A Targetron-Recombinase System for Large-Scale Genome Engineering of Clostridia

Tristan Cerisy, William Rostain, Audam Chhun, Magali Boutard, Marcel Salanoubat, Andrew C. Tolonen

ABSTRACT Clostridia are a group of Gram-positive anaerobic bacteria of medical and industrial importance for which limited genetic methods are available. Here, we demonstrate an approach to make large genomic deletions and insertions in the model Clostridium phytofermentans by combining designed group II introns (targetrons) and Cre recombinase. We apply these methods to delete a 50-gene prophage island by programming targetrons to position markerless lox66 and lox71 sites, which mediate deletion of the intervening 39-kb DNA region using Cre recombinase. Gene expression and growth of the deletion strain showed that the prophage genes contribute to fitness on nonpreferred carbon sources. We also inserted an inducible fluorescent reporter gene into a neutral genomic site by recombination-mediated cassette exchange (RMCE) between genomic and plasmid-based tandem lox sites bearing heterospecific spacers to prevent intracassette recombination. These approaches generally enable facile markerless genome engineering in clostridia to study their genome structure and regulation.

IMPORTANCE Clostridia are anaerobic bacteria with important roles in intestinal and soil microbiomes. The inability to experimentally modify the genomes of clostridia has limited their study and application in biotechnology. Here, we developed a targetron-recombinase system to efficiently make large targeted genomic deletions and insertions using the model Clostridium phytofermentans. We applied this approach to reveal the importance of a prophage to host fitness and introduce an inducible reporter by recombination-mediated cassette exchange.

KEYWORDS clostridia, engineering, prophage, recombinase

The clostridia are Gram-positive obligately anaerobic bacteria that include human pathogens as well as plant-fermenting species critical for healthy functioning of soil and gut microbiomes. Clostridium (Lachnoclostridium) phytofermentans ISDg (1) is a model plant-fermenting Clostridium that breaks down plant biomass using numerous carbohydrate-active enzymes (CAZymes) and ferments the resulting hexose and pentose sugars into ethanol, hydrogen, and acetate (2–4). C. phytofermentans is a member of the Lachnospiraceae family that is abundant in soil (5), dominates the rumen (6), and includes human gut commensals that play important roles in nutrition and intestinal health (7). Because of their ability to directly ferment lignocellulose, plant-fermenting clostridia have industrial potential for the transformation of plant biomass into value-added chemicals. Clostridia have long been the focus of study due to their diverse importance to human health, industry, and the environment (8). However, a lack of methods for genetic manipulation of clostridia has hindered their application in biotechnology and our ability to study the structure and function of their genomes.

The development of targetrons based on the Lactococcus lactis LLItrB group II intron (9) enabled targeted chromosome insertions in C. phytofermentans (10) and other clostridia (11). Targetrons are designed group II introns that can be customized to insert into specific DNA sequences by a retrohoming mechanism with efficiencies approach-
ing 100% of cells (12), obviating the need for antibiotic resistance markers to select for their insertion. Domain IV of the intron can be modified to carry cargo DNA such as single or two tandem lox sites (13). Lox sites are 34-bp elements of two 13-bp palindromic arms separated by an 8-bp spacer that are recognized by Cre, a recombinase that does not require host-encoded factors (14). Cre has previously been applied in clostridia to excise an antibiotic resistance gene integrated by homologous recombination (15). Modified lox sequences expand the utility of the Cre/lox system. For example, lox66 and lox71 sites each contain arm mutations such that their recombination results in a lox72 site with two mutant arms that is no longer recognized by Cre (16). Orthogonal spacer mutants such as lox511 and loxFAS do not recombine with each other (17), permitting simultaneous noninterfering lox recombinations for recombination-mediated cassette exchange (RMCE). RMCE is a method by which a plasmid and genomic cassette can be exchanged by recombination mediated by a site-specific recombinase such as Cre (18).

Here, we describe a way to make large precise deletions (Fig. 1A to D) and insertions (Fig. 1E to H) in clostridial genomes using _C. phytofermentans_ as a model. Targetrons and Cre recombinase have been used together to make genomic insertions in _Escherichia coli_ and deletions in _E. coli_ and _Staphylococcus aureus_ (13), showing that these tools can be used together in Gram-negative and Gram-positive bacteria. We developed a targetron-recombinase system for clostridia and applied it to excise 50 genes (cphy2944 to cphy2993) comprising a 39-kb prophage region in the _C. phytofermentans_ genome, which we chose because mobile elements such as prophages often reduce genomic stability and their removal increases fitness in other Gram-positive bacteria.

