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Delivery of functional Cas:DNA nucleoprotein complexes into recipient bacteria through a type IV secretion system

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CRISPR-associated (Cas) endonucleases and their derivatives are widespread tools for the targeted genetic modification of both prokaryotic and eukaryotic genomes. A critical step of all CRISPR-Cas technologies is the delivery of the Cas endonuclease to the target cell. Here, we investigate the possibility of using bacterial conjugation to translocate Cas proteins into recipient bacteria. Conjugative relaxases are translocated through a type IV secretion system into the recipient cell, covalently attached to the transferred DNA strand. We fused relaxase R388-TrwC with the endonuclease Cas12a and confirmed that it can be transported through a T4SS. The fusion protein maintained its activity upon translocation by conjugation into the recipient cell, as evidenced by the induction of the SOS signal resulting from DNA breaks produced by the endonuclease in the recipient cell, and the detection of mutations at the target position. We further show how a template DNA provided on the transferred DNA can be used to introduce specific mutations. The guide RNA can also be encoded by the transferred DNA, enabling its production in the recipient cells where it can form a complex with the Cas nuclease transferred as a protein. This self-contained setup enables to target wild-type bacterial cells. Finally, we extended this strategy to the delivery of relaxases fused to base editors. Using TrwC and MobA relaxases as drivers, we achieved precise editing of transconjugants. Thus, conjugation provides a delivery system for Cas-derived editing tools, bypassing the need to deliver and express a *cas* gene in the target cells.

CRISPR-Cas | bacterial conjugation | Type IV secretion | base editor | protein translocation

Significance

We have developed a different approach for introducing CRISPR-Cas genetic tools into bacteria. During bacterial conjugation, the relaxase is transferred through the type IV secretion system covalently attached to the DNA. By fusing the Cas protein with the relaxase, we have observed functional Cas activity in the recipient cells, eliminating the need for nuclease expression in these cells. The transferred DNA molecule can supply the guide RNA and donor DNA, enabling seamless genetic modifications through recombination. We have also translocated fusions of relaxases to base editors which are active in recipient cells; these are the largest protein substrates translocated to date. This method could be applied to any recipient cell, in particular wild-type bacterial strains that lack available genetic tools.

The field of genetic engineering has been significantly transformed by CRISPR-Cas technologies, which have provided powerful and precise tools for genome manipulation. Biotechnological applications mostly rely on class 2 systems, where a single Cas protein is sufficient to perform specific cleavage of target nucleic acids (1). Additionally, these endonucleases can be used to deliver fused protein domains to specific genomic locations, leading to site-specific actions such as activation/repression of transcription or histone modification (2–4). A powerful class of engineered Cas proteins are base editors (BE), which consist in the fusion of engineered Cas proteins like nickase Cas or noncatalytic Cas (dead Cas), with a deaminase enzyme, either cytidine or adenine deaminases. This results in cytidine (CBE) or adenine BEs (ABE), respectively. These innovative systems facilitate the targeted alteration of specific nucleotides, enabling the conversion of cytidine to thymidine via CBE or adenine to guanine via ABE at precise genomic positions (5).

The development of CRISPR-Cas tools and therapies relies on the identification of delivery strategies that are both efficient and safe. The most widely used strategy to deliver these tools to bacteria involves the introduction of DNA to express the Cas protein and guide RNA (gRNA) in target cells. In order to achieve DNA delivery, techniques based on bacterial conjugation and phage transduction have been developed and successfully applied to bacterial communities (6–9). However, delivery and expression of the foreign DNA can be challenging in nonmodel bacteria, which often have defensive restriction-modification systems. Moreover, replicative plasmids and selectable markers are frequently not available, while commonly used inducible expression systems and regulatory sequences might not be functional or require optimization (10, 11). The alternative approach involves introducing either the *cas* messenger RNA (mRNA) and the gRNA or a ribonucleoprotein complex, although few studies have been conducted in bacteria using these nanocomplexes and further research is necessary for their broad application (12, 13). Delivering the Cas protein to bacteria directly is attractive in that it would bypass the need to transcribe and translate a large foreign gene in the recipient cells. With this goal in mind, we have tested the possibility of sending Cas proteins fused to conjugative relaxases, proteins that are naturally translocated between bacterial cells during conjugation.

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Bacterial conjugation is an efficient method of introducing DNA into bacteria, including strains and species that can be difficult to transform (14, 15). Donor and recipient bacteria come into physical contact through a type IV secretion system (T4SS), a multiprotein complex that spans the inner and outer membrane of bacterial cells (16). A conjugative relaxase recognizes and cleaves its target (the *oriT* sequence) in the DNA strand to be transferred, making a covalent bond with its 5′ end. This nucleoprotein complex is recruited by the T4SS and translocated to the recipient cell, where the relaxase catalyzes the recircularization of the transferred DNA strand (17). Some broad-host-range conjugative systems, such as that of plasmid RP4, have been used to deliver CRISPR-Cas systems in a large number of genetically amenable bacteria (6, 18–20). A previous work used the VirB/D4 T4SS of *Agrobacterium tumefaciens* to translocate the Cas9 protein fused to the translocation peptide signal VirF into eukaryotic cells (21). In this work, we generate fusion proteins between conjugative relaxases and class 2 Cas12a endonuclease to translocate the protein and introduce mutations at target positions in *Escherichia coli*. Moreover, we also generate fusions between relaxases and BEs, resulting in the precise editing of the target locus in transconjugants. Additionally, we show that the covalently attached transferred DNA strand can be used to encode a gRNA and a template DNA to introduce specific mutations in the target gene of a recipient bacterium. Thus, we provide the proof of concept for a strategy to deliver Cas-derived genetic tools to bacteria that bypasses the need to express the *cas* gene in the target cell. It is also worth noting that the translocated fusion proteins are, to our knowledge, the largest heterologous substrates reported to date for any bacterial secretion system, opening the way to the use of this strategy to deliver large proteins into any potential conjugation recipient cell.

Results

Engineering of a TrwC-Cas12a Fusion Protein. We engineered a gene that encodes the AsCas12a protein with a C-terminal 3×HAtag fused to the C terminus of the relaxase TrwC of the conjugative plasmid R388, resulting in the fusion protein TrwC-Cas12a. This gene was cloned under the regulation of a P*tet* promoter into plasmid pTrwC-Cas12a (Table 1). The stability of the fusion protein was analyzed by western blot. The results (*SI Appendix*, Fig. S1*A*) showed a band with the expected size for the fusion protein (263 kDa), as well as additional bands that likely correspond to degradation products or partially translated proteins. This confirms that while not fully stable, the full-length protein is produced, enabling the transfer of TrwC-Cas12a through the T4SS.

The functionality of TrwC in the fusion protein was tested by complementation assays of a R388 *trwC* deficient mutant (R388*trwC*-). The results (*SI Appendix*, Table S1) showed that the conjugation frequencies were similar to those obtained with TrwC. We then tested whether TrwC-Cas12a maintains RNA-guided DNA cleavage activity by a lethality assay since the introduction of double-strand breaks (DSB) by Cas proteins in the chromosome of *E. coli* leads to cell death (22). We coelectroporated into *E. coli* D1210 plasmids pTrwC-Cas12a and either pgRNA-lacZ or pgRNA-sacB, which encode a gRNA under the control of the isopropyl-ß-D-thiogalactopyranoside (IPTG)-inducible promoter P*lac*, targeting a chromosomal gene (*lacZ*) or a gene not present in the chromosome (*sacB*), respectively. The resulting transformants were selected on plates with or without induction. No transformants were obtained in the presence of the target and upon induction of the fusion protein (*SI Appendix*, Fig. S1 *B* and *C*), confirming that TrwC-Cas12a can cleave target genomic sequences when an appropriate gRNA is present in the cell and that its expression is tightly regulated under the control of the P*tet* promoter.

