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Harnessing the endogenous Type I-C CRISPR-Cas system for genome editing in *Bifidobacterium breve*

Xiao Han,[1,2] Lulu Chang,[1,2] Haiqin Chen,[1,2] Jianxin Zhao,[1,2] Fengwei Tian,[1,2,3] R. Paul Ross,[3,4] Catherine Stanton,[3,5] Douwe van Sinderen,[4] Wei Chen,[1,2,6] Bo Yang[1,2,3]

AUTHOR AFFILIATIONS See affiliation list on p. 14.

ABSTRACT *Bifidobacterium breve*, one of the main bifidobacterial species colonizing the human gastrointestinal tract in early life, has received extensive attention for its purported beneficial effects on human health. However, exploration of the mode of action of such beneficial effects exerted by *B. breve* is cumbersome due to the lack of effective genetic tools, which limits its synthetic biology application. The widespread presence of CRISPR-Cas systems in the *B. breve* genome makes endogenous CRISPR-based gene editing toolkits a promising tool. This study revealed that Type I-C CRISPR-Cas systems in *B. breve* can be divided into two groups based on the amino acid sequences encoded by *cas* gene clusters. Deletion of the gene coding uracil phosphoribosyl-transferase (*upp*) was achieved in five *B. breve* strains from both groups using this system. In addition, translational termination of uracil phosphoribosyl-transferase was successfully achieved in *B. breve* FJSWX38M7 by single-base substitution of the *upp* gene and insertion of three stop codons. The gene encoding linoleic acid isomerase (*bbi*) in *B. breve*, being a characteristic trait, was deleted after plasmid curing, which rendered it unable to convert linoleic acid into conjugated linoleic acid, demonstrating the feasibility of successive editing. This study expands the toolkit for gene manipulation in *B. breve* and provides a new approach toward functional genome editing and analysis of *B. breve* strains.

IMPORTANCE The lack of effective genetic tools for *Bifidobacterium breve* is an obstacle to studying the molecular mechanisms of its health-promoting effects, hindering the development of next-generation probiotics. Here, we introduce a gene editing method based on the endogenous CRISPR-Cas system, which can achieve gene deletion, single-base substitution, gene insertion, and successive gene editing in *B. breve*. This study will facilitate discovery of functional genes and elucidation of molecular mechanisms of *B. breve* pertaining to health-associated benefits.

KEYWORDS *Bifidobacterium breve*, CRISPR-Cas, Type I-C, genome editing

Bifidobacteria are commensal bacteria that colonize the human gut early in life, in some cases constituting more than 90% of the total bacterial population in a given individual (1, 2). Certain *Bifidobacterium* strains have been added to functional foods as bioactive ingredients due to their purported benefits on human health or have been commercially used as adjuvants for drug therapy (2). *Bifidobacterium breve*, a common and abundant component of the gut microbiota in breastfed newborns, has been associated with various beneficial or probiotic activities, such as its ability to alleviate gastrointestinal disorders and its contribution to the maturation of the immune system in infants (3). Although various health benefits have been attributed to *B. breve*, the molecular mechanisms supporting these probiotic activities are poorly understood, which has impeded their regulatory acceptance as probiotics in the European Union and

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Address correspondence to Fengwei Tian, fwtian@jiangnan.edu.cn, or Bo Yang, bo.yang@jiangnan.edu.cn.

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other jurisdictions, while hindering future development and discovery of new functions. In fact, genetic manipulation of *B. breve* can be challenging due to its thick cell wall, oxygen sensitivity, active restriction modification (R-M) systems, and lack of genetic tools (4). The current method commonly used for gene inactivation in *B. breve* is based on homologous recombination using a non-replicating plasmid due to the lack of a functional replicase-encoding *repA* gene or by transposon mutagenesis (5–10). However, these approaches typically introduce antibiotic resistance genes into the genome, not allowing for successive gene editing. Moreover, low transformation frequency and/or low frequency of transposition or homologous recombination events may make it difficult to reliably generate mutants in many strains (11).

The clustered regularly interspaced short palindromic repeats (CRISPR)-associated proteins (Cas) system is one of the immune systems of bacteria against foreign DNA invasion, which are described as two classes, including 6 types and 33 subtypes according to different *cas* genes (12). Since the Type II-A endonuclease Cas9 can result in DNA double-strand breaks under the guidance of a single-guide RNA (sgRNA), these processes have been used for genome editing in a variety of organisms (13). CRISPR-Cas9-based gene editing in bacteria was initially performed in *Escherichia coli* and subsequently implemented in other species (14, 15). However, the large size of the gene encoding SpCas9 (from *Streptococcus pyogenes*, 1368 AA) reduces the transformation efficiency of a plasmid carrying this gene and prevents its efficient delivery to certain bacteria (16). Some studies have shown that SpCas9 may be cytotoxic, leading to abnormal cell morphology and reduced colonies (17, 18). Genome editing using a native CRISPR-Cas system is a potentially less harmful approach. Since its effector protein Cascade is expressed by the strain itself, the size of the plasmid can be greatly reduced to ensure transformation efficiency. Although Type II Cas9 and Type V Cas12a have been widely used in gene editing of animals and plants, Type I is the most abundant CRISPR-Cas type in most bacteria and archaea (12, 19). Endogenous Type I CRISPR-Cas systems, including seven subtypes, have been used as a substitute method in recent years for genetic engineering in bacteria and archaea (20–26). For example, the native I-C CRISPR-Cas system of *Xanthomonas oryzae* pv. *oryzae* can be used as an excellent tool for genetic engineering, facilitating efficient genome editing of precise deletions, insertions, base substitutions, and gene replacements (22). Recently, the native I-G CRISPR-Cas system of *B. animalis* subsp. *lactis* was reprogramed to successfully create large deletions in the genome and deletion in *tetW* to eliminate the tetracycline resistance (26). In addition, following deletion of the *cas3* gene from the *E. coli* Type I CRISPR-Cas system, the effector protein Cascade effectively inhibited transcription without causing DNA cleavage to achieve gene silencing (27). These studies demonstrate the usefulness of native Type I CRISPR-Cas systems for bacterial and archaeal genome editing and gene transcription regulation. The CRISPR-Cas systems encoded by *Bifidobacterium* genomes display high prevalence and diversity (28). A study involving a high number of bifidobacterial genomes (954 publicly available *Bifidobacterium* genomes) identified CRISPR-Cas systems in 57% of the strains, including five CRISPR-Cas subtypes (29). In our previous study, the types of CRISPR-Cas systems and CRISPR loci in *B. breve* were characterized (30). The CRISPR-Cas system widely exists among *B. breve* strains (47%), among which Type I-C accounts for the highest proportion. The protospacer adjacent motif (PAM) sequence was predicted to be 5′-TTC-3′ in the *B. breve* Type I-C system based on previous studies (29, 30). The widely distributed CRISPR-Cas system in *B. breve* genomes and locus architecture characterization from previous reports provides the basis for the development of an endogenous CRISPR-based gene editing toolkit.

In this study, we first characterized the activity of the Type I-C CRISPR-Cas system in *B. breve*. An endogenous CRISPR-based gene editing toolkit was then established by designing a plasmid containing an artificial crRNA and a repair template. Then, gene deletions, single-base substitutions, short DNA insertions, and successive gene editing were performed in *B. breve* using this toolkit. This work thus provides an opportunity for exploring the molecular mechanism of the functional activity of *B. breve*.