**FIG 1** Overview of genomic deletion and insertion in _C. phytofermentans_. (A) pQlox71 is introduced for genomic insertion of a lox71 (L71) site using the LtrA protein encoded by the targetron. (B) pQlox71 is cured and pQlox66 is introduced for genomic insertion of a lox66 (L66) site. (C) pQlox66 is cured, and pQcre1 is introduced for Cre-mediated recombination to delete the sequence between the lox66 and lox71 sites. (D) In the resulting strain, the deletion and lox72 site are confirmed by PCR (arrows show primers). (E) pQadd1 is introduced for genomic delivery of a lox511/71 (L5-71) and loxFAS/66 (LF-66) cassette into the genome. (F) pQadd1 is cured and pQadd2 is introduced, bearing the desired insertion sequence flanked by lox511/66 (LS-66) and loxFAS/71 (LF-71) sites. (G) pQcre2 is introduced for Cre-mediated RMCE. (H) The resulting strain has a genomic copy of the insert sequence flanked by lox511/72 (LS-72) and loxFAS/72 (LF-72) sites in the genome, which is confirmed by PCR (arrows show primers).
TABLE 1  Plasmids and strains used in this study

| Plasmid or Strain | Description | Source or reference |
|-------------------|-------------|---------------------|
| pAT19             | erm (erythromycin resistance), pAMβ1 origin, pUC origin, oriT | 21 |
| pQint             | pAT19 with targetron (LLLtrB-deltaORF intron, ltrA) expressed from P3558 promoter | 10 |
| pRAB1             | Source of PpaqA·cre cassette | 22 |
| pRK24             | RP4 conjugal genes, Tet', Amp' | 10 |
| pMTL82351         | adaB (spectinomycin resistance), pBP1 origin, colEl origin, oriT | CHAIN Biotech |
| pMTC6             | MlsR, Amp', E. coli-Clostridium shuttle vector. PpFBFPm, lac operator, thl terminator | 32 |
| pQlox71F, pQlox71R| pQint with lox71 in either the forward (F) or reverse (R) orientation inserted into MluI site | This study, Addgene 135655, 135656 |
| pQlox66F, pQlox66R| pQint with lox66 in either the forward (F) or reverse (R) orientation inserted into MluI site | This study, Addgene 135657, 135658 |
| pQcre1            | pAT19 with PpaqA·cre inserted between EcoRI and XbaI sites | This study, Addgene 135659 |
| pQcre2            | pMTL82351 with PpaqA·cre cassette from pQcre1 inserted in EcoRI site | This study |
| pQadd1F, pQadd1R  | pQint with lox111/171 and loxFAS/66 cassette inserted into MluI site | This study, Addgene 135660, 135661 |
| pQadd2            | pAT19 with lox111/66-loxFAS/71 cassette inserted between the PstI and EcoRI sites | This study, Addgene 135662 |
| pQadd2_P3368_FbFP | pQadd2 with P3668·FbFP inserted between SphI and Xhol sites of the lox cassette | This study |

| Strains           | Description | Source or reference |
|-------------------|-------------|---------------------|
| E. coli 1100-2    | mcrA0, endA1, mcrB9999 strain used as a conjugal donor | 10 |
| E. coli NEB 5-alpha | Competent E. coli cells used for gene cloning | New England BioLabs |
| C. phytofermentans ISDg | Reference strain | ATCC 700394 |
| C. phytofermentans IntRx2944 | cphy2944·lox11/F.int2944.591s | This study |
| C. phytofermentans IntF2944 | cphy2944·lox71R.int2944.526a | This study |
| C. phytofermentans DI-AS | cphy2944·lox71R.int2993.177a | This study |
| C. phytofermentans DI-SS | cphy2944·lox71F.int2944.526a, cphy2933·lox66F.int2993.177a | This study |
| C. phytofermentans Del-AS | DI-AS-derived cphy2944·cphy2993 deletion strain with palindromic scar | This study |
| C. phytofermentans Del-SS | DI-SS-derived cphy2944·cphy2993 deletion strain with lox72 site | This study |
| C. phytofermentans C. phytofermentans Int1575 | cphy1575·lox111/71·loxFAS/66.96a | This study, Addgene 135662 |
| C. phytofermentans C. phytofermentans Int1575-FbFP | cphy1575·lox111/72_P3368·FbFPloxFAS/72.96a | This study |

(19). We also inserted an anaerobic flavin mononucleotide-based fluorescent protein (20) into the C. phytofermentans genome by RMCE between genomic and plasmid-based tandem lox cassettes and demonstrated that it acts as a carbon source-inducible reporter. We discuss how these methods are an important component of an emerging suite of tools to engineer clostridial genomes.

