

# Programmable Removal of Bacterial Strains by Use of Genome-Targeting CRISPR-Cas Systems

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**ABSTRACT** CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) systems in bacteria and archaea employ CRISPR RNAs to specifically recognize the complementary DNA of foreign invaders, leading to sequence-specific cleavage or degradation of the target DNA. Recent work has shown that the accidental or intentional targeting of the bacterial genome is cytotoxic and can lead to cell death. Here, we have demonstrated that genome targeting with CRISPR-Cas systems can be employed for the sequence-specific and titratable removal of individual bacterial strains and species. Using the type I-E CRISPR-Cas system in *Escherichia coli* as a model, we found that this effect could be elicited using native or imported systems and was similarly potent regardless of the genomic location, strand, or transcriptional activity of the target sequence. Furthermore, the specificity of targeting with CRISPR RNAs could readily distinguish between even highly similar strains in pure or mixed cultures. Finally, varying the collection of delivered CRISPR RNAs could quantitatively control the relative number of individual strains within a mixed culture. Critically, the observed selectivity and programmability of bacterial removal would be virtually impossible with traditional antibiotics, bacteriophages, selectable markers, or tailored growth conditions. Once delivery challenges are addressed, we envision that this approach could offer a novel means to quantitatively control the composition of environmental and industrial microbial consortia and may open new avenues for the development of “smart” antibiotics that circumvent multidrug resistance and differentiate between pathogenic and beneficial microorganisms.

**IMPORTANCE** Controlling the composition of microbial populations is a critical aspect in medicine, biotechnology, and environmental cycles. While different antimicrobial strategies, such as antibiotics, antimicrobial peptides, and lytic bacteriophages, offer partial solutions, what remains elusive is a generalized and programmable strategy that can distinguish between even closely related microorganisms and that allows for fine control over the composition of a microbial population. This study demonstrates that RNA-directed immune systems in bacteria and archaea called CRISPR-Cas systems can provide such a strategy. These systems can be employed to selectively and quantitatively remove individual bacterial strains based purely on sequence information, creating opportunities in the treatment of multidrug-resistant infections, the control of industrial fermentations, and the study of microbial consortia.

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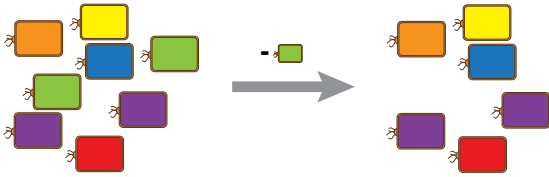
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Microorganisms play critical roles in human health and environmental nutrient cycles and are regularly employed in diverse industrial processes. Within each context, a central challenge is controlling the specific composition of a mixed population. A few strategies can remove some microorganisms but not others (Fig. 1): defined growth conditions, conventional antibiotics, and antimicrobial peptides with some strain specificity, lytic bacteriophages, and the expression of antibiotic resistance genes, auxotrophic markers, or toxins under unique expression systems (1). Unfortunately, most of these approaches offer constrained opportunities to selectively remove individual bacterial strains (e.g., antibiotics and antimicrobial peptides) or require detailed knowledge of the genetics, metabolism, and physiology of each constituent of the population (e.g., selective growth conditions). Lytic bacteriophages often offer exquisite specificity (2). However,

individual bacteriophages must be isolated against each strain and would require additional screening to determine the degree of specificity. Furthermore, lytic bacteriophages replicate as part of the infection cycle, eventually wiping out the entire target population or breeding resistance. What remains elusive is a generalized and programmable strategy that can distinguish between even closely related microorganisms and that allows for fine control over the composition of a microbial population. We proposed that CRISPR-Cas systems could provide such a strategy.

CRISPR-Cas systems are RNA-directed adaptive immune systems in many bacteria and most archaea that recognize nucleic acids of invading plasmids and viruses (3, 4). Recognition is directed by CRISPR RNAs that are processed from transcribed arrays of alternating target-specific “spacer” sequences and identical “repeat” sequences. The spacer region of each CRISPR RNA base



**FIG 1** Selective removal of individual bacterial strains. Approaches are needed that can selectively remove individual constituents (green) but not others within a diverse microbial population.

pairs with complementary nucleic acids, driving cleavage or degradation by the Cas proteins within minutes of invasion (5–7).