RESULTS

Investigating the activity of an endogenous CRISPR-Cas system in *B. breve*

According to our previous research, about 47% of *B. breve* strains encompass an apparently intact, endogenous CRISPR-Cas system, among which Type I-C was shown to be the most prevalent (30). The Cas5, Cas7, and Cas8 proteins constitute a Type I-C Cascade, in which Cas5 is responsible for crRNA processing (31). Following recognition of the PAM sequence by the Cascade, the crRNA base pairs with the target DNA strand to induce R-loop formation. The Cascade then recruits Cas3, which initiates the degradation of the non-target strand (32) (Fig. S1). The Type I-C CRISPR-Cas system was identified in five *B. breve* strains, consisting of a CRISPR array and *cas* gene cluster (Fig. 1A). The Cas4, Cas1, and Cas2 proteins associated with the acquisition of new spacers in the CRISPR locus appear to be conserved among these five *B. breve* strains. The Cas3, Cas5, Cas8, and Cas7 proteins, which are involved in the expression and interference stages of the CRISPR-Cas-mediated defense process, exhibited high homology in the *B. breve* strains FHNXY43M2 and FJSWX38M7. Similarly, a high level of identity was also observed among these proteins in the strains FAHWH21M7, FHuNMY8M2, and ZJHZD20M12. However, between the strains FJSWX38M7 and FAHWH21M7, there was a notable low homology, especially in the Cas5 and Cas8 proteins, where the level of identity was below 30% (the identity of the Cas8 proteins was only 27% at a coverage of 18%, data not shown). The phylogenetic relationships of the Type I-C CRISPR-Cas systems in 61 *B. breve* strains were analyzed based on the sequence similarity of amino acids encoded by the *cas* gene clusters (Fig. 1B). We discovered that, due to the contributions of Cas3, Cas5, Cas8, and Cas7, the Type I-C CRISPR-Cas systems can be divided into two groups. In fact, the strains of each group also clustered on the same branch in the individual phylogenetic trees of the low-homology Cas proteins (Cas3, Cas5, Cas8, and Cas7) (data not shown), demonstrating that the Type I-C CRISPR-Cas systems in *B. breve* may have two distinct evolutionary origins.

In order to investigate the activity of the Type I-C system in *B. breve* FJSWX38M7, a plasmid interference assay was designed. The *E. coli*/*Bifidobacterium* shuttle vector pNZ123, carrying a chloramphenicol resistance gene, was used as a framework for the plasmid interference assay. The PAM sequence of the *B. breve* Type I-C system was predicted to be 5′-TTC-3′ (29, 30). We identified 81,083 instances of the 5′-TTC-3′ or 5′-GAA-3′ motifs in the *B. breve* FJSWX38M7 genome. The number of nucleotides between two neighboring PAM sequences was also counted, with an average of about 64 nucleotides presenting a PAM motif (Fig. S2), allowing for a wide range of genetic manipulations. The last obtained spacer in the FJSWX38M7 CRISPR array was cloned into pNZ123 with or without a PAM sequence, generating plasmids pNZ123-PS82 and pNZ123-S82, respectively. Compared with pNZ123-S82, the transformation efficiency of pNZ123-PS82 was reduced by more than four orders of magnitude, strongly implying that the active CRISPR-Cas system created a barrier to the entry of exogenous DNA with PAM and spacer (Fig. 1C). After the activity of endogenous I-C CRISPR-Cas was demonstrated in *B. breve* FJSWX38M7, an artificial crRNA designed to target arbitrary genes was constructed for gene editing by repurposing the endogenous system. The artificial crRNA included a leader sequence, a spacer insertion site, two repeats, and a rho-terminator. The native leader of the I-C CRISPR array in *B. breve* was first analyzed (Fig. S3), removing strains whose leader sequence were interrupted by mobile elements. The results showed that the leader sequence was relatively conserved, and the nucleic acid with the highest relative frequency at each position was selected to form the final leader sequence. The repeat sequence was predicted using CRISPRCasFinder (https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index) (33) and was conserved between two groups of Type I-C CRISPR-Cas systems in *B. breve*. A short sequence with two BsaI sites was designed in the middle of the two repeat sequences to allow for convenient spacer insertion (Fig. S4; Table S3). Synthetic artificial crRNA was cloned into pNZ123 to generate the gene-editing plasmid pNZ624. The self-targeting experiment was designed to verify the effect of