RESULTS

Plasmids for genomic deletion and insertion. Our strategy to make targeted deletions (Fig. 1A to D) and insertions (Fig. 1E to H) in the C. phytofermentans genome is based on sequential introduction and removal of conditionally replicating plasmids carrying lox sites or Cre recombinase. We constructed plasmids for targetron-mediated delivery of lox sites by inserting the lox cassettes into the unique MluI site in domain IV of the intron, a site that supports DNA insertions while retaining targetron activity (13). Targetrons can be programmed to insert into the genome in either orientation, but both lox linkers must be in the same relative orientation in the genome for Cre to delete the intervening region (Fig. 1C). Similarly, RMCE requires two Cre-based recombinations between tandem lox sites whose orientation determines that of the inserted DNA (Fig. 1E to H). We thus constructed lox-containing targetrons in pQint (10) with either orientation of lox sites: lox71 (pQlox71F and pQlox71R) and lox66 (pQlox66F and pQlox66R) for genome deletions and a tandem lox111/71-loxFAS/66 cassette (pQadd1R and pQadd1R) for genome insertion by RMCE (Table 1; see also Fig. S1 in the supplemental material). These plasmids all contain the erythromycin resistance gene from Streptococcus pneumoniae Tn1545 (21) for selection in E. coli and C. phytofermentans and the pAMβ1 origin that replicates stably in C. phytofermentans but can be cured by serial transfer in liquid medium lacking antibiotics (10).
To express Cre recombinase in *C. phytofermentans* for genomic deletion between *lox71* and *lox66* sites, we constructed pQcre1 (Fig. S1; Table 1) in which *cre* is expressed from the *Bacillus anthracis* *PagA* promoter, a well-characterized constitutive promoter that is widely functional in Gram-positive bacteria (22). Initially, we attempted to deliver a tandem *lox* cassette and *cre* on a single plasmid for RMCE but were unable to construct such a plasmid likely because of interactions between Cre and the *lox* cassette, requiring us to cotransform *C. phytofermentans* with separate plasmids bearing the *lox* cassette and *cre* gene. We found that the pBP1 origin from *Clostridium botulinum* (23) replicates stably in *C. phytofermentans* and can be cured using the same methods as for pAM1/H9252 plasmids and that the *aad9* adenyltransferase gene from *Enterococcus faecalis* (24) confers spectinomycin resistance in *C. phytofermentans*. Moreover, we found that plasmids bearing pAM1/B and pBP1 origins can be simultaneously maintained in the same *C. phytofermentans* cell, provided they have different antibiotic resistance markers. Thus, we constructed pQcre2 in which the *PagA*-*cre* cassette from pQcre1 was inserted into pMTL82351 (Table 1), which carries the pBP1 origin and *aad9*.

**Targetrons position genomic *lox* sites.** Both *lox* sites must be in the same orientation for Cre-based recombination to result in deletion of the intervening DNA (25). To excise the 39-kb prophage region (*cphy2944-2993*) in the *C. phytofermentans* genome (Fig. 2A), pQlox71R was modified to insert a *lox71* targetron at 591 bp from the *cphy2944* start codon in the sense direction relative to gene transcription, yielding pQlox71R. Plasmid pQlox66F.2993 was similarly built to insert a *lox66* targetron in the antisense orientation at 177 bp from the start of *cphy2993*. Both *cphy2944* and *cphy2993* are transcribed on the reverse strand of the genome, such that the *lox71* and *lox66* sites inserted by pQlox71R and pQlox66F.2993 are in the same direction the genome (Fig. 2A).

Conjugal transfer of *lox71* and *lox66* targetron plasmids into *C. phytofermentans* consistently yielded 10 to 20 transconjugant colonies, of which 80% to 100% contained the expected targetron insertion, indicating that the efficiency of delivery and integration of *lox*-containing targetrons is similar to that in previous targetrons studies in *C. phytofermentans* (10, 26, 27). Following sequential delivery and curing of
pQlox71R.2944 and pQlox66F.2993, PCR amplification of cphy2944 and cphy2993 showed targetron insertions in both genes (Fig. 2B), and sequencing of these PCR products established the presence of the expected genomic lox71 in cphy2944 (Fig. 2C) and lox66 in cphy2993 (Fig. 2D). We refer to this double insertion strain as DI-AS (Table 1). Off-site targetron insertions are common (27), which would result in unexpected recombinations. Thus, we applied an inverse PCR assay (27) using primers that anneal to the targetron in order to quantify the number and sequence of genomic targetron insertions. This assay confirmed that DI-AS contains both expected genomic targetrons without any additional off-site insertions (Fig. 2E).

