

Consequences of Cas9 cleavage in the chromosome of Escherichia coli

Lun Cui, David Bikard

▶ **To cite this version:**

Lun Cui, David Bikard. Consequences of Cas9 cleavage in the chromosome of Escherichia coli. Nucleic Acids Research, 2016, 44 (9), pp.4243-4251. 10.1093/nar/gkw223 . pasteur-01967442

HAL Id: pasteur-01967442
https://pasteur.hal.science/pasteur-01967442

Submitted on 3 Jan 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Published online 8 April 2016

Nucleic Acids Research, 2016, Vol. 44, No. 9 ***4243–4251***
doi: 10.1093/nar/gkw223

Consequences of Cas9 cleavage in the chromosome of *Escherichia coli*

Lun Cui and David Bikard*

Synthetic Biology Group, Microbiology Department, Institut Pasteur, Paris 75015, France

Received October 14, 2015; Revised March 20, 2016; Accepted March 22, 2016

ABSTRACT

The RNA-guided Cas9 nuclease from CRISPR-Cas systems has emerged as a powerful biotechnological tool. The specificity of Cas9 can be reprogrammed to cleave desired sequences in a cell's chromosome simply by changing the sequence of a small guide RNA. Unlike in most eukaryotes, Cas9 cleavage in the chromosome of bacteria has been reported to kill the cell. However, the mechanism of cell death remains to be investigated. Bacteria mainly rely on homologous recombination (HR) with sister chromosomes to repair double strand breaks. Here, we show that the simultaneous cleavage of all copies of the *Escherichia coli* chromosome at the same position cannot be repaired, leading to cell death. However, inefficient cleavage can be tolerated through continuous repair by the HR pathway. In order to kill cells reliably, HR can be blocked using the Mu phage Gam protein. Finally, the introduction of the non-homologous end joining (NHEJ) pathway from *Mycobacterium tuberculosis* was not able to rescue the cells from Cas9-mediated killing, but did introduce small deletions at a low frequency. This work provides a better understanding of the consequences of Cas9 cleavage in bacterial chromosomes which will be instrumental in the development of future CRISPR tools.

INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas) genes are the adaptive immune system of bacteria and archaea (1,2). The RNA-guided Cas9 nuclease from *Streptococcus pyogenes* has emerged as a useful and versatile tool (3). The ease with which it can be reprogrammed has in particular been driving its adoption for genome editing applications. Cas9 is guided by a small CRISPR RNA (crRNA) that is processed from the initial transcript of the CRISPR locus by Cas9 together with a trans-activating CRISPR RNA (tracrRNA) and the host RNAseIII (4). Both the tracrRNA and the processed crRNA remain bound to Cas9 and act as a complex to direct interference against target DNA molecules (5). Alternatively, the crRNA and tracrRNA can be fused forming a chimeric single guide RNA (sgRNA) (5). Cas9 scans DNA looking for a short sequence motif known as the protospacer adjacent motif (PAM) (6). Once a PAM is found, DNA is unwound to make base-pair contacts between the crRNA and the target DNA. If base-pairing occurs, a conformational shift in Cas9 brings two nuclease domains in contact with the target DNA leading to the creation of a double strand break (DSB) (7,8).

Genome editing using Cas9 has been reported in a large number of eukaryotes including insects, plants, mammals, yeast, zebrafish, xenopus and nematode (3). Cas9 cleavage in the chromosome can be repaired through homologous recombination (HR) with a template DNA molecule to introduce specific mutations. Alternatively, in the absence of a repair template, eukaryotic cells can survive DSB introduced by Cas9, presumably thanks to repair by the non-homologous end joining (NHEJ) pathway. The error-prone nature of this repair pathway is used in genome editing strategies to introduce small indels at the target site and knockout genes (9,10). In contrast, Cas9 cleavage in the chromosome of bacteria was reported to kill the cell (11). Most bacteria lack an NHEJ system (12,13), which could explain the lethal effect of Cas9 breaks in the chromosome. An exception to this is *Streptomyces coelicolor* whose endogenous NHEJ system was shown to effectively repair Cas9-mediated breaks and introduce indels at the target position (14). However, other bacterial species such as *Clostridium cellulolyticum* are killed by Cas9 breaks despite the fact that they carry NHEJ systems (15). In two recent studies Cas9-mediated killing was used to develop sequence-specific antimicrobials (16,17). Phage capsids were used as vectors to deliver a CRISPR system programmed to target antibiotic resistance or virulence genes in bacterial populations and specifically kill the targeted bacteria. Cas9-mediated killing has also been used in bacterial genome editing strategies where it can help select mutations introduced through HR by eliminating cells carrying the wild-type genotype (11). This strategy has so far only been demonstrated in a few bacteria species where it frequently requires the expression of phage recombinases to promote the introduction of the desired mutation, and has yet to

*To whom correspondence should be addressed. Tel: +33 140 613 924; Email: david.bikard@pasteur.fr

© The Author(s) 2016. Published by Oxford University Press on behalf of Nucleic Acids Research.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

be broadly adopted (15,18–24). In some cases, the DSB introduced by Cas9 seems to promote HR with the template DNA, but this remains to be clearly demonstrated.

The introduction of DSB at unique positions in a genome can also be achieved using other endonucleases such as I-SceI, zinc finger nucleases (ZFN) or transcription activator-like effector nucleases (TALENs) (25). In particular the I-SceI endonuclease has been widely used in bacteria both to probe the consequences of DSB in the chromosome and in genome editing strategies (26,27). In *Escherichia coli*, I-SceI cleavage in the chromosome was reported to kill the cell when no template DNA is available for repair (28). Other reports have shown that *E. coli* can also survive I-SceI cleavage in the chromosome, presumably thanks to a weaker expression (29). Under these conditions not all copies of the chromosome are cut simultaneously leaving an intact sister chromosome available for repair. Alternatively, repair can be achieved by recombination with homologous sequences other than the sister chromosome. I-SceI breaks induce the SOS response and are repaired through the recA/recBCD pathway. The consequences of I-SceI cleavage has also been investigated in *Mycobaterium smegmatis* which carries a NHEJ system (30). Most cells die from the introduction of I-SceI breaks in the chromosome, but a small number of cells can survive by deleting the I-SceI site through NHEJ repair. Repair through HR is also possible when a non-targeted template is present in the cell. Finally RecA independent repair through single stranded annealing was also reported (31).

While the consequences of Cas9 cleavage in the chromosome of *E. coli* are likely similar to what was reported for I-SceI, a systematic study hasn't yet been performed. Different properties of these nuclease could also lead to different outcomes. In particular, Cas9 was reported to stay bound to the DNA ends after cleavage which could affect DNA repair (6). Here, we investigate why DSB introduced by Cas9 leads to cell death in *E. coli* and whether some cells can survive such DNA damage. We show that Cas9 cleavage can kill the cells when all chromosomal copies are cut simultaneously and no intact template is available for homology directed repair. However, not all targets are equal and some positions are being targeted more efficiently than others. Inefficient Cas9 interference can be tolerated through continuous repair by the HR pathway. The inhibition of HR using the phage Mu-Gam protein can restore the CRISPR killing activity. Finally, the introduction of a heterologous NHEJ system from *Mycobacterium tuberculosis* does not rescue cells from Cas9-mediated killing but did enable the introduction of small deletions at the target at low frequencies. These results are consistent with previous data obtained using other nucleases and provided important information for the development of future CRISPR tools in bacteria.