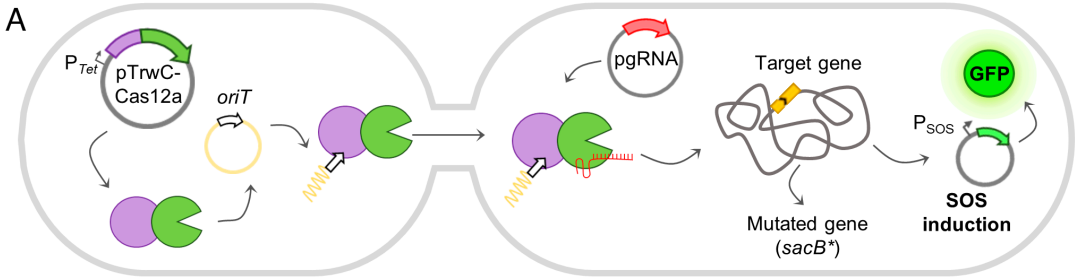
Table 1. Main plasmids used in this work*

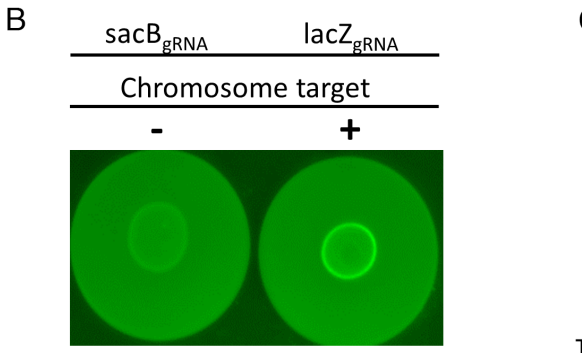
Name in the manuscript	Laboratory name	Main characteristic
pBE	pLG32	P*tet dcpf1-be*
pBE-HA	pAF34	pLG32*:: 3xHA tag*
pBE-linker-TrwC	pLG44	P*tet dCpf1-BE-linker-trwC*;
pCas12a	pLG14	P*tac cas12a*
pErmB	pAF37	P*bad ermB (with start codon)*
pErmB*	pAF20	P*bad ermB with ACG (no start codon)*
pgRNA-ErmB$_{BE}$	pAF23	P*lac ermB$_{gRNA-BE}$*
pgRNA-ErmB$_{BE}$-oriT	pAF26	P*lac ermB$_{gRNA-BE}$::oriT*
pgRNA-lacZ	pLG15	P*lac lacZ$_{gRNA}$*
pgRNA-lacZ$_{BE}$	pLG38	P*lac lacZ$_{gRNA-BE}$*
pgRNA-sacB	pLG19	P*lac sacB$_{gRNA}$*
pgRNA-sacB-oriT	pLG29	*oriT* P*lac sacB$_{gRNA}$*
pHR_oriT	pLG27	*oriTR388+oriVR6K* P*tac::sacB** homologous recombination cassette
pMobA	pAF21	P*tet mobA*
pMobABC	pLG48	P*tet mobABC*
pMobABC-BE	pLG49	P*tet mobAB-be+mobC*
pMobA-BE	pAF18	P*tet mobA-dcpf1-be*
pMobA-BE-HA	pAF33	pAF18*:: 3xHA tag*
pMobA-HA	pAF32	pAF21::*3xHA tag*
poriT	pSU1186	*oriT*
pSOS	pZA31-sulA-GFP	*Psos gfp*
pTrwC	pLG22	P*tac trwC*
pTrwC-BE	pLG33	P*tet trwC-dcpf1-be*
pTrwC-Cas12a	pLG24	P*tet trwC-cas12a*
pTrwC-linker-BE	pAF24	P*tet trwC-linker-dCpf1-BE*;
pUC8	pUC8	Multicopy ApR cloning vector
R388trwC-	pSU1445	R388::*Tn5tac1* in *trwC*
RSF1010_mobA-	pMTX808	pAA58::*ApR* in *mobA*

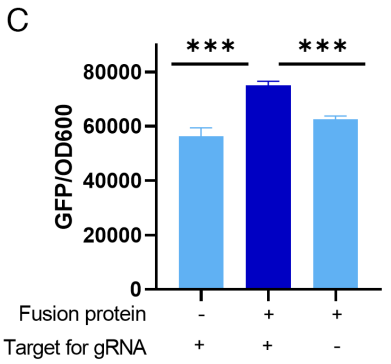
*Detailed description of the plasmids in *SI Appendix* Table S3.

Validating the Endonuclease Activity of Translocated TrwC-Cas12a. Observing TrwC-Cas12a activity after translocation into the recipient cell presents a greater challenge, as only a single or few TrwC-Cas12a proteins are expected to be transferred via conjugation, as opposed to the continuous protein production upon electroporation. To test whether TrwC-Cas12a could be translocated through the T4SS of R388 during conjugation and recover its activity in the recipient, we used two different strategies, as depicted in Fig. 1. Initially, we measured the induction of the SOS response in transconjugants, which is expected to be triggered after the introduction of DSB by Cas12a (22). Subsequently, we isolated mutants that inactivate the target gene and characterized them. Cas12a cleavage should result in a specific mutational signature at the target position.

We conducted conjugation assays using as donor *E. coli* D1210 harboring R388*trwC*- complemented with pTrwC-Cas12a, and as recipient MG1655 harboring the pgRNA and a reporter plasmid with a *gfp* gene under the control of a SOS-responsive promoter (pSOS). In order to discard an increase of the SOS signal triggered by the conjugation process itself (23), we also performed matings using D1210 harboring R388*trwC*-, which cannot conjugate. After the matings, the level of Green Fluorescent Protein (GFP) was visualized in recipients that express either pgRNA-sacB (without chromosomal target) or pgRNA-lacZ (with chromosomal target) (Fig. 1*B*). We detected a significant increase in fluorescence only when TrwC-Cas12a was translocated into recipients expressing the







D

PAM TARGET
TTTGGGACAGCTGGCCATTACAAAACG

WT	…TTTGGGACAGCTGGCCATTACAAAACGCTGACGGCACTG…		TYPE OF MUTATION
TC1	…TTTGGGACAGCTGGCCAT---AAAACGCTGACGGCACTG…	-3bp	ΔTAC
TC2	…TTTGGGACAGCTGGCCAT--CAAAACGCTGACGGCACTG…	-2/3bp	ΔTA/TAC
TC3	…TTTGGGACAGCTGGCCAT-ACAAAACGCTGACGGCACTG…	-1/2bp	ΔT/TA
TC4	…TTTGGGGCAGCTGGCCATTACAAAACGCTGACGGCACTG…	----------	
TC5	…TTTGGGACAGCTGGCCAT--CAAAACGCTGACGGGACTG…	-2/3bp	ΔTA/TAC
TC6	…TTTGGGACAGCTGGCCATT----AACGCTGACGGCACTG…	-4bp	ΔACAA
TF1	…TTTGGGACAGCTGGCCAT---AAAACGCTGACGGCACTG…	-3bp	ΔTAC
TF2	…TTTGGGACAGCTGGCCATTACAAAACGCTGACGGCACTG…	-3bp	ΔTAC/WT
TF3	…TTTGGGACAGCTGGCCATT----AACGCTGACGGCACTG…	-4bp	ΔACAA

250 bp 289 bp

E

WT	Met-[82aa]-VWDSWPLQNADGTVANYHGYHIVFALAGDP-[360aa]-*
ΔTAC	Met-[82aa]-VWDSWP*
ΔTA	Met-[82aa]-VWDSWPSKR*
ΔT	Met-[82aa]-VWDSWPYKTLTALSQTITATTSSLH*
ΔACAA	Met-[82aa]-VWDSWPLTLTALSQTITATTSSLH*