**Cre-mediated prophage deletion.** We introduced Cre recombinase into *C. phytofermentans* strain DI-AS by conjugal transfer of pQcre1 and diagnosed the Cre-mediated deletion using 3 PCR amplifications: amplicons spanning each genomic lox site and an amplicon spanning the genomic region between the two lox sites. Cre-mediated recombination was highly efficient in *C. phytofermentans* such that when DI-AS was transformed with pQcre1, all colonies yielded a product using primers 2944_1/2993_2 spanning the region between lox71 and lox66 and no colonies yielded PCR products using primers flanking each of the individual lox sites (2944_1/2 and 2993_1/2) (Fig. 3A).
We refer to the strain with a Cre-mediated deletion as Del-AS (Table 1). While all 10 Del-AS transconjugants yielded PCR products with primers 2944_1/2993_2, the product was 400 bp rather than the expected 1.5-kb product (Fig. 3A). The sequence of this PCR product revealed that all that remained of the expected lox72-containing targetron was a palindromic fragment corresponding to the first 28 bp of the targetron (Fig. 3B).

Cre-mediated recombination between lox sites in opposing targetrons produces a targetron fragment consisting of a 500-bp inverted repeat that may be unstable. We tested if the relative orientation of the genomic targetrons influences the final product of Cre recombination by generating a second C. phytofermentans strain with both targetrons in the same orientation. To this end, we modified the lox71-targetron of pQlox71F.2944 to insert in cphy2944 in the sense orientation with respect to transcription at 526 bp from the gene start. As the orientation of both the intron targeting and lox71 are reversed relative to that in pQlox71R.2944, the orientation of lox71 in the genome is preserved. We constructed a second double insertion strain, DI-SS (Table 1), with both genomic targetrons in the same orientation by sequential delivery and curing pQlox71F.2944 and pQlox66F.2993 using the same procedures as for DI-AS. Following delivery of pQcre1 to DI-SS, we observed by PCR using primers 2944_1/2993_2 that all 10 transconjugants yielded a 1.5-kb product (Fig. 3A) whose sequence confirmed the expected recombination resulting in a single targetron containing a lox72 site (Fig. 3C). We refer to this strain as Del-SS (Table 1). We thus conclude the Cre-mediated deletion in Del-AS results in an inverted repeat that is excised by homologous recombination to leave only a short palindromic targetron fragment (Fig. 3D), whereas the intact intron containing a lox72 site in Del-SS is stable in the genome (Fig. 3E).

**Prophage excision reduces growth on nonpreferred carbon sources.** Wild-type and DI-SS strains grow similarly on monosaccharides (glucose and xylose), a disaccharide (cellobiose), and a polysaccharide (galactan) (Fig. 4A to D), indicating that targetron insertions in cphy2944 (phage cell wall peptidase) and cphy2993 (phage DNA-binding protein) do not affect growth under these conditions. In contrast, growth of Del-SS was significantly reduced relative to that of the wild-type (WT) on carbon sources other than glucose (Fig. 4A to D), suggesting that the prophage contributes to cell fitness. The organization and sequences of the 50 genes in the C. phytofermentans prophage region are similar to those in Bacillus subtilis bacteriophage SPP1, which has a linear, double-stranded 44-kb DNA genome with all genes transcribed in the same direction (28).

While it is unknown if this C. phytofermentans prophage can form lytic particles, it harbors genes for the major phage functions: bacterial cell wall binding and degradation, phage tail assembly, phage head assembly, and DNA synthesis/replication (see Fig. S2). However, the contribution of these genes to cell fitness on nonglucose carbon sources is not evident from gene annotations.

We compared the mRNA expression in C. phytofermentans WT of the 50 prophage genes on these four carbon sources as measured by transcriptome sequencing (RNA-seq) (3), revealing that cphy2976 transcription is upregulated 68-fold on cellobiose, 15-fold on xylose, and 86-fold on galactan relative to that on glucose (Fig. 4E to H). As such, cphy2976 is among the top 10 most highly expressed genes in the cell on cellobiose and galactan. The cphy2976 gene is cotranscribed with cphy2975 (29) in an operon that opposes all other genes in the prophage region (Fig. S2). Neither gene encodes domains of known function nor has significant similarity to genes in the NCBI database, and so we cannot postulate their roles in the cell. However, our data indicate that deletion of these 50 putative prophage genes unexpectedly decreased C. phytofermentans growth on nonpreferred carbon sources, and the extremely high upregulation of cphy2976 suggests that it plays an important role in cell fitness under these conditions.

