Synthetic genome defenses against selfish DNA elements stabilize engineered bacteria against evolutionary failure

Peng Geng, Sean P. Leonard, Dennis M. Mishler, Jeffrey E. Barrick*

Department of Molecular Biosciences, Center for Systems and Synthetic Biology, The University of Texas at Austin, Austin, TX 78712, USA

***Corresponding Author**

Jeffrey E. Barrick

2500 Speedway A5000

The University of Texas at Austin

Austin, TX 78712

Phone: +1 (512) 471-3247

Email: jbarrick@cm.utexas.edu

Abstract

Mobile genetic elements drive evolution by disrupting genes and rearranging genomes. Eukaryotes have evolved epigenetic mechanisms, including DNA methylation and RNA interference, that silence mobile elements and thereby preserve the integrity of their genomes. We created an artificial reprogrammable epigenetic system based on CRISPR interference to give engineered bacteria a similar line of defense against transposons and other selfish elements in their genomes. We demonstrate that this CRISPR interference against mobile elements (CRISPRi-ME) approach can be used to simultaneously repress two different transposon families in *Escherichia coli*, thereby increasing the evolutionary stability of costly protein expression. We further show that silencing a transposon in *Acinetobacter baylyi* ADP1 reduces mutation rates by a factor of five, nearly as much as deleting all copies of this element from its genome. By deploying CRISPRi-ME on a broad-host-range vector we have created a generalizable platform for stabilizing the genomes of engineered bacterial cells for applications in metabolic engineering and synthetic biology.

Keywords: genome stability, insertion sequence, selfish DNA, reduced mutation cell

Unwanted evolution is a foundational challenge for the many areas of biotechnology that rely on genetically engineered organisms.[1–3] Engineered cells are often less fit than their wild-type progenitors because they divert resources away from cellular replication or otherwise perturb normal physiological processes.[4] Mutations will spontaneously arise in the genomes of some cells in a population that disrupt a DNA-encoded function. Cells with these 'failure mutations' often have a significant competitive growth advantage because the engineered burden has been lifted. They will out-replicate the original engineered cells, resulting in a progressive reduction in the performance of the cell population over time.[5–8] When the aim is to maximize the production of a recombinant protein or a chemical product, these evolutionary failure modes will reduce yields and limit the useful lifetimes of engineered cells. Genetic instability due to evolution is particularly a problem if there is a large fitness burden for the engineered function during the many cell divisions needed to scale an industrial process up to a large bioreactor.[9]

Simple transposons known as insertion sequence (IS) elements are the dominant source of failure mutations in many engineered bacterial cells.[5–8] IS elements are minimal selfish DNA elements: they may consist of just a single transposase gene flanked by inverted repeats.[10] They can cause mutations directly, when new IS copies insert into a target DNA site by cut-and-paste or copy-and-paste mechanisms, and indirectly, when recombination between multiple copies of the same IS element leads to deletions or genome rearrangements. Nicking or cleavage of the chromosome by transposases may also induce mutagenic DNA damage responses,[11] and transposase binding to the β sliding clamp of the DNA polymerase holoenzyme[12] has the potential to decrease the fidelity of DNA replication. IS elements can rapidly proliferate within genomes, and they can invade new cells when they are incorporated into DNA elements that mediate horizontal gene transfer, such as conjugative plasmids. Thus, many bacterial genomes harbor

multiple copies of several different IS families.[13,14]

If one could silence gene expression from all IS elements that are present in a bacterial host, it would be expected to significantly improve the stability of an engineered function in that cell. Yeast and other eukaryotes have evolved a wide array of epigenetic mechanisms—including DNA methylation, chromatin remodeling, and RNAi—that protect the integrity of their genomes.[15] These pathways operate in a flexible manner that enables them to simultaneously silence diverse families of established selfish elements and adapt to newly arrived elements. Many bacteria have defenses, such as RNA-guided nucleases (e.g., CRISPR-Cas9) and restriction-modification systems, that protect their genomes from invasions of new phages, plasmids, and mobile genetic elements.[16] However, bacteria do not have a general capacity to silence selfish elements after they have become entrenched in their genomes that is akin to what takes place in eukaryotes.

Perhaps due to this limitation, many IS elements in bacteria have evolved regulatory mechanisms that repress their own activity.[17,18] Presumably, this self-limiting strategy evolved to prevent an IS element from endangering its own survival by overly proliferating within the host genome and causing deleterious mutations in an entire cell population. For example, an antisense RNA is transcribed from the IS*10* transposase reading frame that binds to and inhibits translation of a transposase mRNA produced by the same or any other copy of IS*10* in the genome. Host factors also impact transposition.[17,18] For instance, *dam* methylation inhibits IS*10* transposition, and the nucleoid-like protein IHF facilitates IS*10* transposition. These interactions often modulate transposition so that it is restricted to certain times during DNA replication or to when cells experience stress. These regulatory interactions may represent 'domestication' of an IS element such that there is indirect selection to maintain its activity in a bacterial genome because it increases the rates of certain types of beneficial mutations,[19,20] including those that disrupt

burdensome plasmids and transgenes added to the genome by human engineering.

Here we describe a reprogrammable plasmid system that prevents evolutionary failures caused by transposons and other mobile elements in genetically engineered bacterial genomes. Our system takes advantage of recently developed CRISPR interference methods[21] and a broad-host-range plasmid backbone[22] that both function in a wide range of bacterial species. Because CRISPR interference can be programmed to bind to and repress specific DNA sequences, one can silence all copies of an active transposon or other selfish element family in a bacterial host genome by adding a single guide RNA to the plasmid. We show that our system for CRISPR interference with mobile elements (CRISPRi-ME) reduces mutation rates in both *Escherichia coli* TOP10 and A*cinetobacter baylyi* ADP1 cells. CRISPRi-ME can be used to give diverse bacterial species a new system of epigenetic protection against pervasive genomic parasites that cause mutations, thereby improving the reliability of genetically engineered versions of these cells.

Results

CRISPR interference from a broad-host range vector. The CRISPRi-ME system uses one or more small guide RNAs (sgRNAs) to target a catalytically dead Cas9 nuclease (dCas9) to bind to specific DNA sequences that silence mobile elements (**Fig. 1**). To implement CRISPRi-ME we started with a well-characterized CRISPR interference (CRISPRi) system that we had previously ported onto a broad-host-range plasmid vector.[22] The basic CRISPRi design is derived from plasmids pTargetF and pCas, which were designed for efficient multiplex genome editing in Enterobacteria.[23] We substituted a dCas9 gene with the canonical deactivating mutations into this system.[21,24] Then, we added these components to a plasmid backbone derived from the broad-host range expression vector pMMB67EH.[25] It contains a low- to medium-copy-number (10-20

plasmids per cell) RSF1010-derived origin of replication that has been shown to function robustly in diverse bacterial species,[26] including *Pasteurella multocida*,[27] *Pseudomonas aeruginosa*,[28] *Acinetobacter baumanni*,[29,30] and *Snodgrassella alvi*.[22] The pMMB67EH backbone also contains an origin of transfer (*oriT*) that enables this plasmid to be conjugated into recipient cells.

To create a customized CRISPRi-ME system (**Fig. 2**), one starts with a plasmid with a single sgRNA targeting unit, a plasmid with the dCas9 transcriptional unit, and a plasmid with the pMMB67EH backbone. Different promoters may be needed to drive transcription of the sgRNA and dCas9 genes to achieve optimal function in different bacterial species, as described in the following sections. Assembly of a CRISPRi-ME plasmid proceeds by first creating one or more variants of the sgRNA plasmid with spacer sequences that target the 5′ end of an essential open reading frame in each mobile element (e.g., IS element transposase) (**Step 1**). These sgRNA modules are joined together into a multiple sgRNA targeting cassette plasmid when one wants to target more than one mobile element family for repression (**Step 2**). Then, all sgRNAs, the dCas9 transcriptional unit, and the pMMB67EH backbone are combined in a final assembly step (**Step 3**). The final CRISPRi-ME plasmid can be purified and transformed into the bacterium of interest or directly transferred into a recipient cell from an *E. coli* strain that encodes the required conjugation machinery (e.g., MFDpir).[31]

CRISPRi-ME reduces mutation rates in *E. coli*. We first tested how effective our CRISPRi system was at silencing gene expression in *E. coli.* We used previously validated promoters to drive expression of each CRISPRi component: the native *Streptococcus pyogenes* Cas9 promoter for dCas9 and the constitutive synthetic promoter pJ23119 for each sgRNA (**Fig. 3a**).[21–23,32] To test the function of this CRISPRi system on the pMMB67EH plasmid backbone, superfolder GFP (sfGFP) under the control of the native *glpT* promoter was integrated into the genome of *E. coli*

TOP10 to create a reporter strain. This CRISPRi configuration strongly repressed expression of sfGFP from the chromosome of this strain when an sgRNA targeting this gene was used (>90%) whereas there was no repression with an off-target sgRNA (**Fig. 3b**).