MATERIALS AND METHODS

Bacterial strains and media

Escherichia coli strains were grown in Luria-Bertani (LB) broth. LB Agar 1.5% was used as solid medium. Different antibiotics (20 ug/ml chloramphenicol, 100 ug/ml carbenicillin, 50 ug/ml kanamycin) were used as needed. Plates containing IPTG (100 uM) and X-gal (40 ug/ml) were used for blue/white screening. *E. coli* strain MG1656 (a ΔlacI-lacZ derivative of MG1655) was used as a cloning strain for plasmid pCas9::lacZ1 and pCas9::lacZ2 (see below). *E. coli* strains MG1656, N4278 (MG1655 recB268::Tn10) (32), MG1655 RecA::Tn10 and JJC443 (lexAind3 MalF::Tn10) (33) are gifts from the Mazel lab. MG1655, ΔsfiA::FRT strain is a gift from Bénédicte Michel.

Plasmid cloning

Plasmid pCRRNA was assembled from the polymerase chain reaction (PCR) amplification products of pCRISPR (18) using primers B299/LC34 and pCas9 (18) using primers LC35/LC36, followed by Gibson assembly (34). Novel spacers were cloned into pCRRNA and pCas9 plasmids as previously described (18). The vector was digested with BsaI, followed by ligation of annealed oligonucleotides designed as follows: 5′-aaac+(target sequence)+g-3′ and 5′-aaaac+(reverse complement of the target sequence)-3′. A list of all spacers tested in this study is provided in (Supplementary Table S1).

The pLCX plasmid was assembled from the pCRISPR backbone amplified using primers LC41/LC42 and two *lacZ* fragments amplified from MG1655 genomic DNA using primers LC38/LC39 and LC37/LC40. The pZA31-sulA-GFP plasmid was assembled from pZA31-Luc (35) linearized with primers LC192/LC193, the sulA promoter fragment amplified with primers LC194/LC196 and GFPmut2 (36) amplified with primers LC191/ LC195. The Mu-Gam expression plasmid pLC13 was constructed through the assembly of pBAD18 amplified with primers LC2/LC296 together with the Mu gam gene fragment amplified from the *E. coli* S17-1 λpir (37) genomic DNA using primers LC397/LC398. All PCR primers used in this study are listed in (Supplementary Table S2).

CRISPR transformation assays

The pCRRNA or pCas9 plasmids (with different spacers) were transformed in recipient *E. coli* strains by chemical transformation using 100 ng of plasmid DNA. Colony forming units (CFU) were counted after plating of serial dilutions on the appropriate selective media. CFUs were normalized by the number of CFUs obtained with a control transformation of pUC19. All transformations were repeated at least three times.

Northern blot analysis

Bacterial total RNAs were prepared using Trizol. Novex® TBE-Urea Gels (10% polyacrylamide gels containing 7 M urea) were used to separate total RNAs. After electrophoresis, the gels were blotted onto Nylon membranes (Invitrogen), which were subsequently cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) buffer (38). The probes were labeled as follow: 100 pmol of oligonucleotide were heat denatured, labeled and phosphorylated by mixing 40 μCi of ^{32}P-γ-ATP and T4 PNK reagents. Finally, the labeled probes were column purified (Macherey-Nagel PCR cleanup kit) and used for overnight hybridization.

Mu Gam

Electrocompetent cells of the *E. coli* strain MG1655 carrying plasmid pLC13 were prepared. Briefly, the cells were grown to an optical density (OD, 600nm) of ∼1.0. Cells were washed twice with ice cold water, then washed once with cold 10% glycerol. The cells were concentrated to about 400 times the original culture volume in 10% glycerol. The pCas9, pCas9::LacZ1 and pCas9::LacZ2 plasmids (40 ng each) were transformed into electrocompetent cells using 1 mm gap cuvette and the GenePulser Xcell (Bio-Rad) set to 1800 V, 25 uF, 200 Ω. Cells were plated for quantification in LB-agar chloramphenicol with or without L-arabinose (0.2%).

SOS response

The pZA31-sulA-GFP plasmid was used to monitor SOS induction (39). The One-Step Integration Plasmid (pOSIP) system (40) was used to integrate *cas9* or *dcas9* under the control of a ptet promoter (41) in the chromosome of strains MG1655, N4278 (MG1655 recB268::Tn10) (32), MG1655 RecA::Tn10 and JJC443 (lexAind3 MalF::Tn10) (33) (see Supplementary Table S3). pCRRNA plasmids with different spacers were introduced into the resulting strains by chemical transformation. Colonies isolated from the transformation plates were re-suspended in 200 μl LB in a 96-well microtiter plate. OD (600 nm) and Green Fluorescent Protein (GFP) signal (excitation filter set to 486 nm and emission filter set to 518 nm) were measured over a 10 h time course in a TECAN infinite M200 Pro. GFP values at OD ∼0.4 are reported.

NHEJ

The pOSIP system (40) was used to integrate the *ligD* and *ku* genes amplified from the genomic DNA of *M. tuberculosis* strain H37Rv in the chromosome of *E. coli* strain N4278 (see Supplementary Table S3). Plasmids pCas9 or pCas9:lacZ2 were transformed by electroporation using 50 ng of plasmid DNA and 50 μl electrocompetent cells. CFU numbers were normalized by pUC19 transformation efficiency. All transformations were repeated at least three times.