Three types of CRISPR-Cas systems, which vary in their specific target and mechanism of action, have been defined. Type I systems cleave and degrade DNA, type II systems cleave DNA, and type III systems cleave DNA or RNA (8). Type I and II systems require two principal factors to effectively target DNA: (i) complementarity between the CRISPR RNA spacer and the target “protospacer” sequence and (ii) a protospacer-adjacent motif (PAM) specific to each CRISPR-Cas system flanking the protospacer (9–11). Effective targeting can occur even for multiple mismatches between the CRISPR RNA and the protospacer, although mismatches within the “seed” region flanking the PAM are more disruptive (9, 12). Similar factors are required for DNA-targeting by type III systems, where these systems evaluate base pairing between the target sequence and the region flanking the protospacer (13).

While these factors help safeguard against accidental targeting of genomic sequences, they provide a simple set of design rules to achieve DNA targeting. This has primarily been exploited with type II systems for genome editing, whereby cleavage is followed by DNA repair through nonhomologous end joining (NHEJ) or homologous recombination (11, 14, 15). However, within microorganisms with poor or absent NHEJ, genome targeting can be lethal. For instance, natural systems that acquired genome-targeting spacers appear to possess inactive systems or mutated target loci (16), potentially explaining the evolution of *Pelobacter carbinolicus* harboring a type I-F CRISPR-Cas system (17). In the industrial bacterium *Streptococcus thermophilus*, cultures under bacteriophage attack rarely integrate genome-targeting spacers and, in such an event, rapidly disappear from the population (18). In the hyperthermophilic archaeon *Sulfolobus solfataricus* harboring a type III-A system, infecting cells with viral particles encoding a genome-targeting spacer slowed the growth of the culture under selecting conditions and led to recombination between the virally encoded spacer and the endogenous CRISPR array (19). In the bacterium *Escherichia coli* expressing a type I-E or II-A system in *trans*, transformation of a plasmid with spacers targeting endogenous genes or a lysogenized bacteriophage led to extremely low recovery of viable transformants (11, 20–23). Similar results were obtained in the bacterial pathogen *Streptococcus pneumoniae* expressing a type II system in *trans*, wherein viable transformants contained mutations or deletions that inactivated CRISPR-Cas-mediated targeting (11, 24). Finally, in the bacterial phytopathogen *Pectobacterium atrosepticum* harboring a native type I-F system, induction of genome-targeting spacers from a tightly regulated plasmid was cytotoxic within a few hours of induction and led to extensive deletions in the target loci (25). This extensive evidence presents an opportunity for the sequence-specific re-

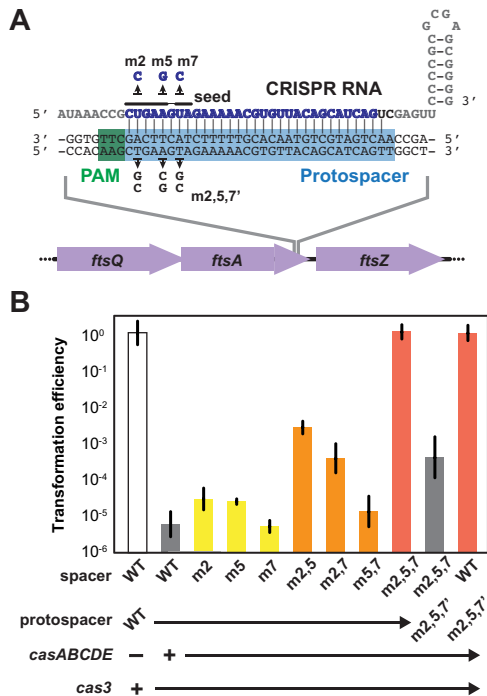
moval of microorganisms by reprogramming CRISPR-Cas systems.