We next tested whether CRISPRi-ME could reduce the rates of inactivating mutations in the *E. coli* chromosome. We examined the rates of mutations yielding D-cycloserine resistance (DCS^R) in cells cultured in LB, which can occur due to inactivating mutations in several *E. coli* genes.[33] We chose to examine a dual CRISPRi-ME plasmid with two sgRNAs (**Fig. 4a**), one targeting IS*10* and one targeting IS*5*, because these were the two most active transposon families in *E. coli* TOP10 cells in our tests of genetic stability using a burdensome plasmid (see next section). IS*10* and IS*5* have also been reported to make sizable contributions to mutagenesis in another *E. coli* strain that is closely related to TOP10.[34] We found that mutation rates to DCS^R were reduced by 46% in a TOP10 strain containing the IS*10*+IS*5* dual-targeted CRISPRi-ME plasmid compared to the wild-type strain with no plasmid (**Fig. 4b**). This mutation rate difference was highly statistically significant ($p = 8.8\times10^{-10}$, likelihood ratio test). In contrast, the mutation rate of the control strain containing a CRISPRi-ME plasmid with dCas9 and an off-target sgRNA was not significantly different from that of the wild-type strain without any CRISPRi-ME plasmid ($p = 0.27$).

Expression of dCas9/sgRNA systems can negatively impact *E. coli* fitness.[35,36] To determine whether there were any trade-offs in terms of host cell performance when employing the plasmid-borne CRISPRi-ME system, we measured the doubling times of each *E. coli* strain during early log-phase growth. For strains with the off-target and IS*10*+IS*5* CRISPRi-ME plasmids, doubling times in LB containing antibiotic to select for plasmid maintenance were 39.8 and 41.8 minutes, respectively. The doubling time of the IS*10*+IS*5* CRISPRi-ME strain was significantly longer than the doubling time of 40.3 minutes determined for the TOP10 strain with no plasmid when it was

cultured in LB with no antibiotic (p = 0.00040, one-tailed t-test). The 3.5% reduction in growth rate for the strain with sgRNAs targeted to the *E. coli* chromosome compared to no noticeable reduction for the strain with a sgRNA targeting a sequence that is not present in the genome is consistent with most of the burden of this CRISPRi-ME system arising from dCas9 strongly binding to the chromosome and forming roadblocks that interfere with DNA replication.[37]

CRISPRi-ME stabilizes burdensome protein expression in *E. coli*. To determine if CRISPRi-ME could also prevent a mobile element from causing inactivating mutations in a costly engineered DNA sequence encoded on a plasmid, we added this synthetic genetic control system to an *E. coli* TOP10 strain containing pSB1C3-sYFP2 (**Fig. 5a**). pSB1C3-sYFP2 is a high copy plasmid constructed from BioBrick parts[38] that strongly expresses a super yellow fluorescent protein variant (sYFP2).[39] Addition of this plasmid exacts a high cost on the host cell. It increases the doubling time of *E. coli* TOP10 by 24%, from 40.3 minutes to 49.9 minutes. Therefore, there is a strong selective pressure favoring cells with plasmids that have mutated to reduce or eliminate sYFP2 expression to alleviate this fitness cost.

In preliminary experiments, we found that mutant cells with IS*10* insertions in sYFP2 rapidly arose and outcompeted fluorescent cells. IS*10* is found in two copies that flank the Tn*10* composite transposon in the genome of the host *E. coli* strain,[40] and it is known to have strong specificity for certain target site sequences.[41] In agreement with this expectation, every independently derived non-fluorescent mutant had an IS*10* insertion at precisely the same site early in the sYFP2 reading frame. We determined that editing this target sequence could prevent IS*10* from inserting at this site. When using this edited plasmid, mutations that eliminated the burden of sfYFP expression still arose, but now they were either point mutations or insertions of an IS*5* element. IS*5* is found in 14 copies in the TOP10 genome. It preferentially inserts in the four-base sequence YTAR,[42]

which occurs many times throughout the engineered sYFP2 gene. Accordingly, inactivating IS*5* element insertions were found to occur at various different positions in the construct. Many bacterial transposons resemble IS*5* more closely than IS*10* in terms of having only a relatively weak specificity for certain target site sequence determinants.[13] It is not feasible to edit all of their possible target sites out of an engineered sequence to eliminate this type of inactivating mutation.

From these preliminary results, we expected that adding a sgRNA targeting the transposase of IS*10* would eliminate the dominant failure mode of pSB1C3-sYFP2 and that adding another sgRNA targeting IS*5* might further stabilize the function of this engineered plasmid. We compared the evolutionary stability of fluorescence in *E. coli* TOP10 cells containing the pSB1C3-sYFP2 plasmid and either a CRISPRi-ME off-target plasmid control, a CRISPRi-ME anti-IS*10* plasmid, or a CRISPRi-ME anti-IS*10*+anti-IS*5* plasmid (**Fig. 5b**). Growth of each replicate population was initiated from all of the cells in a single colony that was brightly fluorescent. All cells from the colony were transferred and grown in liquid cultures overnight to saturation (~35 cell doublings). Then each population was diluted 1:1000 into fresh medium and allowed to regrow for 24 hours to saturation each day (~10 additional cell doublings), while monitoring its fluorescence.

Most of the original fluorescence was lost in populations of the off-target CRISPRi-ME control strain by the fourth day (~65 cell doublings), and these populations were essentially nonfluorescent by the sixth day (~85 cell doublings). In the CRISPRi-ME transposon repression strains, fluorescence was more stable over time. Populations with the dual IS*10*+IS*5* CRISPRi-ME system, maintained more than half their original fluorescence after the fourth day. For both the IS*10* and IS*10*+IS*5* sgRNA strains, fluorescence was significantly greater than in the off-target sgRNA strain after the fourth and subsequent days ($p < 0.0021$, Kolmogorov-Smirnov tests). Fluorescence values for these two strains stabilized by the sixth day at a reduced level rather than decaying all

the way to zero, as was the case for the off-target control strain. Populations of the IS*10*+IS*5* CRISPRi-ME strain were 23% more fluorescent, on average, than populations of the IS*10* and off-target CRISPRi-ME strains on the first day of the experiment, and they remained significantly more fluorescent through the third day ($p < 0.0021$, Kolmogorov-Smirnov tests). The reason for this difference is unknown. Overall, there is a clear improvement in total sYFP2 production integrated over the time course of the experiment in cells with CRISPRi-ME plasmids.

We isolated plasmids from cells that exhibited reduced fluorescence at the end of experiment and sequenced them to determine what types of mutations were responsible for inactivating sYFP2 expression in each case (**Fig. 5c**). In the wild-type *E. coli* strain, sYFP2 was inactivated in 10/10 cases by IS*10* insertions at the hotspot in the sfYFP2 open reading frame. This genetic failure mode explains why these populations lost all fluorescence. In contrast, no IS*10* insertions were found in any of the strains with CRISPRi-ME systems containing an IS*10* sgRNA. An IS*5* insertion was found in 1/10 plasmids from the CRISPRi-ME strain with only the IS*10* sgRNA. In the IS*10*+IS*5* sgRNA strain, there were no IS element insertions in sYFP2. Reduced sYFP2 burden in these strains was nearly always caused by point mutations, in accordance with the observation that most cells in these populations retained some fluorescence. These results show that a compound CRISPRi-ME system, capable of silencing multiple copies of the same IS family and multiple IS families, slowed evolutionary decay of this plasmid's function due to selfish DNA elements.

As was the case when CRISPRi-ME plasmids were added to *E. coli* TOP10 cells to repress chromosomal mutation rates, adding the IS*10*+IS*5* sgRNA plasmid to cells that also contained pSB1C3-sYFP2 significantly inhibited their growth ($p = 1.8 \times 10^{-17}$, one-tailed *t*-test). The doubling time of this strain was 66.8 minutes. This value corresponds to a 25% slower growth rate than that of the strain with only pSB1C3-sYFP2, which has a doubling time of 49.9 minutes. As

before, there was less of a burden on host cells from the off-target sgRNA control plasmid compared to the IS*10*+IS*5* sgRNA plasmid targeting dCas9 to the chromosome. However, adding the off-target sgRNA CRISPRi-ME plasmid did significantly increase the doubling time of cells containing the pSB1C3-sYFP2 plasmid to 57.8 minutes ($p = 5.0 \times 10^{-11}$, one-tailed *t*-test). The greater growth rate cost for adding both types of CRISPRi-ME plasmids to cells with pSB1C3-sYFP2 compared to adding them to TOP10 cells with no other plasmid present is consistent with a synergistic fitness burden resulting from dCas9 and high-level sYFP2 expression both diverting the translational capacity of the host cells away from cellular replication.[4] Cultures of every strain still had sufficient time for the number of doublings needed to reach saturation each day after dilution during the sYFP2 stability experiment. Despite variation in their growth rates, they experienced a uniform number of cell doublings (generations) each day.

CRISPRi-ME reduces mutation rates in *A. baylyi* ADP1. The RSF1010 plasmid origin and dCas9 repression system should enable the CRISPRi-ME system to repress mobile elements in a wide variety of bacterial species. To demonstrate its effectiveness in another context we adapted CRISPRi-ME for use in *Acinetobacter baylyi* ADP1. This γ-proteobacterium is of interest in biotechnology due to its natural transformability and metabolic versatility.[43–45] Acinetobacter species are more closely related to pseudomonads than they are to enterobacteria,[46] and typical *E. coli* plasmids with ColE1-type origins do not replicate reliably in *A. baylyi*.[47,48] The plasmid pMMB67EH, which is the source of the RSF1010 replicon employed in CRISPRi-ME, has been shown to replicate in the related species *Acinetobacter baumanii*.[29,30] Many Acinetobacter species have native type I CRISPR-Cas systems in their genomes,[49] but neither genome editing nor control of gene expression with Cas9-based systems has been demonstrated previously in this genus.

