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Managing the SOS Response for Enhanced CRISPR-Cas-Based Recombineering in *E. coli* through Transient Inhibition of Host RecA Activity

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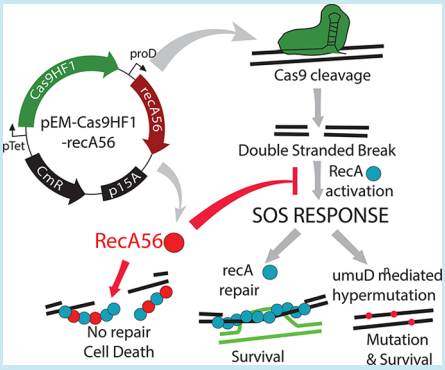
Supporting Information

ABSTRACT: Phage-derived "recombineering" methods are utilized for bacterial genome editing. Recombineering results in a heterogeneous population of modified and unmodified chromosomes, and therefore selection methods, such as CRISPR-Cas9, are required to select for edited clones. Cells can evade CRISPR-Cas-induced cell death through *recA*-mediated induction of the SOS response. The SOS response increases RecA dependent repair as well as mutation rates through induction of the *umuDC* error prone polymerase. As a result, CRISPR-Cas selection is more efficient in *recA* mutants. We report an approach to inhibiting the SOS response and RecA activity through the expression of a mutant dominant negative form of RecA, which incorporates into wild type RecA filaments and inhibits activity. Using a plasmid-based system in which Cas9 and *recA* mutants are coexpressed, we can achieve increased efficiency and consistency of CRISPR-Cas9-mediated selection and recombineering in *E. coli*, while reducing the induction of the SOS response. To date, this approach has been shown to be independent of *recA* genotype and host strain lineage. Using this system, we demonstrate increased CRISPR-Cas selection efficacy with over 10 000 guides covering the *E. coli* chromosome. The use of dominant negative RecA or homologues may be of broad use in bacterial CRISPR-Cas-based genome editing where the SOS pathways are present.



KEYWORDS: *genome editing, recombineering, cas9, RecA, RecA56, SOS*

Escherichia coli is a well-studied model organism with industrial importance in many areas of biotechnology. As such, many tools have been developed to make *E. coli* genetically tractable, including those based on homologous recombination.[1,2] A commonly used and powerful method for homologous recombination is λ-Red "recombineering", which involves induction of three bacteriophage-derived λ-Red proteins, Exonuclease, Beta, and Gamma (Figure 1a),[3,4] to incorporate mutations, deletions, and insertions into the host chromosome in a RecA-independent manner.[5] As Figure 1a illustrates, recombineering inherently produces mixed populations of chromosomes, requiring methods to distinguish edited cells from unedited cells.[6,7]

Recombineering approaches have historically relied on brute-force screening methods or incorporation of selectable markers to identify edited clones. Screening methods, such as PCR, can be laborious in the absence of selection while the use of selectable markers leads to scar sequences or requires further rounds of counterselection to remove and reuse markers.[3,8] As an alternative, Jiang *et al.* demonstrated that the bacterial viral defense mechanism known as CRISPR-Cas could be used to kill bacteria when targeted to genomic sequences and thus could be used to select against wild type cells.[9−12] This easy-to-program, scarless approach has since been refined and applied by a number of laboratories and has exciting potential as a tool for multiplex genome editing and selection.[10,11,13−21] Despite these advances, challenges have remained in the successful and consistent use of CRISPR-Cas9-based genome editing.

A major consequence of CRISPR-Cas-based selection in bacteria is that double stranded DNA breaks (DSBs) are known to induce the SOS response.[15,22,23] The SOS response is a set of global changes leading to not only increases in rates of RecA-dependent repair (utilizing homologous recombination) but also the expression of the error prone *umuDC* polymerase, which can result in greatly increased mutation rates.[22−24] This hypermutation phenotype is likely responsible for CRISPR-Cas "escapes" observed in many genome editing experiments, where mutations in Cas9, targeting guides, and/or the chromosome can lead to the survival of unedited cells.

In addition, complicating the matter, recent work in *E. coli* has shown that gRNA targeting efficacy is also highly variable, with some gRNAs being "ineffective" in killing unedited cells.[13,19] For efficient genome editing, functional and "effective" gRNAs are imperative to selecting cells with the desired modifications. Previous work in eukaryotic cells has

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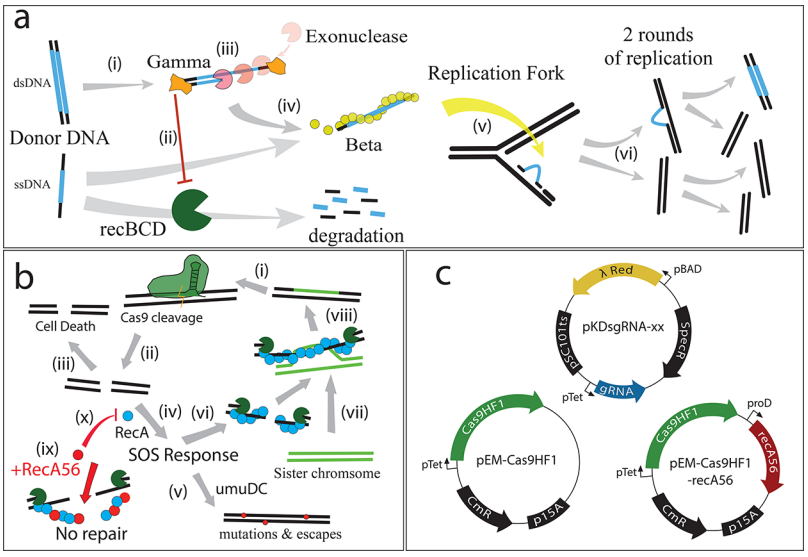