RESULTS

E. coli can survive Cas9 cleavage through homology directed repair

Evidence that CRISPR interference directed against the chromosome leads to cell death first came from the observation that an active CRISPR system and its target cannot coexist in the same cell (11,42,43). Transformation of *E. coli* by a plasmid carrying a CRISPR system targeting the chromosome is very inefficient, typically resulting in a 1000-fold decrease in transformation efficiency compared to a non-targeting control (18,42,44). In a previous study, we took advantage of this to introduce a mutation in the *rpsL* gene of *E. coli* (18). Targeting of the *rpsL* gene by Cas9 killed the cells that did not incorporate a desired mutation provided by an oligonucleotide. To investigate whether this approach could be extended to other loci, we programmed a plasmid-born CRISPR array to target 12 positions spread throughout the *E. coli* chromosome and compared them with the *rpsL* target previously published. All targets were chosen in non-essential genes to ensure that killing by Cas9 would be the result of DNA cleavage and not repression of the target gene (41,45). The pCRRNA plasmid carries the tracrRNA and a minimal CRISPR array consisting of the leader sequence and a single spacer framed by two repeats. This plasmid was transformed in cells containing the pCas9 plasmid expressing Cas9 constitutively (18). Surprisingly, 8 out of 12 spacers could be readily transformed with efficiencies comparable to that of the non-targeting control (Figure 1A and B). Interestingly, three of them (*lacZ1*, *tsuB and wcaH*) resulted in colonies smaller than the control (see Supplementary Figure S1). We hypothesized that Cas9 cleavage in these cells might be inefficient and that competition with the bacteria repair system would stress the cells and slow down colony growth. To test this idea, we repeated this transformation experiment in cells deleted for *recA*. Consistently with our hypothesis, no colonies could be recovered after transformation of spacers *lacZ1*, *tsuB and wcaH*, but also after transformation of all the other spacers. This shows that all spacers are able to direct Cas9 cleavage in the chromosome, including those that can be transformed efficiently, and all spacers induce lethal DSB in the absence of *recA*. However, only some spacers are able to kill cells in the presence of *recA*. This indicates that weak spacers might be tolerated in wild-type cells thanks to the HR pathway.

In order to investigate whether differences in targeting efficiency could be due to differences in expression or processing of the crRNA, we performed a northern blot analysis to compare the CRISPR arrays programmed to target the lacZ1 and lacZ2 positions (Supplementary Figure S2). While targeting lacZ1 leads to small colonies, targeting lacZ2 efficiently kills *E. coli*. Plasmids were transformed in cells deleted for *lacZ* in order to measure CRISPR expression and processing in the absence of active targeting. We could see no difference in the expression level and processing efficiency of the CRISPR arrays carrying spacer lacZ1 and lacZ2. The observed difference in targeting efficiency between these two spacers is therefore likely due to differences in later steps of CRISPR interference such as target binding or cleavage.

HR can only rescue a DSB if an intact sister chromosome is available. This suggests that for some spacers Cas9 cleavage is not efficient enough to cut all copies of the chromosome simultaneously. A corollary is that spacers that do lead to cell death probably kill the cells because no repair template is available. If this is true, then providing an intact repair template during targeting should be able to rescue the cells. To test this hypothesis we constructed a plasmid, pLCX, carrying a 2-kb fragment homologous to the target region of spacer *lacZ2*, but with a point mutation in the PAM motif blocking CRISPR interference (Figure 1C). Transformation of the *lacZ2* spacer led to ∼100× more colonies in the presence of pLCX than in cells carrying a control empty plasmid, and no colonies could be recovered in the *recA* mutant (Figure 1D). The *lacZ* gene of the recovered colonies was sequenced and confirmed to carry the point mutation provided by the pLCX plasmid (see Supple-

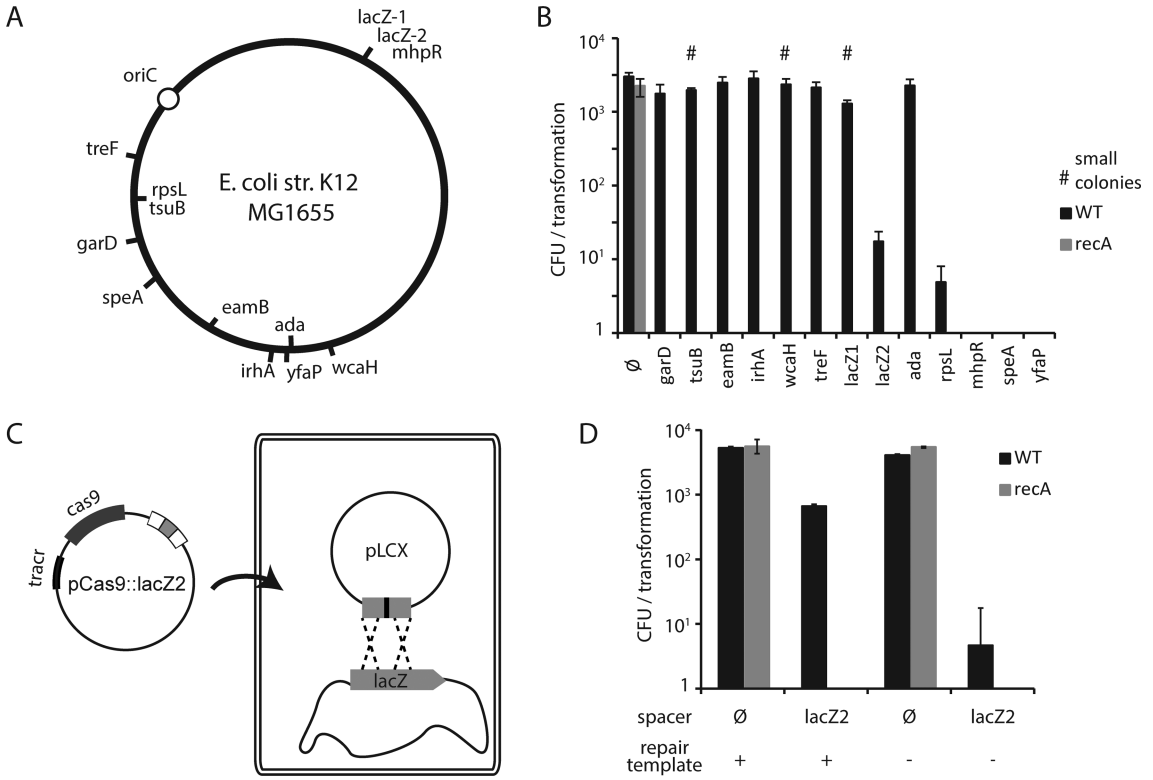


Figure 1. Weak self-targeting CRISPR-Cas9 systems can be tolerated through homology directed repair. (**A**) Position of the targets on the *Escherichia coli* chromosome. Targets on the inside of the circle are on the non-template strand of the gene, targets on the outside are on the template strand. (**B**) The pCRRNA carrying different spacers was transformed in cells expressing Cas9 constitutively. Average CFU numbers are reported for transformation in wild-type cells (black bars) and *recA*-cells (gray bars), showing that some spacers can be tolerated in the presence of *recA* but not in the *recA*-strain (mean ± s.d., $n \geq 3$). Transformation events yielding small colonies are marked with a hashtag (see Supplementary Figure S1 for colony size measurments). (**C**) Schematics of the transformation assay performed to demonstrate homology directed repair. The pCas9 plasmid carrying Cas9, the tracrRNA and a CRISPR array was programmed to target a position within the *lacZ* gene. The resulting plasmid pCas9::lacZ2 was transformed in cells carrying a plasmid with homologies to the target region but carrying a mutation preventing Cas9 cleavage (pLCX). (**D**) CFU numbers are reported after transformation either in wild-type (black bars) or *recA*-cells (gray bars), showing that the presence of a repair template rescues cells targeted by Cas9 at the lacZ2 position (mean ± s.d., $n \geq 3$).

mentary Figure S3), showing that it was indeed used as a template for HR.