We found that the RSF1010 backbone used in CRISPRi-ME reliably replicated in *A. baylyi*

ADP1. However, it was necessary to change the promoters driving expression of dCas9 and sgRNAs in order for CRISPRi to function effectively from this platform in ADP1 (**Fig. 6a**). For dCas9 we used a promoter that has been used to drive the *tdk* gene, which is used as a counter-selectable marker in this organism.[50] For the sgRNA, we used the T5 promoter, which has previously been shown to yield robust constitutive expression in ADP1.[51] With these modifications, there was near-complete repression of an sfGFP gene integrated into the chromosome when the CRISPRi-ME plasmid was used with an on-target sgRNA (**Fig. 6b**).

A. baylyi ADP1 has six copies of one type of transposable element, IS*1236*, and this element is a dominant source of genetic instability in this strain.[52–54] Therefore, we designed a CRISPRi-ME plasmid that represses the IS*1236* transposase (**Fig. 7a**). To determine if silencing IS*1236* stabilized the ADP1 genome against evolution, we used Luria-Delbrück fluctuation assays to measure mutation rates. Loss-of-function mutations in a copy of the *tdk* counterselectable marker inserted into the bacterial chromosome confer resistance to the chain-terminating base analogue, azidothymidine (AZT).[45] Therefore, the mutation rate to AZT resistance (AZT^R) yields an aggregate estimate of the risk that an engineered DNA construct inserted into the ADP1 genome has of becoming inactivated by IS*1236* activity or by other mutations.

We measured mutation rates to AZT^R in two ADP1 host strains that had the *tdk* mutational reporter gene integrated at different locations in the bacterial chromosome (**Fig. 7b**). We found that the presence of the anti-IS*1236* CRISPRi-ME system reduced the rates of inactivating mutations in the *tdk* gene by a factor of five at both sites whereas strains with the off-target sgRNA exhibited no change in mutation rates. The five-fold reduction in mutation rates indicates that there is near-complete suppression of IS*1236* activity, as mutation rates in strains with the CRISPRi-ME system were almost as reduced as they were in a positive-control 'clean-genome' ADP1-ISx

strain in which all six IS*1236* elements were deleted from the genome.[54]

Both the off-target and anti-IS*1236* CRISPRi-ME systems significantly slowed growth of the *A. baylyi* ADP1 mutational reporter strain ($p = 6.9 \times 10^{-5}$ and $p = 7.6 \times 10^{-6}$, respectively, one-tailed *t*-tests). The doubling times of these strains were 52.1 and 58.0 minutes, respectively, compared to 44.6 minutes for the strain with no plasmid. The detrimental effects of both types of CRISPRi-ME plasmids on *A. baylyi* fitness were similar to what we observed in *E. coli* containing the burdensome pSB1C3-sYFP2 plasmid. The fitness cost of the CRISPRi-ME system in ADP1 may be due to excessive expression of dCas9 in this host coupled with further growth inhibition specifically when an sgRNA targeting dCas9 to bind the chromosome is also present. Additionally, the genome of *A. baylyi* ADP1 encodes a type I-F CRISPR-Cas system.[49] If this system is expressed, unlike the cryptic CRISPR-Cas system in the *E. coli* chromosome that is transcriptionally silenced by H-NS in K-12 laboratory strains,[55] then some of the additional burden for the CRISPRi-ME plasmid configurations tested in ADP1 could also be due to crosstalk between dCas9 and sgRNAs on the plasmid and this bacterium's native CRISPR-Cas system.

Discussion

In this study, we developed and employed CRISPR interference against mobile elements in bacteria. This CRISPRi-ME approach reduced the detrimental effects of IS elements on the continued production of a target biomolecule and significantly stabilized genetically engineered DNA sequences. Specifically, we prevented inactivating mutations that result in the loss of burdensome protein expression from a plasmid in the *E. coli*, and we reduced mutation rates in the bacterial chromosome by as much as 5-fold in *A. baylyi*. A similar dCas9 system on an *E. coli* plasmid with a p15A origin of replication was recently shown to repress multiple IS elements simultaneously

and prevent mutations in this bacterium.[56] Because our CRISPRi-ME system employs broad-host-range components (the RSF1010 replicon and the dCas9 catalytically inactivated RNA-guided nuclease), it can be readily reprogrammed to implement this approach in diverse bacterial species.

To completely prevent loss-of-function mutations generated by insertion sequences, 'clean-genome' bacterial strains have been constructed in which one or more IS element families and sometimes other selfish elements, like prophage, have been deleted from the chromosome. Examples of clean-genome strains include *Escherichia coli* MDS42,[5] *Pseudomonas putida* EM383,[57] *Corynebacterium glutamicum* WJ004 and WJ008,[58] and *Acinetobacter baylyi* ADP1-ISx.[54] Engineering projects that begin in these strain backgrounds do not have to worry about IS element activity. However, many strains of bacteria used in research and industrial applications already exist that have been subjected to extensive genome editing efforts or directed evolution during which many beneficial mutations have accumulated in their genomes.[59–62] Preventing IS elements from compromising the functions of these highly engineered strains is nontrivial. One must either identify the mutations that are important for the strain's function and re-engineer them into a clean-genome strain background or repeat the process of sequentially deleting selfish elements from the engineered strain's genome, which is labor-intensive.[5,54]

In eukaryotic cells that have efficient nonhomologous end joining (NHEJ), it is possible to simultaneously inactivate many members of a single selfish DNA element that contributes to genome instability by targeting them for cleavage with an RNA-guided nuclease.[63] NHEJ processes do not exist or are inefficient in most bacteria,[64] including *E. coli*,[65] but it may be possible in the future to heterologously express a NHEJ system to achieve multiplex editing that could be used to inactivate selfish DNA elements in bacterial genomes.[66] Alternatively, the process of re-cleaning a new bacterial genome can be accelerated by using a related clean-genome strain as a

donor for transduction and existing multiplex genome editing methods.[67]

The CRISPRi-ME approach is to silence the expression of mobile elements, rather than to delete them from the bacterial chromosome. It resembles how eukaryotic genomes have evolved defenses to maintain genome integrity against abundant selfish DNA elements in their genomes. In the context of bacterial genetic engineering, the CRISPRi-ME system can be used to rapidly prototype whether silencing a particular mobile element family will increase the stability of an engineered function before investing in the time-consuming process of deleting all of its copies from a genome. There was only a modest (3.5%) growth rate cost for adding this exogenous silencing control system to *E. coli* cells to reduce chromosomal mutation rates, so this approach could also be useful for directly stabilizing certain bioproduction strains. Our results in *A. baylyi* emphasize that further optimization of dCas9 and sgRNA promoters may be necessary in new bacterial species for a CRISPRi-ME system to achieve optimal mobile element repression without imposing a greater fitness burden on cells. Recently Tn*7* and ICE element based tools for integrating CRISPRi systems into bacterial genomes have become available.[68] CRISPRi-ME could also be implemented using these systems when maintaining a genetic control plasmid is not desirable and for adding compatibility with even more bacterial species. CRISPRi-ME gives bacteria a synthetic line of defense against endogenous mobile DNA elements, thereby stabilizing the function of genetically engineered cells.

Methods

Bacterial strains and growth conditions. *E. coli* strains were cultured at 37°C in Lysogeny Broth (LB) (10 g NaCl, 10 g tryptone, and 5 g yeast extract per liter). We used *E. coli* DH5α for all cloning steps. *A. baylyi* was cultured in LB at 30°C. Both bacteria were incubated with orbital

shaking at 200 r.p.m. over a 1-inch diameter. Media amendments were added at the following concentrations when specified: spectinomycin (Spec), 60 μg/ml; carbenicillin (Crb), 100 μg/ml; chloramphenicol (Cam), 20 μg/ml; D-cycloserine (DCS), 60 μg/ml; 3′-azido-2′,3′-dideoxythymidine (AZT), 200 μg/ml.

Broad-host-range CRISPRi platform. Lee et al. constructed a versatile yeast toolkit (YTK) for Golden Gate assembly of plasmids,[69] and we extended it to enable genetic engineering of bacteria from the bee gut microbiome (BTK).[22] These kits designate particular restriction enzyme overhangs for promoters, coding sequences, terminators, and connecters that allow plasmids to be hierarchically assembled using Golden Gate assembly. We followed the basic design principles used in the BTK for CRISPRi-ME plasmid construction as illustrated in **Fig. 2**. The five component plasmids needed to assemble the CRISPRi-ME systems validated in *E. coli* and *A. baylyi* in this study and their DNA sequences are available from the Addgene plasmid repository.

The first two component plasmids for the single sgRNA targeting unit and dCas9 transcriptional unit plasmids were created by cloning these genes into pYTK095, which has a ColE1 origin.[69] The sgRNA targeting unit plasmid contains connectors ConLS and ConR1 flanking a sgRNA transcriptional unit. Megaprimer PCR of Whole Plasmids (MEGAWHOP) cloning[70] was used to change the 20-base sgRNA target region in this plasmid to the sequences given in **Dataset S1** for different experiments. These sgRNA sequences were designed to target dCas9 binding to regions within the first sixty bases of the reading frame being silenced. Each *E. coli* sgRNA was checked for potential off-target binding sites in the bacterial genome using the Cas-Designer web tool.[71] For the multiple target CRISPRi system, the sgRNA transcriptional units from two such plasmids were assembled into the pYTK095 plasmid backbone using Gibson assembly with an arbitrary DNA linker added between them to maintain terminal ConLS and

ConR1 linkers. The dCas9 transcriptional unit is flanked by ConL1 and ConRE connectors in its plasmid. The coding sequence for dCas9 was derived from plasmid pdCas9[24] with the removal of an internal BsmBI site. The sgRNA and dCas9 transcriptional units were assembled together with the RSF1010 origin from pMMB67EH using BsmBI Golden Gate assembly.