Figure 1. Enhanced CRISPR-Cas9-based Recombineering in *E. coli*. (a) An overview of λ-Red recombineering. (i) Gamma binds the ends of linear dsDNA and inhibits degradation by the host RecBCD nuclease (ii). (iii) Exonuclease degrades dsDNA in a 5′ to 3′ manner, leaving ssDNA to which Beta can then bind (iv). Linear ssDNA can also serve as a donor. (v) Once bound, Beta facilitates recombination by promoting annealing to homologous DNA at genomic replication forks. A mixed population of edited and unedited chromosomes is generated. (b) Model of CRISPR-Cas9-mediated selection. (i) The Cas9 nuclease is directed to the desired target site by a programmable sequence specific guide RNA (gRNA), where it creates double-strand breaks (DSBs) (ii). If DSBs are not repaired, cell death occurs (iii). Alternatively, RecA can activate the SOS Response (iv). The SOS response can induce the mutagenic *umuDC* polymerase leading to mutations and survival (v). In addition, the SOS response increases RecA-dependent homologous recombination, which using sister chromatids as templates (vi−viii), can lead to repair and cell survival. The expression of RecA56 protein inhibits RecA-dependent repair as well as induction of the SOS response, improving the likelihood of cell death. (c) Plasmids used for improved CRISPR-Cas genome editing throughout this study. (Top) gRNA expression plasmids pDsgRNA-xx, containing a temperature sensitive pSC101(ts) origin, arabinose inducible λ-red gene (from the pBAD promoter), a tetracycline inducible gRNA (pTet promoter) and a spectinomycin resistant cassette.[18] (Lower Left) A medium copy plasmid for Cas9 expression pEM-Cas9HF1, containing a p15a origin, a tetracycline (pTet promoter) inducible high fidelity Cas9 variant tagged with a C-terminal ssrA tag, to maintain low expression levels and a chloramphenicol resistance cassette (this study - adapted from Reisch and Prather 2015[18]). (Lower Right) Plasmid pEM-Cas9HF1-recA56, the same as pEM-Cas9HF1, modified for the expression of RecA56 from a high level constitutive proD promoter (this study).[35]

identified numerous gRNA features that may impact Cas9 cleavage.[25−27] Despite this, rules for reliably choosing robust guide sequences are still not fully understood. For example, Xu *et al.* reported that their algorithm was unable to predict 40% of ineffective gRNAs despite being trained from multiple published data sets covering different cell lines and organisms.[27] While gRNA-specific cleavage efficiency, or cutting rate, is one important factor in Cas9-based selection in *E. coli*, in this work we demonstrate the equally important impact of host responses, mainly the SOS response, in evading Cas9 killing. As there exists no NHEJ pathway in bacteria, *E. coli* cells rely on either homologous recombination or mutation to evade CRISPR-Cas-based selection. Homologous recombination involves sister chromosomes as well as RecA and RecBCD proteins to repair DSBs.[28] A rapidly growing exponential phase *E. coli* can have up to 8 copies of its chromosome, providing ample templates for repair.[29] If Cas9 cleavage is efficient and cleaves all available chromosomal copies at the desired location faster than RecA-led chromosomal repair can occur, the cell will eventually die.[15] If, however, repair rates are faster, cells can evade Cas9-based killing (Figure 1b). Alternatively, the accumulation of mutations can either render the CRISPR-Cas system ineffective or generate resistant mutants.

To evaluate the impact of the SOS response and host repair, as well as to further improve Cas9-mediated selection in recombineering experiments, we report the design and validation of a system to transiently inhibit host RecA functions. This is performed using a dominant negative form of RecA (RecA56), which temporarily inhibits RecA activity, DSB repair, and induction of the SOS response (Figure 1b).[30] Mutant *recA* alleles, including *recA56*, produce proteins that bind with wild type RecA monomers in RecA filaments and prevent them from being able to bind to ssDNA and perform repair.[31] To implement this approach, we constructed a plasmid-based system (Figure 1c) enabling coexpression of a "High Fidelity" Cas9 (Cas9HF1) variant, which is tagged for proteolytic degradation *via* a C-terminal ssrA tag, in addition to the expression of the dominant negative RecA56 protein.[18,32]

RESULTS AND DISCUSSION

Recently, Cas9 cleavage in *E. coli* was demonstrated to induce the SOS response and lead to RecA-mediated repair of DSBs.[15] A similar effect has been described with other nucleases targeted to the host genome.[33,34] As a result, Cas9-based selection was improved in *recA* mutants.[15] RecA is not only directly involved in recombination-based repair but also in the cascade of events leading to induction of the SOS response.[22]

We first sought to compare the impact of coexpressing RecA56 (a dominant negative mutant RecA with an R60C mutation) on CRISPR-Cas-based killing to the impact of a complete *recA* knockout.[30] Two promoters were initially evaluated for the expression of *recA56*. A strong constitutive promoter "proD" was found to be more effective than an inducible promoter (refer to Supporting Information, Figure S1).[35] When compared to an in-frame *recA* knockout (from the Keio collection[36]), expression of RecA56 was as effective in preventing RecA-mediated repair (Figure 2) leading to efficient killing.

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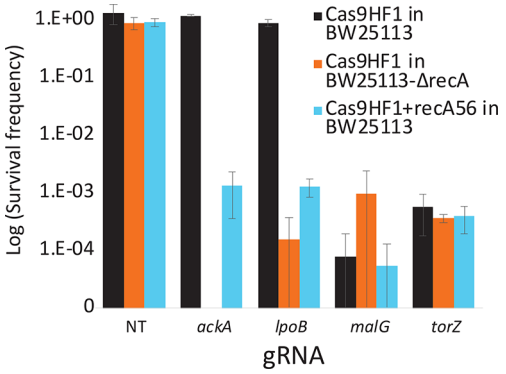

Figure 2. RecA56 expression mimics a recA deletion. A comparison of survival frequency after Cas9 induction in a wild type *recA* host (BW25113, black) a *recA* deletion (orange) and a wild type recA host with coexpression of RecA56 (blue). Several gRNAs were evaluated including a nontargeting control (NT), and gRNAs targeting the following genes: *ack*, *lpoB*, *malG* and *torZ*. Survival frequencies were calculated by comparing colony counts with and without Cas9 induction.