Cas9 cleavage leads to SOS induction

Spacers that can be tolerated likely result in constant Cas9 cleavage and RecA mediated repair. This should lead to an elevated level of SOS induction (46). To test this we integrated *cas9* in the chromosome under the control of a ptet promoter and monitored SOS levels with a GFP reporter plasmid (Figure 2A). Spacers were provided on the pCRRNA. Targeting with the *lacZ1* spacer led to elevated GFP fluorescence levels when aTc was added to the media, but more surprisingly also in the absence of induction (Figure 2B and Supplementary Figure S4). This demonstrates that the ptet promoter controlling Cas9 is leaky and that the small amount of Cas9 proteins produced can already lead to the introduction of DSB resulting in SOS induction. Consistently with an induction of the SOS pathway, no fluorescence could be observed in *recA*, *recB* or *lexA(ind-)* mutants (Figure 2B). Mutations in the catalytic sites of Cas9 also abolished SOS induction showing that cleavage of DNA and not mere binding is the cause of the SOS induction (Figure 2B, dCas9). We further measured the SOS response triggered by all 13 spacers (Figure 2C). Interestingly, the strength of SOS induction correlates well with the ability of the spacers to kill the cells. This corroborates the idea that efficient cleavage of all copies of the chromosome is responsible for cell death.

The SOS induction leads to cell filamentation through the action of the SulA (also known as SfiA) protein which blocks cell division (47). This division block allows DNA repair to happen and ensures that all daughter cells receive an intact copy of the chromosome. Nonetheless filamentation itself can lead to cell death, and inactivation of *sulA* can improve the growth of mutants that strongly induce the SOS response (48). In order to ensure that Cas9-mediated killing is not the result of the SOS induction and division block itself, we performed Cas9 targeting experiments in a *sulA* knockout strain. The transformation efficiency of the *lacZ2* spacer was identical in the WT and *sulA* cells (Supplementary Figure S5). This is consistent with the hypothesis that cell death results from the inability to repair simultaneous breaks at all copies of the chromosome.

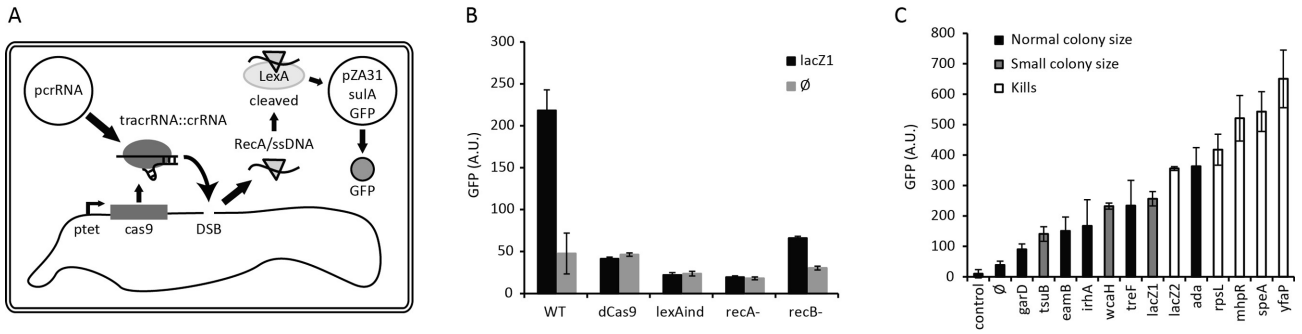


Figure 2. Cas9 cleavage in the chromosome induces the SOS response. (**A**) Schematics of the SOS reporter assay. Cas9 is expressed under the leaky control of a non-induced ptet promoter in the chromosome. The tracrRNA and crRNA are expressed from the pCRRNA plasmid. Cas9-mediated breaks lead to the formation of recA/ssDNA filaments which activate the self-cleavage of the LexA repressor. This releases the repression of *GFPmut2* controlled by the sulA promoter. (**B**) The pCRRNA plasmid programmed to target the *lacZ1* position (black bars) or a control empty pCRRNA (gray bars) were introduced in different cell backgrounds. GFP fluorescence was measured during exponential growth (mean ± s.d., $n \geq 3$). (**C**) SOS response resulting from CRISPR targeting with different spacers. The bar marked as 'control' indicates the auto-fluorescence level of *Escherichia coli* without the pZA31-sulA-GFP plasmid. Spacers that cannot be transformed under constitutive Cas9 expression from the pCas9 (see Figure 1B) are shown in white. Spacers that can be transformed but lead to the formation of small colonies (see Figure 1B) are shown in gray. Finally, spacers that can be transformed in the presence of pCas9 and form colonies of regular size (see Figure 1B) are shown in black (mean ± s.d., $n \geq 3$).

Mu-Gam restores killing by weak spacers

CRISPR-Cas9 genome editing tools and sequence-specific antimicrobials both rely on the ability of Cas9 to efficiently kill bacteria. In order to make Cas9 killing more reliable, we investigated methods to prevent DNA repair and restore Cas9's ability to kill *E. coli* even when directed by a weak crRNA (Figure 3A). The Gam protein of phage Mu binds double stranded ends and protects the phage DNA from degradation by host exonulceases (49). It was shown that upon UV exposure, the survival of cells expressing Gam is similar to that of a *recB* mutant, demonstrating that Gam blocks DNA repair (50). We cloned the *Mu-gam* gene under the control of a pBAD promoter and measured the transformation efficiency of pCas9 programmed with the *lacZ1* or *lacZ2* spacers in the presence or absence of arabinose. Both spacers target the *lacZ* gene but show different transformation efficiencies (Figure 1B). The expression of Gam reduced the number of colonies recovered after transformation of both pCas9::lacZ1 and pCas9::lacZ2 by a factor of 242× and 15× respectively (Figure 3B). The Mu-Gam protein can thus promote Cas9-mediated killing when directed by a weak crRNA and improve killing efficiency when directed by an already strong crRNA. Unexpectedly, colonies obtained after induction of Gam and transformation of a control plasmid were markedly smaller than without Gam induction. This indicates that Gam overexpression itself is toxic to the cells and that adjusting Gam expression might be required for its use in future CRISPR tools.