GFP repression assays. An *E. coli* MG1655 derivative constitutively expressing sfGFP from the chromosome was created using λ Red recombination.[72] Briefly, we generated a DNA fragment with the native *E. coli glpT* promoter controlling sfGFP linked to an adjacent chloramphenicol resistance gene via PCR reactions that also added 50-bp extensions homologous to regions adjacent to the *lacZ* gene. This product was electroporated into cells induced to express the λ Red proteins from plasmid pKD46 as previously described.[73] A fluorescent colony was selected on LB-Cam agar and then cured of the temperature-sensitive pKD46 plasmid to isolate strain MG1655-sfGFP.

For *A. baylyi* we used natural transformation to add a similar cassette to the chromosome at a neutral location (Site 2) as previously described.[54] Briefly, a double-stranded DNA fragment which contained sfGFP under control of the Tac promoter, a chloramphenicol resistance gene, and two 1-kb chromosomal flanking homology regions was constructed by PCR. Then, *A. baylyi* ADP1 was transformed with this DNA fragment as previously described.[74] A fluorescent colony was selected after plating these cells on LB-Cam agar and designated strain ADP1-sfGFP.

CRISPRi-ME plasmids were transformed into MG1655-sfGFP and ADP1-sfGFP to test the effectiveness of gene silencing. Entire colonies were scraped from agar plates and inoculated into 10 ml of LB in 50 ml Erlenmeyer flasks. After incubation for 12 hours, the absorbance at 600 nm (OD600) and fluorescence (excitation 488 nm, emission 525 nm) were measured for 100 μl samples taken from these cultures using a Tecan Infinite M200 PRO plate reader. The off-target

sgRNA used in these tests was targeted to a different fluorescent protein variant, GFP optim-1.[22]

Mutation rate measurements. For each *E. coli* strain, an initial overnight culture was grown in LB-Spec for strains carrying a CRISPRi-ME plasmid or LB for other strains. Then, nineteen independent 1 ml cultures per strain in 18 × 150 mm test tubes in the same media were each inoculated with ~5000 cells from the overnight culture. These new replicate cultures for the fluctuation test were then allowed to grow for 24 h. To estimate the total number of cell numbers in the final cultures, dilutions in sterile saline from three of the tubes were plated on nonselective LB agar plates. 25 µl of the other sixteen tubes were plated separately on selective LB-DCS agar plates. Colonies on nonselective and selective plates were counted after incubation at 37°C for 24 h or 48 h, respectively. The off-target sgRNA used in this experiment targeted the GFP optim-1 sequence, as above. Mutation rates, confidence intervals, and *p*-values for the significance of differences in mutation rates between strains were estimated from cell counts on selective and nonselective plates via maximum likelihood methods using rSalvador (version 1.7).[75]

The procedure for *A. baylyi* was similar. In this case, we inoculated fourteen independent 100 µl test tube cultures per strain with ~500 cells from overnight cultures. After overnight growth to saturation (~16 h), we plated dilutions from two of the tubes on nonselective LB agar plates, and the entire volumes of each of the other twelve tubes on a selective LB-AZT agar plate. Colonies on both selective and nonselective plates were counted after incubation at 30°C for 24 h.

Doubling time measurements. For both *E. coli* and *A. baylyi* we revived cells from frozen stocks in 10 ml LB plus appropriate antibiotics in 50 ml Erlenmeyer flasks. After overnight growth under standard conditions, 200 µl of each culture was diluted into 10 ml of fresh medium in new flasks. These cultures were incubated for 1.5 h under the same growth conditions. Then, we transferred 200 µl from each culture into twelve separate wells of a 96-well plate (Costar #3598).

We monitored growth in this plate, recording the absorbance at 600 nm (OD600) every 17.2 min in a Tecan Infinite 200 PRO plate reader with continuous shaking during incubation. Doubling times were estimated for each of the twelve replicate wells from their growth curves by performing nonlinear least squares fits to an exponential growth model with lag time parameter. We took the lowest doubling time (maximum growth rate) found when fitting this model to every set of three consecutive measurements (spanning an interval of 34.4 minutes) during exponential phase. Growth curve fits and *t*-tests comparing the resulting sets of twelve doubling time estimates for each strain were performed using the R statistical programming language.[76]

Monitoring decay of plasmid function. The *E. coli* reporter plasmid was constructed by BioBrick assembly of promoter (J23100), ribosome binding site (B0034), and sYFP2 fluorescent protein (K864100) parts obtained from the iGEM Registry of Standard Biological Parts.[38] There were two six-base-pair repeats (TACTAG) located upstream and downstream of the ribosome binding site in this initial plasmid that mediated a deletion that dominated among the mutations leading to non-fluorescent cells after IS*10* silencing in preliminary experiments. To eliminate this mutational hotspot, we modified the upstream copy of the repeat to GTATAG.

For each strain tested, ten different strongly fluorescent colonies from an LB agar plate were transferred into test tubes containing 5 ml of LB. After 24 hours of growth (designated day 1), 5 µl of culture was transferred from each test tube into 5 ml of fresh LB in a new test tube. This serial transfer procedure was repeated for eight additional days. Fluorescence (excitation 495 nm, emission 530 nm) and OD600 were monitored as in the section describing the GFP repression assays. The off-target sgRNA control in this experiment was targeted to the *A. baylyi* ADP1 IS*1236* sequence. Statistical tests comparing fluorescence normalized to OD600 for the ten populations of each strain were performed in R.[76]

Supporting Information

Dataset S1: Sequences of plasmids and sgRNA spacers in GenBank format.

Author Contributions

P.G, S.P.L., and J.E.B conceived the study. P.G., S.P.L, and D.M.M. created plasmids. All authors designed experiments. P.G. performed experiments. P.G. and J.E.B analyzed data, created figures, and wrote the manuscript. All authors edited the manuscript.

Acknowledgments

We thank Kelsey Hu and the UT Austin "Microbe Hackers" Freshman Research Initiative stream for their work identifying the evolutionary failure modes of the sYFP2 plasmid and Gabriel Suárez for advice and materials for working with *A. baylyi* ADP1. This project was funded by the Defense Advanced Research Projects Agency (HR0011-15-C0095) and the National Science Foundation (CBET-1554179).

References

(1) Arkin, A. P., and Fletcher, D. A. (2006) Fast, cheap and somewhat in control. *Genome Biol. 7*, 114.

(2) Canton, B., Labno, A., and Endy, D. (2008) Refinement and standardization of synthetic biological parts and devices. *Nat. Biotechnol. 26*, 787–793.

(3) Renda, B. A., Hammerling, M. J., and Barrick, J. E. (2014) Engineering reduced evolutionary potential for synthetic biology. *Mol. BioSyst. 10*, 1668–1678.

(4) Borkowski, O., Ceroni, F., Stan, G.-B., and Ellis, T. (2016) Overloaded and stressed: whole-cell considerations for bacterial synthetic biology. *Curr. Opin. Microbiol. 33*, 123–130.

(5) Umenhoffer, K., Fehér, T., Balikó, G., Ayaydin, F., Pósfai, J., Blattner, F. R., and Pósfai, G. (2010) Reduced evolvability of *Escherichia coli* MDS42, an IS-less cellular chassis for molecular and synthetic biology applications. *Microb. Cell Fact. 9*, 38.

(6) Sleight, S. C., Bartley, B. A., Lieviant, J. A., and Sauro, H. M. (2010) Designing and engineering evolutionary robust genetic circuits. *J. Biol. Eng. 4*, 12.

(7) Sleight, S. C., and Sauro, H. M. (2013) Visualization of evolutionary stability dynamics and competitive fitness of *Escherichia coli* engineered with randomized multigene circuits. *ACS Synth. Biol. 2*, 519–528.

(8) Rugbjerg, P., Myling-Petersen, N., Porse, A., Sarup-Lytzen, K., and Sommer, M. O. A. (2018) Diverse genetic error modes constrain large-scale bio-based production. *Nat. Commun. 9*, 787.

(9) Sandoval, C. M., Ayson, M., Moss, N., Lieu, B., Jackson, P., Gaucher, S. P., Horning, T., Dahl, R. H., Denery, J. R., Abbott, D. A., and Meadows, A. L. (2014) Use of pantothenate as a metabolic switch increases the genetic stability of farnesene producing *Saccharomyces cerevisiae*. *Metab. Eng. 25*, 1–12.

(10) Vandecraen, J., Chandler, M., Aertsen, A., and Van Houdt, R. (2017) The impact of insertion sequences on bacterial genome plasticity and adaptability. *Crit. Rev. Microbiol. 43*, 709–730.

(11) Lane, D., Cavaillé, J., and Chandler, M. (1994) Induction of the SOS response by IS*1* transposase. *J. Mol. Biol. 242*, 339-350.

(12) Díaz-Maldonado, H., Gómez, M. J., Moreno-Paz, M., Martín-Úriz, P. S., Amils, R., Parro,

V., and De Saro, F. J. L. (2015) Transposase interaction with the β sliding clamp: Effects on insertion sequence proliferation and transposition rate. *Sci. Rep. 5*, 1–13.