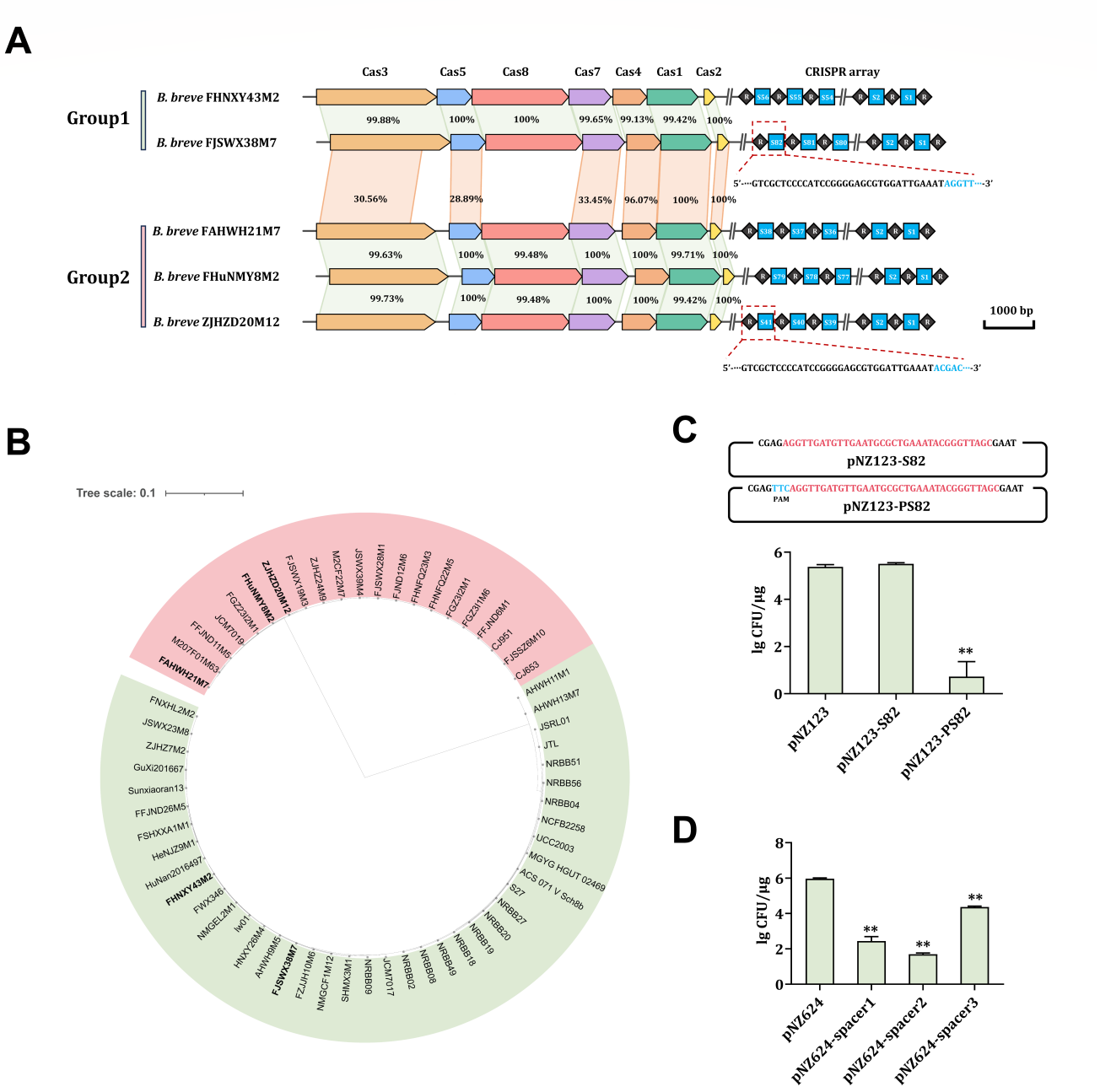

FIG 1 Endogenous Type I-C CRISPR locus in *B. breve* and the activity of CRISPR-Cas system in *B. breve* FJSWX38M7. (A) Schematic representation of the Type I-C CRISPR array and *cas* loci in *B. breve*. The *cas* genes were indicated by arrows of different colors. The percentage amino acid (aa) identity was indicated. Repeats were indicated by black diamonds. Spacers were indicated by blue squares, and the first spacer-acquired named S1 was shown on the right. (B) Phylogenetic tree based on the amino acid sequences of Cas proteins. Cas proteins were spliced in order and aligned to construct a phylogenetic tree, showing two evolutionary branches. (C) Plasmid interference assay. pNZ123-PS82 contained the spacer 82 and PAM sequence in the FJSWX38M7 CRISPR array, while pNZ123-S82 only contained spacer 82 without the PAM sequence. The transformation efficiencies of these plasmids were represented by $\log_{10}$ CFU/μg. The values were mean ± SD of three independent experiments. The empty plasmid pNZ123 was used as a control. $^{**}P < 0.01$ was determined by an unpaired *t*-test. (D) Self-target assay. pNZ624-sgRNA1-3 contained three different sgRNAs targeting the *upp* gene, respectively. sgRNA2 targeted the forward strand, and sgRNA1/sgRNA3 targeted the reverse strand. The empty plasmid pNZ624 was used as a control. $^{**}P < 0.01$ was determined by an unpaired *t*-test.

artificial crRNA. The number of different spacer lengths in the *B. breve* I-C CRISPR-Cas system was analyzed. The vast majority of spacers were 35 nucleotides long, and there was no significant difference in the average spacer length between both groups, which was a reference for the length design of the spacer design for gene editing purposes (Fig.

S5). Three different spacers were designed based on the *upp* gene as the target gene, among which spacer1 and spacer3 targeted the non-coding strand and spacer2 targeted the coding strand. Three spacers were ligated to BsaI-digested pNZ624 to generate pNZ624-spacer1, pNZ624-spacer2, and pNZ624-spacer3, respectively. The transformation efficiency of FJSWX38M7 decreased significantly when using these three plasmids when compared with pNZ624, in particular, the transformation efficiency of pNZ624-spacer2 was reduced by more than 10,000-fold (Fig. 1D). These results indicate that the nuclease Cas3 of the strain cuts its own genome, being under the guidance of the Cascade containing spacer, thus leading to cell death, which demonstrated the potential of the endogenous CRISPR-Cas system in gene editing.

Establishment of the Type I-C CRISPR-based gene deletion toolkit for *B. breve*

DNA single-strand breaks or double-strand breaks pose a deadly threat to bacteria. Different from eukaryotes, which often use non-homologous end joining (NHEJ) to repair DNA damage, NHEJ is not ubiquitous in prokaryotes (34). The repair of DNA damage in bacteria mainly relies on the homologous recombination pathway, which is a more stable and precise method compared with NHEJ. Therefore, the repair template provided to the bacteria can trigger DNA homology-directed repair.

Two 500-bp DNA fragments corresponding to upstream and downstream regions of the *upp* gene in the FJSWX38M7 genome were cloned and ligated by overlap extension PCR to generate a repair template. Since pNZ624-spacer2 had the highest genome cleavage efficiency in the self-targeting experiment, the repair template was ligated to pNZ624-spacer2 to generate pNZ624-Δ*upp*-spacer2, allowing the strain to repair DNA damage caused by Cas3 cleavage (Fig. 2A). Plasmid pNZ624-Δ*upp*-spacer2 was delivered into FJSWX38M7 by electrotransformation, followed by random picking of colonies that had grown on mMRS agar supplemented with chloramphenicol. The deletion event of the *upp* gene in FJSWX38M7 was verified using PCR analysis. One of the samples yielded a smaller PCR product (2,351 bp). Sanger sequencing also showed that this sample had a 642-bp deletion as expected, compared with the wild type. Uracil phosphoribosyl-transferase (UPRT), encoded by the *upp* gene, can convert 5-fluorouracil (5-FU) into 5-fluorouridine monophosphate (5-FUMP), which is then converted into 5-fluorodeoxyuridine monophosphate (5-FdUMP), thereby inhibiting the activity of thymidine nucleotide synthase, leading to cell death (35). Therefore, strains harboring an intact and expressed *upp* gene cannot grow in media supplemented with 5-FU. Overnight cultures of the FJSWX38M7 wild type and FJSWX38M7Δ*upp* were dropped onto mMRS agar supplemented with 5-FU. As expected, the strain with the deletion of the *upp* gene grew normally on agar containing 5-FU, whereas the wild type failed to grow (Fig. 2C).

To explore whether genetic manipulation can also be performed in other *B. breve* strains that harbor the endogenous CRISPR-Cas system using this toolkit, the four *B. breve* strains (FHNXY43M2 from group 1, FAHWH21M7, ZJHZD20M12, and FHuNMY8M2 from group 2) were used as target strains to accomplish the *upp* gene knockout. PCR screening confirmed that the *upp* gene was also successfully deleted in each of these strains, though exhibiting different editing efficiencies (2/7 for FHNXY43M2, 1/9 for FAHWH21M7, 2/20 for ZJHZD20M12, and 1/23 for FHuNMY8M2) (Fig. 2D). These results demonstrated that the endogenous I-C CRISPR-based toolkit was effectively applicable in genomic engineering for both groups of *B. breve*, despite the significant differences in the sequences of their Cas proteins.

Precise single-base substitutions and short DNA insertion in *B. breve* using the endogenous CRISPR gene editing toolkit

Point mutations occur widely during bacterial replication, including substitutions, insertions, and deletions of single bases. Point mutations can have a variety of deleterious or beneficial functional consequences, including changes in gene expression or alterations in encoded proteins (36). Single-base substitution of genes by genetic engineering technology is an important method to regulate gene expression, explore