We next sought to compare the impact of coexpressing RecA56 on CRISPR-Cas-based SOS induction. Toward this goal, we leveraged a previously described SOS reporter, based on the promoter for the ColD plasmid-borne *cda* gene.[37] We constructed a GFP-based SOS reporter plasmid and followed fluorescence after the induction of several inducible Cas9 variants. Results are given in Figure 3. In the presence of targeting gRNAs, wild type Cas9 led to an elevated SOS response even in the absence of Cas9 induction, whereas an ssrA-tagged variant had a reduced "leaky" SOS response. This is presumably due to decreased levels of Cas9 attributable to the ssrA degron tag which targets Cas9 for proteolytic degradation by the clpXP protease.[38] This is consistent with previously reported results describing the "toxicity" of Cas9 constructs lacking a degron tag.[18] Interestingly, "escape" behavior was observed with wild type Cas9 constructs lacking an ssrA tag (Figure S2), where SOS induction decreased after a lag phase followed by a recovery of growth. A high-fidelity variant of Cas9 (Cas9HF1, also with an ssrA tag) was also evaluated for its ability to induce the SOS response. As several reports suggest that Cas9HF1 has a slower catalytic activity when compared to the wild type enzyme,[39,40] we expected to see reduced SOS induction. However, at least for the two guides evaluated in this

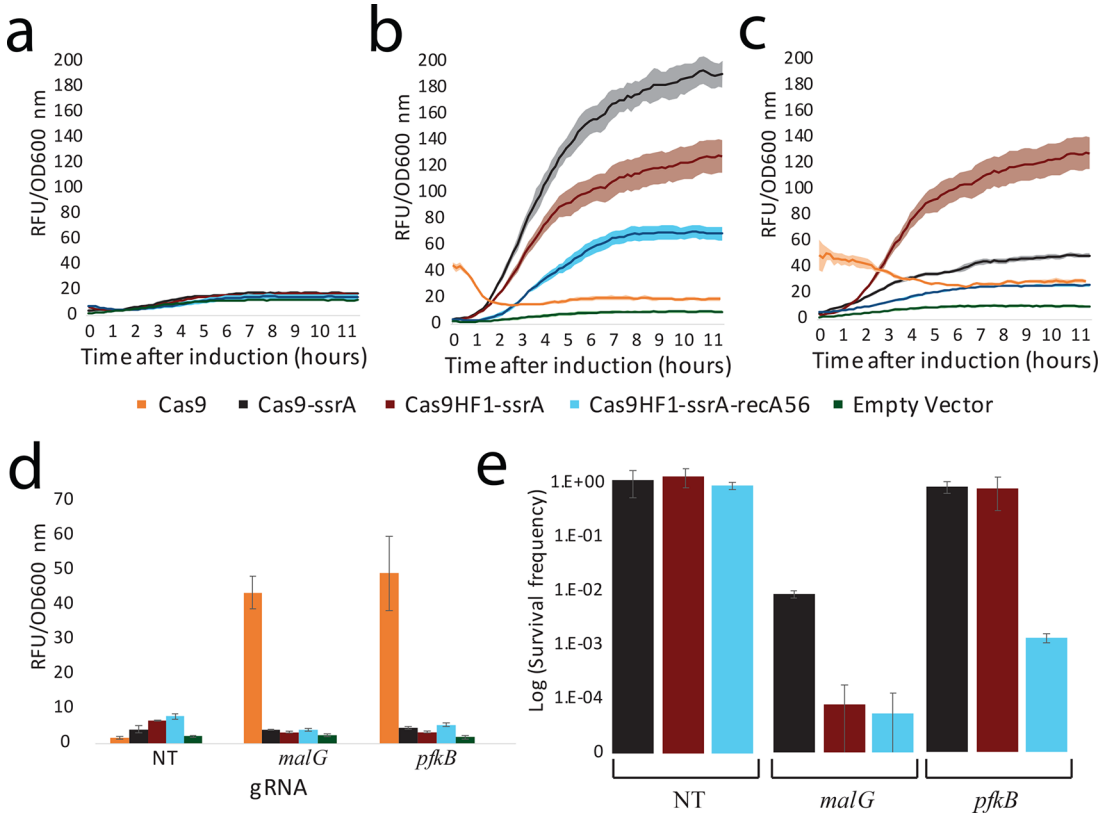

Figure 3. RecA56 expression inhibits the induction of the SOS response. (a–c) A GFP reporter measuring SOS induction was used to assess induction of the SOS response with several Cas9 constructs: empty vector control (green), wild type Cas9 (Cas9, orange), wild type Cas9 with an ssrA tag (Cas9-ssrA, Cas9-CR4 from Reisch and Prather 2015,[18] black), high fidelity Cas9 with an ssrA tag (Cas9HF1-ssrA, red), and high fidelity Cas9 with an ssrA tag, coexpressing the *recA56* allele (Cas9HF1-ssrA-recA56, blue). All Cas9 variants were under the control of the anhydrotetracycline (aTc) inducible pTet promoter. Relative fluorescent units per OD600 nm (RFU/OD600 nm) are plotted as a function of time post induction with aTc. SOS induction with three gRNAs were compared (a) a nontargeting (NT) gRNA, (b) a gRNA targeting the *malG* gene and (c) a gRNA targeting the *pfkB* gene. Shaded areas represent the standard deviation. In the case of wild type Cas9, SOS induction is seen prior to aTc addition. Cells plated at the end of these Cas9 cultures comprised "escapes". (d) The preinduction SOS response was compared for the "NT", "malG" and "pfkB" gRNAs and all Cas9 constructs. (e) Killing efficiency was measured for the ssrA-tagged constructs and all three guides. The *malG* gRNA effectively kills its host with all three constructs, while *pfkB* is ineffective with either Cas9-ssrA or Cas9HF1-ssrA. Killing efficiency is rescued using Cas9HF1-ssrA-recA56.

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experiment, the level of the SOS response was dependent not only on the Cas9 variant but the specific guide as well. We next evaluated the impact of the coexpression of the *recA56* allele on the SOS response. As can be seen in Figure 3, the addition of *recA56* significantly reduced the level of SOS induction. This was expected as previous studies demonstrated that RecA filaments incorporating the RecA56 protein have an impaired protease activity. This protease activity is required to activate LexA, which in turn is needed to induce the SOS response.[22,41]

We next compared killing efficiency or survival with SOS induction levels (Figure 3a–c). While all three Cas9 constructs effectively killed with a gRNA targeting the *malG* gene, ssrA-tagged Cas9 and Cas9HF1 were ineffective at killing using a gRNA targeting the *pfkB* gene. Importantly, the level of SOS induction did not correlate with survival, supporting that the Cas9 variant and/or gRNA specific cutting rates and loci repair kinetics play an important role in gRNA specific selection. The Cas9HF1-recA56 construct recovered killing with the *pfkB* gRNA indicating that reducing repair rates can improve the selection efficiency of "ineffective" guides.

