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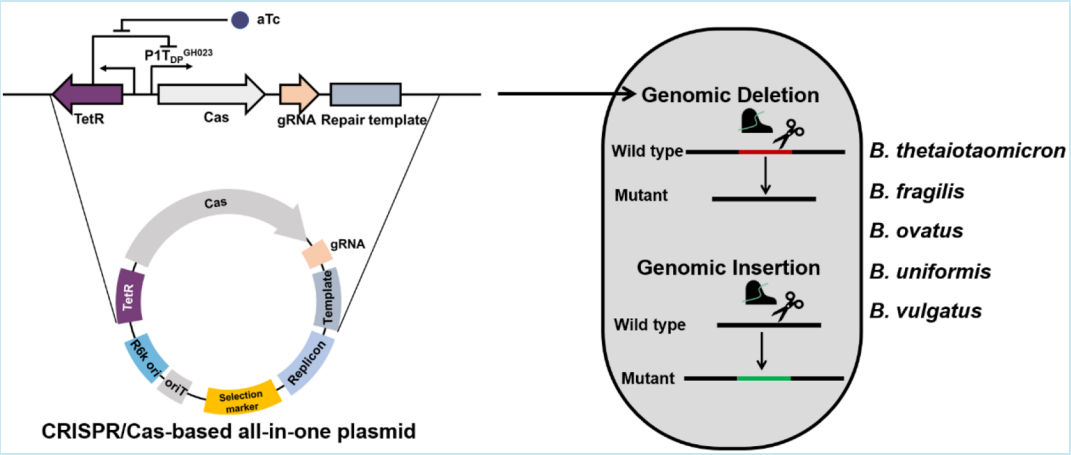
CRISPR/Cas-Based Genome Editing for Human Gut Commensal *Bacteroides* Species

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ABSTRACT: *Bacteroides* is the most abundant genus in the human gut microbiome and has been increasingly used as model organisms for studying the function and ecology of the gut microbiome. However, genome editing tools for such commensal gut microbes are still lacking. Here we developed a versatile, highly efficient CRISPR/Cas-based genome editing tool that allows markerless gene deletion and insertion in human gut *Bacteroides* species. We constructed multiple CRISPR/Cas systems in all-in-one *Bacteroides*–*E. coli* shuttle plasmids and systematically evaluated the genome editing efficiency in *Bacteroides thetaiotaomicron*, including the mode of Cas protein expression (constitutive, inducible), different Cas proteins (FnCas12a, SpRY, SpCas9), and sgRNAs. Using the anhydrotetracycline (aTc)-inducible CRISPR/FnCas12a system, we successfully deleted large genomic fragments up to 50 kb to study the function of metabolic gene clusters. Furthermore, we demonstrated that CRISPR/FnCas12a can be broadly applied to engineer multiple human gut *Bacteroides* species, including *Bacteroides fragilis*, *Bacteroides ovatus*, *Bacteroides uniformis*, and *Bacteroides vulgatus*. We envision that CRISPR/Cas-based genome editing tools for *Bacteroides* will greatly facilitate mechanistic studies of the gut commensal and the development of engineered live biotherapeutics.

KEYWORDS: *Bacteroides, CRISPR/Cas, genome editing, Cas12a, human gut microbiome, engineered live biotherapeutics*

INTRODUCTION

Bacteroides is the most abundant genus in the human gut microbiome[1] and plays an important role in the development and maintenance of a healthy gut microbiome. *Bacteroides* species have been associated with various diseases, such as obesity,[2] inflammatory bowel disease,[3,4] and colorectal cancer.[5,6] Moreover, *Bacteroides* species are increasingly used as model organisms to study the physiology and function of gut commensal microbes, such as polysaccharide utilization loci (PULs),[7−9] pathogenicity,[10,11] bile acid metabolism,[12,13] as well as the ecology and evolution of the gut microbiome.[14] Nevertheless, functional studies of *Bacteroides* are still in their infancy. Development of highly efficient genome editing tools for commensal gut microbes such as *Bacteroides* is of great value for functional dissection of the gut microbiome.

Currently available genetic tools for *Bacteroides* suffer from several limitations. One genome editing approach is based on the mobilizable *Bacteroides* elements NBU1 and NBU2.[15−20] The NBU-based method has been used to insert DNA fragments as large as 70 kb into the *Bacteroides* genome.[15,21]