Fig. 1. Endonuclease activity of TrwC-Cas12a in the recipient cell. (*A*) Schematic representation of Cas12a activity assays in the recipient cell. In the donor cell, pTrwC-Cas12a (in gray) will express TrwC-Cas12a. Thanks to its relaxase activity, the fusion protein will cleave and bind covalently to the *oriT* (white arrow), and the complex will be recruited and translocated through the T4SS into the recipient cell. In the recipient, pgRNA-lacZ or pgRNA-sacB (in red) will produce a gRNA targeting a gene on the chromosome. Thanks to its endonuclease activity, the incoming TrwC-Cas12a will process the gRNA generating a complex, which will be guided to the target gene, where it will produce a DSB. (*B*) Induction of SOS response by TrwC-Cas12a in the recipient cell. Visualization of conjugation filters under a fluorescent lector system after 3 h of mating under induction conditions. Filter on the *Right* (gRNA with a target on the chromosome) shows increased fluorescence level in comparison with the filter on the *Left* (gRNA without target on the chromosome). (*C*) GFP fluorescence relative to OD_{600} levels, measured with a TECAN Infinite M200 Pro. Data correspond to the mean of three independent assays (***$P < 0.0005$). (*D*) Analysis of the *sacB* mutations in sucrose-resistant colonies. Alignment of the *sacB* region close to the protospacer adjacent motif (PAM) and target sites for Cas12a-gRNA. The *sacB* region was PCR-amplified from sucrose-resistant transconjugants (TC1 to TC6) and from sucrose-resistant transformants (TF1 to TF3). The *sacB* sequence in strain MG1655::*sacB* was also determined and is shown at the *Top* for comparison (WT). The PAM sequence and the spacer sequence are shown at the *Top*. The red triangle marks Cas12a cleavage site in the shown DNA strand. Nucleotides in red mark the site where the DNA sequence splits into two overlapping traces (see text). (*E*) Amino acid sequences of SacB variants resulting from the different mutations. The deletions are indicated at the *Left*, in blue. The amino acids shown in green and underlined are encoded by the PAM sequence. Stop codons are shown as *.

gRNA against *lacZ* (Fig. 1*C*). These data indicate that Cas12a induces the SOS response and that TrwC-Cas12a is active as a site-specific endonuclease in the recipient cell after translocation through the T4SS.

To obtain direct evidence for Cas12a mutagenic activity in the recipient cell, we aimed to select mutations introduced as a result of DNA repair following Cas12a cleavage at the target position. Sequence rearrangements have been reported following Cas9 cleavage in the chromosome of *E. coli* (22). To investigate whether such rearrangements could occur after cleavage by TrwC-Cas12a, we selected transconjugants resistant to sucrose in a *sacB*-containing strain. The expression of this gene in the presence of sucrose is lethal in bacteria (24), so the transconjugants will only survive if there is a mutation inactivating the gene. We conducted several matings translocating the fusion protein or the relaxase into target or nontarget recipients (MG1655::*sacB* and MG1655, respectively) and no differences in conjugation frequencies were observed among the different conditions (*SI Appendix*, Table S1). However, when we selected for sucrose-resistant transconjugants, several colonies appeared when TrwC-Cas12a was translocated into the recipient containing *sacB.* After confirming by PCR amplification that the *cas12a* gene was not present in the transconjugants, 11 sucrose-resistant transconjugants were analyzed by PCR amplification of the 5′ end of the *sacB* gene. In five cases, we did not observe any visible PCR product, possibly due to large deletions encompassing the region amplified with the primers. The sequence of the PCR products of the six remaining transconjugants was determined (Fig. 1*D*). All transconjugants but one showed 1 to 4-nt deletions in the target DNA sequence. Interestingly, the sequences of TC2, TC3, and TC5 showed a mixture of two DNA sequences after the Cas12a cleavage site, which corresponded to two different deletions (shown in red in Fig. 1*D*). These mixed colonies likely indicate the generation of different mutations on different copies of the chromosome in the recipient cell. All the deletions produced an early stop codon on the *sacB* sequence (Fig. 1*E*). The three unique sucrose-resistant colonies obtained in the TrwC control did not show any mutation in the sequenced *sacB* region. To compare the *sacB* mutations observed in the transconjugants with the ones produced by the cleavage of Cas12a alone, we coelectroporated pCas12a and pgRNA-sacB into MG1655::*sacB,* and plated the transformants in sucrose-containing medium. Three sucrose surviving transformants were selected. We amplified the *sacB* region and determined the DNA sequence (Fig. 1*D*, TF1 to TF3). We detected 3-nt and 4-nt deletions at the target sequence, which coincided with the ones observed in several transconjugants. Thus, we confirm that neither conjugative DNA transfer nor TrwC activity is involved in the type of mutations obtained, which are solely derived from Cas12a endonuclease activity. In summary, these results provide direct proof of the Cas12a activity of TrwC-Cas12a in the recipient cell after translocation through the T4SS.

Delivering gRNA or a DNA Template in a Mobilizable Plasmid. Since the relaxase is translocated covalently attached to a DNA strand during conjugation, we used this DNA as a source for the other elements of the genetic modification machinery: the gRNA or a template DNA to promote homologous recombination (HR)-mediated gene editing. This method allows us to target unmodified recipient cells since we send all the components from the donor bacteria (Fig. 2*A*).

We constructed a mobilizable plasmid encoding the gRNA against the *sacB* gene (pgRNA-sacB-oriT). This plasmid is translocated covalently attached to TrwC-Cas12a into the recipient cell, where the gRNA will be expressed. We generated donor bacteria carrying three plasmids: R388*trwC*-, pTrwC-Cas12a or pTrwC (negative control for Cas12a activity), and pgRNA-sacB-oriT or poriT (a mobilizable plasmid that does not encode a gRNA for Cas12a). As recipient strains, we used MG1655::*sacB* or MG1655, with and without a target gene, respectively. The results (Fig. 2*B*) showed a significant decrease in the number of transconjugants only when the gRNA was translocated by TrwC-Cas12a into a recipient cell containing the target gene (*sacB*). This reduction likely results from the death of transconjugants mediated by Cas12a cleavage of the target gene in the chromosome. It is also worth mentioning that we tested the translocation of the protein–gRNA complex directly from the donor, by placing the gRNA-encoding plasmid in the donor but with no *oriT*. However, we did not see any effect. This is likely because the bond between the fusion protein and the gRNA is not strong enough to withstand the translocation and unfolding through the T4SS.

The CRISPR-Cas gene editing system can edit a cell without leaving a scar if provided with a homologous template carrying the desired mutation. To test whether the DNA template could be provided in the donor DNA, we constructed plasmid pHR_oriT. This is a mobilizable suicide plasmid that contains a *sacB* HR cassette under the control of a P*tac* promoter and with the PAM sequence mutated to generate an early stop codon in the gene (*SI Appendix*, Fig. S2*A*). Mobilization of the plasmid using TrwC or TrwC-Cas12a was equally efficient (*SI Appendix*, Table S1). If TrwC-Cas12a promotes recombination, we should detect an increase in the editing efficiency in comparison with the spontaneous insertion of the template by HR. We performed matings using DH5αpir as donors, mobilizing pHR_oriT with TrwC or TrwC-Cas12a. As recipient cells, we used MG1655*::sacB* harboring either pgRNA-sacB or pUC8. Table 2 summarizes the results of three independent assays. We did not observe differences in the frequency of sucrose-resistant recipients when TrwC-Cas12a was delivered to the cell compared to TrwC alone (Table 2, Sucrose^R frequency column). However, when we analyzed the colonies for edition, we found a substantial increase in the fraction of sucrose-resistant colonies that had incorporated the desired mutation. Cm-sensitive colonies were selected to discard integrants (as schematized in *SI Appendix*, Fig. S2*B*), and the target region was amplified to determine whether the colonies had incorporated the PAM mutation from the recombination cassette. Fig. 2*C* shows the editing ratio, calculated as the number of colonies which had incorporated the desired mutation, divided by the total number of sucrose-resistant colonies analyzed for that condition. Over 40% of the colonies had incorporated the desired mutations when both TrwC-Cas12a and pgRNA-sacB were present, while less than 10% were edited in the absence of either factor, due to HR with the donor DNA in the absence of DNA cleavage by Cas12a. Overall, these results demonstrate that TrwC-Cas12a promotes recombination of the HR template that was codelivered with it in the recipient cell in the presence of the specific gRNA.