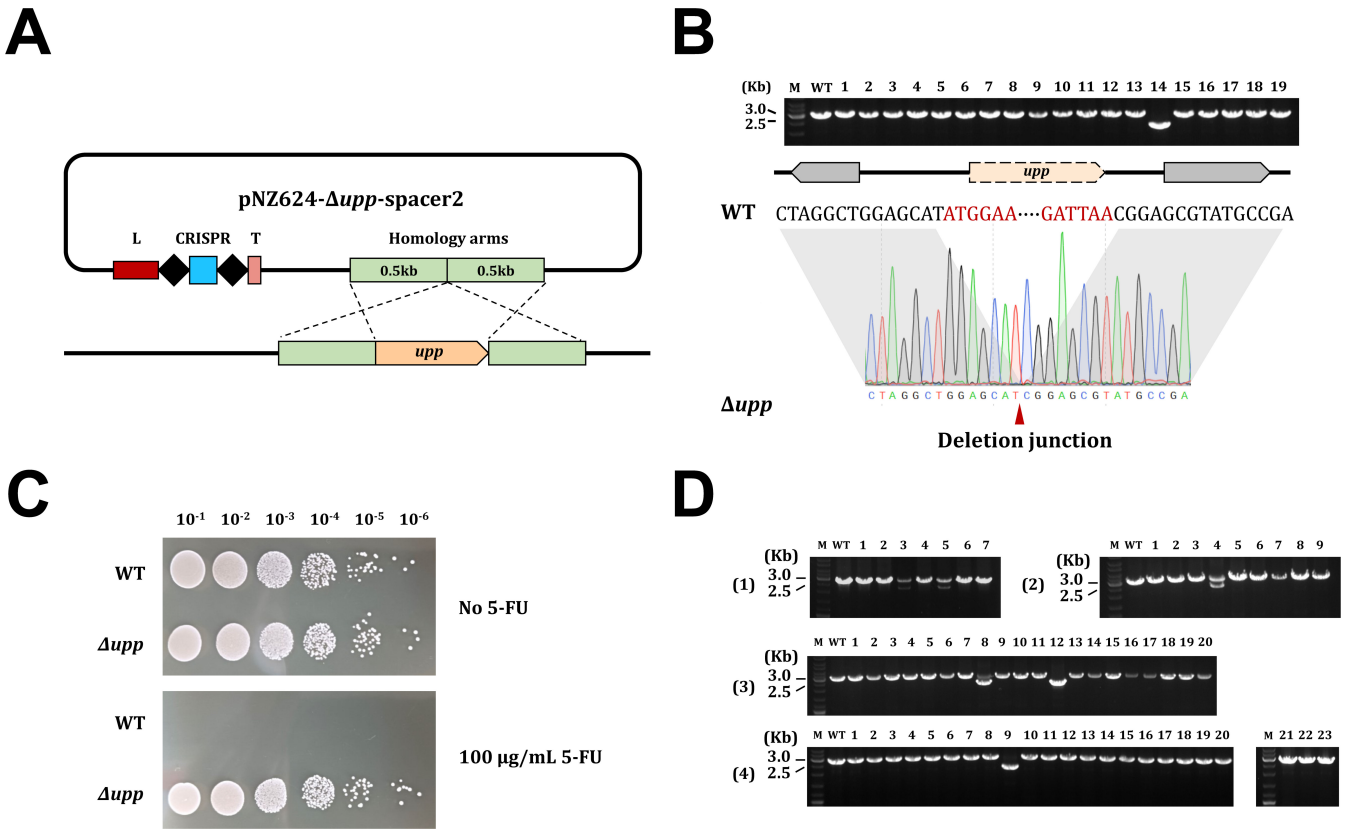

FIG 2 The *upp* gene deletion in *B. breve* using the endogenous Type I-C CRISPR-Cas system. (A) Schematic diagram of the *upp* gene deletion mediated by the endogenous I-C CRISPR-Cas system. The gene editing was constructed based on pNZ123, including a native leader, an artificial CRISPR array, a terminator, and a 1-kb repair template. (B) Successful deletion of the *upp* gene in *B. breve* FJSWX38M7 was confirmed in one of the 19 randomly selected colonies by PCR and Sanger sequencing. M, marker; WT, wild type. (C) The FJSWX38M7Δ*upp* mutant exhibited resistance to 100 μg/mL 5-FU while growing similarly on plates without 5-FU compared with the WT. Overnight liquid cultures of FJSWX38M7 WT and Δ*upp* mutants were adjusted to the same optical density at 600 nm (OD_{600}) and then diluted to different concentrations, and 5 μL of each dilution was spotted on plates with or without 5-FU. The plates were incubated at 37°C for more than 36 h and then photographed. (D) PCR screening of Δ*upp* mutants in different *B. breve* strains. 1, FHNXY43M2; 2, FAHWH21M7; 3, ZJHZD20M12; 4, FHuNMY8M2. The *upp* deletion was observed in 2 out of 7 tested colonies of FHNXY43M2, 1 out of 9 tested colonies of FAHWH21M7, 2 out of 20 tested colonies of ZJHZD20M12, and 1 out of 23 tested colonies of FHuNMY8M2.

the key active sites of proteins, or change the activity of enzymes. To explore the potential of the CRISPR-based *B. breve* toolkit for precise single-base substitutions, a spacer targeting the *upp* gene in strain FJSWX38M7 and a repair template containing a single-base substitution (193A>T) were cloned into plasmid pNZ624 (Fig. 3A). Forty transformants were screened by PCR, and Sanger sequencing results showed that the expected single-base substitution appeared in 16 transformants, showing a high editing efficiency (Fig. 3C and D).

Gene insertion may allow strains to acquire new functions or completely disrupt gene function. In fact, horizontal gene transfer occurs frequently in bacteria and is an important driving force for their adaptation to the environment and evolution (38). To further expand *B. breve* genome editing capability, the short DNA insertion assay was performed in *B. breve* FJSWX38M7 using the endogenous I-C CRISPR-based toolkit. Three stop codons were designed in the repair template and ligated into pNZ624-spacer2 to generate plasmid pNZ624-*upp*STOP-spacer, which is aimed at targeting the *upp* gene in FJSWX38M7 (Fig. 3B). Sanger sequencing demonstrated that the efficiency of stop codon insertion was 3/40, including a pure mutant and two mixed mutants (Fig. 3E).

In the single-base substitution assay, the lysine (**A**AG) of UPRT in FJSWX38M7 was mutated to a stop codon (**T**AG), resulting in the translation of UPRT being terminated (Fig. 3C). Similarly, the insertion of three consecutive stop codons into the *upp* gene in