Cas9 cleavage induces large chromosomal deletions mediated by HR

We further studied the consequences of chromosomal cleavage by spacers *lacZ1* and *lacZ2*. Cells transformed with pCRRNA::lacZ1 and pCRRNA::lacZ2 in the presence of pCas9 were plated on Xgal to report mutations in *lacZ*. Interestingly, 1.6% of the *lacZ1* and 51.7% of the *lacZ2* transformants formed white colonies (Figure 4A). Furthermore, the small blue colonies resulting from *lacZ1* transformation in some cases formed large white colonies when restreaked, a phenomenon that was never observed when restreaking clones in the absence of Cas9 targeting (see Supplementary Figure S6). This is a clear indication that mutations at the target site are induced by Cas9 cleavage and not just selected from pre-existing mutants in the population. No colonies were obtained after transformation in *recA*- cells, suggesting that HR is involved in the formation of these mutants (Figure 1B). We genotyped 14 white colonies through PCR and sequencing (Figure 4B, Supplementary Figure S7 and Table S4). All of them showed large deletions of the *lacZ* region ranging from 12.9 to 35.0 kb. In 11/14 cases the borders of the deletions implicated repeat regions known as repetitive extragenic palindromic (REP) sequences. Several insertion sequences have been shown to integrate into these elements which have also been implicated in a variety of functions including gene regulation and control of DNA structure (51). Interestingly a recombination event between REP elements surrounding the *lac* operon was at the origin of the F'128 plasmid which was widely used to study the *lac* operon (52). Out of these 11 deletions involving REP elements, seven can be explained by recombination events between 35 bp repeats (see Supplementary Table S4). The four other events involve either micro-homologies or in one case no sequence homology at all. Finally in three cases recombination happened outside of the REP elements but also involve micro-homologies.

Repair of Cas9-mediated DSB through NHEJ

Repair of DSBs in the absence of a sister chromosome can be achieved through the NHEJ repair pathway. The presence of such systems in most eukaryotic cells is presumably the reason why they are able to survive Cas9 breaks better than bacteria. NHEJ is indeed absent from many widely studied bacterial strains including *E. coli* (12,13). In bacteria the NHEJ function is carried out by two proteins, Ku and LigD. Ku binds to DNA ends, protects them from nucleases, and recruits LigD to process and ligate the ends. The NHEJ system from *M. tuberculosis* has already been

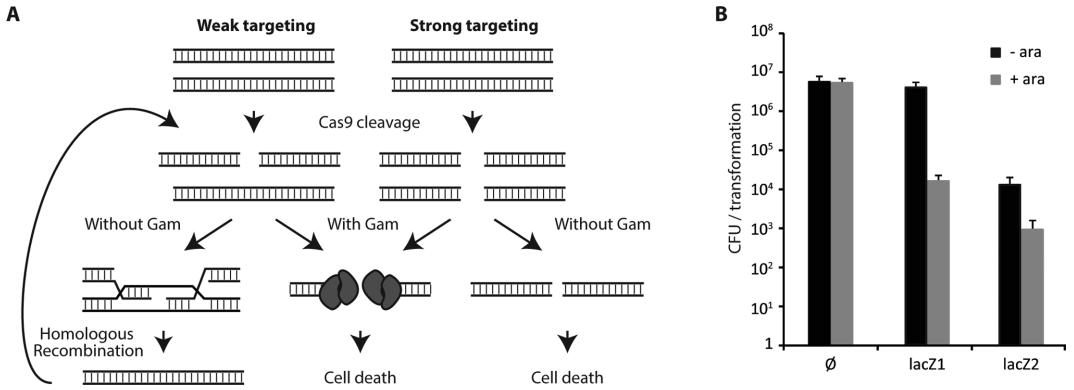


Figure 3. Mu Gam enhances Cas9-mediated killing. (**A**) Schematics showing the outcome of weak or strong Cas9 cleavage activity in the chromosome of *Escherichia coli*. Weak targeting leaves a chromosomal copy intact that can be used as a template to repair the broken chromosome through HR. The repaired chromosome can then be cut again, thus entering a cycle of cleavage and repair, which constitutively induces the SOS response. Strong targeting leads to the simultaneous cleavage of all copies of chromosome which cannot be repaired through HR and thus leads to cells death. The Mu Gam protein can specifically bind to double stranded ends and block repair through HR, thereby promoting cell death even under weak targeting conditions. (**B**) Plasmid pCRRNA carrying the *lacZ1*, *lacZ2* spacers or a spacer-less control was transformed in cells carrying plasmid pLC13 expressing Mu Gam under the control of an arabinose inducible promoter. The number of transformants obtained in the presence or absence of arabinose is reported (mean ± s.d., $n \geq 3$). Upon Gam expression both weak and strong targeting leads to efficient killing.

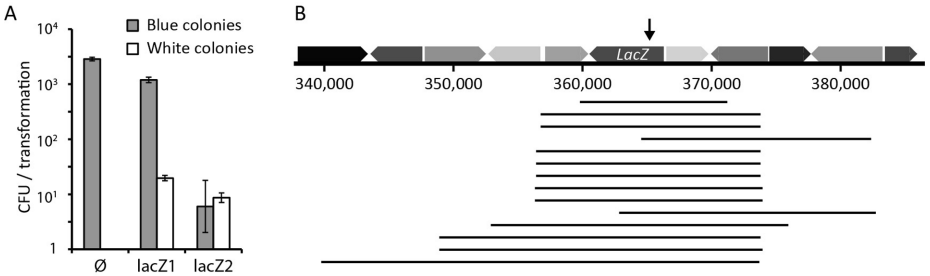


Figure 4. Cas9 cleavage of the *lacZ* gene induces the formation of large deletions. (**A**) The pCRRNA plasmid carrying the *lacZ1*, *lacZ2* spacers or a spacer-less control was transformed in cells expressing Cas9 constitutively from the pCas9 plasmid. The number of white and blue CFU on Xgal are reported (white bars and grey bars), indicating the number of colonies where *lacZ* was inactivated (mean ± s.d., $n \geq 3$). (**B**) Schematics of the *lac* operon genomic region. The target position is shown by a black arrow. The deletions mapped in 14 white colonies are depicted by black bars.

shown to enable the circularization of linear plasmids in *E. coli* when the *recB* exonuclease is inactivated (53). In the presence of RecB, double-stranded ends are presumably resected too fast for NHEJ to take place. To investigate whether this system could repair DSB introduced by Cas9, we integrated the *ku* and *ligD* genes from *M. tuberculosis* under the control of a constitutive promoter in the chromosome of *E. coli* strain N4278, a *recB* mutant of strain MG1655. CRISPR interference directed by *lacZ2* resulted in the death of the vast majority of cells as evidenced by the low transformation efficiency compared with a non-targeting control (Figure 5A). The total number of colonies recovered after transformation with or without NHEJ was similar, showing that the *M. tuberculosis* NHEJ system is not able to rescue cells from Cas9-mediated death.

The majority of cells recovered after transformation of the *lacZ2* spacer were blue regardless of the presence of NHEJ. We could show that these blue colonies carry a defective CRISPR system (Supplementary Figure S9). Nonetheless, 10× more white colonies could be recovered in the presence of the NHEJ system, suggesting that the NHEJ system is functional and can repair Cas9 breaks but with an efficiency lower than the frequency of background mutations in the CRISPR system. Analysis of 8 blue and 13 white colonies confirmed that small deletions were introduced in *lacZ* for 13/13 white colonies and 1/8 blue colonies (Figure 5B and C, Supplementary Figure S8 and Table S5). This blue colony carried a 9-bp in-frame deletion which can explain its phenotype.