Expanding the Tool: Translocation of BEs Fused to Relaxases. In order to extend the relaxase-Cas approach to other conjugative relaxases and other Cas-related genetic editing tools, our subsequent goal was to employ our delivery system to deliver a CBE. For this purpose, we used the dCpf1-BE system (hereafter referred to as BE), which comprises the cytidine deaminase APOBEC1, dLbCas12a, and a Uracil Glycosylase Inhibitor (25). We fused this BE to either TrwC or to the MobA relaxase from plasmid RSF1010 (plasmids pTrwC-BE and pMobA-BE). The latter was chosen due to its smaller size, promiscuity, and ability to

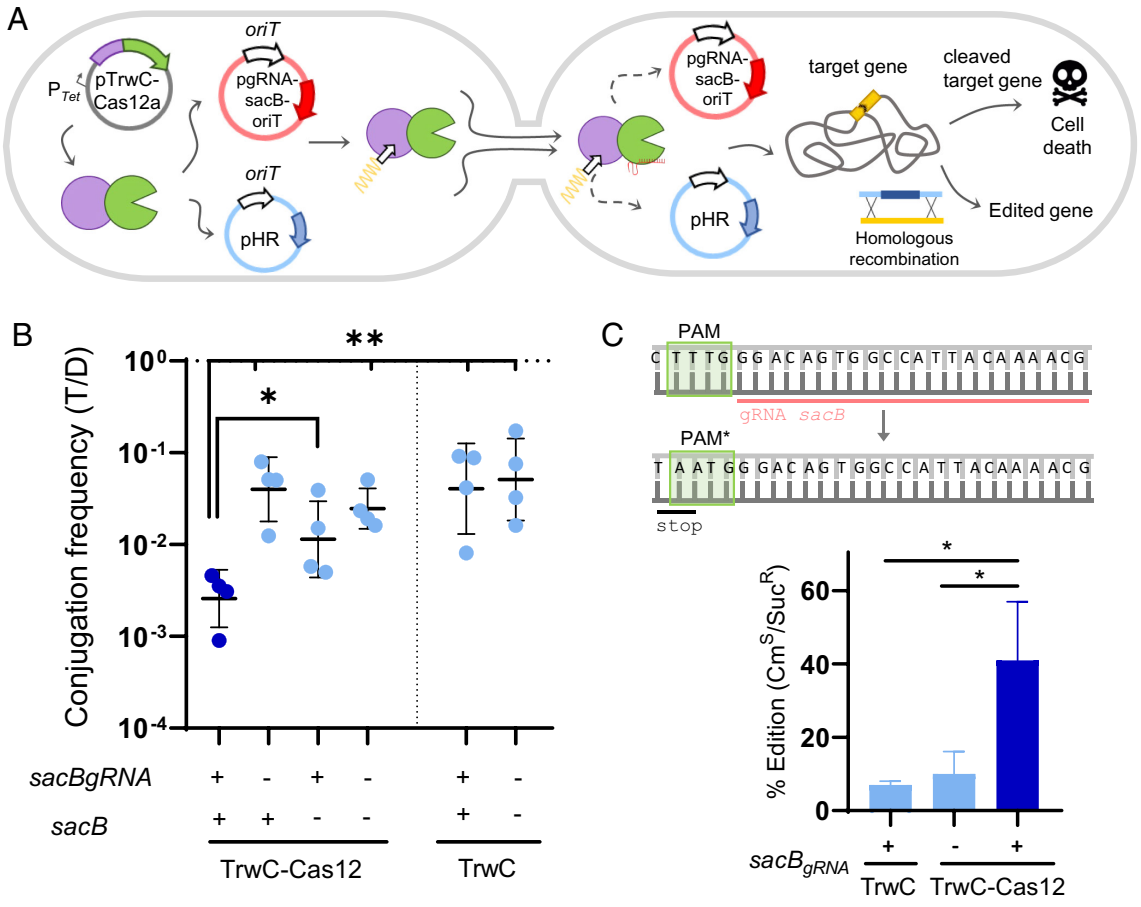


Fig. 2. Translocation of donor DNA encoding the gRNA or a DNA template. (*A*) Schematic representation of the assays. In the donor cell, pTrwC-Cas12a (in gray) will express TrwC-Cas12a. Thanks to its relaxase activity, the fusion protein will cleave and bind covalently to the pgRNA-sacB-oriT plasmid (in red) or to the pHR_oriT (in blue), and the complex will be recruited. The complex formed by the fusion protein and the mobilizable ssDNA of interest (represented as squiggly black line) will be translocated through the T4SS into the recipient cell. In the recipient cell, if the attached DNA is the pgRNA-sacB-oriT, the incoming complex will be guided to the target gene, where it will produce a DSB, which is lethal to the bacteria. On the other hand, if the translocated plasmid is the pHR_oriT, the DSB produced in the chromosome of the recipient bacteria will be repaired through HR, utilizing the HR cassette provided by pHR_oriT, generating specific editions in this gene. (*B*) Lethality in transconjugants upon mobilization of a plasmid encoding the gRNA. T/D, transconjugants/donor. Data correspond to four independent assays (**$P < 0.005$; *$P < 0.05$). (*C*) Introduction of seamless mutations using a HR cassette. (*Top*) The genome sequence of MG1655::*sacB* and the design of the *sacB* HR cassette, where the PAM sequence was mutated to generate an early STOP codon (PAM*). (*Bottom*) Percentage of edition with TrwC or TrwC-Cas12a. The editing rate is calculated as the fraction of chloramphenicol sensitive colonies which had incorporated the *sacB* mutations from the HR cassette per sucrose-resistant colony analyzed. The data represent the results of three independent experiments (*$P < 0.05$).

be fused to translocation signals while retaining its functionality (26–28). Following the same approach used for the TrwC-Cas12a fusion, we inserted the BE gene at the 3′ end of the relaxase genes, eliminating the stop codon, downstream of the P*tet* promoter. As controls, we also cloned the BE and MobA separately under the regulation of the same promoter (pBE and pMobA), adding a C-terminal 3×HA tag (pMobA-HA, pMobA-BE-HA, and pBE-HA). We then verified the expression and stability of the relaxase-BE fusions (*SI Appendix*, Fig. S3*A*). We could observe the fusion proteins in all cases, albeit in low amounts. In the case of TrwC fusions, the TrwC moiety was clearly visible, suggesting instability of the fusion proteins (*SI Appendix*, Fig. S3*A*). In an attempt to increase the stability, we inserted between the relaxase and the BE a linker previously used for TrwC-Cre fusions (29), and we also changed the order of both moieties (pBE-linker-TrwC, pTrwC-linker-BE). The resulting fusion proteins showed no improvement in stability (*SI Appendix*, Fig. S3*B*).

To test the relaxase activity in the fusion proteins, mating assays were conducted. We tested TrwC and TrwC-BE complementation of R388*trwC*- plasmid, and MobA and MobA-BE complementation of RSF1010*mobA*- in a strain providing the RP4 T4SS from the chromosome. Results demonstrated that both relaxase-CBE fusions exhibited the ability to mobilize a plasmid through complementation at a frequency comparable to that achieved with TrwC or MobA alone (*SI Appendix*, Table S1).