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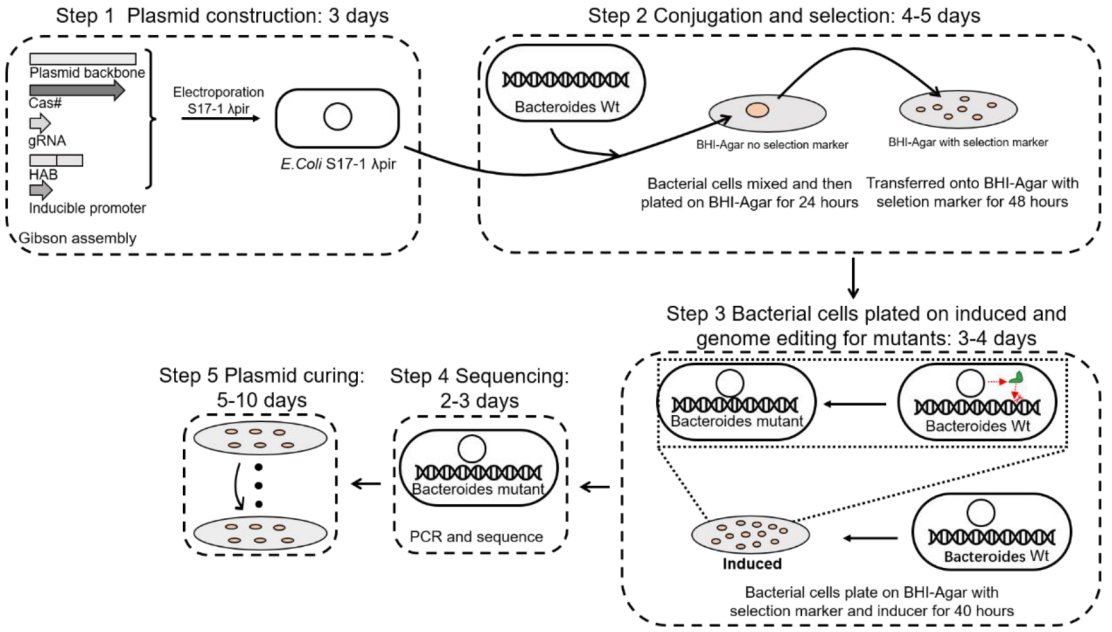


Figure 1. Workflow of CRISPR/Cas genome editing in *Bacteroides*. A flowchart showing the experimental process of all-in-one CRISPR/Cas-based genome editing system in *Bacteroides* strains. In Step 1, the *B. thetaiotaomicron*–*E. coli* shuttle plasmid was constructed using the Gibson assembly method. In Step 2, the plasmid was conjugated into *Bacteroides* and transformants were isolated by selection markers. In Step 3, expression of the Cas protein was induced to activate the gene editing process. In Step 4, *Bacteroides* mutants were confirmed by PCR and sequencing. In Step 5, the plasmid of the mutant strain was cultured in antibiotics-free medium for plasmid curing.

However, this approach can only insert genes at specific NBU sites in the genome, and the insertion is not markerless (i.e., with selection markers). Allelic exchange is another commonly used method for gene editing in *Bacteroides*.[18,22−25] Originally, the methods based on allelic exchange required the creation of a mutant background (e.g., deletion of thymidine synthase (Δ*tdk*),[18] thymidylate synthetase (Δ*thyA*),[25] or mutation of phenylalanyl-tRNA synthetase (*pheS**)[26]) for counterselection. Recent development of selection methods based on inulin utilization[24] or inducible effectors[23] have overcome this limitation, yet the genome editing efficiency is not high (Table S1).

The CRISPR/Cas system has been used in genome editing and transcriptional regulation of various organisms.[27−31] Deactivated Cas9 (dCas9)-based CRISPR interference has been successfully applied to *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) for target-specific knockdown.[32] On the basis of previous studies, we propose that the CRISPR/Cas system can be used for genome editing of *Bacteroides* species. While the Cas9 nuclease is most widely used in genome editing systems, additional Cas proteins have been developed.[33−36] SpRY, a Cas protein derived from SpCas9, can target almost all protospacer-adjacent motif (PAM) sites in human cells.[34] FnCas12a, a single-strand RNA-guided endonuclease of the Class II CRISPR-Cas system, cleaves targeted DNA with features distinct from those of Cas9, such as utilizing a T-rich (PAM) and cutting in staggered ends.[37] Both CRISPR/Cas9 and CRISPR/Cas12a have been widely used for genome editing in bacteria,[38−42] and thus, they may serve as tools for targeted genome editing of *Bacteroides*, but have not been investigated so far.

Here, we developed a CRISPR/Cas-based markerless genome editing tool for human gut *Bacteroides* species. We systematically evaluated the genome editing efficiency of three different CRISPR/Cas systems, namely FnCas12a, SpRY, and SpCas9, in *B. thetaiotaomicron*; among them the CRISPR/FnCas12a system had the highest genome editing efficiency. To demonstrate the advantage of our system in functional studies, we deleted large genomic fragments (up to 50 kb) of metabolic gene clusters in *B. thetaiotaomicron*. Finally, we showed that the CRISPR/FnCas12a system can be broadly applied to engineer multiple *Bacteroides* species. Taken together, this versatile, highly efficient CRISPR/Cas-based system can be used to genetically manipulate human gut *Bacteroides* for functional investigations and development of engineered probiotics.