(13) Siguier, P., Gourbeyre, E., and Chandler, M. (2014) Bacterial insertion sequences: Their genomic impact and diversity. *FEMS Microbiol. Rev. 38*, 865–891.

(14) Touchon, M., and Rocha, E. P. C. (2007) Causes of insertion sequences abundance in prokaryotic genomes. *Mol. Biol. Evol. 24*, 969–981.

(15) Slotkin, R. K., and Martienssen, R. (2007) Transposable elements and the epigenetic regulation of the genome. *Nat. Rev. Genet. 8*, 272–285.

(16) Koonin, E. V., Makarova, K. S., and Wolf, Y. I. (2017) Evolutionary genomics of defense systems in archaea and bacteria. *Annu. Rev. Microbiol. 71*, 233–261.

(17) Nagy, Z., and Chandler, M. (2004) Regulation of transposition in bacteria. *Res. Microbiol. 155*, 387–398.

(18) Kleckner, N. (1990) Regulation of transposition in bacteria. *Annu. Rev. Cell Biol. 6*, 297–327.

(19) Chao, L., Vargas, C., Spear, B. B., and Cox, E. C. (1983) Transposable elements as mutator genes in evolution. *Nature 303*, 633–635.

(20) Fehér, T., Bogos, B., Méhi, O., Fekete, G., Csörgo, B., Kovács, K., Pósfai, G., Papp, B., Hurst, L. D., and Pál, C. (2012) Competition between transposable elements and mutator genes in bacteria. *Mol. Biol. Evol. 29*, 3153–9.

(21) 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*, 1173–83.

(22) Leonard, S. P., Perutka, J., Powell, J. E., Geng, P., Richhart, D. D., Byrom, M., Kar, S.,

Davies, B. W., Ellington, A. D., Moran, N. A., and Barrick, J. E. (2018) Genetic engineering of bee gut microbiome bacteria with a toolkit for modular assembly of broad-host-range plasmids. *ACS Synth. Biol. 7*, 1279–1290.

(23) Jiang, Y., Chen, B., Duan, C., Sun, B., Yang, J., and Yang, S. (2015) Multigene editing in the *Escherichia coli* genome via the CRISPR-Cas9 system. *Appl. Environ. Microbiol. 81*, 2506–2514.

(24) 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.

(25) Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M., and Scherzinger, E. (1989) Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. *Gene 75*, 271–288.

(26) Fürste, J. P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P., Bagdasarian, M., and Lanka, E. (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene 48*, 119–131.

(27) Lee, M. D., and Henk, A. D. (1997) RSF1010-based shuttle vectors for cloning and expression in *Pasteurella multocida*. *Vet. Microbiol. 54*, 369–374.

(28) Uwate, M., Ichise, Y. ki, Shirai, A., Omasa, T., Nakae, T., and Maseda, H. (2013) Two routes of MexS-MexT-mediated regulation of MexEF-OprN and MexAB-OprM efflux pump expression in *Pseudomonas aeruginosa*. *Microbiol. Immunol. 57*, 263–272.

(29) Tucker, A. T., Nowicki, E. M., Boll, J. M., Knauf, G. A., Burdis, N. C., Trent, M. S., and Davies, B. W. (2014) Defining gene-phenotype relationships in *Acinetobacter baumannii* through one-step chromosomal gene inactivation. *mBio 5*, e01313-14.

(30) Tucker, A. T., Leonard, S. P., DuBois, C. D., Knauf, G. A., Cunningham, A. L., Wilke, C. O., Trent, M. S., and Davies, B. W. (2018) Discovery of next-generation antimicrobials through bacterial self-screening of surface-displayed peptide libraries. *Cell 172*, 618–628.

(31) Ferrières, L., Hémery, G., Nham, T., Guérout, 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.

(32) Jiang, Y., Qian, F., Yang, J., Liu, Y., Dong, F., Xu, C., Sun, B., Chen, B., Xu, X., Li, Y., Wang, R., and Yang, S. (2017) CRISPR-Cpf1 assisted genome editing of *Corynebacterium glutamicum*. *Nat. Commun. 8*, 15179.

(33) Baisa, G., Stabo, N. J., and Welch, R. A. (2013) Characterization of *Escherichia coli* D-cycloserine transport and resistant mutants. *J. Bacteriol. 195*, 1389–1399.

(34) Durfee, T., Nelson, R., Baldwin, S., Plunkett, G., Burland, V., Mau, B., Petrosino, J. F., Qin, X., Muzny, D. M., Ayele, M., Gibbs, R. A., Csörgo, B., Pósfai, G., Weinstock, G. M., and Blattner, F. R. (2008) The complete genome sequence of *Escherichia coli* DH10B: insights into the biology of a laboratory workhorse. *J. Bacteriol. 190*, 2597–606.

(35) Nielsen, A. A., and Voigt, C. A. (2014) Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks. *Mol. Syst. Biol. 10*, 763–763.

(36) Cui, L., Vigouroux, A., Rousset, F., Varet, H., Khanna, V., and Bikard, D. (2018) A CRISPRi screen in *E. coli* reveals sequence-specific toxicity of dCas9. *Nat. Commun. 9*, 1–10.

(37) Whinn, K., Kaur, G., Lewis, J. S., Schauer, G., Müller, S., Jergic, S., Maynard, H., Gan, Z. Y., Naganbabu, M., Bruchez, M. P., O'Donnell, M., Dixon, N. E., van Oijen, A. M., and Ghodke, H. (2018) Nuclease dead Cas9 is a programmable roadblock for DNA replication.

bioRxiv doi: https://doi.org/10.1101/455543.

(38) Shetty, R. P., Endy, D., and Knight, T. F. (2008) Engineering BioBrick vectors from BioBrick parts. *J. Biol. Eng. 2*, 5.

(39) Kremers, G. J., Goedhart, J., Van Munster, E. B., and Gadella, T. W. J. (2006) Cyan and yellow super fluorescent proteins with improved brightness, protein folding, and FRET förster radius. *Biochemistry 45*, 6570–6580.

(40) Foster, T. J., Davis, M. A., Roberts, D. E., Takeshita, K., and Kleckner, N. (1981) Genetic organization of transposon Tn10. *Cell 23*, 201–13.

(41) Bender, J., and Kleckner, N. (1992) Tn*10* insertion specificity is strongly dependent upon sequences immediately adjacent to the target-site consensus sequence. *Proc. Natl. Acad. Sci. U. S. A. 89*, 7996–8000.

(42) Lee, H., Doak, T. G., Popodi, E., Foster, P. L., and Tang, H. (2016) Insertion sequence-caused large-scale rearrangements in the genome of *Escherichia coli*. *Nucleic Acids Res. 44*, 7109–19.

(43) Young, D. M., Parke, D., and Ornston, L. N. (2005) Opportunities for genetic investigation afforded by Acinetobacter baylyi, a nutritionally versatile bacterial species that is highly competent for natural transformation. *Annu. Rev. Microbiol. 59*, 519–551.

(44) Elliott, K. T., and Neidle, E. L. (2011) *Acinetobacter baylyi* ADP1: transforming the choice of model organism. *IUBMB Life 63*, 1075–1080.

(45) Metzgar, D., Bacher, J. M., Pezo, V., Reader, J., Döring, V., Schimmel, P., Marlière, P., and de Crécy-Lagard, V. (2004) *Acinetobacter* sp. ADP1: an ideal model organism for genetic analysis and genome engineering. *Nucleic Acids Res. 32*, 5780–5790.

(46) Williams, K. P., Gillespie, J. J., Sobral, B. W. S., Nordberg, E. K., Snyder, E. E., Shallom, J.

M., and Dickerman, A. W. (2010) Phylogeny of gammaproteobacteria. *J. Bacteriol. 192*, 2305–14.

(47) Hunger, M., Schmucker, R., Kishan, V., and Hillen, W. (1990) Analysis and nucleotide sequence of an origin of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for *Escherichia coli* shuttle plasmids. *Gene 87*, 45–51.

(48) Gralton, E. M., Campbell, A. L., and Neidle, E. L. (1997) Directed introduction of DNA cleavage sites to produce a high-resolution genetic and physical map of the *Acinetobacter* sp. strain ADP1 (BD413UE) chromosome. *Microbiology 143*, 1345–1357.

(49) Touchon, M., Cury, J., Yoon, E.-J., Krizova, L., Cerqueira, G. C., Murphy, C., Feldgarden, M., Wortman, J., Clermont, D., Lambert, T., Grillot-Courvalin, C., Nemec, A., Courvalin, P., and Rocha, E. P. C. (2014) The genomic diversification of the whole *Acinetobacter* genus: origins, mechanisms, and consequences. *Genome Biol. Evol. 6*, 2866–2882.

(50) de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., Samair, S., Lechaplais, C., Gyapay, G., Richez, C., Durot, M., Kreimeyer, A., Le Fèvre, F., Schächter, V., Pezo, V., Döring, V., Scarpelli, C., Médigue, C., Cohen, G. N., Marlière, P., Salanoubat, M., and Weissenbach, J. (2008) A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Mol. Syst. Biol. 4*, 174.

(51) Murin, C. D., Segal, K., Bryksin, A., and Matsumura, I. (2012) Expression vectors for *Acinetobacter baylyi* ADP1. *Appl. Environ. Microbiol. 78*, 280–283.

(52) Gerischer, U., D'Argenio, D. A., and Ornston, L. N. (1996) IS*1236*, a newly discovered member of the IS*3* family, exhibits varied patterns of insertion into the *Acinetobacter calcoaceticus* chromosome. *Microbiology 142*, 1825–1831.