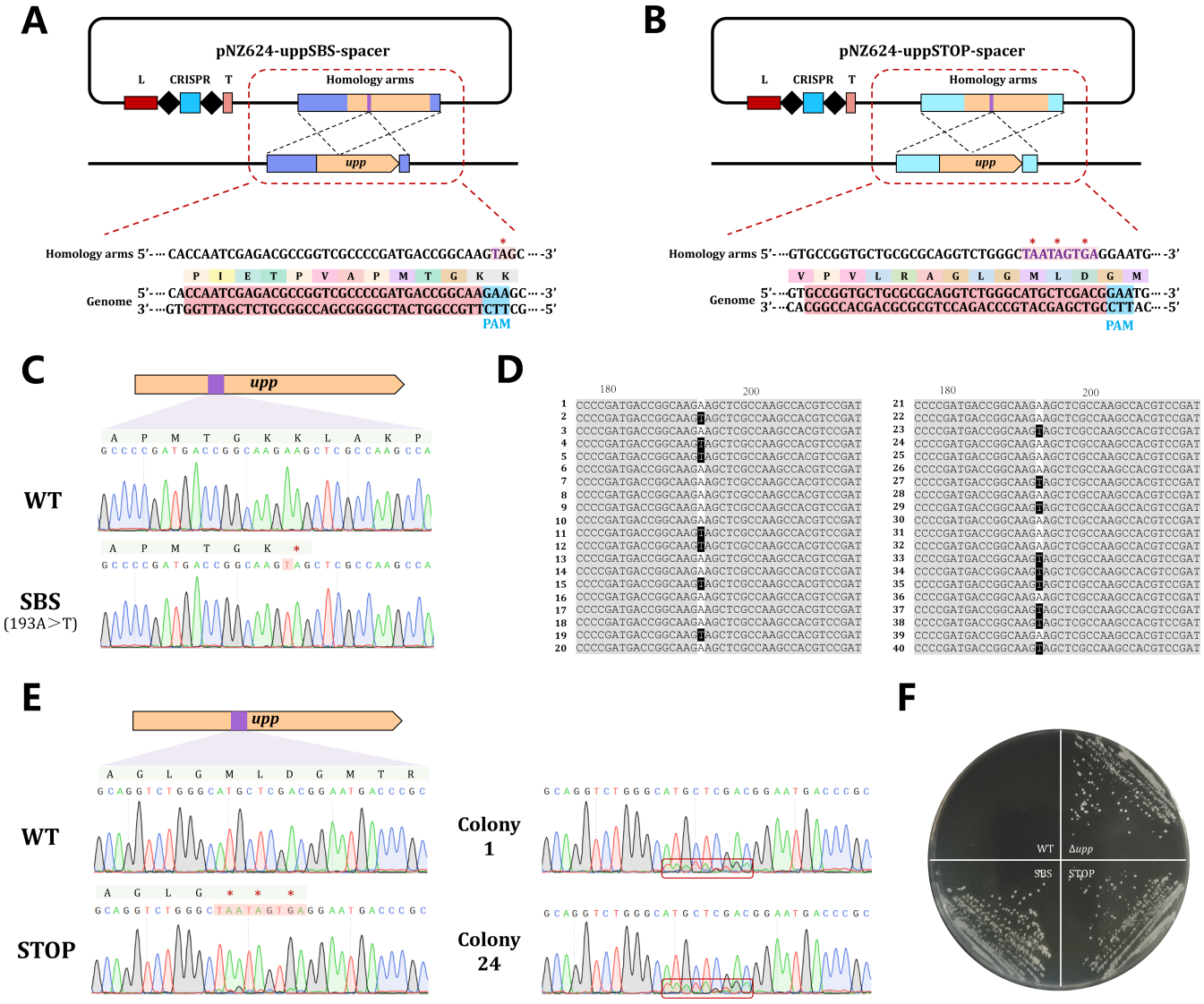

FIG 3 Single-base substitution and insertion of stop codons in *B. breve* using the endogenous CRISPR gene editing toolkit. (A) Schematic diagram of the single-base substitution mediated by the endogenous I-C CRISPR-Cas system. Single-base substitution sites were designed in the PAM region to prevent plasmid self-targeting. (B) Schematic diagram of stop codon insertion mediated by the endogenous I-C CRISPR-Cas system. Three consecutive stop codons were introduced in the *upp* gene by the repair template. (C) Sanger sequencing revealed that colonies on antibiotic plates carried 193A>T mutations in the *upp* gene that turned a lysine into a stop codon. (D) Multiple sequence alignment of Sanger sequencing results of 40 colonies was performed using GeneDoc (37). Black squares indicated that the colony was shown to contain a single-base substitution. (E) The chromatogram revealed the successful insertion of three consecutive stop codons in the *upp* gene. The chromatograms of colonies 1 and 24 showed the stop codon insertion region with double peaks, indicating that they were mixed deletion mutants. (F) The FJSWX38M7 mutant with the single-base substitution or insertion of stop codon could grow on the plate containing 100 μg/mL 5-FU, which proved that the translation of uracil phosphoribosyl-transferase was successfully terminated.

the short DNA insertion assay also caused premature termination of translation (Fig. 3E). The mutant strains FJSWX38M7-*upp*SBS and FJSWX38M7-*upp*STOP obtained from the above experiments were inoculated on plates supplemented with 5-FU and were shown to grow in the presence of this toxic uracil analog as expected (Fig. 3F).

Successive gene deletions mediated by endogenous Type I-C CRISPR-Cas system in *B. breve*

In the study of gene clusters, it is often necessary to knock out different gene combinations to verify gene function, which requires gene editing tools to have the ability to

edit multiple genes. For successive gene editing, the self-targeting plasmid must be cured from the edited cells before the next gene editing. To characterize the curing of the pNZ624-based gene editing plasmid, FJSWX38M7Δ*upp* harboring the plasmid was continuously cultured for nine passages in mMRS broth without adding antibiotics. Cultures were counted using the pour plate method in agar with or without antibiotics. Since cells without the plasmid cannot grow on the agar supplemented with chloramphenicol, the difference between counts on agar plates with and without antibiotics was assumed to represent the number of cells in which the plasmid had been cured. According to the enumeration results, the number of viable cells of FJSWX38M7Δ*upp* could reach ~10^9 CFU/mL stably in mMRS broth, but the number of cells containing the plasmid decreased by an order of magnitude every two generations (Fig. 4A). After one generation of culture in broth without antibiotics, the proportion of cells containing plasmids to the total number of cells dropped to 6%, and after three generations, it was even lower than 0.5% (Fig. 4B). Hence, pNZ624-derived editing plasmids were easily cured by culturing in non-selective mMRS medium, ensuring the feasibility of successive gene editing in *B. breve*.

To validate the gene knock system beyond the *upp* gene and to establish if a strain with multiple successive gene knockouts can be constructed, we next targeted the *bbi* gene. Conjugated linoleic acid (CLA) is a functional fatty acid widely used in food, and *B. breve* is the species with strong biotransformation of CLA among bifidobacteria (39). The *bbi* gene in *B. breve* encodes a linoleic acid isomerase that directly converts the substrate linoleic acid (LA) to CLA in a one-step reaction without other intermediates