A major advantage of using a plasmid-based system to transiently inhibit RecA is the applicability independent of host strain. To demonstrate this, we evaluated the effectiveness of our construct in improving Cas9-based killing in three commonly used *E. coli* strains: BW25113, MG1655, and BL21(DE3) (Figure 4a–c). In these experiments, cells were first transformed with the desired gRNA plasmid pKDsgRNA-xx[18] containing the gRNA targeted to the site of interest (*i.e.*, pKDsgRNA-malG targets the "malG" gene). They were then transformed with either pEM-Cas9HF1 (a control plasmid expressing Cas9HF1, Figure 1c) or pEM-Cas9HF1-recA56 (coexpressing RecA56, Figure 1c), recovered for 2 h, and plated in serial dilutions to evaluate killing. Using ten gRNAs and a nontargeting control (NT), we demonstrated a high variability between target sites within a single strain as well as variability at the same target site between strains in the presence of normal RecA activity. For example, the *ackA* gRNA appears to be ineffective in BW25113 and MG1655 while efficiently directing Cas9 targeting in BL21(DE3). When coexpressed with *recA56*, all ten gRNAs efficiently cleaved their target sites, leading to host cell death.

When designing these experiments, we wanted to evaluate gRNAs that had been previously published. Reisch *et al.* demonstrated the *ackA* gRNA was "effective" in MG1655 and Bassalo *et al.* observed that the *hisG* gRNA did not result in cell death in BW25113.[13,18] Interestingly, in the absence of *recA56*, using Cas9HF1, we were unable to observe cell death from the *ackA* gRNA in MG1655 or BW25113, although it did kill in BL21(DE3). Furthermore, *hisG* targeting was "effective" in BW25113 and MG1655 but not in BL21(DE3). While the ackA data suggest that the use of Cas9HF1 might be responsible for the lack of cell death, the *hisG* data with Cas9HF1 shows that this is not the complete story. This suggests further variability in gRNA efficacy and guide specific cutting rates, or loci repair rates, between host strains, that may also be Cas9 variant specific. As shown in Figure 3, coexpression of *recA56* rescued the efficacy of not only these two guides across all three strains, but others as well.

Our main goal was to develop a tool for improved CRISPR-Cas-based selection in recombineering. To assess the performance of our construct we selected a gRNA that worked without the *recA56* allele (*malG* in BW25113) and several gRNAs that only worked alongside *recA56*. We tested integration of double-

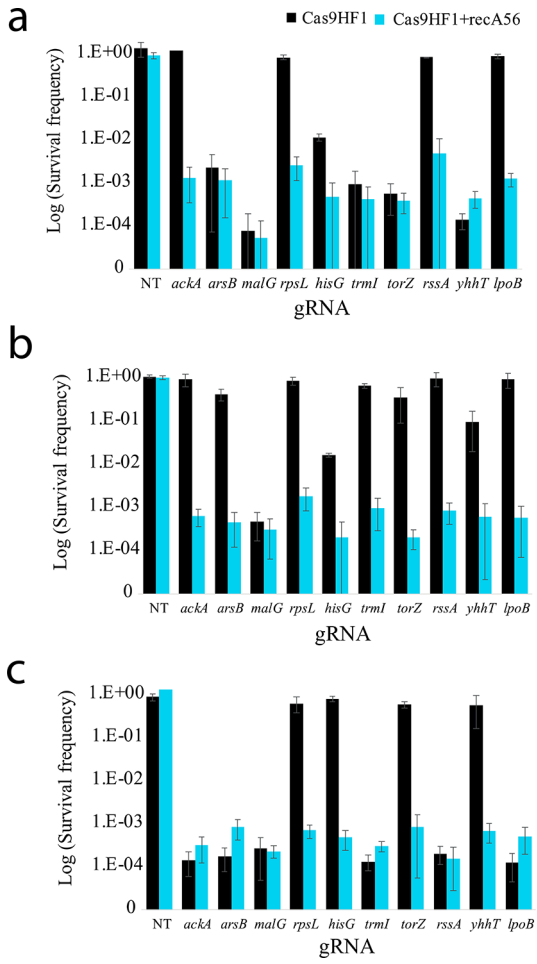

Figure 4. RecA56 improves Cas9 selection independent of host strain A comparison of survival frequency after Cas9 induction in wild type *recA* hosts (a) BW25113, (b) MG1655 and (c) BL21(DE3), with RecA56 (blue) and without RecA56 (black) expression. Several gRNAs were evaluated including a nontargeting control (NT), and gRNAs targeting the following genes: *ack*, *arsB*, *malG*, *rpsL*, *hisG*, *trmI*, *torZ*, *rssA*, *yhhT* and *lpoB*. Survival frequencies were calculated by comparing colony counts with and without Cas9 induction.

stranded DNA (dsDNA) and single-stranded DNA (ssDNA) donors to create deletions of these loci, assessing the impact of *recA56* expression. As shown in Figure 5, in the case of *malG* we see efficient editing with no significant decrease in efficiency when expressing *recA56*. In respect to the "ineffective" gRNAs, we demonstrate the importance of *recA56* coexpression. In the presence of normal RecA-mediated repair, selection is inefficient and leads to a very low editing efficiency. *recA56* expression rescues gRNA efficacy improving selection and thus editing efficiency. Routine use of this approach was validated not only in BW25113 but also strain BL21(DE3), where deletions were constructed in the following genes: *ldhA, sspB, adhE*, and *ompT*. (Refer to Table S1 for primers and donor DNA used in these edits). For routine use of this method, only two colonies are picked after Cas9HF1 induction on plates and evaluated for the desired gene deletion by confirmatory PCR.