RESULTS

Construction of CRISPR/Cas-Based Genome Editing Systems for *Bacteroides*. We developed a streamlined and markerless genome editing approach in human gut commensal *Bacteroides* species (Figure 1). Briefly, the experimental process of all-in-one CRISPR/Cas-based genome editing system in *Bacteroides* strains required the following steps (see Materials and Methods): (1) Step 1: plasmid construction. The *Bacteroides*–*E. coli* shuttle plasmid was constructed using Gibson assembly. The all-in-one plasmid encodes a Cas protein, a custom single guide RNA (sgRNA), a repair template, and genetic elements for plasmid replication and conjugation. (2) Step 2−3: genome editing. The plasmid was conjugated into *Bacteroides* and transformants were isolated by selection markers. Expression of the Cas protein was induced to activate the gene editing process. (3) Step 4−5: mutant validation. *Bacteroides* mutants were confirmed by PCR, sequencing, and phenotypic assays. The plasmid of the mutant strain was cultured in an antibiotics-free medium for plasmid curing.

The promoter has been shown to be a key component of CRISPR/Cas systems for genome editing in bacteria. Inducible promoters have been used to improve CRISPR/Cas system

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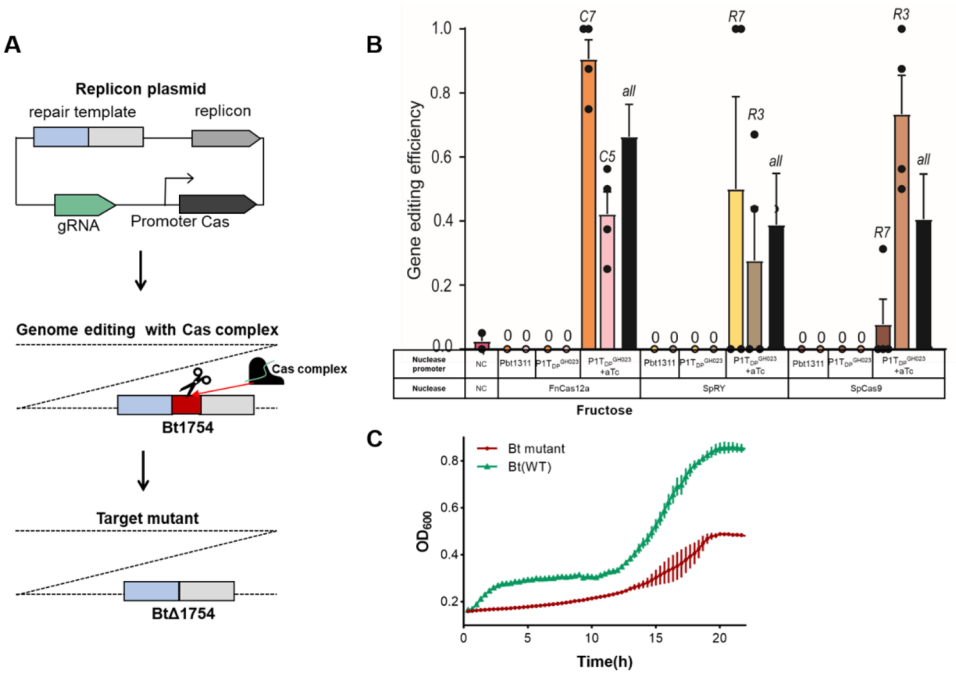


Figure 2. Evaluation of CRISPR/Cas-based genome editing systems in *B. thetaiotaomicron*. (A) The all-in-one plasmid approach for gene deletion via the CRISPR/Cas system. (B) Gene editing efficiency (deletion of *Bt1754*) after transformation with all-in-one plasmids containing a combination of three Cas proteins (FnCas12a, SpRY, SpCas9), two promoters (Pbt1311: constitutive; $P1T_{DP}^{GH023}$: inducible), and two different sgRNAs. NC: negative control, the replication plasmid p134 lacking Cas proteins and promoters. Error bars represent standard errors of the mean (n = 4, biological replicates). (C) The growth curve of *B. thetaiotaomicron* (Bt (WT, wild type)) and BtΔ1754 (Bt mutant) in minimal media with fructose. Error bars represent standard deviation (n = 3, biological replicates).