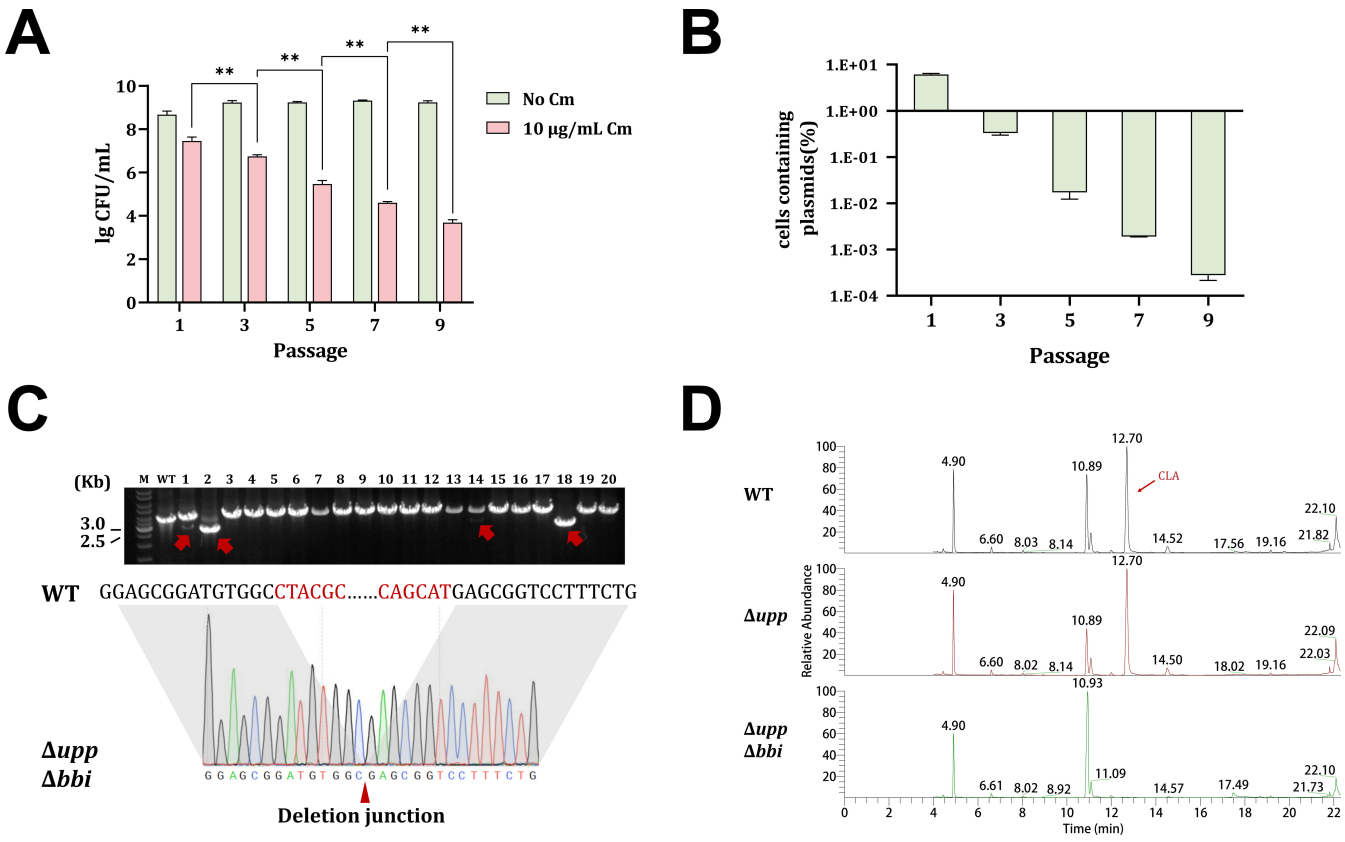

FIG 4 Successive gene editing in *B. breve* using the endogenous Type I-C CRISPR-Cas system. (A) Plasmid curing. FJSWX38M7Δ*upp* containing pNZ624-Δ*upp*-sgRNA2 was continuously cultured for nine generations in broth without antibiotics, and the number of colonies that could grow on the antibiotic plate gradually decreased, which proved that the plasmid was gradually eliminated. $^{**}P < 0.01$ was determined by an unpaired *t*-test. (B) The proportion of cells containing plasmids to the total number of cells. (C) Successfully identified four *bbi* gene deletion mutants out of 20 randomly screened FJSWX38M7Δ*upp* transformants by PCR and Sanger sequencing, including three mixed mutants. (D) Gas chromatogram of conjugated linoleic acid production. The retention time of conjugated linoleic acid was 12.70 min.

(40). Therefore, the *bbi* gene in FJSWX38M7Δ*upp* was selected as the next target gene. The FJSWX38M7Δ*upp*-containing plasmid was cultured for two generations in mMRS broth without antibiotics, then isolated, and purified on mMRS agar without antibiotics. The colonies that had been plasmid cured were screened by multiplex PCR (Fig. S6). pNZ624-Δ*bbi*-spacer was introduced into the plasmid-cured FJSWX38M7Δ*upp* to generate a strain with successive deletions of the *upp* gene and the *bbi* gene. PCR analysis showed that colonies 1, 2, 14, and 18 carried a 993-bp deletion, showing an editing efficiency of 20% (4/20) (Fig. 4C). The CLA transformation ability of mutants was analyzed using GC-MS. The results showed that the wild type or FJSWX38M7Δ*upp* is able to convert LA into CLA, while the presence of CLA was not detected in the fermentation broth of FJSWX38M7Δ*upp*Δ*bbi* (Fig. 4D). These results demonstrate that successive gene editing of *B. breve* is feasible based on its endogenous I-C CRISPR-Cas system.

DISCUSSION

B. breve is one of the dominant species in the gut of breast-fed newborns and is important for maintaining health in early life (3). Evidence from animal model studies and human clinical trials had previously shown that *B. breve* plays an important role in the prevention and treatment of gastrointestinal diseases (41), neurological diseases (42), and allergic diseases (43). However, the lack of genetic tools has so far hampered the investigation of the molecular mechanisms behind their purported benefits (4, 44). It has been reported that pORI19 lacking a *repA* gene can be used to accomplish insertional mutagenesis in *B. breve* (45). However, the non-replicative plasmids used in these strategies will introduce antibiotic resistance genes into the *B. breve* genome, which complicates successive gene editing and increases the risk of horizontal transfer of resistance genes. In contrast, editing methods based on a CRISPR-Cas system can achieve efficient and seamless deletion of genes. A recent study showed that the exogenous CRISPR-Cas9-based editing system can be successfully applied to gene knockout in *B. animalis* AR668 (46). Another study restructured the endogenous I-G type system and introduced the exogenous base editor in *B. animalis* subsp. *lactis* to achieve gene deletion and single-base mutation, providing a solid basis for attempting a similar editing strategy based on the CRISPR-Cas system in *B. breve* (26).

The widespread presence of an endogenous CRISPR-Cas system in the *B. breve* genome presented this system as an attractive opportunity for gene editing. Type I-C is the predominant subtype in *B. breve* and relies on the degradation of the non-target strand by the nuclease Cas3 to form a DNA nick (47). In fact, DNA double-strand breaks caused by Cas9 may be irreparable by the host (48), and even the expression of Cas9 was strongly toxic in some strains (17, 18). The advantage of the endogenous CRISPR-Cas system editing strategy is that only the spacer and the repair template need to be provided by the plasmid since the nuclease is expressed by the host. This can greatly reduce the length of the plasmid, which is conducive to its efficient delivery.

Our analysis revealed a high conservation of Cas4, Cas1, and Cas2 proteins associated with new spacer acquisition in the Type I-C CRISPR-Cas system of *B. breve*. In contrast, Cas3, Cas5, Cas8, and Cas7 proteins, involved in the expression and interference stages, showed significant divergence, with two distinct groups emerging based on their homogeneity, highlighting a potential evolutionary adaptation within these systems. Although there were differences in Cas proteins, the conservation of PAM sequences, leader sequences, repeat sequences, and spacer lengths provided the possibility for gene editing based on the endogenous I-C CRISPR-Cas system in *B. breve*. Plasmid pNZ123 was used as the basis for gene editing vectors and was shown to be efficiently electro-transformed into *B. breve* (49). Plasmid interference assay demonstrated the activity of the endogenous I-C CRISPR-Cas system in FJSWX38M7, and then self-targeting experiments were designed to explore the feasibility of gene editing based on the endogenous I-C CRISPR-Cas system. A native leader and an artificial CRISPR array were used to transcribe crRNA to better mimic the expression and maturation stages of the CRISPR-Cas system. The crRNA serves as a guide that directs the Cascade to their target nucleic acid

sequence. In other species, such as *Thermus thermophilus* (50), *Streptococcus pyogenes* (51), and *Xanthomonas oryzae* (51), the Cas5d protein cleaves their pre-crRNA at the 3' base of the predicted stem-loop structure. However, the Cas5-mediated pre-crRNA processing site of the Type I-C CRISPR-Cas system in *B. breve* remains unknown and needs further investigation. The non-essential gene *upp* encoding UPRT, was selected as the target gene since the deletion mutants of *upp* can easily be distinguished from the wild-type by growing on agar supplemented with 5-FU (52). Three different spacers showed different cleavage efficiencies; similar phenomena are commonly observed in gene editing of animals and plants based on CRISPR-Cas9, which may be related to the GC content of the spacer and the location of the targeted gene (53, 54). The *upp* gene deletion was successfully achieved in five different *B. breve* strains from two groups using pNZ624-spacer2 supplemented with the repair templates. However, escaping from CRISPR targeting was also observed. Escape from targeting may not be related to the mutations of PAM-protospacer regions in the genome (25). The reasons for observed escape transformants have been speculated to be as follows: (i) Surviving transformants escaped CRISPR targeting due to the mutation or deletion of *cas* operators (55, 56); (ii) Low gene editing efficiency was due to the genetic recalcitrance of *B. breve* (57); (iii) This may be attributed to insufficient expression of Cascade and/or crRNA (48).

Due to the lack of NHEJ repair mechanisms, it is necessary to introduce repair templates in *B. breve*. The mutated site and inserted sequence can be designed in the repair template to achieve gene mutation and seamless insertion. Here, point mutations and short DNA insertions in the *upp* gene were successfully achieved using the endogenous CRISPR gene editing toolkit, and the gene single-base substitution showed high efficiency.