**Genomic insertion by RMCE.** To effectuate targeted genomic insertions in C. phytofermentans by RMCE, the targetron of pQadd1R (Fig. S1) was customized to insert tandem lox sites with incompatible linkers (lox511/71 and loxFAS/66) at 96 bp from the start of cphy1575 (Fig. 5A) to produce pQadd1R.1575. The cphy1575 gene is homolo-
gous to *B. subtilis* AprX, a nonessential S8 subtilisin serine protease that degrades heterologous protein in stationary phase (30, 31). The *cphy1575* gene is contained in a cluster of 6 genes with 50% or greater amino acid similarity (*cphy1571* to *cphy1576*) that likely have overlapping function, further suggesting that inserting heterologous DNA into *cphy1575* would have minimal impact on the cell. We transformed *C. phytofermentans* with pQadd1R.1575, confirmed the insertion of the intron into *cphy1575* by PCR (Fig. 5B), and cured the plasmid to yield strain int1575 (Table 1). We confirmed that int1575 did not contain any additional off-site targetron insertions by inverse PCR, which yielded only the expected 5.1-kb band corresponding to the insertion in *cphy1575* (Fig. 5C).

pQadd2 contains tandem *loxS11/66* and *loxFAS/71* sites (Fig. S1) to facilitate RMCE with the complementary cassette integrated in the genome of strain int1575 (Fig. 5A). The *lox* sites in pQadd2 are separated by SpeI and XhoI sites into which we cloned a version of the FbFP oxygen-independent green fluorescent protein from *Pseudomonas putida* that has been codon optimized for clostridia (32). The FbFP gene is expressed from the promoter of *cphy3368* (P3368), encoding a GH48 cellobiohydrolase (33) whose transcription is highly upregulated on cellobiose relative to that on glucose (3). The resulting pQadd2 plasmid bearing the P3368-FbFP cassette was called pQadd2.P3368-FbFP. We sequentially transformed strain int1575 with pQadd2.P3368-FbFP and pQcre2. As these plasmids have different origins of replication and antibiotic resistance, they can be maintained simultaneously in the *C. phytofermentans* cell (Fig. 5D).

Following transformation of strain 1575 with pQadd2.P3368-FbFP and pQcre2, PCR amplification of the *cphy1575* genomic region (Fig. 5E) of 10 transconjugants showed
that they all contained the intended insertion of the P3368-FbFP cassette (Fig. 5F). The sequence of this PCR product confirmed the insertion of the P3368-FbFP cassette flanked by lox511/71 and loxFAS/66 site. As such, Cre-based recombination for RMCE is highly efficient in C. phytofermentans. We cured the plasmids yielding strain int1575_FbFP (Table 1), and compared its fluorescence to that of the WT. The fluorescence of WT and int1575_FbFP cells was similar when grown on glucose, whereas int1575_FbFP showed elevated fluorescence relative to that of the WT on cellobiose (Fig. 5G). These results show that FbFP functions as a fluorescent reporter in C. phytofermentans and that P3368 can be used to regulate transcription of heterologous genes in a cellobiose-inducible manner.

**DISCUSSION**

This study describes a strategy to make genomic deletions and insertions by leveraging two well-characterized genetic tools that are efficient and independent of host factors. Targetrons delivered both single and tandem lox sites into the C. phytofermentans genome in more than 80% of transconjugants, showing that splicing lox sites into the targetron does not significantly reduce its efficiency. Further, Cre effec-tuated recombination between lox sites for genomic deletion and RMCE-mediated insertion in 100% of colonies. Targetrons and Cre recombinase have already been used separately in other clostridial species, supporting that this approach can be generally applied in clostridia. Unexpectedly, we found that if the two genomic targetrons are in opposite orientations, the lox72 site and most of the targetron sequence are excised to leave only a short scar following Cre-mediated recombination. While this secondary recombination can be prevented by controlling the orientation of the lox sites or potentially by introducing two nonhomologous targetrons based on Ll.LtrB and EclV, it is potentially preferable to reduce the size of the scar relative to a lox-containing targetron.