We next turned to evaluate the impact of *recA56* expression on Cas9-based selection on a genome-wide scale. Toward this goal, we constructed a library of 12 471 gRNAs targeting the genomes of *E. coli* strains BW25113 and MG1655. After transformation of this library into both strains, the amount of

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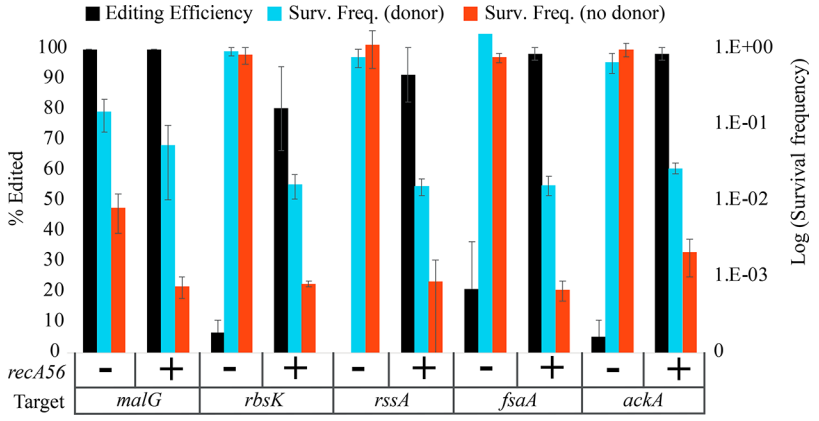

Figure 5. RecA56 improves Recombineering with "ineffective" guides. dsDNA donor was used for in recombineering with CRISPR-Cas9 selection for the deletion of the *malG* and *rbsK*, while ssDNA donor was used for the deletion of *rssA*, *fsaA* and *ackA* genes. A comparison of survival frequency and editing efficiency in BW25113 using an "effective" guide (targeting *malG*) and ineffective guides targeting *rbsK*, *rssA*, *fsaA* and *ackA*, with and without RecA56 expression. Survival frequencies were calculated by comparing colony counts with and without Cas9 induction with and without the addition of donor DNA. Editing efficiencies were calculated on the basis of colony PCR of surviving clones, to confirm gene deletions.

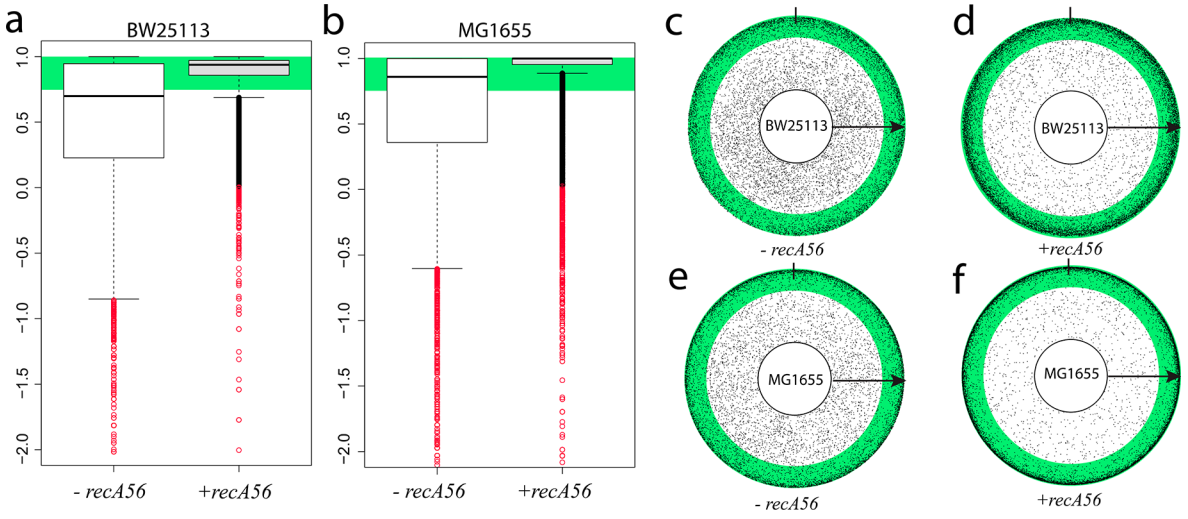

Figure 6. RecA56 improves gRNA effectiveness genome-wide. Box plots represent the Cas9 killing efficiency of a library of 12 471 gRNAs targeting the chromosome of *E. coli* strains BW25113 (a) and MG1655 (b) with and without the expression of recA56. Red dots indicate guides whose frequencies increase after Cas9 induction, indicating no Cas9 cutting. Circular genome-wide plots show the impact of RecA56 expression on gRNA killing efficiencies for all 12 471 guides positioned around the chromosomes of *E. coli* strains BW25113 (c *vs* d) and MG1655 (e *vs* f). The chromosomal target of each gRNA (black dot) is represented by the clockwise distance from the start of the genome (12 o'clock hash mark). The killing efficiency for each gRNA is plotted from 0 to 1, from the inner circle to the outer circle (arrow). gRNAs with killing efficiencies <0 were assigned values of zero (at the inner circle) for visualization purposes. The green bands indicate "effective" gRNAs with killing efficiencies >0.75.

each gRNA sequence within the library pool (counts) was tracked pre- and post-Cas9HF1 induction and selection using next generation sequencing. Killing efficiencies were calculated-based on changes in gRNA levels pre- and postinduction as compared to a nontargeting control. A killing efficiency of 1 indicates 100% killing (*i.e.*, complete gRNA depletion from the library after selection), whereas killing efficiencies <0 indicate a higher number of reads postselection, or propagation and survival of the gRNA. (Refer to Materials and Methods for calculation of killing efficiencies). Experiments were performed to compare selection with Cas9HF1 alone and in combination with expression of *recA56*. As Figure 6 demonstrates, expression of *recA56* significantly improves the proportion of all gRNA sequences "effective" in killing, which we defined as a killing efficiency >0.75 (Figure 6a and b, refer to Materials and Methods for cutoff determination). To directly compare library killing efficiencies with the survival frequencies of gRNAs when tested individually, a subset of library guides were evaluated in individual killing experiments. These data are given in Table 1. Improved selection was uniformly distributed along the chromosome of both strains (Figure 6c–f), with no obvious location dependence. On average, in both strains, the expression of *recA56* resulted in an increase in the number of "effective" gRNAs. In MG1655, out of a total of 12 471 gRNAs tested, 6517 were considered "effective" with Cas9HF1 expression alone, while 10 238 were "effective" in the presence of *recA56*. The impact in BW25113 was more dramatic with "effective" gRNA increasing from 5656 to 10 235. These results represent a set of ∼10 200 usable gRNA sequences at a resolution of ∼400 bp around the *E. coli* chromosome, which have been demonstrated to be "effective" for Cas9-based selection in two host strains of *E. coli*. These gRNAs are provided as a practical starting point for those planning future

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Table 1. Comparison of Library Killing Efficiencies with Single Experiment Survival Frequencies[a]

	single guide survival frequency		library killing efficiency	
gRNA	Cas9HF1	Cas9HF1-recA56	Cas9HF1	Cas9HF1-recA56
pfkB-2	0.80018	0.94302	−0.118	0.300
fsaA	0.78459	0.00068	0.195	0.834
pfkB	0.764363	0.001296	0.220	0.900
rssA	0.85172	0.00498	0.502	0.946
ackA	1.20096	0.00135	0.698	0.866
lpoB	0.89543	0.00129	0.713	0.951
trmI	0.00095	0.00042	0.767	0.965
malG	0.00008	0.00006	0.882	0.866
yhhT	0.00014	0.00045	0.884	0.975
hisG	0.01186	0.00048	0.917	0.895
torZ	0.00058	0.00040	0.982	0.986

[a]All data from strain BW25113.