(53) Renda, B. A., Dasgupta, A., Leon, D., and Barrick, J. E. (2015) Genome instability mediates

the loss of key traits by *Acinetobacter baylyi* ADP1 during laboratory evolution. *J. Bacteriol. 197*, 872–881.

(54) Suárez, G. A., Renda, B. A., Dasgupta, A., and Barrick, J. E. (2017) Reduced mutation rate and increased transformability of transposon-free *Acinetobacter baylyi* ADP1-ISx. *Appl. Environ. Microbiol. 83*, e01025-17.

(55) Pul, U., Wurm, R., Arslan, Z., Geissen, R., Hofmann, N., and Wagner, R. (2010) Identification and characterization of *E. coli* CRISPR-cas promoters and their silencing by H-NS. *Mol. Microbiol. 75*, 1495–512.

(56) Nyerges, Á., Bálint, B., Cseklye, J., Nagy, I., Pál, C., and Tamás, F. (2018) CRISPR-interference based modulation of mobile genetic elements in bacteria. *bioRxiv* doi: https://doi.org/10.1101/428029.

(57) Martínez-García, E., Nikel, P. I., Aparicio, T., and de Lorenzo, V. (2014) *Pseudomonas* 2.0: genetic upgrading of *P. putida* KT2440 as an enhanced host for heterologous gene expression. *Microb. Cell Fact. 13*, 159.

(58) Choi, J. W., Yim, S. S., Kim, M. J., and Jeong, K. J. (2015) Enhanced production of recombinant proteins with *Corynebacterium glutamicum* by deletion of insertion sequences (IS elements). *Microb. Cell Fact. 14*, 207.

(59) Lajoie, M. J., Rovner, A. J., Goodman, D. B., Aerni, H.-R., Haimovich, A. D., Kuznetsov, G., Mercer, J. A., Wang, H. H., Carr, P. A., Mosberg, J. A., Rohland, N., Schultz, P. G., Jacobson, J. M., Rinehart, J., Church, G. M., and Isaacs, F. J. (2013) Genomically recoded organisms expand biological functions. *Science 342*, 357–360.

(60) Burgard, A., Burk, M. J., Osterhout, R., Van Dien, S., and Yim, H. (2016) Development of a commercial scale process for production of 1,4-butanediol from sugar. *Curr. Opin. Biotechnol.*

42, 118–125.

(61) Winkler, J. D., Halweg-Edwards, A. L., Erickson, K. E., Choudhury, A., Pines, G., and Gill, R. T. (2016) The resistome: a comprehensive database of *Escherichia coli* resistance phenotypes. *ACS Synth. Biol. 5*, 1566–1577.

(62) Garst, A. D., Bassalo, M. C., Pines, G., Lynch, S. A., Halweg-Edwards, A. L., Liu, R., Liang, L., Wang, Z., Zeitoun, R., Alexander, W. G., and Gill, R. T. (2017) Genome-wide mapping of mutations at single-nucleotide resolution for protein, metabolic and genome engineering. *Nat. Biotechnol. 35*, 48–55.

(63) Yang, L., Güell, M., Niu, D., George, H., Lesha, E., Grishin, D., Aach, J., Shrock, E., Xu, W., Poci, J., Cortazio, R., Wilkinson, R. A., Fishman, J. A., and Church, G. (2015) Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science 350*, 1101–1104.

(64) Pitcher, R. S., Brissett, N. C., and Doherty, A. J. (2007) Nonhomologous end-joining in bacteria: a microbial perspective. *Annu. Rev. Microbiol. 61*, 259–282.

(65) 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.

(66) Su, T., Liu, F., Gu, P., Jin, H., Chang, Y., Wang, Q., Liang, Q., and Qi, Q. (2016) A CRISPR-Cas9 assisted non-homologous end-joining strategy for one-step engineering of bacterial genome. *Sci. Rep. 6*, 37895.

(67) Umenhoffer, K., Draskovits, G., Nyerges, Á., Karcagi, I., Bogos, B., Tímár, E., Csörgő, B., Herczeg, R., Nagy, I., Fehér, T., Pál, C., and Pósfai, G. (2017) Genome-wide abolishment of mobile genetic elements using genome shuffling and CRISPR/Cas-assisted MAGE allows the efficient stabilization of a bacterial chassis. *ACS Synth. Biol. 6*, 1471–1483.

(68) Peters, J. M., Koo, B.-M., Patino, R., Heussler, G. E., Hearne, C. C., Qu, J., Inclan, Y. F.,

Hawkins, J. S., Lu, C. H. S., Silvis, M. R., Harden, M. M., Osadnik, H., Peters, J. E., Engel, J. N., Dutton, R. J., Grossman, A. D., Gross, C. A., and Rosenberg, O. S. (2019) Enabling genetic analysis of diverse bacteria with Mobile-CRISPRi. *Nat. Microbiol. 4*, 244-250.

(69) Lee, M. E., DeLoache, W. C., Cervantes, B., and Dueber, J. E. (2015) A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synth. Biol. 4*, 975–986.

(70) Miyazaki, K. (2011) MEGAWHOP cloning: A method of creating random mutagenesis libraries via megaprimer PCR of whole plasmids. *Methods Enzymol. 498*, 399–406.

(71) Park, J., Bae, S., and Kim, J. (2015) Cas-Designer: a web-based tool for choice of CRISPR-Cas9 target sites. *Bioinformatics 31*, 4014–4016.

(72) Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A. 97*, 6640–6645.

(73) Doublet, B., Douard, G., Targant, H., Meunier, D., Madec, J. Y., and Cloeckaert, A. (2008) Antibiotic marker modifications of λ Red and FLP helper plasmids, pKD46 and pCP20, for inactivation of chromosomal genes using PCR products in multidrug-resistant strains. *J. Microbiol. Methods 75*, 359–361.

(74) Renda, B. A., Chan, C., Parent, K. N., and Barrick, J. E. (2016) Emergence of a competence-reducing filamentous phage from the genome of *Acinetobacter baylyi* ADP1. *J. Bacteriol. 198*, 3209–3219.

(75) Zheng, Q. (2017) rSalvador: An R package for the fluctuation experiment. *G3: Genes, Genomes, Genet. 7*, 3849–3856.

(76) R Core Team. (2018) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

(77) Cox, R. S., Madsen, C., McLaughlin, J., Nguyen, T., Roehner, N., Bartley, B., Bhatia, S.,

Bissell, M., Clancy, K., Gorochowski, T., Grünberg, R., Luna, A., Le Novère, N., Pocock, M., Sauro, H., Sexton, J. T., Stan, G.-B., Tabor, J. J., Voigt, C. A., Zundel, Z., Myers, C., Beal, J., and Wipat, A. (2018) Synthetic Biology Open Language Visual (SBOL Visual) Version 2.0. *J. Integr. Bioinform. 15*, 20170074.

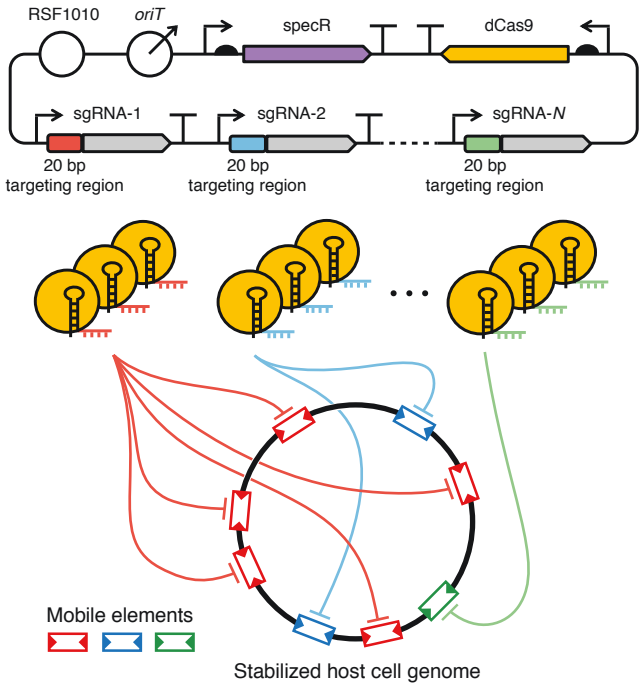

Figure. 1. CRISPRi-ME stabilization of a genome against mobile element instability. In the CRISPR interference with mobile elements (CRISPRi-ME) system, one expresses the catalytically inactive dCas9 protein and one or more small guide RNAs (sgRNAs) targeting it to repress genes (e.g., transposases) required for the mobilization of different selfish element families in a host cell. Repressing the activity of mobile DNA elements prevents mutations that commonly inactivate genes required for an engineered function. The pictured configuration uses a broad-host-range plasmid based on the RSF1010 replicon that functions in diverse bacterial species. Plasmid maps in this and other figures are represented using SBOL visual glyphs.[77]