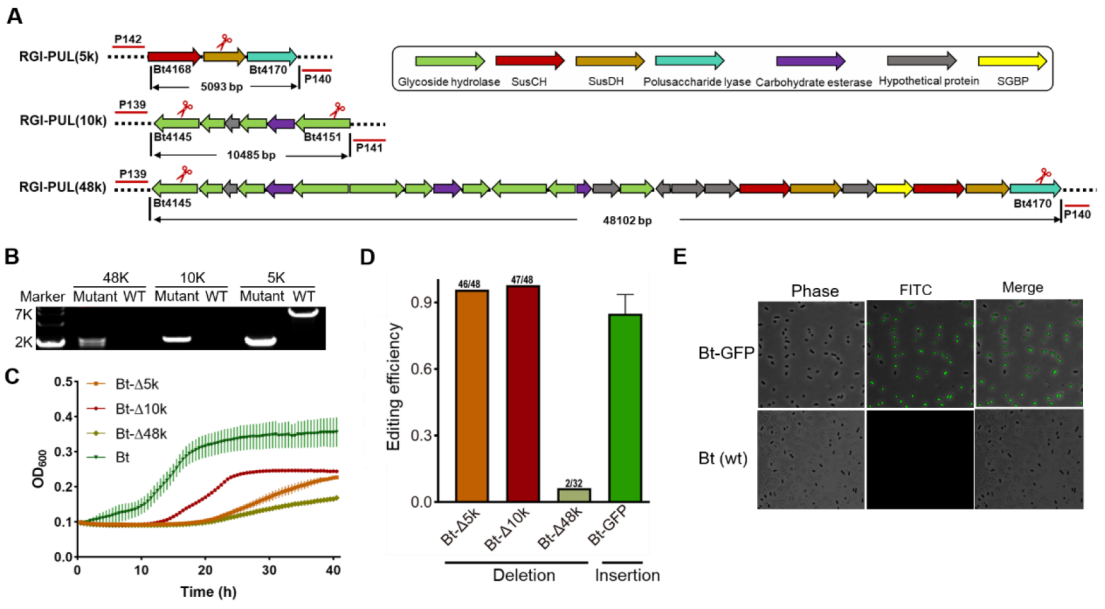


Figure 3. Markerless genome editing in *B. thetaiotaomicron* using the CRISPR/FnCas12a system. (A) Deletion of large fragments (48, 10, and 5 kb) in RGI PUL. Two sgRNAs are designed for deletion of the 48 kb or 10 kb fragments, and one sgRNA is designed for deletion of the 5 kb fragment. Genes encoding proteins of known or predicted functionalities are color-coded. (B) Colony PCR results from RGI-PUL deletion mutants generated with CRISPR-FnCas12a recombineering. (C) The growth curve of Bt-Δ5k, Bt-Δ10k, Bt-Δ48k, and Bt (wild type) in Rhamnogalacturonan minimal medium. Error bars indicate standard deviation (n = 3, biological replicates). (D) The efficiency of PULs deletion and GFP insertion in *B. thetaiotaomicron*. Error bars indicate standard deviation (n = 3, biological replicates). (E) The *gfp* gene was inserted into the genome at locus *Bt1754*. Epifluorescent microscopy of *B. thetaiotaomicron* (Bt (WT, wild type)) and *B. thetaiotaomicron* expressing GFP (Bt-GFP), grown under anaerobic condition and exposed aerobic condition for 5 h.

genome editing efficiency in *Clostridium*.[42,43] We therefore tested both inducible promoters (P_{LacO23} and $P1T_{DP}^{GH023}$) and constitutive promoters (Pbt1311 and PcfxA)[44] as candidates for Cas protein expression in *B. thetaiotaomicron*. We tested the strength of these promoters with the NanoLuc luciferase gene as a reporter[44,45] (Figure S1A). Of these promoters,