Subsequently, we proceeded to test the functionality of dCpf1-BE. To achieve this, we engineered the pErmB* plasmid, incorporating a target that would allow us to identify a "gain-of-function"

Table 2. Homologous recombination assays

Relaxase*	Recipient†	Sucrose^R frequency‡	Cm^S/Suc^R§	%Edition¶
TrwC	$sacB_{gRNA}$	$3.8 \times 10^{-5} \pm 3.5 \times 10^{-5}$	23/42	7.1
TrwC-Cas12a	$sacB_{gRNA}$	$1.7 \times 10^{-5} \pm 1.3 \times 10^{-5}$	34/42	40.5
	no gRNA	$9.7 \times 10^{-6} \pm 4.2 \times 10^{-6}$	35/42	9.5

The data represent the results of four to six experiments.
*Donor cells were DH5αpir harboring pHR_oriT and R388*trwC*- complemented with pTrwC or pTrwC-Cas12a.
†MG1655::*sacB* was used as recipient cell, harboring pUC8 (no gRNA) or pgRNA-sacB ($sacB_{gRNA}$).
‡Expressed as sucrose-resistant recipients per recipient.
§Cm-sensitive per sucrose-resistant colonies.
¶Percentage of Cm^S edited colonies per sucrose-resistant colonies.

mutation. The ATG start codon of the erythromycin (Em) resistance gene (*ermB*), under the regulation of an arabinose-inducible promoter, had been switched to ACG. Plasmid pgRNA-ermB$_{BE}$ encoded a gRNA encompassing this mutation, so that edition would restore the ATG codon by changing C to T. Therefore, edited cells would be Em-resistant (EmR) (Fig. 3 *A*, *Top*). We first assessed the BE activity of the constructs in *E. coli* by electroporation of the components of the system. Plasmids encoding either BE, TrwC-BE or MobA-BE, and MobA or TrwC as negative controls, were electroporated into DH5α cells containing plasmids pgRNA-ErmB$_{BE}$ and the target pErmB*. These cells were then selected with Em and appropriate inducers. EmR colonies appeared on plates containing the BE, either on its own or as a fusion protein with a relaxase. The *ermB* gene was PCR-amplified, and DNA sequencing showed that 94 to 100% had edited pErmB as expected, while no edition was ever observed in the occasional EmR colonies that emerge in the negative controls with no BE. The editing frequency, calculated as the percentage of EmR transformants, was 100% for the BE and TrwC-BE fusion and 88% for MobA-BE (Fig. 3*B*).

We also tested the system by editing the genomic *lacZ* gene. In this case, plasmid pgRNA-lacZ$_{BE}$ encoded a gRNA with a C within the editing window which, upon changing to T, would result in a premature stop codon (Fig. 3 *A*, *Bottom*). This plasmid was cotransformed with BE, relaxase-BE, or relaxase plasmids into the *lacZ*-bearing strain D1210 and selected on plates containing X-Gal, so that successfully edited colonies would be white. We were able to detect lighter-shaded colonies in cases where the BE or relaxase-BE were present, and sequencing of the *lacZ* region showed that on average 97 to 100% of those colonies were edited. Both BE and relaxase-BE fusions exhibited a preference for editing a C within the editing window which did not give rise to a stop codon. Nevertheless, the resultant colonies displayed a lighter phenotype, possibly attributable to a CRISPR interference phenomenon (30). Interestingly, when we extended the incubation of the plates for 3 d, a majority of the colonies eventually developed a lighter halo, indicating that edition was still ongoing after plating. For both *ermB* and *lacZ* edition, edited colonies showed a mixture of edited and unedited sequences. However, the edited one predominated after successive passages. Such mixed sequences have previously been reported in BE-induced mutations (7, 31–33). Altogether, these findings demonstrate that the BE and its fusions to TrwC and MobA relaxases are active in *E. coli*, targeting both genomic and plasmidic genes.

Next, we aimed to assess the editing activity of the fusion proteins upon translocation into the recipient bacteria containing a gRNA targeting a chromosomal *lacZ* or plasmidic *ermB* gene, as depicted in Fig. 4*A*. For the matings targeting *lacZ*, donor bacteria harbored the R388*trwC*- plasmid, complemented by nonmobilizable plasmids pTrwC or pTrwC-BE. Selection of the transconjugants was done in X-Gal containing media (Fig. 4*B*). We sequenced the target position in 10 lighter-shaded transconjugants and detected modifications in 52% of them. Next, in order to test the MobA-BE fusion against the plasmidic target *ermB*, donor cells had the RP4 system integrated into their chromosome plus mobilizable plasmid RSF1010*mobA*-, complemented with MobA or MobA-BE. EmR transconjugants appeared in matings with MobA-BE at a frequency of 8×10^{-4}, but none were detected in matings with MobA. DNA sequencing of these transconjugants revealed that, on average, 89% of them were edited as intended (Fig. 4*C*).

Similar to the results observed upon electroporation of BE, many of the clones analyzed contained a mixture of edited and wild-type (WT) sequences. We noticed again a preference for editing the C that does not generate a stop codon in *lacZ* (Fig. 4*D*). For *ermB*, we identified 3.6% of double editing events, simultaneously at the desired position 12 after the PAM, and at position 16, close to the canonical editing window (Fig. 4*D*).

Finally, we wanted to check whether the gRNA could be encoded in a mobilizable plasmid translocated by the relaxase-BE fusion. To achieve this, we generated plasmid pgRNA-ErmB$_{BE}$-oriT, which includes the *oriT* sequence from RSF1010 and can be mobilized by MobA. The donors therefore contained this plasmid and pMobABC-BE, or pMobABC as a negative control. As a recipient, we used DH5α with the target plasmid pErmB*. After conjugation, the rate of Em-resistance among transconjugants was 1.9×10^{-3} in the case where the relaxase-BE fusion was present, while no EmR clones were obtained in the negative control. We analyzed 7 to 10 EmR colonies per replicate. We confirmed by PCR amplification of the *oriT* that these transconjugants contained the pgRNA-ErmB$_{BE}$-oriT plasmid, and sequencing showed that 100% of them had been correctly edited restoring the ATG codon of *ermB*.

In conclusion, our findings demonstrate that relaxase-BE fusions can also be translocated via conjugation into a recipient

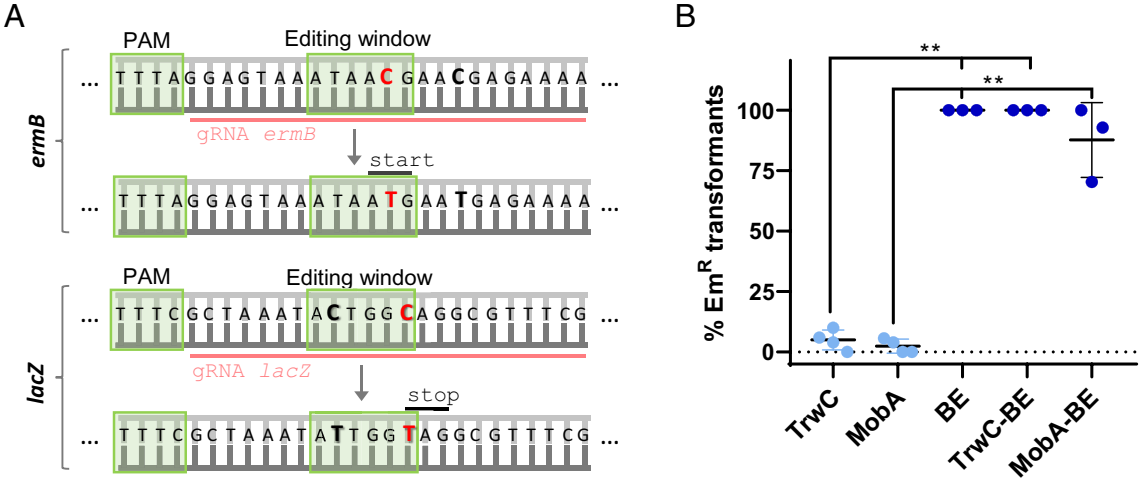


Fig. 3. Activity of relaxase-BE fusions in *E. coli*. (*A*) Sequence of mutated *ermB* gene present in plasmid pErmB* (*Top*) and D1210 *lacZ* gene (*Bottom*). The sequences complementary to the gRNAs against *ermB* and *lacZ* are indicated (red line). The PAM sequences and the editing windows are also highlighted (green boxes). The nucleotides where editing is expected to occur are marked in red, as well as the resulting stop or start codons. Other C nucleotides where mutations were detected are highlighted in bold and larger font. (*B*) Percentage of EmR transformants. Mean ± SD is represented. $^{**}P < 0.005$.