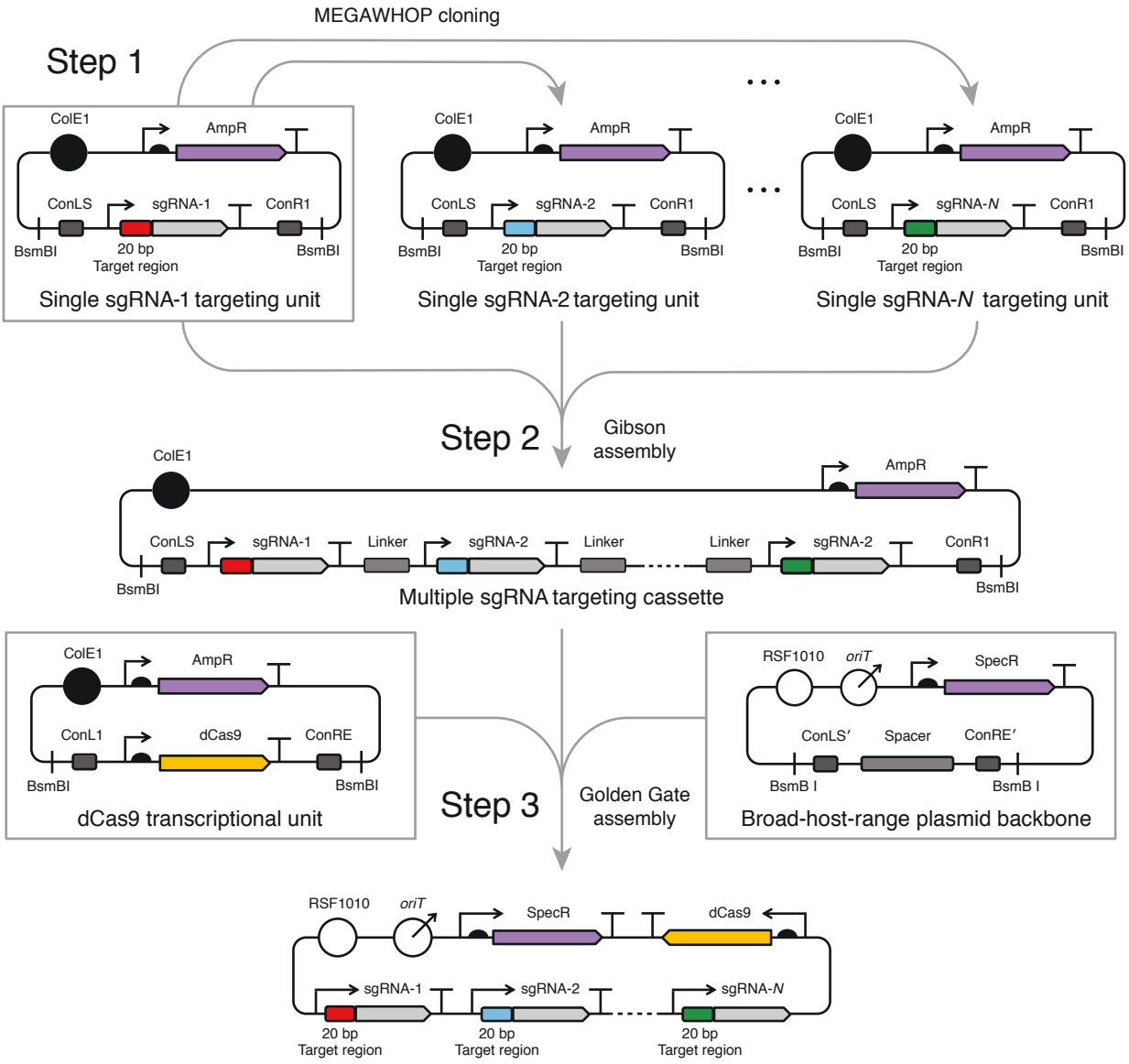

Figure. 2. Construction of CRISPRi-ME transcriptional units and plasmids. To create a broad-host-range CRISPRi-ME plasmid, one first constructs a series of plasmids containing individual sgRNA transcriptional units targeted to different mobile elements by changing the 20-base-pair target region of a template plasmid by a method such as MEGAWHOP cloning (**Step 1**). Next, the sgRNA transcriptional units from each of these plasmids are composed into a multiple sgRNA targeting cassette by a sequence-independent cloning method such as Gibson assembly through the addition of unique linker sequences between sgRNA units (**Step 2**). Finally, the dCas9 transcriptional unit and the multiple sgRNA targeting cassette are assembled onto the broad-host-range (RSF1010) plasmid backbone by BsmBI Golden Gate Assembly (**Step 3**). The three boxed plasmids are provided as genetic parts for implementing a custom CRISPRi-ME system. Multiple versions of the single sgRNA targeting unit and dCas9 transcriptional unit plasmids, with different promoters driving sgRNA and dCas9 expression, were created and tested to achieve optimal function in two different bacterial species in this study.

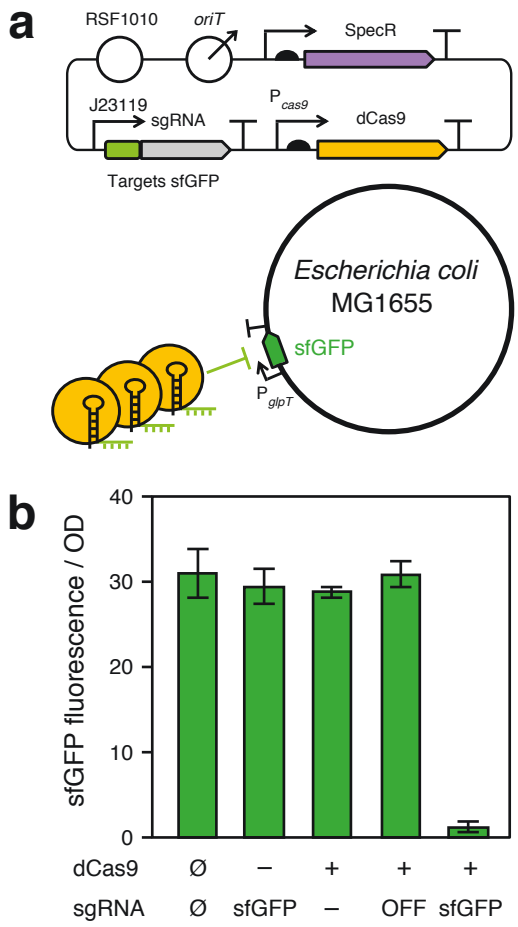

Figure. 3. CRISPRi silences sfGFP expression in *E. coli*. (**a**) Design of experiment testing CRISPRi-mediated silencing of a genomically encoded sfGFP reporter gene in *E. coli* MG1655 from a broad-host-range plasmid with an RSF1010 origin. (b) Expression of sfGFP is repressed by this CRISPRi configuration when the sgRNA is targeted to this gene versus in cells with no plasmid (Ø) and in control experiments with plasmids missing either dCas9 or the sgRNA or with dCas9 and an off-target sgRNA (OFF). Wild-type *E. coli* exhibits no fluorescence. Error bars are standard deviations from nine biological replicates.

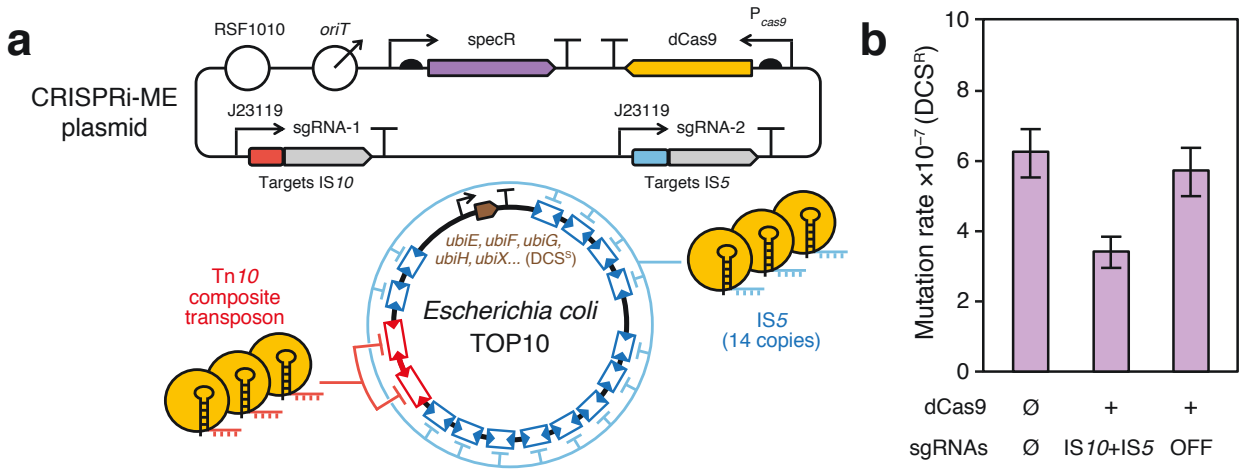

Figure. 4. CRISPRi-ME reduces the rates of inactivating mutations in *E. coli.* (**a**) Design of experiment targeting CRISPRi-ME against multicopy IS*10* and IS*5* transposons in the *E. coli* TOP10 genome to reduce chromosomal mutation rates. Loss of function mutations in several different genes (e.g., *ubiE*) can yield DCS resistance (DCS^{R}) in *E. coli* cultured in LB.[33] (**b**) Mutation rates to DCS^{R} of strains containing the dual IS*10*+IS*5* or off-target CRISPRi-ME plasmids estimated from Luria-Delbrück fluctuation tests compared to the mutation rate of a control strain with no CRISPRi-ME plasmid (Ø). Error bars are 95% confidence intervals.