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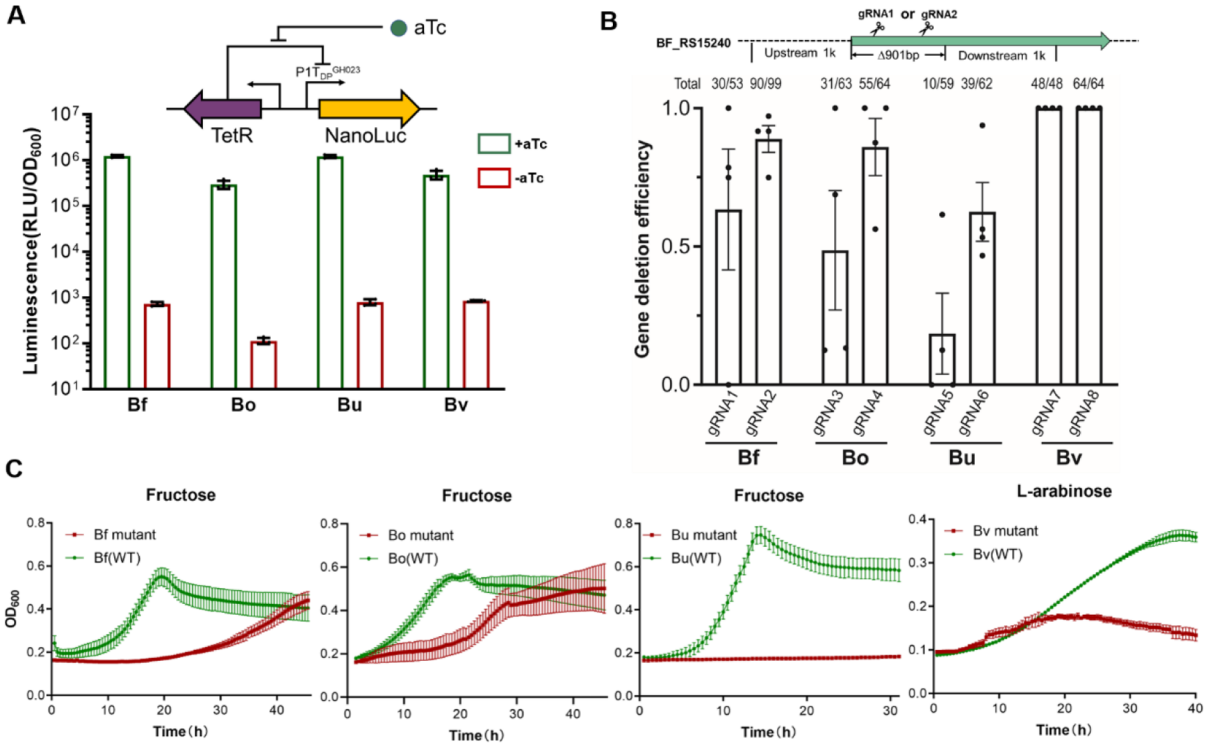


Figure 4. Application of CRISPR/FnCas12a genome editing system in multiple *Bacteroides* species. (A) Design and evaluation of the aTc inducible promoter in *Bacteroides* species (measured by the NanoLuc reporter, plasmid p134-TetR- $P1T_{DP}^{GH023}$-Nluc). (B) Gene deletion efficiency in *Bacteroides* species. Two different sgRNAs were tested for each species. Error bars are standard errors of the mean (n = 4, biological replicates). (C) The growth curve of wild type and mutant strains in minimal media with fructose or L-arabinose. Error bars indicate standard deviation (n = 3, biological replicates). *B. fragilis*: Bf, *B. ovatus*: Bo, *B. vulgatus*: Bv, *B. uniformis*: Bu.

$P1T_{DP}^{GH023}$ increased luminescence over 900-fold in response to the inducer aTc. In contrast, P_{LacO23} increased only 13-fold in response to the inducer IPTG. For constitutive promoters, we found that the strength of promoter Pbt1311 (~22 000 RLU/OD_{600}) was roughly 50% higher than PcfxA (~15 000 RLU/OD_{600}). Thus, $P1T_{DP}^{GH023}$ and Pbt1311 were chosen as candidate promoters for Cas protein expression.

To evaluate which CRISPR/Cas system (FnCas12a, SpRY, and SpCas9) has the highest genome editing efficiency in our system, we designed a series of all-in-one plasmids (Figure 2). *Bt1754*, which is required for the use of fructose-based carbohydrates in *B. thetaiotaomicron*,[44,46] was selected as a target gene (Figure 2A). Two different sgRNAs were designed for each CRISPR/Cas system. The plasmid p134-sgRNA-HAB was regarded as a control for recombination efficiency. Unexpectedly, no mutant was generated by Cas proteins under the control of Pbt1311 (Figure 2B). In contrast, when Cas expression was controlled by the inducible $P1T_{DP}^{GH023}$ promoter, upon induction of aTc the editing efficiency of the FnCas12a, SpRY, and SpCas9 proteins were nearly 60%, 40%, and 40%, respectively. (Figure 2B, 2C). Our results show that the inducible CRISPR/Cas systems can be used for genome editing in human gut *Bacteroides* species, and suggest that FnCas12a may have the highest efficiency.

The CRISPR/FnCas12a System Enables Efficient Gene Deletion and Insertion in *B. thetaiotaomicron*. Gene clusters such as Polysaccharide Utilization Loci (PUL) are key to understanding the metabolic functions of gut microbiome and metabolite-mediated ecological interactions.[47] Existing genome editing tools of gut commensal bacteria, however, can rarely achieve targeted deletion of large genomic fragments[26,48,49] for mechanistic investigation of metabolic gene clusters. To demonstrate the application of our CRISPR/Cas-based system in deletion of large genomic fragments, we selected the PUL known to be involved in the utilization of rhamnogalacturonan-I (RGI-PUL, encoding genes *Bt4145-Bt4183*).[47] We performed deletion of three RGI-PUL fragments with varying sizes: 5 kb (*Bt4168-Bt4170*), 10 kb (*Bt4145-Bt4151*), and 48 kb (*Bt4145-Bt4170*) (Figure 3A). All three fragments of RGI-PUL were successfully deleted using the CRISPR/FnCas12a system (Figure 3B). The deletion efficiency of the 5 kb and 10 kb fragments was nearly 100%; in contrast, the efficiency dropped to ~6% for the 48 kb fragment (Figure 3D). The deletion mutants showed different levels of decrease in growth rate compared to the wild type, when grown on medium with rhamnogalacturonan as the sole carbon source (Figure 3C).