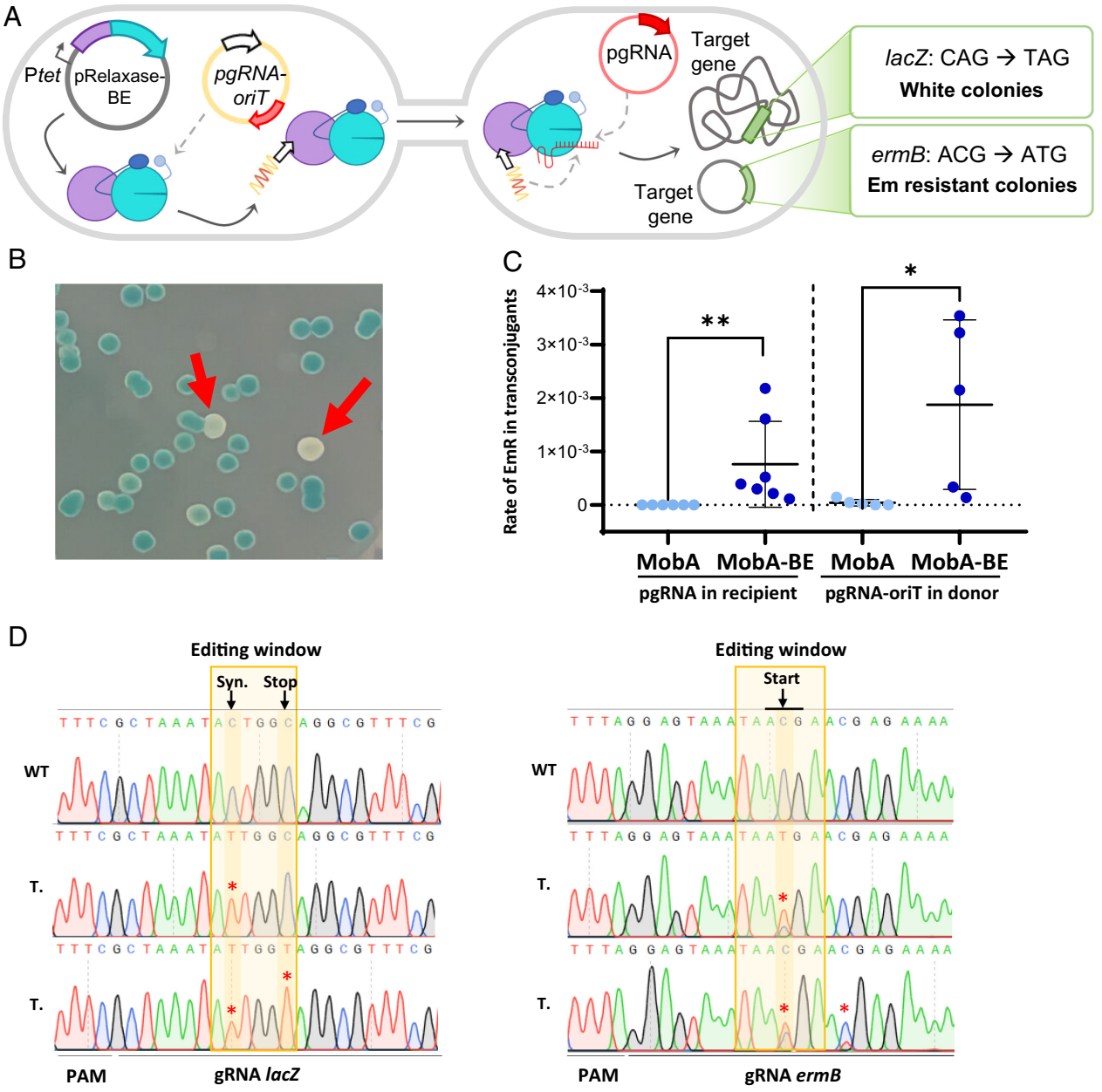


Fig. 4. Activity of relaxase-BE fusions delivered by conjugation. (*A*) Schematic representation of the conjugative delivery and activity of relaxase-BE fusions in the recipient cell. In the donor cell, pTrwC-BE or pMobA-BE express the relaxase-BE fusions, which are then translocated to the recipient cell. After delivery, the fusion protein binds to a gRNA produced either by a pgRNA plasmid present in the recipient or by a pgRNA-oriT plasmid translocated from the donor by the fusion protein. Then the complex targets a specific gene, where it performs base conversion. (*B*) Transconjugants obtained by the translocation of TrwC-BE and selected in the presence of X-Gal. Red arrows indicate two light-colored colonies that were found to be edited. (*C*) Rate of EmR transconjugants edited by MobA-BE when the gRNA is in the recipient or is delivered by conjugation from the donor bacteria. The mean ± SD is represented. **P* < 0.05; ***P* < 0.005. (*D*) Chromatograms obtained from the sequencing of transconjugants (T.) in which *lacZ* is targeted by TrwC-BE (*Left*) or *ermB* is targeted by MobA-BE (*Right*). The wild-type (WT) sequence is provided as a reference. Edited nucleotides are marked with asterisks. The effect of the mutation in the protein product is indicated at the *Top*: Syn. generates a synonymous codon, Stop introduces a stop codon, and Start restores the mutated start codon.

cell, where they can refold and regain their gRNA-guided editing activity. We have also verified that the gRNA can be encoded in the plasmid mobilized by the fusion protein.

Discussion

Targeted genetic modification of bacteria is fostering an increasing number of biotechnological and biomedical applications. The CRISPR-Cas technology has boosted the field, making targeted mutations a routine task for many model microorganisms (34). This technology has allowed metabolic engineering of different bacteria such as *E. coli* (35), *Clostridium spp.* (36), or *Cyanobacteria spp.* (37), improving their use as cell factories. The system has also been used for biomedical research to study different pathogens such as *Mycobacterium tuberculosis, Yersinia pestis,* or *Klebsiella pneumoniae* (34). CRISPR-Cas can also target specific bacterial or plasmid populations, enabling their use as antimicrobials (9, 38, 39). In spite of its success, the technology still faces significant limitations. A critical step to accomplish a genetic modification is the delivery of the endonuclease, gRNA, and template DNA to the target cell. Electroporation of plasmid DNA can be used to transform a wide range of bacteria, but protocols have to be fine-tuned for each new target strain, success

is not guaranteed, and efficiencies are often very low. Phages have also been successfully engineered for CRISPR-Cas or BE delivery into bacteria (7, 40), but the restricted host range of phage vectors and bacterial resistance to infection can pose a significant limitation. Bacterial conjugation, a natural means of delivering DNA complexes into a wide range of bacteria, has been used to introduce the CRISPR-Cas genetic system into different species (20, 41, 42), avoiding the need for electroporation. Still, ensuring expression of the endonuclease in the recipient cell can be challenging in poorly characterized microorganisms, while overexpression can lead to toxicity and off-target activity (43).

In this work, we take advantage of the fact that the conjugative machinery delivers a nucleoprotein complex (relaxase:DNA) to the recipient cell. The rationale was to fuse the Cas endonuclease to the conjugative relaxase, so that the Cas protein itself is delivered to any bacteria that can be reached by conjugation, bypassing the need for transcription and translation of a *cas* gene. In addition, the limited amount of Cas protein minimizes the off-target effects observed when Cas12a or Cas12a-BE are overproduced in the target cell (25, 44, 45). Furthermore, the relaxase-Cas protein can be translocated with a DNA strand coding for the gRNA or a HR cassette, still significantly shorter than a *cas*-encoding DNA (a ≈ 3 kb plasmid instead a ≈ 7 to 8 kb plasmid), helping to bypass the host restriction system. While the gRNA has to be expressed from the recipient, it is not toxic so any wide range promoter will likely be effective for this purpose. The closest approach carried out to date involves a Cas9-VirD2 fusion used to promote Cas9-mediated HR in target cells, but the relaxase-like moiety was not used as a delivery system (46).