All in all, sequencing revealed deletion events ranging from 9 to 298 bp (Figure 5C). Surprisingly the right end of the deletion was always located between the cleavage site and the PAM motif, while the other end varied greatly. This likely indicates some protective role of Cas9 on the end of DNA containing the PAM motif. We also analyzed six white colonies obtained in the absence of Ku and LigD. In all cases, the deletions introduced were larger than 1 kb, in stark contrast with the small deletions obtained by NHEJ. We precisely mapped two of these deletions that were 17.7 and 27.8 kb and resulted from recombination events between REP elements (see Supplementary Table S6). In the absence of RecB, these events are likely not the result of HR, but might have been mediated by the TnpA transposase (51). These colonies could also be the result of an alternative end-joining repair mechanism relying on the endogenous *ligA* ligase previously reported in *E. coli* (54). This mechanism generates large deletions up to 40 kb using micro-

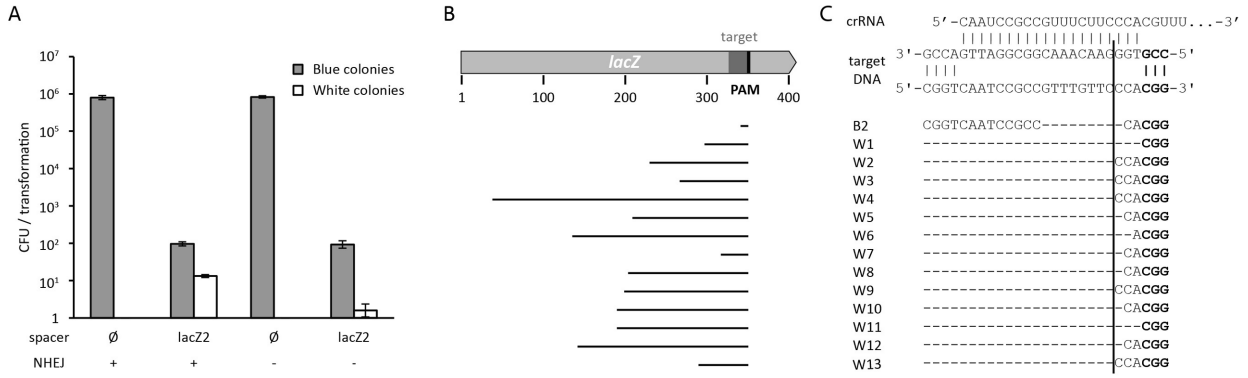


Figure 5. NHEJ repair of Cas9 induced DSB in the genome of *Escherichia coli.* (**A**) Plasmid pCas9 programmed with spacer *lacZ2* or a spacer-less control was transformed in *recB*-cells with or without the NHEJ system (Ku and LigD) from *Mycobacterium tuberculosis*. The number of blue and white CFUs on Xgal plates is reported (grey and white bars), indicating colonies where *lacZ* was inactivated. (**B**) Schematics showing the beginning of the *lacZ* open-reading frame. Deletions mapped in one blue and 13 white colonies are depicted by black bars. Base numbers from the start codon are shown. (**C**) The right edge of the mapped deletions is aligned below the crRNA and target DNA for spacer *lacZ2*. The cleavage point is shown by a vertical black bar and bold characters indicate the PAM motif.

homologies as joining points in a RecA and RecB independent manner.

DISCUSSION

The ability of Cas9 to kill bacteria when directed to cut in their chromosome has been used as a counter-selection tool for the purpose of gene editing and for the development of sequence-specific antimicrobials (16–18). However, the mechanism of Cas9-mediated cell death has so far remained unclear. Here, we show that not all targets are equal and *E. coli* can survive active targeting at some positions. Cas9-induced breaks activate the SOS response and can be repaired through the HR pathway. This enables *E. coli* to tolerate the presence of weak self-targeting CRISPR systems. Other targets can be cleaved efficiently leading to the introduction of DSB in all copies of the chromosome simultaneously. In the absence of a template for HR, extensive recession of the DNA ends by RecBCD and other nucleases is likely the cause of cell death.

Variations in the efficiency of Cas9 cleavage between different targets have been reported previously (9,55,56). The ability to predict the efficacy of guide RNAs is of prime importance for all applications of Cas9 technologies. High-throughput screens of sgRNA libraries in human or mouse cells have allowed identifying good targets (55,57,58), and were used to build predictive models for the design of highly active sgRNAs. However, the recent model from Jong *et al.* (57) gave very poor prediction for the activity of the 13 targets that were used in our study (see Supplementary Figure S10). This could stem from differences in the requirements for efficient Cas9 targeting between mammalian cells and *E. coli*, as well as the fact that these screens were performed using sgRNAs instead of the dual crRNA and tracrRNA system. In particular some features that influence the expression of the sgRNA, loading of the sgRNA on Cas9, or the accessibility of the target DNA are likely not generalizable to our system. This highlights the necessity to perform similar screens in bacteria. A better knowledge of what makes a good CRISPR target will be critical for the development of reliable genome engineering tools as well as CRISPR antimicrobials. In the absence of good predictive models of a guide RNA efficiency, we demonstrated that inhibition of HR by the Mu-Gam protein allows to kill *E. coli* even when using a weak target.

Interestingly cell death is not the only possible outcome of efficient Cas9 cleavage in the chromosome of *E. coli*. Large deletions can be introduced through recombination between distant homologous sequences or between microhomologies in a *recA* dependent manner. This is consistent with rearrangements observed in a previous study where a mRFP gene integrated in the genome was targeted by Cas9 (41). These results are in clear contrast with eukaryotic systems where Cas9 mediated DSB can be repaired by the NHEJ pathway leading to the introduction of small indels. We show here that the NHEJ system from *M. tuberculosis* is able to repair DSB introduced by Cas9 in *E. coli*, albeit with a very low efficiency. The presence of the NHEJ system does not allow cells to tolerate Cas9 cleavage and survive even when a weak target is used. Nonetheless, around 10% of the colonies recovered in the presence of NHEJ carried a small deletion at the target position, the rest of the colonies being mutants of the CRISPR system. Interestingly, the asymmetrical deletions that we obtained were markedly different from what was reported in *Mycobaterium* using I-SceI (30), and indicate a protective role of Cas9 against exonucleases on the DNA end containing the PAM. The possibility to repair Cas9 mediated DSB through NHEJ in *E. coli* paves the way toward the easy introduction of knockout mutations at any locus of interest, provided that the repair efficiency can be improved. This strategy should also make it possible in the future to achieve knockout screens in bacteria similar to those performed in mammalian cells (55,56).

Finally, studying the interplay between DNA repair pathways and CRISPR systems is interesting not just from the perspective of biotechnological applications but also as a way to better understand CRISPR evolution. Indeed, the ability of recBCD or NHEJ to repair Cas9-mediated breaks could make CRISPR-Cas9 interference against phage inefficient. Further studies of the interactions between DNA repair and CRISPR immunity might help understand the

selective pressures that determine the success of absence of CRISPR systems in bacterial species.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENT

We are grateful to the laboratory of Didier Mazel, Benedicte Michel and Jean-Marc Guigo for providing mutant strains of *E. coli* and to Brigitte Gicquel for her gift of *M. tuberculosis* H37Rv genomic DNA. Florence Depardieu and Antoine Vigouroux provided experimental assistance.