Here, we investigated the potential of CRISPR-Cas systems for the sequence-specific targeting and selective removal of individual strains of bacteria. Using the *E. coli* type I system as a model, we found that targeting the *E. coli* genome led to potent removal of cells as long as the target sequence contained a PAM and was complementary to the spacer. In contrast to targeting of bacteriophages and plasmids, genome targeting accommodated multiple mutations in the seed region. Furthermore, targeting was similarly effective regardless of genomic location, strand, or transcriptional activity. Finally, using genomic sequence information, we could selectively remove closely related bacterial strains whether in pure or mixed cultures. The extent of removal could even be modulated by mixing targeting and nontargeting plasmids. Our findings open the possibility of quantitatively controlling the composition of microbial consortia and selectively treating multidrug-resistant infections, particularly with ongoing advances in the delivery of nucleic acids to microorganisms.

## RESULTS

**Genome targeting with the type I-E CRISPR-Cas system in *E. coli*.** We first evaluated the impact of targeting a natural genomic locus with the type I-E CRISPR-Cas system from *Escherichia coli* K-12, one of the best-characterized CRISPR-Cas systems to date. This system encodes six *cas* genes in two operons (*casABCDE* and *cas3*) required for CRISPR RNA processing and the cleavage and degradation of target DNA (26). Because the *casABCDE* operon is repressed by H-NS in *E. coli* K-12 under normal growth conditions (27), we used a previously developed system consisting of two plasmids (pCasA-E and pCas3) (see Fig. S1 in the supplemental material) that inducibly express all six *cas* genes (26). In addition, we generated a third plasmid, encoding an altered version of the endogenous CRISPR1 array in *E. coli* K-12 that accommodates the sequential insertion of engineered spacer sequences (pCRISPR) (see Fig. S1 and S2). pCRISPR plasmids encoding engineered, genome-targeting spacers were transformed into *E. coli* K-12 substrain BW25113 cells equipped with inducible expression of the T7 polymerase (BW25113-T7) and harboring the two *cas*-expressing plasmids (pCasA-E and pCas3) (see Fig. S1). As part of the assay, we measured the transformation efficiency, a proxy for removal of strains in pure cultures, by dividing the number of viable transformants for each genome-targeting CRISPR plasmid by the number of viable transformants for the original pCRISPR plasmid. Lower ratios or transformation efficiencies indicate a greater extent of removal.

We began with a spacer that is complementary to the template strand of the essential *ftsA* gene, involved in cell division (Fig. 2A). The selected protospacer was immediately downstream of AAG, one of the four PAMs for this CRISPR-Cas system (9). The resulting anti-*ftsA* ( $\alpha$ -*ftsA*) plasmid exhibited transformation efficiency  $\sim 10^5$ -fold lower than that of the original pCRISPR plasmid (Fig. 2B), paralleling the transformation efficiency of plasmids encoding prophage-targeting CRISPR RNAs (21, 22). In the absence of the *casABCDE* operon, the  $\alpha$ -*ftsA* plasmid and the original pCRISPR plasmid yielded similar transformation efficiencies (Fig. 2B), ruling out transformation issues and confirming the role of the *casABCDE* operon. Forced expression of the chromosomally encoded *cas* genes through deletion of the *hns* gene also resulted in a low transformation efficiency (see Fig. S3A in the



**FIG 2** Potent and sequence-specific removal through genome targeting with CRISPR-Cas systems. (A) Design of a CRISPR RNA targeting the *ftsA* gene in *E. coli* K-12. The 32-nt spacer sequence is in blue, and the repeat sequence is in gray. The last two nucleotides of the spacer (in black) are fixed to introduce restriction sites used for cloning additional repeat-spacer pairs. Flanking the protospacer (highlighted in blue) is the protospacer-adjacent motif (PAM) (highlighted in green) required for DNA targeting. Point mutations within the established seed region of the spacer (9) and the protospacer tested in panel B are shown. (B) Transformation efficiencies of  $\alpha$ -*ftsA* plasmids containing different mutations in the seed region of the spacer. Single, double, and triple mutations of the spacer sequence are shown in yellow, orange, and red, respectively. The transformations were carried out in BW25113-T7 (wild type [WT]) or BW25113-T7m257' (m2,5,7'), each harboring two plasmids: pCas3 (+*cas3*) and either pCasA-E (+*casABCDE*) or pCasA-E' (-*casABCDE*). Figure S1 in the supplemental material illustrates the general transformation procedure. Transformation efficiency was calculated as the number of transformants for each tested plasmid divided by the number of transformants for the original pCRISPR plasmid for the same culture. Values represent the geometric means and SEM of data from three independent experiments.

supplemental material) (27). In total, these results demonstrate the potency of genome targeting using a type I CRISPR-Cas system.