The pNZ624-based gene editing plasmids were easily cured by continuous culture in medium (both broth and plate) without supplementation of antibiotics, which ensured their feasibility for successive genome editing in *B. breve*. A commonly used plasmid curing method is to connect the sucrose-sensitive gene *sacB* to the plasmid, and the survivors on the sucrose plate are the colonies without the plasmid (58). However, this method is not suitable for *B. breve*, because the preparation of electroporation competent cells of *B. breve* needs to be preserved in sucrose ammonium citrate solution. Another approach is to use plasmids with thermosensitive replicons, but given the lack of genetic tools in bifidobacteria, more plasmids that can be used in bifidobacteria need to be developed. A recent study provided a strategy for plasmid elimination using a lactose-induced second sgRNA targeting the resistance gene of this plasmid (46). Cas9 was guided by the first sgRNA to cleave the target gene when lactose was not added. The addition of lactose-induced transcription of the second sgRNA directed Cas9 to cleave the plasmid itself when target gene deletion was complete.

In the current study, the gene editing efficiency based on the endogenous I-C CRISPR-Cas system in *B. breve* was similar to that implemented using the endogenous I-G CRISPR-Cas system in *B. animalis* subsp. *lactis*, and there is still further room for improvement, such as (i) construction of dual spacers; since Cas3 will cut non-target DNA strands, using spacers targeting the coding strand and the non-coding strand separately will cause DNA double-strand breaks and reduce escape targeting; (ii) changing the length of the repair template; studies have shown that an appropriate repair template length was beneficial to improve editing efficiency (25, 46); and (iii) introducing the auxiliary repair system. Recombinase systems, such as lambda Red, RecAb, and Rac-RecET, can improve the homologous recombination efficiency of repairing templates and genomes to increase the positive rate of transformants (58).

Overall, this study successfully achieved instances of gene deletion, single-base substitution, gene insertion, and successive gene editing in *B. breve* using the endogenous CRISPR gene editing toolkit. It represents the first report of gene editing in *B. breve* based on an endogenous CRISPR-Cas system, providing a new tool for functional gene mining in *B. breve* and opening up opportunities for the development of engineered biotherapeutics through genetic engineering.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S1, and the oligonucleotides used are listed in Table S2. *E. coli* was cultured on Luria-Bertani (LB) agar or in LB broth at 37°C with shaking. *B. breve* was propagated anaerobically (80% N_2, 10% CO_2, and 10% H_2) at 37°C on modified de Man Rogosa Sharpe (mMRS) agar or in mMRS broth. The composition of mMRS was as follows: 10 g/L tryptone, 10 g/L beef extract, 5 g/L yeast extract, 20 g/L D-(+)-glucose, 2 g/L K_2HPO_4, 2 g/L diammonium hydrogen citrate, 2 g/L sodium acetate, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $MnSO_4{\cdot}H_2O$, 1 g/L Tween80, and 1 g/L L-cysteine. If necessary, 10 µg/mL chloramphenicol was supplemented to the medium for selecting *E. coli* recombinants or *B. breve* mutants. 5-FU (final concentration 100 µg/mL) was added to mMRS agar to culture *B. breve* Δ*upp* mutants.

Phylogenetic analysis

All genome sequences used in this study were available on the National Center for Biotechnology Information (NCBI) Genome database or SRA database (30). Orthologous genes were aligned using MAFFT version 7.3 (59). Subsequently, the construction of the phylogenetic tree was conducted with PhyML version 3.1 (60), employing the neighbor-joining method. Visualization and enhancement of the resultant tree were facilitated through ITOL (https://itol.embl.de) (61).

DNA manipulations

B. breve genomic DNA was extracted using the TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China), and plasmid DNA was extracted with the TIANprep Mini Plasmid Kit (TIANGEN, Beijing, China) according to the instructions of the manufacturer. The oligonucleotides used in this study were synthesized by Shanghai Sangon. DNA fragments were amplified using 2× Phanta Flash Master Mix (Vazyme, Nanjing, China) following standard PCR procedures. PCR products were analyzed on 1% agarose gels, and DNA fragments were purified using the GeneJET Gel Extraction Kit (Thermo Scientific, Massachusetts, USA). The digested plasmid and DNA fragment (if necessary) were ligated with T4 DNA ligase (Thermo Scientific) or One Step Cloning Kit (YEASEN, Shanghai, China) and then transformed into *E. coli*. The correct recombinants were confirmed by PCR using specific primers. Sanger sequencing service was provided by Azenta (Suzhou, China).

Oligonucleotides were phosphorylated and annealed as previously described (62). Briefly, paired oligonucleotides were incubated at 37°C for 30 min in a reaction mixture supplemented with T4 PNK (NEB, Massachusetts, USA) and T4 Ligation Buffer (NEB, Massachusetts, USA). The reaction mixture was incubated at 95°C for 5 min and then ramped down to room temperature at 5°C/min. Diluted annealed oligonucleotides and digested plasmids were ligated overnight at 16°C using T4 DNA ligase and then transformed into *E. coli*.

Construction of plasmids

Plasmid pNZ123 is a replication shuttle vector which is functional in *E. coli* and various bifidobacterial species. Diluted annealed S82-123-F/S82-123-R or PS82-123-F/PS82-123-R and XhoI-EcoRI digested pNZ123 were ligated to obtain interference plasmids pNZ123-S82 or pNZ123-PS82. Endogenous CRISPR-based editing vector pNZ624 was obtained by ligation of artificial crRNA synthesized from GenScript and NspI-digested pNZ123, wherein the artificial crRNA included a leader sequence, a spacer insertion site, two repeats, and a rho-terminator. The annealed oligonucleotides uppspacer1-F/R were ligated with BsaI-digested pNZ624 to obtain pNZ624-spacer1 for self-targeting experiments. pNZ624-spacer2 and pNZ624-spacer3 were also obtained using the method described above. The upstream and downstream homology arms of the *upp*

gene in FJSWX38M7 were amplified using primers 21uppHAL-F/R and 21uppHAR-F/R, respectively. The two purified fragments were ligated by overlap extension PCR to obtain a 1-kb repair template. Both the repair template and pNZ624-spacer2 were digested by XhoI-EcoRI and ligated to obtain the *upp* gene knockout plasmid pNZ624-Δ*upp*-spacer2. The *upp* gene knockout plasmid pNZ624-Δ*upp*-spacer6, pNZ624-Δ*upp*-spacer15, pNZ624-Δ*upp*-spacer16, and pNZ624-Δ*upp*-spacer38 were obtained by using pNZ624-spacer2 as the backbone and ligating the repair templates cloned from *B. breve* FAHWH21M7, FHNXY43M2, FHuNMY8M2, and ZJHZD20M12, respectively.

In order to obtain plasmid pNZ624-*upp*SBS-spacer, primers 21HALSBS-F/R and 21HARSBS-F/R were used to amplify the upstream and downstream homology arms, and the mutated bases were introduced by primers 21HALSBS-R and 21HARSBS-F. The upstream and downstream homology arms were ligated by overlap extension PCR to generate a repair template containing single-base substitutions. Finally, the repair template and the XhoI-EcoRI-digested plasmid containing spacer (BsaI-digested pNZ624 ligated with annealed uppspacerSBS-F/R) were ligated to obtain the single-base substitution plasmid. Similarly, homology arms designed to introduce three stop codons in the *upp* gene were amplified by PCR using the primers 21HALSTOP-F/R and 21HARSTOP-F/R. The overlap extension PCR-amplified repair template was cloned into XhoI-EcoRI-digested pNZ624-spacer2 to generate plasmid pNZ624-*upp*STOP-spacer. The construction method of *bbi* gene knockout plasmid pNZ624-Δ*bbi*-spacer was the same as that of pNZ624-Δ*upp*-spacer2, and the oligonucleotides used in this process were bbispacer-F/R, 21bbiHAL-F/R, and 21bbiHAR-F/R.