Engineering of clostridia with streamlined genomes will enhance our ability to optimize the production of target molecules and understand the contributions of nonessential genes to cell fitness. Deletion of one or a few genes by double-crossover homologous recombination-based integration of an antibiotic resistance marker has been achieved in certain clostridial species (34-36). Moreover, CRISPR is proving to be an efficient way to select for clostridia with markerless, homologous recombination-mediated gene deletions by killing nonrecombinant cells using Cas nucleases (37-39). CRISPR methods can select for small genomic changes because Cas nucleases can be
programmed to cleave any genomic site containing a PAM sequence (40). However, CRISPR methods are still limited by the probability of initially introducing the desired mutation by homologous recombination, because CRISPR-mediated DNA cleavage only modestly induces recombination in bacteria (40) and most bacterial taxa lack the proteins for nonhomologous end joining (41). As the efficiency of homologous recombination decreases exponentially with distance between recombination sites (42), CRISPR methods are generally confined to making genomic changes of a few kilobases or less. Furthermore, the double-stranded DNA cleavage activities of Cas nucleases are toxic, making them difficult to introduce into clostridial species with low transformation efficiencies (41). In contrast, targetrons and Cre-lox are independent of homologous recombination, do not cause double-stranded DNA breaks, and are efficient such that large changes can be easily made without selection.

Insertion of heterologous genes and pathways into clostridia is fundamental to being able to endow them with novel phenotypic and metabolic properties. The C. phytofermentans int1575 strain containing a genomic loxS11/71-loxFAS/66 cassette is a chassis that can be generally applied for facile RMCE-mediated integration of genes and pathways of interest. Genomic insertion by RMCE does not require selectable markers, and the length of the inserted DNA is only limited by that which can be cloned into pQadd2. A previous method for genomic insertion in clostridia termed allele-coupled exchange (ACE) links expression of a selectable marker gene to the formation of a double-crossover recombinant chromosome (43). ACE is advantageous relative to RMCE in permitting serial accumulation of larger insertion fragments but disadvantageous in requiring a uracil auxotrophy or genomic insertion of antibiotic resistance genes. In conclusion, the targetron-recombinase method described here expands our capabilities to engineer clostridia by enabling large markerless genomic deletions and insertions. This approach complements other recent technologies such as CRISPR and ACE to make a suite of tools to understand the biology of clostridia and exploit their usefulness in industry and medicine.

MATERIALS AND METHODS

Cell cultivation and conjugation. C. phytofermentans ISDg (ATCC 700394) was cultured anaerobically at 30°C in GS medium (44) containing 3 g liter-1 of either glucose (GS767; Sigma), xylose (X3877; Sigma), cellobiose (C7252; Sigma), or galactan (P-GALLU; Megazyme). Growth on different carbon sources was measured in 400-μl cultures in 100-well microtiter plates (9502550; Bioscreen) that were sealed in the anaerobic chamber (2% H2, 98% N2) as previously described (45). Cell densities were measured using a Thermo Scientific Bioscreen C as the optical density at 600 nm (OD600) with 30 s of shaking before each reading.

Plasmids were introduced into C. phytofermentans by conjugal transfer from E. coli strain 1100-2 (pRK24) (10), and plasmids were maintained using erythromycin (200 μg ml-1 in liquid medium, 40 μg ml-1 in solid medium) or spectinomycin (600 μg ml-1 in liquid medium and solid medium). Following conjugation, ten transconjugant colonies were picked, and the presence of the plasmid was confirmed by PCR using primers pAMB1_1/2 for pAM and pRAB1 (44) using primers PpagA-Cre_1/2 and pRAB1 (44). PCR and sequencing using primers flanking the genomic insertion site (primers are listed in Table S1 in the supplemental material).