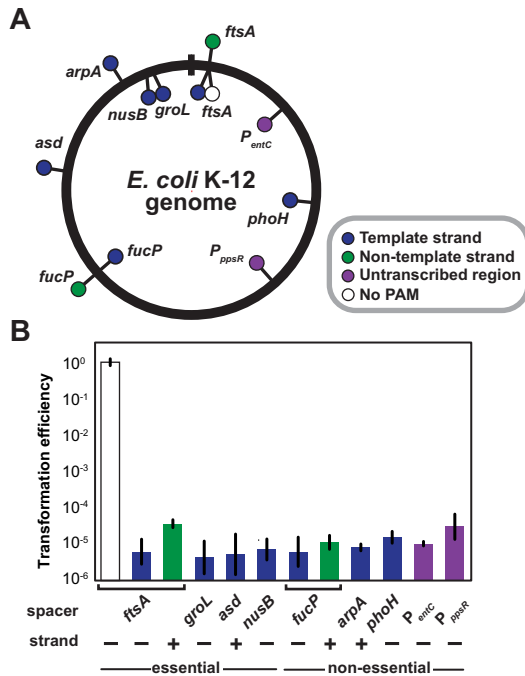
Each experiment yielded a few colonies that presumably escaped genome targeting. These colonies may have escaped through (i) alteration to the target genomic site or (ii) disruption of the expression/activity of the CRISPR RNAs or Cas proteins (16, 28). To initially explore the basis of escape, we inoculated 22 colonies for storage and further analysis. Less than half of the colonies (10/22) exhibited substantial growth after 13 h in liquid culture selecting for all three plasmids, suggesting that the rate of survival is overestimated. We first sequenced the genomic *ftsA* locus of the 10 viable colonies plus that of 4 additional colonies from a previous experiment. Interestingly, the locus was unaltered in all 14 isolates, unlike previous examples of escape (9, 20). Turning our attention to the expression plasmids, we found that the isolated plasmids conferred resistance to all three antibiotics when transformed into a plasmid-free strain, ruling out integration of

the resistance cassette into the *E. coli* genome. Finally, we sequenced the CRISPR locus on the  $\alpha$ -*ftsA* plasmid, which revealed various deletions that removed the  $\alpha$ -*ftsA* spacer (see Fig. S4 in the supplemental material). In total, alterations to the CRISPR RNAs appear to principally account for surviving colonies, at least within our experimental setup.

We next evaluated the sequence specificity of CRISPR-Cas-mediated removal. We introduced different point mutations into the seed region of the  $\alpha$ -*ftsA* spacer (Fig. 2A), where the seed region for the type I-E CRISPR-Cas system in *E. coli* was previously identified as the first through fifth, seventh, and eighth nucleotides flanking the PAM (9, 12). Prior work demonstrated that single point mutations within this region of the spacer for type I CRISPR-Cas systems disrupted immunity against bacteriophages (9). However, we found that point mutations to the second (m2), fifth (m5), or seventh (m7) nucleotide of the wild-type (WT)  $\alpha$ -*ftsA* spacer only marginally disrupted removal (Fig. 2B). Pairing point mutations (m2,5; m2,7; m5,7) further disrupted removal, while only the combination of all three point mutations (m2,5,7) fully disrupted removal.

To further probe the specificity of removal, we introduced compensatory mutations within the native *ftsA* gene (m2,5,7') (see Fig. S5 in the supplemental material). The matched pairing of the m2,5,7 spacer and the m2,5,7' strain resulted in a large extent of removal, albeit less than that seen with the pairing of the WT spacer and the WT strain (Fig. 2B). Separately, the mismatched pairing of the WT spacer and the m2,5,7' strain exhibited negligible removal (Fig. 2B), excluding the possibility of unintended targeting at other genomic loci.