Preparation of *E. coli* competent cells and transformation

A fresh 37°C overnight culture of *E. coli* TOP10 was inoculated into 50 mL of LB broth. The culture was grown at 37°C with shaking (200 rpm) until OD_{600} reached 0.5. And then, the culture was frozen on ice for 10 min. The cells were harvested at 4,000 rpm for 10 minutes at 4°C. The cell pellet was resuspended in 10 mL of 0.1 mol/L $CaCl_2$ solution and frozen on ice for 20 min, followed by cell harvesting by centrifugation (4,000 rpm, 10 minutes at 4°C). The cell pellet was again resuspended in $CaCl_2$ and chilled on ice, followed by centrifugation steps. Finally, the pellet was resuspended in 1 mL of 0.1 mol/L $CaCl_2$ glycerol solution (1 mol/L $CaCl_2$ solution and 22% glycerin solution were mixed at a volume ratio of 1:9) and aliquoted in 100-µL volumes into pre-cooled 1.5-mL centrifuge tubes and stored at −80°C until use.

Ten microliters of the ligation product or 200 ng of the plasmid was added to 100 µL of *E. coli* competent cells, mixed by flicking, and incubated on ice for 30 minutes. The mixture was heated at 42°C for 90 s and frozen again on ice for 2 min. Eight hundred ninety microliters (preheated at 37°C) of LB broth was added to the mixture and incubated at 37°C with shaking (200 rpm) for 60 minutes. An appropriate volume of cultures was spread on selective LB agar and incubated overnight at 37°C. Primers pNZ123-F/R were used for PCR screening of recombinant plasmids to confirm that the gene fragments and vectors were correctly ligated.

Preparation of *Bifidobacterium* competent cells and transformation

Preparation and transformation of competent *B. breve* cells were based on a previously described protocol with modifications (49). A fresh 5-mL overnight culture of *B. breve* was inoculated into 45 mL of mMRS broth and grown anaerobically at 37°C until the OD_{600} reached between 0.6 and 0.9. After the culture was chilled on ice for 20 min, the cells were harvested by centrifugation (6,000 rpm, 10 minutes at 4°C). The cell pellet was washed twice with pre-cooled sucrose citrate buffer (0.5M sucrose and 1 mM ammonium citrate, pH 5.8) under the same centrifugation conditions. The final harvested cell pellet was resuspended in 200 µL ice-cold sucrose-citrate buffer and distributed according to the volume of 50 µL per tube.

Two hundred nanograms of plasmid was added to 50 µL *B. breve* competent cells, flicked to mix well, and then transferred to a pre-cooled 2-mm electroporation cuvette.

The electroporation cuvette was pulsed with 2,000 V using an Eppendorf electroporator. After electrotransformation, 950 µL mMRS broth (preheated at 37°C) was added to the cells and incubated at 37°C for 2.5 h under anaerobic conditions. An appropriate volume of culture was plated on selective mMRS agar and grown anaerobically at 37°C for 36–72 h. Primers used to identify mutations or deletions in the obtained transformants were listed in Table S2.

Plasmid curing

FJSWX38M7Δ*upp* containing the plasmid was inoculated at 2% (vol/vol) in the mMRS broth without antibiotics, cultured anaerobically at 37°C for 24 h, and then inoculated the next generation by the same operation, repeated nine times. The cultures of each passage were counted using the pour plate method in a medium with or without antibiotics. The cure ratio of the plasmid was recorded as the difference between the counts of the two plates divided by the counts of the non-selective plates.

B. breve recombinants containing the plasmid were cultured in mMRS broth without antibiotics for two passages and then streaked on mMRS agar without antibiotics. Single colonies were streaked onto mMRS agar with or without antibiotics, and uppYZ-F/uppYZ-R primer pair and pNZ123-F/pNZ123-R primer pair were used to confirm the deletion of the *upp* gene and the presence of the plasmid pNZ624-Δ*upp*-spacer2. Growth failure on antibiotic agar and the absence of the plasmid-specific amplification product in agarose gel electrophoresis were taken as proof that the corresponding plasmid had been cured.

Extraction and analysis of conjugated linoleic acid

CLA in the culture was extracted and analyzed as previously described (63). Briefly, *B. breve* was cultured for 72 h in mMRS broth containing 0.5 mg/mL linoleic acid. Two milliliters of isopropanol was added to 3 mL of culture and mixed by vigorous shaking. Three milliliters of hexane was added to the mixture and shaken vigorously. After standing, the supernatant was transferred to a clean glass bottle and dried using the nitrogen blower. Then, 400 µL of methanol was added to the glass bottle and shaken thoroughly. An appropriate amount of trimethylsilylated diazomethane was added to the bottle until the yellow color of the solution remained unchanged for 15 min, indicating that the methyl esterification was complete. The above samples were dried again under nitrogen. One milliliter of hexane was added to the glass bottle, shaken thoroughly, and then centrifuged at 5,000 rpm for 5 min. Eight hundred microliters of the supernatant was transferred to a 2-mL vial for detection by GCMS-QP2010 Ultra (Thermo Scientific, Massachusetts, USA). The specific parameters were as follows. A gas phase column RT-5MS (30 m × 0.25 mm × 0.25 µm) was used. The initial temperature of the program was 180°C and kept for 3 min and then raised to 190°C at a rate of 10°C/min and kept for 3 min. Then, the temperature was raised to 220°C at 5°C/min and kept for 1 min. Finally, the temperature was raised to 230°C at a rate of 2°C/min. The temperature of the injection port and the interface was both 240°C, and the temperature of the ion source was 230°C.

Statistical analysis

Statistical analysis was performed using an unpaired two-tailed Student's *t*-test. $*P < 0.05$ and $**P < 0.01$ were considered statistically significant.

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AUTHOR AFFILIATIONS

[1]State Key Laboratory of Food Science and Resources, Jiangnan University, Wuxi, Jiangsu, China
[2]School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, China
[3]International Joint Research Center for Probiotics & Gut Health, Jiangnan University, Wuxi, Jiangsu, China
[4]APC Microbiome Ireland, University College Cork, Cork, Ireland
[5]Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland
[6]National Engineering Research Center for Functional Food, Jiangnan University, Wuxi, Jiangsu, China

AUTHOR ORCIDs

Haiqin Chen http://orcid.org/0000-0002-2359-2622
Fengwei Tian http://orcid.org/0000-0002-8511-4546
Douwe van Sinderen http://orcid.org/0000-0003-1823-7957
Wei Chen http://orcid.org/0000-0003-3348-4710
Bo Yang http://orcid.org/0000-0002-1347-3718

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AUTHOR CONTRIBUTIONS

Xiao Han, Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft | Lulu Chang, Writing – review and editing | Haiqin Chen, Writing – review and editing | Jianxin Zhao, Data curation, Resources | Fengwei Tian, Writing – review and editing | R. Paul Ross, Writing – review and editing | Catherine Stanton, Writing – review and editing | Douwe van Sinderen, Writing – review and editing | Wei Chen, Conceptualization, Funding acquisition, Project administration, Supervision | Bo Yang, Conceptualization, Data curation, Funding acquisition, Investigation, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Tables S1 to S3 (AEM02074-23-s0001.docx). Strains, plasmids, oligonucleotides, and sequences used in this study.

Fig. S1 to S6 (AEM02074-23-s0002.docx). Fig. S1 (Derived model for Type I-C Cascade-mediated DNA interference), Fig. S2 (Occurrence frequency of Type I-C PAM sequences in the *B. breve* FJSWX38M7 genome), Fig. S3 (Leader sequence of Type I-C CRISPR array in *B. breve* using the WebLogo server), Fig. S4 (Construction steps of the gene editing vector based on pNZ624), Fig. S5 (The spacer lengths of Type I-C CRISPR-Cas system in *B. breve*), and Fig. S6 (Multiplex PCR to determine plasmid curing in *B. breve* FJSWX38M7Δupp).

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