Furthermore, to demonstrate the application of our CRISPR/Cas-based system in targeted insertions, we inserted fluorescent reporter *gfp* in *B. thetaiotaomicron*, which has been shown to be functional in human gut *Bacteroides*.[50] As expected, insertion of *gfp* was successful (Figure 3E); the insertion efficiency was above 80% (Figure 3D). Taken together, our results show that the CRIPSR/FnCas12a system enables efficient gene deletion and insertion in *B. thetaiotaomicron*.

Genome Editing in Multiple *Bacteroides* Species. To determine whether the CRISPR/FnCas12a system can be applied to different human gut commensal *Bacteroides* species, we constructed a set of all-in-one plasmids for engineering *B. fragilis*, *B. ovatus*, *B. vulgatus*, and *B. uniformis* (Supporting Table S3). First, we evaluated the aTc-inducible promoter in

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different *Bacteroides* species, and found that the expression of NanoLuc reporter increased ~1000-fold upon aTc induction (Figure 4A). Then, we selected the orthologous genes of *Bt1754* in *B. fragilis*, *B. ovatus*, and *B. uniformis* for targeted deletions.[44] These orthologous genes are highly conserved among *Bacteroides* species (Figure S3A) and are predicted to be a hybrid two-component sensor that regulates the utilization of fructose. Because *B. vulgatus* cannot grow in minimal medium with fructose as the sole carbon source (Figure S3B), we selected a different target gene *BVU_2320,* which has been reported to be an essential gene for L-arabinose metabolism.[51,52] For each targeted gene deletion, we designed two different sgRNAs (Figure S3C).

We found that the inducible CRISPR/FnCas12a system successfully deleted the target gene in each *Bacteroides* species. Overall, the genome editing efficiency was above 60%; the efficiency was 100% in *B. vulgatus* (Figure 4B). Compared to the wild type, the deletion mutants showed a significant decrease in growth rate when grown in minimal medium with fructose as the single carbon source (or L-arabinose for *B. vulgatus*) (Figure 4C). Finally, we tested plasmid curing in different *Bacteroides* species. The fraction of bacterial cells harboring plasmids decreases significantly after 5 rounds of serial transfer in medium without antibiotics selection (Figure S4). Taken together, our results show that the CRISPR/FnCas12a system can be broadly applied to engineer *Bacteroides* species.

DISCUSSION

Bacteroides is the most abundant genus in the human gut and plays an important role in the gut microbiome.[1] Here, we developed CRISPR/Cas-based systems for versatile and highly efficient genetic manipulation of *Bacteroides* species, allowing targeted, markerless deletions and insertions in the genome. As an example, we successfully deleted PULs fragments of varying sizes (up to ~50 kb) in *B. thetaiotaomicron*. For deletion of large gene clusters, proteins that facilitate recombineering in bacteria (e.g., RecA,[53] RecT[54]) can be used to further improve the genome editing efficiency. Importantly, we found that the inducible FnCas12a system could achieve successful gene editing for different *Bacteroides* species, highlighting the broad applicability of our method. We also demonstrated plasmid curing in *Bacteroides* species, which would allow for iterative rounds of genome editing.

The all-in-one CRISPR/Cas system developed in this study is a single plasmid (~12 kb) comprising the p134 replicon, Cas gene, sgRNA, repair template, and other necessary genetic elements. In addition, we evaluated the feasibility of a double-plasmid system in *B. thetaiotaomicron*, which consisted of the integration plasmid and the replication plasmid (Figure S2). The Cas gene and inducible promoter were integrated into the bacterial genome by the integration plasmid, followed by the introduction of sgRNA and repair templates on the replication plasmid. Overall, we found that the gene editing efficiency of the all-in-one plasmid system (Figure 2B) was higher than or similar to the double-plasmid-system (Figure S2B).