To show proof of concept of this strategy, we have selected the Cas endonuclease Cas12a. Cas12a has emerged as an interesting alternative to Cas9. Its ability to process the primary transcript of the CRISPR array on its own simplifies the multiplex editing of several targets (47). Its smaller size could also facilitate its translocation through the T4SS channel. For the relaxase moiety, we chose TrwC, the well-characterized relaxase from the broad-host-range plasmid R388 (17), for several reasons. TrwC has been successfully fused to other polypeptides without losing its relaxase activity (48, 49). It can be translocated into the recipient cell either alone or linked to DNA, and its activity in the recipient cell has been directly demonstrated (50, 51). Furthermore, TrwC can be translocated, by its own or by heterologous T4SS (28, 48, 50), into a wide range of organisms, including most proteobacterial species (52), Gram-positive bacteria (53), cyanobacteria (54), and even mammalian cells (55). TrwC can also catalyze low-frequency integration of an incoming *oriT* into a resident chromosomal *oriT* copy (10^{-7} integrants/donor); while it is formally possible that cryptic *oriT* sequences exist in a recipient chromosome, the integration efficiency drops by two logs when minimal *oriT* sequences were tested (56), so off-target integration events on short, nic-like *oriT* sequences in the chromosome will be negligible.

For TrwC to function in the recipient cell, it must partially unfold during secretion, and in fact, the inclusion of an unfolding-resistant domain was shown to preclude protein translocation (29). The detection of Cas12a activity in the recipient cell after translocation of TrwC-Cas12a (Figs. 1 and 2) confirms that the complete fusion protein, despite its size of 263 kDa, can be translocated through the T4SS, and that the CRISPR endonuclease recovers its native structure after unfolding. We therefore conclude that there are no unfolding-resistant domains in Cas12a interfering with the translocation of the fusion protein.

We have demonstrated the application of TrwC-Cas12a, delivered via conjugation, for targeted mutagenesis (Fig. 1*D*) in the recipient cell. Sucrose-resistant transconjugants were only obtained when the appropriate gRNA was also expressed in the recipient. The *sacB* region revealed small deletions at the expected TrwC-Cas12a cleavage site, providing unequivocal evidence that the mutations were triggered by the RNA-guided endonuclease activity of TrwC-Cas12a. The mutation pattern resulting from Cas12a-induced DSB repair in bacteria is not well documented in the literature. The absence of amplification of *sacB* in some mutants suggests the loss of the *sacB* locus by large deletions, as previously described after Cas9 cleavage in the chromosome of *E. coli* (22) as well as after Cas12a cleavage (57). The detected mutations consist of small 1 to 4 nt deletions at the cleavage site and are also produced by the action of Cas12a alone (Fig. 1*D*, compare TC with TF1 to 3), ruling out a possible effect of the relaxase moiety. A similar pattern of Cas12a-induced mutations has been identified in other prokaryotes, such as *Amycolatopsis mediterranei* (58), but not in *E. coli*. The introduction of small deletions proves to be a useful strategy for gene knockout.

To develop a delivery system capable of modifying WT recipient bacteria, it is necessary to introduce not only the Cas endonuclease but also the gRNA. We used the conjugatively transferred DNA strand to encode the gRNA, so that both the nuclease and the gRNA would be provided from the donor cell. In this case, to our surprise, we observed an increase in the efficiency of Cas12a, as inferred by the observed lethality in the transconjugants (Fig. 2*B*). We hypothesize that the increase in Cas12a cleavage efficiency may be attributed to the physical proximity of the nuclease and the gRNA-encoding DNA in the translocated nucleoprotein complex.

We have also used conjugatively delivered TrwC-Cas12a::DNA complexes to introduce the template DNA carrying the desired mutation into the target bacteria, in order to accomplish seamless targeted mutations by HR. The HR template introduced a point mutation in the PAM sequence, which generated a premature STOP codon in *sacB*. Although the number of sucrose-resistant colonies obtained was similar under the different conditions tested, the ratio of edited cells was significantly higher when both TrwC-Cas12a and the gRNA were present (Fig. 2*C*). Our results demonstrate a proof of concept for the use of bacterial conjugation to deliver relaxase-Cas fusion proteins, along with the covalently bound DNA template, into a recipient cell. However, the editing efficiency is currently too low for practical application, with only about 1 in 50,000 transconjugants resulting in edited colonies. Thus, this approach would require the selection of edited cells, which is rarely possible outside model systems like the one used in this study.

CRISPR-Cas editing strategies in bacteria typically hinge on the principle that unless the desired mutation is incorporated, cleavage of the target sequence by the Cas nuclease will result in the death of the target bacteria. This allows for the introduction of mutations without the need for a selectable phenotype. The cleavage of a target sequence in the *E. coli* chromosome will only result in bacterial death if the cleavage is rapid enough to prevent homology-directed repair with an intact copy of the chromosome (22). While the delivery of the TrwC-Cas12a protein through the T4SS was not sufficient to kill target bacteria, likely because the T4SS most likely delivers a small number of Cas12a proteins to a single recipient, the TrwC-Cas12a:DNA complex encoding the gRNA did induce about 90% lethality in the transconjugants. This opens up the possibility of obtaining mutations without prior selection. A Cas protein must find two molecular partners in the recipient cell: the gRNA and then the target DNA. This lengthy search affects the speed of cleavage (59). By linking covalently Cas to the DNA encoding the gRNA, the first part of this search is

likely significantly shortened. Future strategies that leverage this fact and increase the amount of Cas proteins delivered, may lead to a more efficient cleavage which provides the typical counterselection mechanism for CRISPR-Cas genome editing strategies in bacteria.

Counterselection of the WT genotype is only necessary when the editing efficiency is low, as is often the case with commonly used recombineering strategies. Novel strategies have been developed that do not depend on the introduction of double-strand breaks to perform targeted genetic modifications. These strategies, such as base editing or prime editing, appear to work at high efficiencies (5). To demonstrate the versatility of our delivery system, we have also tested the ability to send base editing tools via bacterial conjugation. BE has been previously tested in eukaryotes (25), but not in bacteria. To the N terminus of this fusion protein, we added either the conjugative relaxase TrwC or the MobA relaxase of RSF1010. The latter is another well-characterized relaxase known to be active in recipients after translocation through different T4SS (60, 61). In addition, MobA uses the T4SS of plasmid RP4, which is known for its promiscuity, especially among distantly related donor/recipient pairs (62–64), thus increasing the range of potential recipient cells.

We have designed two systems to detect base editing, based on either a loss (*lacZ*) or gain (*ermB*) of function. By using both selection systems, we verified that the vast majority of the selected transconjugants in these experiments had incorporated the expected mutation (Fig. 4). BE as a fusion behaves similarly to its standalone form, with the same limitations, such as lower efficiency when editing C that are found after a G, or occasional editing outside of what is strictly defined as the editing window (65). The frequency of edition in the absence of selection is around 0.08% of transconjugants and is increased up to 0.19% when the gRNA is encoded in the delivered plasmid. This frequency is 100-fold higher than DSB-induced homology repair, still too low to be useful for the generation of mutations without selection, but a significant step forward. Future work might improve this efficiency by assessing the properties of other BE designs, optimizing the stability of fusion proteins, and ensuring that a higher number of BE proteins are transferred through the T4SS. An increasing number of Cas protein variants are being characterized and will be tested in the future, such as the miniature Cas12n proteins (66), whose primitive nature and smaller size may prove to facilitate stability, translocation, and refolding, increasing the efficiency of the system.

Finally, it is worth noting that these large multidomain proteins, which are close to 300 kDa in size, can be transported through the T4SS and regain activity in recipient cells, as evidenced by the base editing activity. To our knowledge, this is the largest heterologous substrate translocated through a bacterial secretion system (67). These findings suggest that unfolding through the T4SS is not extensive. Indeed, recent studies have revealed that when TrwC is covalently attached to a ssDNA and translocated through the T4SS, the complex undergoes seven discrete steps of cotranslocational unfolding, each stage representing a different translocation intermediate at various stages of unfolding (68). Therefore, the T4SS could serve as an effective delivery channel for large proteins that would not be able to regain activity if they were completely unfolded during translocation, as is the case with T3SS-mediated translocation (69).