FUNDING

Pasteur-Roux Fellowship (to L.C.); French Government's Investissement d'Avenir program; Laboratoire d'Excellence 'Integrative Biology of Emerging Infectious Diseases' [ANR-10-LABX-62-IBEID]. Funding for open access charge: Laboratoire d'Excellence 'Integrative Biology of Emerging Infectious Diseases' [ANR-10-LABX-62-IBEID].
Conflict of interest statement. None declared.

REFERENCES

1. Sorek,R., Lawrence,C.M. and Wiedenheft,B. (2013) CRISPR-mediated adaptive immune systems in bacteria and archaea. *Annu. Rev. Biochem.*, **82**, 237–266.
2. Marraffini,L.A. (2015) CRISPR-Cas immunity in prokaryotes. *Nature*, **526**, 55–61.
3. Hsu,P.D., Lander,E.S. and Zhang,F. (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, **157**, 1262–1278.
4. Deltcheva,E., Chylinski,K., Sharma,C.M., Gonzales,K., Chao,Y., Pirzada,Z.A., Eckert,M.R., Vogel,J. and Charpentier,E. (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*, **471**, 602–607.
5. Jinek,M., Chylinski,K., Fonfara,I., Hauer,M., Doudna,J.A. and Charpentier,E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, **337**, 816–821.
6. Sternberg,S.H., Redding,S., Jinek,M., Greene,E.C. and Doudna,J.A. (2014) DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature*, **507**, 62–67.
7. Jinek,M., Jiang,F., Taylor,D.W., Sternberg,S.H., Kaya,E., Ma,E., Anders,C., Hauer,M., Zhou,K., Lin,S. *et al.* (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science*, **343**, 1247997.
8. Anders,C., Niewoehner,O., Duerst,A. and Jinek,M. (2014) Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature*, **513**, 569–573.
9. Cong,L., Ran,F.A., Cox,D., Lin,S., Barretto,R., Habib,N., Hsu,P.D., Wu,X., Jiang,W., Marraffini,L.A. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, **339**, 819–823.
10. Mali,P., Yang,L., Esvelt,K.M., Aach,J., Guell,M., DiCarlo,J.E., Norville,J.E. and Church,G.M. (2013) RNA-guided human genome engineering via Cas9. *Science*, **339**, 823–826.
11. Bikard,D., Hatoum-Aslan,A., Mucida,D. and Marraffini,L.A. (2012) CRISPR interference can prevent natural transformation and virulence acquisition during in vivo bacterial infection. *Cell Host Microbe*, **12**, 177–186.
12. Shuman,S. and Glickman,M.S. (2007) Bacterial DNA repair by non-homologous end joining. *Nat. Rev. Microbiol.*, **5**, 852–861.
13. Bowater,R. and Doherty,A.J. (2006) Making ends meet: repairing breaks in bacterial DNA by non-homologous end-joining. *PLoS Genet.*, **2**, e8.
14. Tong,Y.J., Charusanti,P., Zhang,L.X., Weber,T. and Lee,S.Y. (2015) CRISPR-Cas9 based engineering of actinomycetal genomes. *ACS Synth. Biol.*, **4**, 1020–1029.
15. Xu,T., Li,Y., Shi,Z., Hemme,C.L., Li,Y., Zhu,Y., Van Nostrand,J.D., He,Z. and Zhou,J. (2015) Efficient genome editing in Clostridium cellulolyticum via CRISPR-Cas9 nickase. *Appl. Environ. Microbiol.*, **81**, 4423–4431.
16. Bikard,D., Euler,C.W., Jiang,W., Nussenzweig,P.M., Goldberg,G.W., Duportet,X., Fischetti,V.A. and Marraffini,L.A. (2014) Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nat. Biotechnol.*, **32**, 1146–1150.
17. Citorik,R.J., Mimee,M. and Lu,T.K. (2014) Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nat. Biotechnol.*, **32**, 1141–1145.
18. Jiang,W., Bikard,D., Cox,D., Zhang,F. and Marraffini,L.A. (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat. Biotechnol.*, **31**, 233–239.
19. Pyne,M.E., Moo-Young,M., Chung,D.A. and Chou,C.P. (2015) Coupling the CRISPR/Cas9 system to lambda Red recombineering enables simplified chromosomal gene replacement in Escherichia coli. *Appl. Environ. Microbio.* , **81**, 5103–5114.
20. Jiang,Y., Chen,B., Duan,C.L., Sun,B.B., Yang,J.J. and Yang,S. (2015) Multigene editing in the Escherichia coli genome via the CRISPR-Cas9 system. *Appl. Environ. Microbiol.*, **81**, 2506–2514.
21. Oh,J.H. and van Pijkeren,J.P. (2014) CRISPR-Cas9-assisted recombineering in Lactobacillus reuteri. *Nucleic Acids Res.*, **42**, e131.
22. Wang,Y., Zhang,Z.T., Seo,S.O., Choi,K.J., Lu,T., Jin,Y.S. and Blaschek,H.P. (2015) Markerless chromosomal gene deletion in Clostridium beijerinckii using CRISPR/Cas9 system. *J. Biotechnol.*, **200**, 1–5.
23. Huang,H., Zheng,G.S., Jiang,W.H., Hu,H.F. and Lu,Y.H. (2015) One-step high-efficiency CRISPR/Cas9-mediated genome editing in Streptomyces. *Acta Bioch Bioph Sin.* , **47**, 231–243.
24. Cobb,R.E., Wang,Y. and Zhao,H. (2014) High-efficiency multiplex genome editing of streptomyces species using an engineered CRISPR/Cas system. *ACS Synth. Biol.* , **4**, 723–728.
25. Kim,H. and Kim,J.S. (2014) A guide to genome engineering with programmable nucleases. *Nat. Rev. Genet.*, **15**, 321–334.
26. Rosenberg,S.M., Shee,C., Frisch,R.L. and Hastings,P.J. (2012) Stress-induced mutation via DNA breaks in Escherichia coli: a molecular mechanism with implications for evolution and medicine. *Bioessays*, **34**, 885–892.
27. Posfai,G., Kolisnychenko,V., Bereczki,Z. and Blattner,F.R. (1999) Markerless gene replacement in Escherichia coli stimulated by a double-strand break in the chromosome. *Nucleic Acids Res.*, **27**, 4409–4415.
28. Pennington,J.M. and Rosenberg,S.M. (2007) Spontaneous DNA breakage in single living Escherichia coli cells. *Nat. Genet.*, **39**, 797–802.
29. Meddows,T.R., Savory,A.P. and Lloyd,R.G. (2004) RecG helicase promotes DNA double-strand break repair. *Mol. Microbiol.*, **52**, 119–132.
30. Gupta,R., Barkan,D., Redelman-Sidi,G., Shuman,S. and Glickman,M.S. (2011) Mycobacteria exploit three genetically distinct DNA double-strand break repair pathways. *Mol. Microbiol.*, **79**, 316–330.
31. Gupta,R., Ryzhikov,M., Koroleva,O., Unciuleac,M., Shuman,S., Korolev,S. and Glickman,M.S. (2013) A dual role for mycobacterial RecO in RecA-dependent homologous recombination and RecA-independent single-strand annealing. *Nucleic Acids Res.*, **41**, 2284–2295.
32. Meddows,T.R., Savory,A.P., Grove,J.I., Moore,T. and Lloyd,R.G. (2005) RecN protein and transcription factor DksA combine to promote faithful recombinational repair of DNA double-strand breaks. *Mol. Microbiol.*, **57**, 97–110.
33. Bierne,H., Seigneur,M., Ehrlich,S.D. and Michel,B. (1997) uvrD mutations enhance tandem repeat deletion in the Escherichia coli chromosome via SOS induction of the RecF recombination pathway. *Mol. Microbiol.*, **26**, 557–567.
34. Gibson,D.G., Young,L., Chuang,R.Y., Venter,J.C., Hutchison,C.A. 3rd and Smith,H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods*, **6**, 343–345.
35. Lutz,R. and Bujard,H. (1997) Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O