CRISPR-Cas9-based genome editing experiments in *E. coli* (Table S2).

In conclusion, we have developed a plasmid system for efficient and robust (consistent) CRISPR-Cas9-mediated recombineering in *E. coli*. This system is reliant on the transient overexpression of the dominant negative mutant *recA56* allele. This approach not only inhibits the competitive repair process responsible for cell survival but also minimizes the induction of the SOS response, differentiating this approach from prior methods. The SOS response is known to lead to increased mutations rates, providing additional routes for potential escapes. This method simplifies and improves CRISPR-Cas9-based genome engineering in potentially any *E. coli* host, highlighting the importance of competitive repair pathways and induction of the SOS response in optimizing CRISPR-Cas-based selection. These results indicate that observations of "ineffective" gRNA sequences are a consequence of the summation of not only cutting rates but also rates of repair, which can vary with Cas9 variant, specific gRNA sequence, and even host strain. "Ineffective" gRNA sequences can, in most cases, be made "effective" by inhibiting host repair. However, we still observe certain gRNAs that are "ineffective" in CRISPR-Cas9-based selection. For example, a second gRNA targeting the *pfkB* gene (pfkB-2) was found to result in nearly 100% survival even in the presence of *recA56* (Figure S3). The determinants and mechanisms explaining these remaining "ineffective" gRNA sequences are an area for future study.

Other studies have attempted to inhibit host recombination to improve CRIPSR-Cas9-based editing, specifically through overexpression of the λ-Red Gamma protein or *E. coli* RecX protein.[15,42] The λ-Red Gamma protein binds to dsDNA breaks inhibiting the RecBCD nuclease, preventing both ssDNA and RecA filament formation, which should result in inhibition of both recombination-based repair as well as SOS induction. Unfortunately, Gamma overexpression was reported to have toxic effects minimizing the potential of this approach.[15] The *E. coli* RecX protein is a native inhibitor of RecA activity, normally involved in turning off the SOS response.[43,44] As a result, the *recX* gene is normally induced as part of the SOS response.[43,45] Despite its ability to inhibit RecA, RecX has been shown to have no impact on SOS induction.[46] Dominant negative *recA* alleles, such as *recA56*, may be more suited to inhibit host repair processes as they not only inhibit RecA-mediated recombination, but also minimize SOS induction. In the future, additional strategies targeting other components of the SOS response, potentially including *lexA* and *dinB*, could be considered.

The current approach overcomes many of the practical issues arising from factors impacting strain-to-strain and experimental variability of CRISPR-Cas9-based selection in *recA* proficient hosts. This variability is presumably due to differences in host strain and guide specific SOS induction, *recA*-mediated repair rates, Cas9 expression levels and cutting rates, all of which can vary greatly between experimental protocols. Specifically, our results suggest that in most cases, minimizing the competitive repair pathways will enable efficient selection using most gRNAs. Specific genome editing protocols and the order of "cutting" and "pasting" must also be considered for optimal results. The longer Cas enzymes are induced, the more likely escapes will be observed due to the accumulation of mutations. Prolonged expression should be avoided particularly when multiple rounds of edits are desired. The potential accumulation of unwanted mutations may be of even more concern when multiplexing or performing multiple rounds of edits. It is advisable to edit or "paste" donor DNA prior to selection with CRISPR-Cas when possible.

While this work has focused on improving CRISPR-Cas-based selection of edited clones, the importance of recombineering efficiency cannot be overlooked. If recombineering or the "pasting" of donor DNA is inefficient (a frequency below $\sim 10^{-4}$, see Figure 5) unedited clones may still dominate the small numbers of surviving colonies. In our hands, it is good practice to perform parallel editing experiments both with and without donor DNA. Significant increases in total colony counts in the presence of donor indicate successful recombineering and a high likelihood of obtaining a desired edit. When total colony counts with donor are no more than a control without donor DNA, failed recombineering is likely.

Furthermore, *recA* and the SOS response are ubiquitous in bacteria. The current plasmid-based system can serve as a starting point for broader host range CRISPR-Cas genome editing tools utilizing previously reported broad host range expression constructs.[47] As Cas9HF1 has been shown to reduce off-target cutting, the use of this variant or newer high fidelity Cas9 variants may be desired.[32] Recently, 35% of all experimentally validated off-target sites were shown to have >4 bp mismatches in the gRNA.[48] By this definition, almost all of the guides evaluated in our *E. coli* gRNA library have at least one off-target site for wild type Cas9. Using Cas OFFinder[49] we evaluated the number potential off-target sites for all of the gRNAs individually tested in this work (see Table S3) . Although off-target cutting rates are slower than target cutting rates, the potential impact of off-targets may be greater in bacteria and other microbial species with larger genomes.

In addition, RecA has key homologues in every kingdom of life, suggesting the potential utility of using dominant negative RecA homologues in any species with homologous recombination-based repair.[50,51] In eukaryotes, such as diploid *S. cerevisiae* strains, recombination-based repair may compete with CRISPR-Cas cleavage in organisms where RAD51 takes on the function of *recA*.[52] Fortuitously, a dominant negative RAD51 mutant (RAD51-K191A) has already been identified.[53] In all cases, careful consideration of host responses to CRISPR-Cas-based selection will likely improve future genome editing tools.

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MATERIALS AND METHODS

Reagents and Media. Low salt Luria broth (LB Lennox, Genesee Scientific, San Diego, CA) was used for cloning and strain propagations in both liquid cultures and agar plates. Other reagents were obtained from Sigma-Aldrich (St. Louis, MO). Antibiotic concentrations, unless otherwise stated, were as follows: chloramphenicol (35 μg/mL), spectinomycin (50 μg/mL), kanamycin (35 μg/mL). Anhydrotetracycline (aTc; used to induce Cas9 and gRNA expression) was prepared in filtered 70% ethanol at a 1000× stock and used at a final working concentration of 100 ng/L. Arabinose (used to induce λ-Red expression) was added at a final concentration of 7.5 g/L.