**Potent removal by targeting diverse locations throughout the genome.** Programming of CRISPR-Cas systems to remove individual strains would greatly benefit from the ability to readily target any PAM-flanking sequence throughout a genome. Previous examples of genome targeting successfully targeted different genes on both strands of the genome (11, 19, 20, 25). However, a comprehensive and quantitative investigation of genome targeting has not been conducted. Toward this goal, we designed 10 additional spacers that target different protospacers flanked by a PAM throughout the *E. coli* K-12 genome (Fig. 3A). The corresponding protospacers covered a diverse range of locations, including the positive and negative strands of the genome, template strands and nontemplate strands of genes, and within untranscribed regions. Furthermore, we targeted both essential and non-essential genes because of their relative capacities to tolerate mutations or deletions. In all cases, the extent of removal was statistically similar to that of the original  $\alpha$ -*ftsA* spacer (*P* values between 0.05 and 0.88) (Fig. 3B), suggesting that removal is based on chromosomal injury rather than on perturbing the natural function of the target locus. Furthermore, in the absence of the *casABCDE* operon, each plasmid and the original pCRISPR plasmid yielded similar transformation efficiencies (see Fig. S6 in the supplemental material). The PAM was an essential feature, similar to findings of previous studies (11, 20, 25), since targeting a separate site within the *ftsA* gene lacking a PAM resulted in negligible removal (Fig. 3B). Based on these results, we conclude that potent removal can be achieved by targeting diverse locations throughout the genome as long as a PAM is present. Interestingly, the simultaneous targeting of multiple locations (*asd*, *msbA*, *ftsA*, and *nusB*) exhibited extents of removal similar to those with targeting of only one of the locations (*ftsA*) (*P* = 0.48) (see Fig. S7).

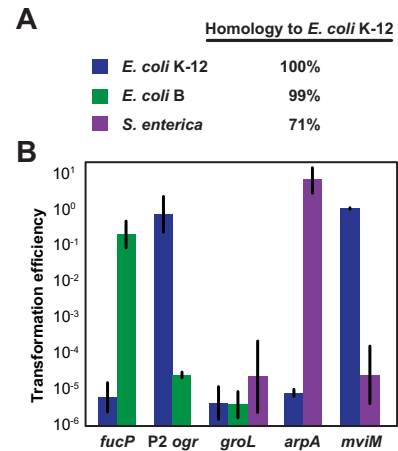


To explore the broad potential of our approach through native CRISPR-Cas systems outside of *E. coli*, we explored the impact of genome targeting in the Gram-positive bacterium *Streptococcus thermophilus*. In particular, we assessed genome targeting through the two native type II CRISPR-Cas systems (CRISPR1 and CRISPR3) previously shown to be active under normal growth conditions (4, 29). The transformation efficiencies of plasmids encoding CRISPR1 and CRISPR3 RNAs targeting the *lacZ* gene were near the limit of detection ( $\sim 10^3$ -fold lower than that of the empty plasmid) (see Fig. S3B and Table S1 in the supplemental material). Therefore, potent removal can be achieved through different native CRISPR-Cas systems.

**Sequence-specific removal of individual strains.** The flexibility and sequence specificity of genome targeting open the intriguing possibility of using CRISPR-Cas systems to specifically remove individual microbial species and strains. To begin exploring this possibility, we focused on two substrains of *E. coli*: *E. coli* K-12 (BW25113-T7) and *E. coli* B [BL21(DE3)] (Fig. 4A). Because the genomes of these strains bear more than 99% sequence homology and almost all cellular processes are identical (30), selectively removing one of the strains would be extremely difficult with antimicrobial agents or under defined growth conditions. However, the distinguishing sequences afford ample opportunities to selectively target either strain with programmed CRISPR-Cas systems. Using *in silico* genomic analyses, we identified one PAM-flanking

sequence unique to *E. coli* K-12 (within the *fucP* gene, involved in the transport of L-fucose), one PAM-flanking sequence unique to *E. coli* B (within the *ogr* gene, located within the P2 prophage), and one PAM-flanking sequence shared by both strains (within the *groL* gene, involved in protein folding). We subsequently designed CRISPR spacers that recognize PAM-adjacent protospacers in each gene and measured removal in pure cultures harboring pCasA-E and pCas3 (see Fig. S1 in the supplemental material). As expected, targeting *fucP* removed only the K-12 strain, targeting *ogr* removed only the B strain, and targeting *groL* removed both strains (Fig. 4B).