and AraC/I1-I2 regulatory elements. *Nucleic Acids Res.*, **25**, 1203–1210.
36. Cormack,B.P., Valdivia,R.H. and Falkow,S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene*, **173**, 33–38.
37. Ferrieres,L., Hemery,G., Nham,T., Guerout,A.M., Mazel,D., Beloin,C. and Ghigo,J.M. (2010) Silent mischief: bacteriophage Mu insertions contaminate products of Escherichia coli random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad- host- range RP4 conjugative machinery. *J. Bacteriol.*, **192**, 6418–6427.
38. Pall,G.S. and Hamilton,A.J. (2008) Improved northern blot method for enhanced detection of small RNA. *Nat. Protoc.*, **3**, 1077–1084.
39. Cole,S.T. (1983) Characterisation of the promoter for the LexA regulated sulA gene of Escherichia coli. *Mol. Gen. Genet.*, **189**, 400–404.
40. St-Pierre,F., Cui,L., Priest,D.G., Endy,D., Dodd,I.B. and Shearwin,K.E. (2013) One-step cloning and chromosomal integration of DNA. *ACS Synth. Biol.*, **2**, 537–541.
41. Qi,L.S., Larson,M.H., Gilbert,L.A., Doudna,J.A., Weissman,J.S., Arkin,A.P. and Lim,W.A. (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*, **152**, 1173–1183.
42. Edgar,R. and Qimron,U. (2010) The Escherichia coli CRISPR system protects from lambda lysogenization, lysogens, and prophage induction. *J. Bacteriol.*, **192**, 6291–6294.
43. Stern,A., Keren,L., Wurtzel,O., Amitai,G. and Sorek,R. (2010) Self-targeting by CRISPR: gene regulation or autoimmunity? *Trends Genet.*, **26**, 335–340.
44. Gomaa,A.A., Klumpe,H.E., Luo,M.L., Selle,K., Barrangou,R. and Beisel,C.L. (2014) Programmable removal of bacterial strains by use of genome-targeting CRISPR-Cas systems. *Mbio*, **5**, doi:10.1128/mBio.00928-13.
45. Bikard,D., Jiang,W., Samai,P., Hochschild,A., Zhang,F. and Marraffini,L.A. (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res.*, **41**, 7429–7437.
46. Sutton,M.D., Smith,B.T., Godoy,V.G. and Walker,G.C. (2000) The SOS response: recent insights into umuDC-dependent mutagenesis and DNA damage tolerance. *Annu. Rev. Genet.*, **34**, 479–497.
47. Mukherjee,A., Cao,C. and Lutkenhaus,J. (1998) Inhibition of FtsZ polymerization by SulA, an inhibitor of septation in Escherichia coli. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 2885–2890.
48. Nurse,P., Zavitz,K.H. and Marians,K.J. (1991) Inactivation of the Escherichia coli priA DNA replication protein induces the SOS response. *J. Bacteriol.*, **173**, 6686–6693.
49. Akroyd,J. and Symonds,N. (1986) Localization of the gam gene of bacteriophage-Mu and characterization of the gene-product. *Gene*, **49**, 273–282.
50. Shee,C., Cox,B.D., Gu,F., Luengas,E.M., Joshi,M.C., Chiu,L.Y., Magnan,D., Halliday,J.A., Frisch,R.L., Gibson,J.L. *et al.* (2013) Engineered proteins detect spontaneous DNA breakage in human and bacterial cells. *Elife*, **2**, e01222.
51. Ton-Hoang,B., Siguier,P., Quentin,Y., Onillon,S., Marty,B., Fichant,G. and Chandler,M. (2012) Structuring the bacterial genome: Y1-transposases associated with REP-BIME sequences. *Nucleic Acids Res.*, **40**, 3596–3609.
52. Kofoid,E., Bergthorsson,U., Slechta,E.S. and Roth,J.R. (2003) Formation of an F' plasmid by recombination between imperfectly repeated chromosomal Rep sequences: a closer look at an old friend (F'(128) pro lac). *J. Bacteriol.*, **185**, 660–663.
53. Malyarchuk,S., Wright,D., Castore,R., Klepper,E., Weiss,B., Doherty,A.J. and Harrison,L. (2007) Expression of Mycobacterium tuberculosis Ku and Ligase D in Escherichia coli results in RecA and RecB-independent DNA end-joining at regions of microhomology. *DNA Repair*, **6**, 1413–1424.
54. Chayot,R., Montagne,B., Mazel,D. and Ricchetti,M. (2010) An end-joining repair mechanism in Escherichia coli. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 2141–2146.
55. Wang,T., Wei,J.J., Sabatini,D.M. and Lander,E.S. (2014) Genetic screens in human cells using the CRISPR-Cas9 system. *Science*, **343**, 80–84.
56. Shalem,O., Sanjana,N.E., Hartenian,E., Shi,X., Scott,D.A., Mikkelsen,T.S., Heckl,D., Ebert,B.L., Root,D.E., Doench,J.G. *et al.* (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*, **343**, 84–87.
57. Doench,J.G., Hartenian,E., Graham,D.B., Tothova,Z., Hegde,M., Smith,I., Sullender,M., Ebert,B.L., Xavier,R.J. and Root,D.E. (2014) Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat. Biotechnol.*, **32**, 1262–1267.
58. Doench,J.G., Fusi,N., Sullender,M., Hegde,M., Vaimberg,E.W., Donovan,K.F., Smith,I., Tothova,Z., Wilen,C., Orchard,R. *et al.* (2016) Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat. Biotechnol.*, **34**, 184–191.