Strains. Strain *E. cloni* (Lucigen, Middleton WI) was used for cloning and plasmid propagation. Strains BW25113, MG1655, and JW2669−1, an in-frame recA deletion mutant of strain BW25113 from the Keio Collection, were obtained from the Yale Coli Genetic Stock Center (http://cgsc.biology.yale.edu).[36] Strain BL21(DE3) was obtained from ThermoFisher Scientific (Cat. #C600003).

Plasmids. Plasmids pCas9-CR4 and pKDsgRNA (AddGene #62655 and # 62656) were used as starting constructs.[18] Plasmid pKDsgRNA-ack was used as a template for making new gRNA plasmids. gRNA sequences were cloned through Q5 Site-Directed Mutagenesis Kit from New England BioLabs, per manufacturer's instructions. All primers and DNA used in this study are listed in Table S1. The same reverse primer (EM01) was used for these reactions. The forward primer was designed as 5′-[20 bp spacer sequence]-GTTTTAGAGCTAGAAATAGCAAG-3′ (EM02-EM19). pCas9-CR4, which contains an aTc induced promoter (pTet) to drive expression of both *cas9*, was used as a template to create pEM-Cas9HF1 and pEM-Cas9noSsrA. For pEM-Cas9HF1, "High fidelity" changes (N497A/R661A/Q695A/Q926A) were made to reduce off-target effects,[32] using Q5 Mutagenesis. RecA56, purchased as a synthetic gBlock from IDT (Coralville, IA), was subsequently cloned behind pEM-Cas9HF1 using NEB HiFi DNA Assembly (Catalog #E2621S) to create pEM-Cas9HF1-indRecA56. pEM-Cas9HF1 was linearized by PCR prior to cloning (Primers EM30/31 in Table S1). pEM-Cas9HF1-recA56 was then created by inserting the proD promoter *via* Q5 Mutagenesis (EM32/33 Table S1). pSMART-SOS-GFPuv was ordered as a plasmid from Twist Biosciences (San Francisco, CA). The empty vector control used in the SOS response assay was pACYC184 (Novagen, Inc., Madison, WI). Sequencing was performed on purified plasmid DNA by Eton Biosciences (Research Triangle Park, NC) and Eurofins Genomics (Louisville, KY). The following plasmids are available on AddGene: pEM-Cas9HF1 (AddGene #89961), pEM-Cas9FH1-recA56 (AddGene #89962), pEM-Cas9HF1-indRecA56 (AddGene #102294), pEM-Cas9noSsrA (AddGene #102285), pSMART-SOS-GFPuv (AddGene #102283), as well as all gRNA plasmids (AddGene #89951−88960, #102284, #102286−102291).

SOS Response Assays. Using *E. coli* strain BW25113, cells were transformed with pSMART-SOS-GFPuv alongside the appropriate Cas9, gRNA, and empty vector constructs. Cells were inoculated into 5 mL LB with appropriate antibiotics and grown to OD600 > 2. Cells were concentrated to OD600 = 10 and diluted in half with 10% glycerol to OD600 = 5. These samples were either frozen for future use or used right away. To compare pEM-Cas9noSsrA, pCas9-CR4, pEM-Cas9HF1, and pEM-Cas9HF1-recA56, growth and fluorescence were obtained in a BioLector (m2p-labs, Baesweiler, Germany) using a high mass transfer FlowerPlate (CAT#: MTP-48-BOH, m2p-labs, Germany). For pCas9-CR4, pEM-Cas9HF1, and pEM-Cas9HF1-recA56, 16 μL of the OD normalized culture was inoculated into 784 μL of LB with appropriate antibiotics. Due to challenges growing cells containing pEM-Cas9noSsrA, seeds were made by scraping colonies from plates into 5 mL LB with appropriate antibiotics. After 3 h at 30 °C, cells were concentrated to OD600 = 5 and used right away. For these samples, 75 μL of OD normalized culture was inoculated into 725 μL of LB with appropriate antibiotics. BioLector settings were as follows: Biomass gain = 20, GFPuv gain = 50, shaking speed = 1300 rpm, temperature = 30 °C, and humidity = 85%. For induction of the Cas9 and gRNA constructs, 0.8 μL of stock aTc was added in exponential phase, OD600 between 1.0 and 2.5. For pEM-Cas9noSsrA, 0.8 μL of stock aTc was added 3.5 h after inoculation. Biomass and GFP fluorescence were smoothed using a three-point moving average. BioLector biomass readings (light scattering) were corrected to OD600 using the following formula:

$$\mathrm{OD600}_t = (\mathrm{OD600}^*_t - \mathrm{OD600}^*_{to}) \times \frac{(\mathrm{OD600}_{tf} - \mathrm{OD600}_{to})}{(\mathrm{OD600}^*_{tf} - \mathrm{OD600}^*_{to})} + 0.25$$

where OD600 refers to an offline measurement and OD600* refers to BioLector biomass readings. *to* is the original time point, *tf* is the final measurement, and *t* is the individual measurement being converted. Data are presented as RFU per OD600 and are averages of triplicate experiments.

Cas9 Killing Assays. For each strain, cells were transformed with pKDsgRNA-xx containing the desired gRNA. pKDsgRNA-xx is temperature sensitive and grows at 30 °C so all subsequent experiments were performed at 30 °C. The cells were grown to midexponential phase OD600 ~0.5, concentrated 50 times, and made electrocompetent following standard protocols.[54] Electrocompetent cells were then transformed with either pEM-Cas9HF1 or pEM-Cas9HF1-recA56, recovered for 2 h in low salt LB, and plated in serial dilutions on both chloramphenicol and spectinomycin. For Cas9 induction, plates also contained aTc. Survival frequency was calculated by counting colony forming units (CFUs) on plates with aTc and dividing by the number of CFUs on plates without aTc. Survival frequencies were averaged over three transformations.