Plasmid construction. Enzymes were purchased from New England BioLabs, and cloning was performed using NEB 5'-alpha Competent E. coli cells (C2987T; NEB). The lox66 and lox71 sites were cloned by annealing complementary 5’ phosphorylated oligonucleotides (L71_1/2 and L66_1/2) and cloning them into the unique MluI site in domain IV of the Ll.LtrB-ΔORF intron of pQint (10). To enable positioning of lox sites in either orientation in the genome relative to the intron insertion, lox66 and lox71 sites were cloned into pQint in both orientations yielding 4 plasmids: pQlox66F, pQlox66R, pQlox71F, and pQlox71R. Using crossover PCR, the pQlox71F intron was targeted to insert in the antisense orientation at 526 bp from the cphy2944 start codon (pQlox71F/2944), the pQlox71R intron was targeted to insert at 591 bp from the cphy2944 start codon (pQlox71R/2944), and the pQintL66F intron was targeted to insert in the antisense orientation at 177 bp from the start of cphy2993 (pQlox66F/2993). pQcre1 was constructed by PCR amplifying the Ppaga-cre cassette from pRAB1 (22) using primers PpagaA-Cre_1/2 and inserting it between the EcoRI and XbaI sites of pAT19 (21). PCR and sequencing using primers OK_PpagaA-Cre_1/2 confirmed the presence of the PpagaA-cre cassette in pQcre1. pQadd1F/R were constructed by annealing complementary 5’ phosphorylated oligonucleotides 2ML4_1/2 encoding a double lox cassette (loxS11/71 and loxFAS/66) and cloning it into the unique MluI site of pQint. The pQadd1R intron was subsequently targeted to insert into cphy1575 in the antisense.
orientation at 96 bp from the start codon to make plasmid pQadd1R.1575, pQadd2 was constructed by annealing and PCR extending oligonucleotides 2ML9_1/2 encoding a double lox cassette (loxS11/66 plus loxFAS/71), and cloning the resultant product between the unique PsiI and EcoRI sites of pAT19. The P3368-FbFP cassette was inserted into pQadd2 by PCR amplifying the cPHY3368 promoter region from C. phytofermentans genomic DNA using primers P3368_1/2 and cloning the product into the SpeI and Xhol sites between the loxS11/66 and loxFAS/71 sites of pQadd2. The PpFbFpM gene was PCR amplified from pMTC6 (32) using primers P3368_FbFP_1/2 and cloned between the XbaI and Xhol sites directly downstream of P3368 to make pQadd2.P3368-FbFP. To enable cotransformation of plasmid pQadd2.P3368-FbFP and a cre plasmid into the same cell, pQcre2 was built by PCR amplifying the Ppaga-cre cassette from pQcre1 using primers Cre2_1/2 and subcloning it into the unique EcoRI site of pMTL82351 (CHAIN Biotech). The sequences of all plasmids were confirmed by Sanger sequencing.

Genomic insertion of lox sites. The C. phytofermentans strains carrying lox71 and lox66 sites for prophage deletion were made by sequential conjugal delivery and curing of plasmids pQlox71R.2944 and pQlox66F.2993 (strain DI-AS) or plasmids pQlox71F.2944 and pQlox66F.2993 (strain DI-SS). Following delivery of each plasmid, we confirmed the intended targetron insertion in 10 transconjugants by PCR using primers flanking the insertion site, cured the plasmid from 3 transconjugants, and demonstrated plasmid loss by PCR and restoration of erythromycin sensitivity. Following delivery and curing of plasmids for both lox sites, PCR and sequencing confirmed the presence and orientation of both the lox71 site at 591 bp from the cPHY2944 start in strain DI-AS and at 526 bp from the cPHY2944 start in strain DI-SS (primers OK_2944_1/2). Similarly, the location and orientation of the lox66 site at 177 bp from the cPHY2993 start were confirmed in both strains (OK_2993_1/2). The C. phytofermentans int1575 strain was made by conjugal delivery and curing of pQadd1R.1575. The presence and antisense orientation of the loxS11/71/loxFAS/66 cassette at 96 bp from the start of cPHY1575 in the int1575 genome was confirmed in 8 of 10 transconjugants by PCR and sequencing (OK_1575_1/2).

The absence of additional, off-site genomic targetron insertions in strains DI-SS, DI-AS, and int1575 was confirmed by inverse PCR (27). To this end, genomic DNA was extracted and purified from 5 ml of log-phase culture using the GenElute Bacterial Genomic DNA kit (NA2110; Sigma). DI-SS and DI-AS genomic DNA (1 μg) was digested with HindIII; int1575 genomic DNA was digested with EcoRI. Digested DNA was purified with QiAquick PCR purification kit (28104; Qiagen) and ligated with T4 DNA ligase (M0202S; NEB). Intron insertion sites were PCR amplified by Q5 polymerase (M0491; NEB) using primers int_1/2 with a 7-min extension time. Targetron insertion sites were identified by gel extraction and sequencing of the PCR products.

Genomic deletion and insertion using Cre recombinase. To effectuate Cre-mediated genomic deletions, pQcre1 was conjugated into C. phytofermentans strains DI-SS and DI-AS, and its presence in 10 transconjugants was confirmed by PCR (primers pAMB1_1/2). Liquid cultures of the ten transconjugants were grown in liquid medium containing erythromycin, and deletion of the 38.9-kb region between the lox71 and lox66 sites was diagnosed using 3 PCRs: two reactions to confirm the absence of products using primers 2944_1/2 and 2993_1/2 and a reaction to amplify a product spanning the deleted region using primers 2944_1/2993_2. pQcre1 was cured from Del-SS and Del-AS as described above.