In summary, we present a proof of concept demonstrating that bacterial conjugation can be utilized to deliver active Cas endonucleases and BEs to recipient bacteria. This method eliminates the need for endonuclease expression in the recipient cell. The nucleoprotein complex can include any DNA of interest, which could encode the gRNA or the template DNA for HR. The delivery system is flexible enough to accommodate different relaxases and genetic editing tools of large size, as evidenced by the successful delivery of a BE system. While this approach is necessarily limited by conjugation efficiency, we benefit from the fact that efficient conjugation has been described from *E. coli* to most Gram-negative and even some Gram-positive bacteria. Conjugation could be widely used for the targeted genetic modification of prokaryotes, especially WT strains which are difficult to transform, and poorly characterized genera for which gene expression tools are underdeveloped (53).

Materials and Methods

Bacterial Strains and Plasmids. Bacterial strains used in this work are listed in *SI Appendix*, Table S2. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) broth, supplemented with agar for solid culture. Details of the strains used for the different experiments and the construction of the *E. coli* screening strain FD3 are explained in *SI Appendix, Materials and Methods*.

Bacterial plasmids used in this work are listed in *SI Appendix*, Table S3. Plasmid constructions and primers are detailed in *SI Appendix*, Table S4 and *Materials and Methods*. Table 1 summarizes the main features of key plasmids used in this work.

Mating Assays. Mating assays were performed as described in ref. 70 with the modifications detailed in *SI Appendix, Materials and Methods*. Briefly, cultures of donor and recipient strains, induced as indicated to express the relaxase-Cas fusion proteins and/or the gRNA, were mixed on LB plates, and mating plates were incubated at 37 °C for 1 to 4 h, depending on the experiment. Dilutions were plated on selective media for donors, recipients, transconjugants, or edited colonies, as required. Plates were supplemented with the specific inducer when indicated. Conjugation frequencies are expressed as the number of transconjugants per donor cell.

Measurement of Cas12a Cleavage Activity in Bacteria. In order to detect Cas12a cleavage activity in prokaryotic cells, we measured SOS induction and DSB-induced lethality.

SOS response assay. In order to detect induction of the SOS response upon translocation of TrwC-Cas12a, the plasmids pSOS, pgRNA-lacZ, or pgRNA-sacB were introduced by electroporation into the recipient strain MG1655. After the matings, GFP levels were detected directly on the conjugation plates in an Azure Biosystems c400 imaging system. Next, conjugation was stopped by introducing the filter in 2 mL LB broth. Then, 100 µL was added on a 96-well black flat microtiter plate. GFP signal (excitation filter: 475 nm and emission filter: 515 nm) and bacterial cell density (OD_{600} nm) were measured with a TECAN infinite M200 Pro plate reader.

Lethality assay. First, 100 ng of each plasmid encoding Cas12a and gRNA (*lacZ* or *sacB*) was electroporated into D1210, and cells were plated on antibiotic-containing media supplemented with IPTG 500 µM for gRNA expression. aTc 200 ng/mL for induction of *cas12a* expression was added when indicated. Lethality was measured by comparing the number of colony forming unit with/without induction or with/without target for the gRNA. In order to measure Cas12a-induced lethality upon translocation into the recipient cell, we compared the number of transconjugants obtained under these conditions.

RNA-guided mutations in sacB. Plasmids encoding the nuclease and *sacB* gRNA were electroporated into MG1655::*sacB*, (strain FD3, *SI Appendix*, Table S2), and cells were plated on antibiotic-containing media supplemented with IPTG 500 µM and 1% sucrose to counterselect *sacB* activity. To detect mutations upon translocation of TrwC-Cas12a into the recipient MG1655::*sacB* strain the matings were directly plated on 1% sucrose-containing plates. Sucrose-resistant transconjugants were directly picked for PCR amplification of the *sacB* region using sacB_F and sacB_R primers (*SI Appendix*, Table S4). The size of the amplicon was checked by agarose gel electrophoresis. Amplicons were purified and their DNA sequence was determined (STAB VIDA). Sequence alignments were performed with BioEdit Sequence Alignment Editor.

Measurement of Seamless Editing Rate. After matings, sucrose-resistant colonies were replicated in Cm-containing plates to discard integrants (Cm-resistant). Then, a fragment of 357 bp from *sacB* containing the PAM region was amplified from the Cm-sensitive transconjugants using the oligonucleotides sacB_F and sacB_HR_R (*SI Appendix*, Table S4). Amplicons were purified and their DNA sequence was determined (STAB VIDA). Sequence alignments were performed with BioEdit Sequence Alignment Editor.

The editing rate was calculated as the number of Cm-sensitive colonies incorporating the desired mutation (stop codon) divided by the number of sucrose-resistant colonies analyzed in each assay.

Analysis of BE-Edited Colonies. To test the activity of BE and its fusions by electroporation, 100 ng of plasmids containing BE, relaxases, or relaxase-BE fusions and 100 ng of pgRNA-lacZ$_{BE}$ were coelectroporated in D1210. Cells were plated on antibiotic-containing media supplemented with X-Gal 60 μg/mL, IPTG 500 μM, and aTc 200 ng/mL (except in the case of pTrwC, where aTc is not added). Light-colored transformants (visually detected) were passaged 1 to 3 times to fix the edition. Afterward, the *lacZ* region was PCR-amplified using lacZ_F and lacZ_R primers (*SI Appendix*, Table S4). For assays targeting plasmid-borne *ermB**, 100 ng of the same plasmids were electroporated in DH5α containing plasmids pErmB* and pgRNA-ErmB$_{BE}$. Transformants were selected on erythromycin, ampicillin, arabinose (Em Ap Ara) plates and grown for 48 h. EmR colonies were replicated again in the same medium and picked for PCR amplification of the *ermB* region with araBAD_F and pBAD33_seq_R primers (*SI Appendix*, Table S4). The size of the amplicons was checked by agarose gel electrophoresis. All PCR products were purified, and their DNA sequence was determined (STAB VIDA). In order to determine the ratio between EmR and total transformants, transformants were selected in media containing only antibiotics for plasmid selection and then transferred to Em Ara Ap plates. Then, the percentage of EmR colonies was calculated.

To verify edition following the translocation of relaxases and their fusions by conjugation, transconjugants were plated on selective media. In the case of *lacZ*, the medium contained the antibiotics needed for transconjugant selection, along with X-Gal and IPTG. To detect edition of *ermB*, transconjugants were selected on two types of media: one with the necessary antibiotics for their selection, to obtain the total number of transconjugants, and another with Em Ap Ara to selectively isolate and count those resistant to Em. Selection of the colonies, passages, PCR amplification of the target genes, and sequencing was done in the same way as in the case of electroporation.

Western Blot. Total protein extracts were obtained as described in ref. 71. *E. coli* D1210 cells containing plasmids pTrwC, pCas12a, pTrwC-Cas12a, pBE, pTrwC-BE, pTrwC-linker-BE, pBE-linker-TrwC, pMobA-HA, pMobA-BE-HA, or pBE-HA were grown overnight. The cultures were diluted 1:20 and induced with IPTG 500 μM or aTc 200 ng/mL for 3 h. Then, 1 mL of each culture was collected, centrifuged, and resuspended in 1/10 volume of 2× SDS-gel loading buffer. Samples were stored at −20 °C for at least overnight. Samples were boiled for 5 min and loaded on 9% acrylamide Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. After the run, the gels were transferred to nitrocellulose membranes. Primary antibody [anti-TrwC (72) or anti-HA-tag (HAtag Rabbit Poly Ab, Proteintech)] and secondary antibody (Anti-Rabbit IgG IRDye®800CW, Li-Cor) were used at 1:10,000 dilution. Detection was performed with an Odyssey Clx. NZYColour Protein Marker II and PageRule Plus Prestained Protein Ladder (Thermo Fisher) were used as molecular weight marker.

Statistical Analysis. Student's *t* test was employed to identify statistically significant discrepancies between the means of three or more independent results. The nonparametric Mann–Whitney *U* test and Kruskal–Wallis test were used for data that did not follow a Gaussian distribution. All statistical analyses were conducted using GraphPad Prism 8 software.

Data, Materials, and Software Availability. All primer sequences, repair template sequences, and analysis targets are included in the manuscript and/or *SI Appendix*.

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