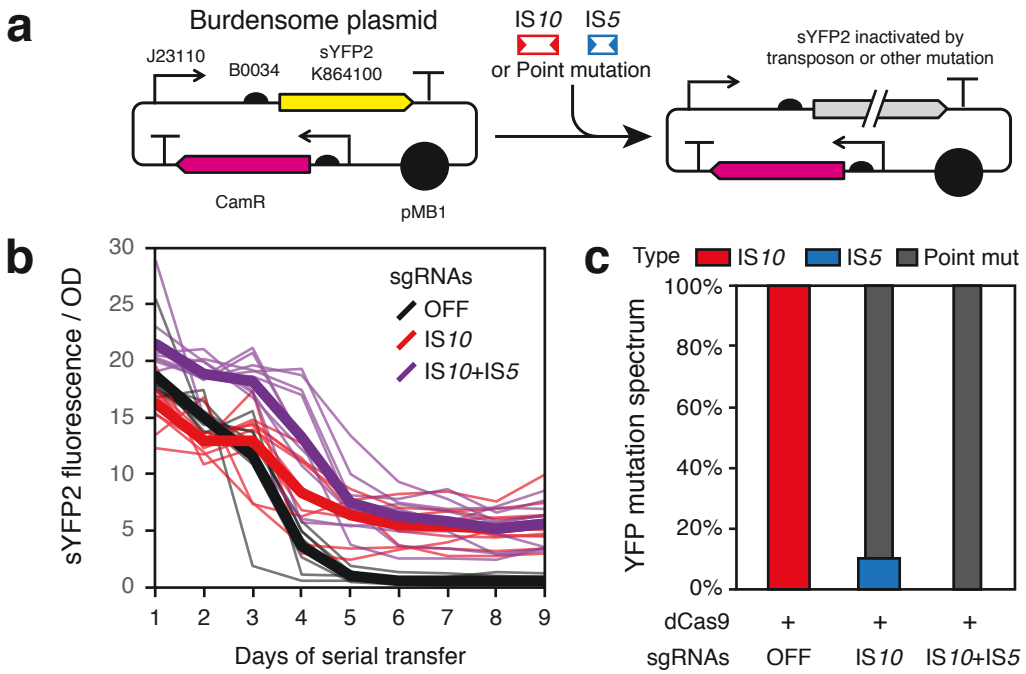

Figure. 5. CRISPRi-ME prevents evolutionary failure of a burdensome plasmid in *E. coli*. (a) Burdensome plasmid used for testing CRISPRi-ME against IS*10* and IS*5* transposons in the *E. coli* TOP10 genome. The evolutionary lifetime of sYFP2 expression from this high-copy plasmid and the types of failure mutations in the presence of different CRISPRi-ME plasmids. (**b**) sYFP2 fluorescence was monitored over multiple days of serial transfer and regrowth in ten independent cell populations with each CRISPRi-ME plasmid (thin lines). The mean for each treatment at each time point is also shown (thick lines). The CRISPRi-ME plasmids tested contained an off-target sgRNA (OFF), an sgRNA targeting IS*10*, or two sgRNAs targeting IS*10* and IS*5*. (**c**) Types of mutations that led to a loss of sYFP2 fluorescence in cells containing each CRISPRi-ME plasmid. One evolved sYFP2 plasmid per population was isolated and analyzed at the conclusion of the experiment shown in panel **b**.

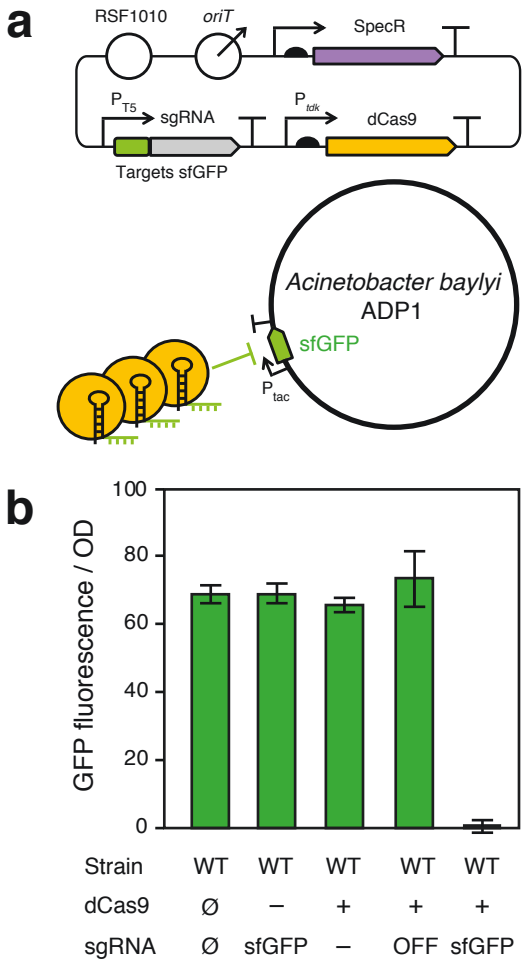

Figure. 6. CRISPRi silences sfGFP expression in *A. baylyi*. (**a**) Design of experiment testing CRISPRi-mediated silencing of genomically encoded sfGFP in *A. baylyi* ADP1 from a broad-host-range plasmid with an RSF1010 origin. (**b**) Expression of sfGFP is repressed by this CRISPRi configuration when the sgRNA is targeted to this gene versus in cells with no plasmid (Ø) and in control experiments with plasmids missing either dCas9 or the sgRNA or with dCas9 and an off-target sgRNA (OFF). Error bars are standard deviations from nine biological replicates.

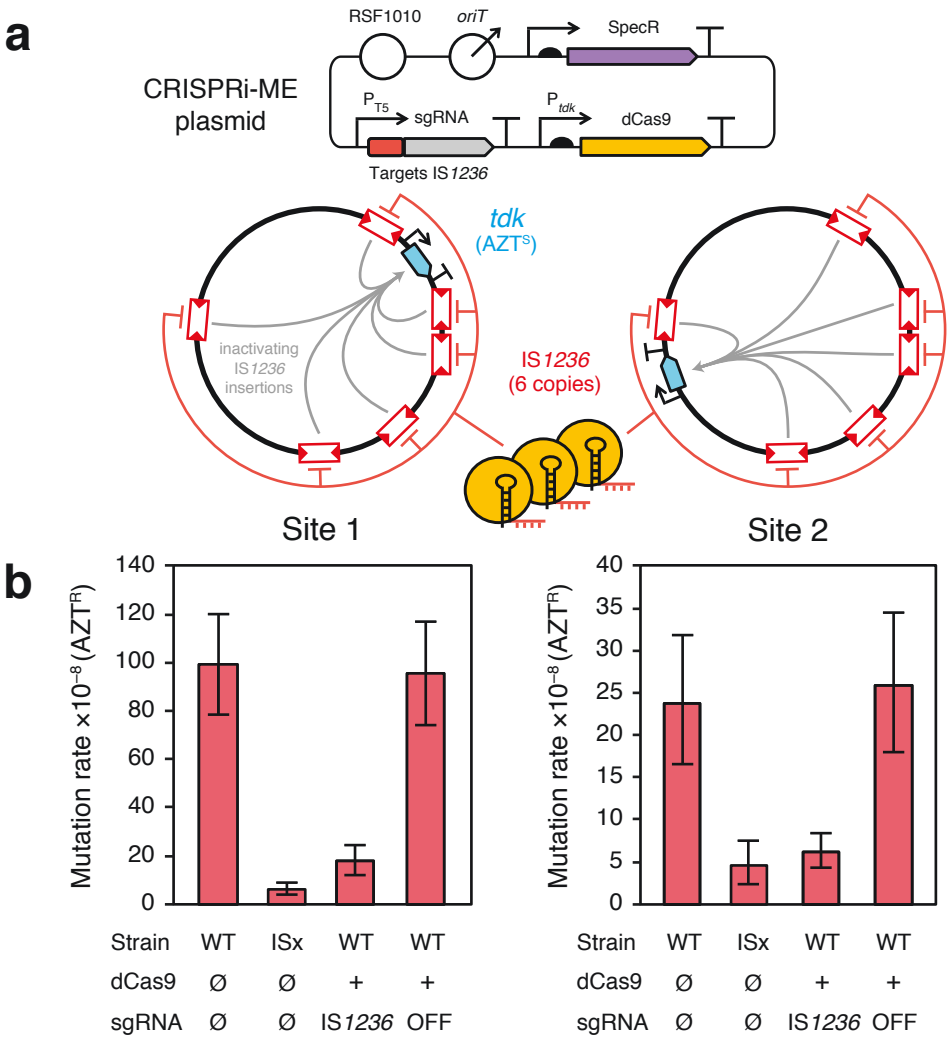

Figure. 7. CRISPRi-ME reduces the rates of inactivating mutations in *A. baylyi*. (**a**) Design of experiment using CRISPRi-ME to silence IS*1236* in *A. baylyi* ADP1. A counter-selectable *tdk* mutational reporter gene was integrated into the ADP1 chromosome at different sites in two strains. Expression of the *tdk* gene results in toxic incorporation of AZT during DNA replication. If an inactivating mutation occurs in *tdk*, it enables cells to grow on selective agar containing AZT. (**b**) Mutation rates to AZT resistant (AZT^R) of strains containing anti-IS*1236* or off-target CRISPRi-ME plasmids estimated from Luria-Delbrück fluctuation tests compared to the mutation rates of control strains with no CRISPRi-ME plasmid (Ø). ISx is a variant of wild-type *A. baylyi* ADP1 (WT) with all five IS*1236* elements deleted from its chromosome. Error bars are 95% confidence intervals.