Our study provides a systematic evaluation of the gene editing efficiency of multiple CRISPR/Cas-based systems in *Bacteroides*, including the mode of Cas protein expression, different Cas proteins and sgRNAs. We found that the promoter controlling Cas expression is a key factor in gene editing efficiency. Surprisingly, no mutant was generated if constitutive promoters were used for all three tested Cas proteins (Figure 2B). One possible explanation is that the capacity of homologous recombination is weak during conjugation, and thus bacterial cells with double-strand breaks (DSB) cannot repair with the template quickly enough, leading to cell death.[43] The expression of Cas protein complex may also impose a burden in bacterial growth.[55,56]

While different Cas proteins (FnCas12a, SpRY, and SpCas9) can achieve targeted deletions in *B. thetaiotaomicron*, the deletion efficiency of CRISPR/FnCas12a was overall higher than SpRY and SpCas9 (Figure 2, Figure S2). Consistent with this observation, we found a significant decrease in *B. thetaiotaomicron* transformants carrying the FnCas12a plasmid without repair templates (Figure S1B), indicating that the expression of FnCas12a introduced more DSBs than other Cas proteins. SpRY[57,58] and SpCas9[38,40] have been utilized broadly in genome editing of many bacterial species, and the reason for their low efficiency in *Bacteroides* is still unclear. Possible explanations for the difference in genome editing efficiencies across bacterial species include variations in the toxicity, activity, or stability of Cas proteins.[59,60] Regardless, SpRY and SpCas9 also can be valuable tools for editing genes that lack suitable PAM sites for FnCas12a.

Previous studies have demonstrated that specific aspects of sgRNA design, including the position of specific nucleotides in the target sequence, the accessibility of the target site, and the sequence of flanking regions, play important roles in genome editing.[55,61,62] We evaluated a set of sgRNAs for FnCas12a, SpRY, and SpCas9 in *B. thetaiotaomicron* (Supporting Table S5). Indeed, our results showed that the use of different sgRNAs can lead to substantial variations in gene editing efficiency (Figure S2B).

While our CRISPR/Cas-based genome editing system displayed higher efficiency than previous methods based on allelic exchange[23,24,26] (Table S1), the time required for one round of genetic manipulation is similar (2−3 weeks, see Figure 1). One advantage of the CRISPR/Cas system is multiplexed genes editing.[63] In our system, we used two sgRNAs to successfully delete 10 kb and 48 kb DNA fragments (Figure 3), showing the potential of multiplexed gene editing in *Bacteroides*. Lastly, although our current method still depends on antibiotics resistance selection, it can be readily combined with other selection methods such as insulin utilization[24] to expand the applicability to wild *Bacteroides* strains.

In this study, we developed a versatile and highly efficient genome editing tool for *Bacteroides*, one of the most important residents in human gut microbiome. The lack of genome editing tools for gut commensal bacteria has hampered mechanistic investigations. We envision that CRISPR/Cas-based genome editing tools would greatly facilitate our understanding of the function of the gut microbiome at the molecular level,[30] as well as the development of engineered probiotics with long-term colonization in the gut.[64]

MATERIALS AND METHODS

Bacterial Culture. Bacterial strains used in this study are listed in Supporting Table S2. Wild type strains of *Bacteroides* (*B. thetaiotaomicron*, *B. fragilis*, *B. ovatus*, *B. uniform* and *B. vulgatus*) were grown in Brain Heart Infusion (BHI, HKM) liquid medium with hemin and VK3. Bacteria were cultured in anaerobic chamber (Coy, specify model; 85% N_2, 10% H_2 and 5% CO_2) at 37 °C. *E. coli* strain S17 were cultured in LB medium with ampicillin at 37 °C. Antibiotics concentrations

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used in this study: ampicillin at 100 μg/mL, gentamicin at 200 μg/mL, tetracycline at 2 μg/mL, and erythromycin at 25 μg/mL. To assay the growth of mutant strains, minimal medium (MM)[19] supplemented with appropriate carbohydrates as previously described.[19,65] Fructose, L-arabinose, and rhamnogalacturonan was added as the sole carbon source at a final concentration of 0.3%, 1% and 1%, respectively. *Bacteroides* strains were grown in BHI medium for 7−10 h and diluted 1:100 in minimal medium. Cultures were incubated anaerobically at 37 °C in clear flat bottom 96-well plates and growth was monitored (OD_{600}) every 30 min in a microplate reader (BioTek Eon).

Plasmid Construction. The plasmids used in this study are listed in Supporting Table S3. All plasmids were sequenced by Sangon Biotech. Plasmids were constructed using the backbone of pNBU2-bla-ermGb plasmid.[32] Using Gibson assembly, the attN2 site of pNBU2 plasmid was replaced by replicon p134[66,67] or p143[68] previously identified in *Bacteroides* plasmids. The DNA fragments (Supporting Table S4) of Cas genes or sgRNA or sgRNA-homologous arms (1 kb repair templates) were cloned into the p134 backbone by NEBuilder HiFi DNA Assembly Master Mix (NEB, E2621S). The Cas genes (SpCas9/SpRY/FnCas12a) and sgRNAs genes were synthesized by Sangon Biotech. Oligonucleotides sgRNAs sequence used for gene deletion or insertion are listed in Supporting Table S5. And sgRNAs were designed using CHOPCHOP.[61]