Recombineering. Recombineering was performed with arabinose-induced λ-Red on the plasmid pKDsgRNA-xx following published protocols.[18] Cells were transformed with the pKDsgRNA-xx containing the gRNA targeting the part of the genome to be modified or deleted. As before, these cells were grown to midexponential phase OD600 ~0.5. λ-Red expression was induced by addition of arabinose to the culture at a final concentration of 7.5 g/L. Cells continued to grow for 20 min following addition of arabinose. Cells were then made electrocompetent and concentrated 50 times. After cells were prepared, they were cotransformed with donor DNA (200 ng of double-stranded DNA or a final concentration of 20 μM of oligonucleotides) and either pEM-Cas9HF1 or pEM-Cas9HF1-recA56. They were plated as described earlier on *Cm*/Spec or *Cm*/Spec/aTc. Survival frequency was calculated from CFUs as described previously. Editing efficiency was calculated-based on results confirmed by colony PCR of 21−24 colonies per transformation and then averaged over 3 transformations.

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(Refer to Supporting Information Table 1, for confirmation primers used for colony PCR).

Guide Library Design and Construction. A guide library was generated from the *E. coli* genome, which contains over 540 000 Cas9 PAM sites ("NGG"), of which approximately 530 000 are adjacent to unique 20 bp target sequences. From these sequences, 12 471 unique gRNA were selected by applying three key criteria, including: (1) Guides must have a unique "seed" sequence (defined as the 12 bp most adjacent to the 3′ end of the guide). (2) Guides chosen were a minimum of 320 bp away from the nearest target site of any other guide in the library. (3) Each guide chosen was present and unique in both *E. coli* strains BW25113 and MG1655. The library was constructed by adapting established protocols.[55] A BsmBI site, overhang (underlined), was added onto the ends of each spacer sequence, and primer binding sites were added as follows:

5′-AGGCACTTGCTCGTACGACGCGTCTCAGCAC-[20 bp spacer sequence]-GTTTCGAGACGATGTGGG-CCCGGCACCTTAA-3′.

These guide sequences were ordered as a single library of 12 471 oligos from Twist Biosciences (San Francisco, CA). The pooled library was PCR amplified using EM30 and EM31. pKDsgRNA-NT was designed as both a nontargeting control and a cloning vector containing BsmBI sites to insert the gRNA library. Golden Gate Assembly was performed to ligate the amplified gRNA pool into the pKDsgRNA-NT backbone. Briefly, Esp3I (Thermo Fisher Scientific Cat# ER0451) was used in the cleavage step while T4 Ligase (New England BioLabs, # M0202S) was used for ligation. The library was then transformed into Lucigen's 10G Elite electrocompetent *E. coli*. The Carbon-Clarke equation was used to calculate how many colonies were needed to ensure a 99% confidence that all guides in the library was represented after each transformation (~60 000 colonies).[56] Colonies were scraped and midi-prepped (Zyppy Plasmid Midiprep Kit, Zymo Research, Irvine, CA) in preparation to be transformed into the desired strains.

Library Selection. BW25113 and MG1655 electrocompetent cells were transformed with the purified library plasmid pool. Again, enough transformations were done to ensure greater than 99% confidence that all sequences were present. Cells from six transformations were scraped into LB, diluted to OD600 0.2, and grown back up to OD600 0.6. In parallel, control plasmid pKDsgRNA-NT, pKDsgRNA-ack, and pKDsgRNA-malG were grown to OD600 ~0.5 and each mixed into the library at 2.5% of the total cells by OD. These guides were chosen as a nontargeting guide, a guide that only works with recA56, and a guide that works without recA56, respectively. The library and controls were then made electrocompetent and transformed with either pEM-Cas9HF1 or pEM-Cas9HF1-recA56. Transformations were recovered for 2 h and plated half on *Cm*/Spec and half on *Cm*/Spec/aTc. Enough transformations were done to ensure 99% confidence that all sequences were present on *Cm*/Spec. Plasmids were purified as described earlier.

Next Generation Sequencing. gRNA sequences for each sample were amplified from the purified plasmid preps as described previously.[55] Amplification was done using Q5 Hot Start Polymerase (NEB, M0493L) in one 50 μL reaction per sample. Amplification was performed following manufacturer's instructions, using 20 cycles at an annealing temperature of 60 °C. Primer EM32 was used along with barcoded reverse primers as follows (EM33-EM40): 5′-CAAGCAGAAGAC-GGCATACGAGAT-[6 bp barcode sequence]-GACTCG-GTGCCACTTTTTCAA-3′. (See Table S1 for full sequences, including barcodes). The amplified samples were purified using gel extraction (QIAquick Gel Extraction Kit, Qiagen). Each sample was quantified after purification with the Qubit dsDNA High Sensitivity assay kit (Thermo Fisher, Q32854). Samples were pooled and sequenced on a MiSeq (Illumina) with 21-bp paired-end sequencing using custom read and index primers EM41-EM43.

Data Processing and Analysis. We aligned NGS output FASTQ files to custom indexes (generated from the bowtie2-build function) using Bowtie 2,[57] with the options −p 32 −end-to-end −very-sensitive −3 1 −I 0 −X 200. Counts for each gRNA in the population were extracted and used for further analysis. First, expected gRNA counts were calculated by measuring the enrichment of the nontargeting spike in control gRNA (which was set to 100% survival, 0 killing). The difference between expected and observed counts was then divided by the expected counts to calculate killing efficiency. We defined "effective" gRNA as having a killing efficiency >0.75. This value is based on a comparison of measured gRNA killing efficiency in the library experiments with the performance of gRNAs evaluated individually (Figures 2, 3 and 4). The lowest killing efficiency of any gRNA that was "effective" in individual experiments was 0.76. gRNAs with killing efficiencies lower than 0.75 may still be useful.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00174.

Table S1: Primers and Plasmids used in this study (XLSX)
Table S2: Guide RNA Killing Efficiency (XLSX)
Table S3: Potential Off-Targets for Individually Evaluated gRNAs (XLSX)
Figure S1: Comparison of inducible and constitutive *recA56* constructs (PDF)
Figure S2: Cas9 mediated escape (PDF)
Figure S3: An example ineffective gRNA (PDF)

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B. Hoover, A. Yaseen, N. Valyasevi, Z. Roecker, R. Menacho-Melgar, and E. Moreb performed killing/selection and editing experiments. E. Moreb performed SOS induction experiments. E. Moreb and M. Lynch designed experiments and analyzed results. E. Moreb and M. Lynch prepared the manuscript.

Notes

The authors declare the following competing financial interest(s): Patent applications have been filed by authors.

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