitational sedimentation of the cells, which facilitates easier downstream processing (Ishikawa *et al.* 2022).

Challenges and innovations in CRISPR/Cas9-based genome editing in protists

Despite these advancements, the application of CRISPR/Cas9 in protists presents several technical challenges. Unlike many eukaryotes, protists exhibit diverse DNA repair mechanisms that influence the efficiency and accuracy of CRISPR-induced modifications. Some species primarily rely on homology-directed repair (HDR), while others predominantly use microhomology-mediated end joining (MMEJ), leading to variability in gene-editing out-comes (Lander *et al.* 2016a). Additionally, low transfection efficiency in certain protists, such as *Trichomonas vaginalis*, poses a significant hurdle, necessitating further optimization of delivery methods to enhance genome-editing success rates (Pal and Dam 2022). In addition to gene knockouts, CRISPR/Cas9 has been utilized for knock-in strategies, where exogenous sequences, such as epitope tags, are integrated into the genome for protein detection and functional studies. Epitope tags such as HA, His, Y-fast, and Myc enable protein tracking using commercially available antibodies in Western blotting, immunoprecipitation, and fluorescence microscopy (Vinopalová *et al.* 2024; Horáčková *et al.* 2022; Kangassu-Marcolino *et al.* 2021). Kangassu-Marcolino *et al.* (2021) successfully implemented a single-vector transformation strategy in *Entamoeba histolytica*, introducing a destabilizing domain of dihydrofolate reductase (ddDHFR) fused with a Myc-tag, allowing for regulated protein expression in response to trimethoprim (Kangassu-Marcolino *et al.* 2021). A

similar strategy was employed in *Giardia intestinalis*, where CRISPR-mediated knock-in epitope tagging demonstrated effectiveness even in polyploid organisms, where genetic modification is typically more challenging (Horáčková *et al.* 2022). Further optimization of donor DNA with homologous arms has improved the specificity and efficiency of epitope tag integration, ensuring accurate genome modifications (Vinopalová *et al.* 2024). Additionally, high-throughput CRISPR screening approaches, as demonstrated by Yadav *et al.* (2023), have opened new avenues for parallel testing of multiple gene modifications, significantly enhancing functional genomics research. By employing these knock-in strategies, CRISPR/Cas9 enables precise and efficient genetic modifications, allowing researchers to study gene function and protein localization with higher accuracy. This methodological advancement not only facilitates fundamental biological research in protists but also enhances the development of novel therapeutic and biotechnological applications (Yadav *et al.* 2023).

Emerging innovations and future directions

Recent advancements in CRISPR/Cas9 technology have significantly expanded its applications in protist research. One of the most notable innovations is CRISPR interference (CRISPRi), a technique that enables reversible gene silencing without introducing permanent mutations. This approach has been successfully employed in *T. gondii*, providing a valuable tool for investigating essential genes and regulatory pathways (Markus *et al.* 2021). In addition to CRISPRi, base editing and prime editing have emerged as promising alternatives to conventional genome-editing methods. These techniques allow for precise nucleotide modifications without generating double-strand breaks, reducing the risk of unintended mutations and increasing editing accuracy (Gilbert *et al.* 2014). Such innovations hold great potential for functional genomics studies and therapeutic research, particularly in protists with complex genome architectures. As CRISPR/Cas9 technology continues to advance, future research is expected to focus on enhancing gene-editing efficiency, developing species-specific

delivery methods, and integrating CRISPR-based tools with single-cell sequencing and transcriptomics. These advancements will enable a deeper understanding of protist biology, host-pathogen interactions, and metabolic regulation (Lander *et al.* 2016a). The application of CRISPR/Cas9 in protists has already led to transformative discoveries, contributing to major breakthroughs in parasite biology, molecular pathogenesis, and metabolic engineering. Moving forward, the continued refinement of CRISPR-based techniques will further drive the development of novel therapeutic strategies and biotechnological applications in fields such as drug discovery, disease modelling, and bioengineering (Faktorová *et al.* 2020).

Conclusions

The CRISPR/Cas9 system has revolutionized genome editing, providing an efficient, precise, and versatile tool for modifying genetic material across various biological systems. This technology has significantly advanced molecular genetics, biomedical research, and biotechnological applications, demonstrating transformative potential in human health, microbial genome engineering, and environmental biotechnology. In oral microbiome research, CRISPR-based approaches offer promising strategies for disease prevention, microbial regulation, and antibiotic resistance management. Similarly, its applications in protist genome editing have provided valuable insights into host-pathogen interactions, parasite biology, and metabolic pathways, contributing to novel therapeutic and industrial applications. Despite its remarkable progress, CRISPR/Cas9 technology still faces several challenges, including off-target effects, delivery system limitations, immunogenicity, and variability in DNA repair mechanisms. Addressing these challenges requires the development of high-fidelity Cas variants, optimized delivery methods, and advanced detection systems to enhance precision and efficiency. Furthermore, ethical and regulatory considerations must be carefully navigated before widespread clinical applications can be realized. By highlighting its role in human health, oral microbiome regulation, microbial genome

engineering, and protist research. With continuous advancements, CRISPR/Cas9 is poised to redefine genetic engineering, offering innovative solutions to complex biological challenges. As research progresses, the continued refinement of CRISPR-based techniques will pave the way for groundbreaking discoveries across multiple scientific disciplines, shaping the future of genetic research and biotechnological innovation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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