Conjugation. Plasmids were transformed into donor *E. coli* S17 and conjugated into *Bacteroides* as previously described,[44] with minor modifications. Overnight cultures of *E. coli* S17−1 donor strains were diluted 100-fold in LB medium containing ampicillin, and *Bacteroides* recipients were diluted 100-fold in BHI medium containing gentamicin. Donor and recipient strains, each grown to exponential phase (OD_{600} = 0.4−0.6), were mixed by volume at a 1:5 ratio (*B. thetaiotaomicron*) or 1:1 ratio (*B. fragilis*, *B. ovatus*, *B. vulgatus*, and *B. uniformis*), centrifuged at 5500 rpm for 15 min, resuspended in 150 μL of BHI liquid medium, and plated as a dime-sized puddle on nonselective BHI-blood agar plates for 24 h at 37 °C under aerobic condition to allow for conjugation. Bacterial lawns were resuspended in 900 μL BHI medium with no selective marker and transconjugants selected by plating serial dilutions (such as 10^0, 10^1, 10^2, 10^3) on BHI-blood agar plates containing gentamicin and erythromycin or tetracycline. The mixed donor and recipient strains (150 μL) were plated on BHI-blood agar plates for the selection step, and then after 24 h anaerobic incubation at 37 °C, the transconjugants were selected on gentamicin and erythromycin as above.

Construction of Mutant Strains. The all-in-one plasmids, containing inducible promoter, the Cas gene, sgRNA, and repair template, conjugated into *Bacteroides* strains. *Bacteroides* strains containing all-in-one plasmid were incubated overnight at 37 °C in BHI medium with gentamicin and erythromycin under anaerobic conditions. And then the *Bacteroides* strains were diluted 1:100 in BHI medium with aTc induced for 24 h (aTc final concentration 100 ng/mL). Cultures were diluted by 10^0, 10^1, 10^2 and 10^3-fold and spread onto BHI-aTc agar plate and incubated at 37 °C for 40 h. Colonies were identified by PCR and sequencing. For plasmid curing, *Bacteroides* strains were cultured in BHI liquid medium without antibiotics, and passaged five times with a dilution factor of 100-fold (every 20−24 h).

Luciferase Assay. The NanoLuc luciferase assay was performed as previously described[32,44] with minor modifications. *Bacteroides* strains were grown to mid exponential phase (OD_{600} = 0.4−0.6). 500 μL of the *Bacteroides* cell culture was centrifuged at 12 000 rpm for 5 min, resuspended in 500 μL 1× PBS and centrifuged at 12 000 rpm for 5 min, and then resuspended in 50 μL 1× BugBuster (Novagen) at 26 °C for 10 min. Lysates were cleared of cellular debris by centrifugation at 21 130*g* for 10 min at 4 °C. 50 μL of the supernatant was mixed with an equal volume of NanoLuc Reaction Buffer using the Nano-Glo Luciferase Assay System (Promega) and incubated for 10 min at 26 °C to induce luminescence production. Luciferase activities were measured with an integration time of 1 s at a gain setting of 100 in a BioTek Synergy H1 Hybrid Reader. Luciferase values were normalized to the OD_{600} of 200 μL of culture at the time of harvest (BioTek Synergy H1 Hybrid Reader).

Confocal Microscopy. The imaging of green fluorescent protein was performed as previously described[50] with minor modifications. Cells were grown nonaerobically and spotted on sealed glass slide pads, and images were acquired by a confocal laser scanning microscope (Nikon, A1) with Plan Apo λ 100× oil objective lens (Nikon 1.5 NA). Field of views were collected by excitation with laser 488 nm. Constant focus during imaging was achieved by Nikon PFS autofocus.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00543.

Supporting Figure S1: Evaluation of promoters and Cas proteins in *B. thetaiotaomicron*; Supporting Figure S2: Gene editing efficiency of the double-plasmid system in *B. thetaiotaomicron*; Supporting Figure S3: sgRNA design for gene deletion in *Bacteroides* species; Supporting Figure S4: Plasmid curing for *Bacteroides* species; Supporting Table S1: Comparison of genome editing efficiency with previous studies; Supporting Table S2: Bacterial strains used in this study; Supporting Table S3: Plasmids used in this study; Supporting Table S4: Sequence of plasmid elements used in this study; Supporting Table S5: Oligos and gRNA spacers used in this study (PDF)

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#L.Z. and Y.T. contributed equally to this work. L.Z., Y.T., W.Z., and L.D. designed the experiments. L.Z. performed the experiments and data analysis. Y.T., Y.H., J.S., Z.Q., and X.C. assisted with the experiments. L.Z. and L.D. wrote the manuscript with input from all coauthors.

Notes

The authors declare the following competing financial interest(s): The results described in this manuscript support patent application CN202111225679.0.

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