To make a Cre-mediated genomic insertion by RMCE, pQadd2.P3368-FbFP and pQcre2 were sequentially conjugated into strain int1575, and the simultaneous presence of both plasmids in 10 transconjugant colonies was confirmed by PCR using primers pBP1_1/2 for pQcre2 and pAMB1_1/2 for pQadd2.P3368-FbFP. After 1 transfer in medium containing erythromycin and spectinomycin, PCR using primers OK_1575_1/2 diagnosed insertion of the P3368-FbFP cassette into the int1575 genome, yielding strain int1575_FbFP.

Measurement of FbFP fluorescence. Cellular fluorescence was compared between triplicate cultures of C. phytofermentans WT and strain int1575_FbFP. Cultures were grown to log phase (OD600 of 0.6) in glucose or cellobiose medium. One milliliter of culture was centrifuged, and the cell pellet was washed once in phosphate-buffered saline PBS and resuspended in 500 μl PBS. The OD600 of each culture was confirmed, and the cellular fluorescence was measured in black 96-well Costar plates using a Safas Xenius XMA (excitation, 448/20 nm; emission, 495/20 nm; photomultiplier, 850 V). Background fluorescence of PBS was subtracted from measurements, and the fluorescence measurements were normalized to culture density (OD600).

Data availability. Plasmids from this study have been submitted to the Addgene Plasmid Repository and are available using the accession numbers listed in Table 1.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00710-19.

FIG S1, EPS file, 0.2 MB.
FIG S2, EPS file, 0.1 MB.
TABLE S1, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS
We thank Ralph Bertram and Christopher F. Schuster from the University of Tuebingen, Germany, for providing pRAB1, Corinne Cruaud for DNA sequencing, and Peter Enyeart for helpful discussions.
36. Leang C, Ueki T, Nevin KP, Lovley DR. 2013. A genetic system for Clostridium ljungdahlii: a chassis for autotrophic production of biocommodities and a model homoacetogen. Appl Environ Microbiol 79:1102–1109. https://doi.org/10.1128/AEM.02891-12.
37. Wang Y, Zhang Z-T, Seo S-O, Choi K, Lu T, Jin Y-S, Blaschek HP. 2015. Markerless chromosomal gene deletion in Clostridium beijerincki using CRISPR/Cas9 system. J Biotechnol 200:1–5. https://doi.org/10.1016/j.jbiotec.2015.02.005.
38. Wasels F, Jean-Marie J, Collas F, López-Contreras AM, Lopes Ferreira N. 2017. A two-plasmid inducible CRISPR/Cas9 genome editing tool for Clostridium acetobutylicum. J Microbiol Methods 140:5–11. https://doi.org/10.1016/j.mimet.2017.06.010.
39. Cañadas IC, Groothuis D, Zygouropoulou M, Rodrigues R, Minton NP. 2019. RiboCas: a universal CRISPR-based editing tool for Clostridium. ACS Synth Biol 8:1379–1390. https://doi.org/10.1021/acsynbio.9b00075.
40. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 31:233–239. https://doi.org/10.1038/nbt.2508.
41. McAllister KN, Sorg JA. 2019. CRISPR genome editing systems in the genus Clostridium: a timely advancement. J Bacteriol 24:e00219-19. https://doi.org/10.1128/JB.00219-19.
42. Kung SH, Retchless AC, Kwan JY, Almeida R. 2013. Effects of DNA size on transformation and recombination efficiencies in Xylella fastidiosa. Appl Environ Microbiol 79:1712–1717. https://doi.org/10.1128/AEM.03525-12.
43. Heap JT, Ehsaan M, Cooksley CM, Ng Y-K, Cartman ST, Winzer K, Minton NP. 2012. Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. Nucleic Acids Res 40:e59. https://doi.org/10.1093/nar/gkr1321.
44. Cavedon K, Leschne SB, Canale-Parola E. 1990. Cellulase system of a free-living, mesophilic clostridium (strain C7). J Bacteriol 172:4222–4230. https://doi.org/10.1128/jb.172.8.4222-4230.1990.
45. Cerisy T, Soutere T, Torres-Romero I, Boutard M, Dubois I, Patrouix J, Labadie K, Berrabah W, Salanoubat M, Doring V, Tolonen A. 2017. Evolution of a biomass-fermenting bacterium to resist lignin pheno- lics. Appl Environ Microbiol 83:e00289-17. https://doi.org/10.1128/AEM.00289-17.