**Selective and titratable removal of individual strains in mixed cultures.** We next proceeded from pure cultures to mixed

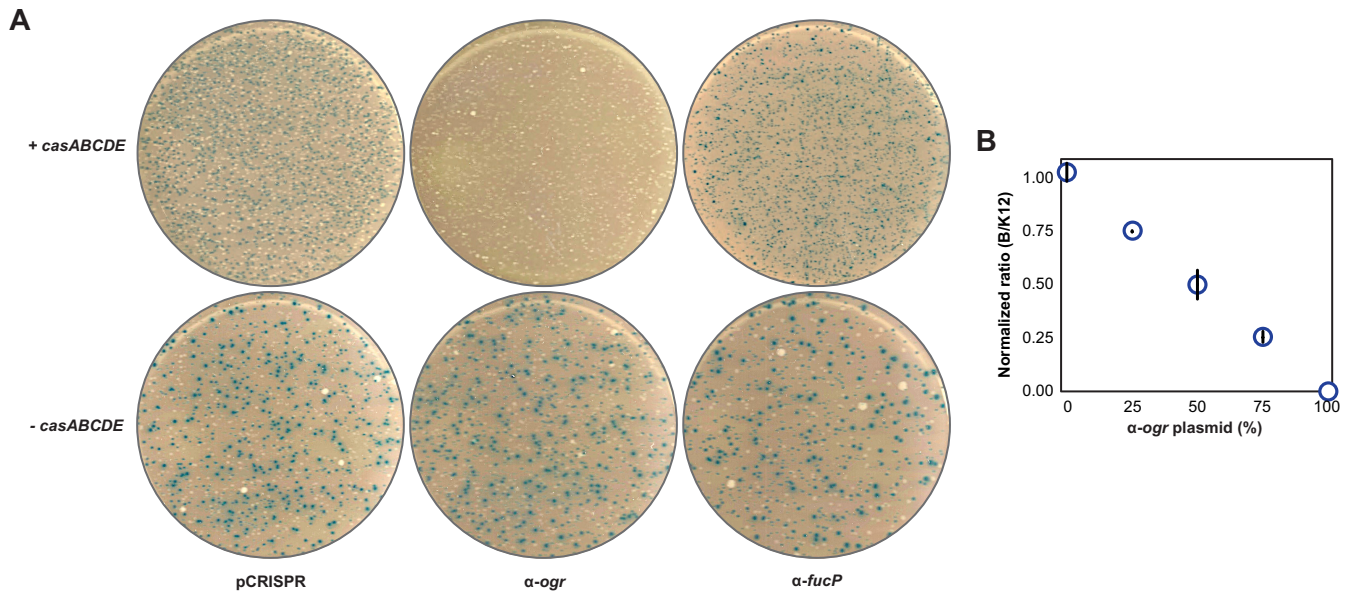


sequence unique to *E. coli* K-12 (within the *fucP* gene, involved in the transport of L-fucose), one PAM-flanking sequence unique to *E. coli* B (within the *ogr* gene, located within the P2 prophage), and one PAM-flanking sequence shared by both strains (within the *groL* gene, involved in protein folding). We subsequently designed CRISPR spacers that recognize PAM-adjacent protospacers in each gene and measured removal in pure cultures harboring pCasA-E and pCas3 (see Fig. S1 in the supplemental material). As expected, targeting *fucP* removed only the K-12 strain, targeting *ogr* removed only the B strain, and targeting *groL* removed both strains (Fig. 4B).

One potential application of programmable removal with CRISPR-Cas systems is targeting pathogenic bacteria while sparing commensal bacteria. Toward this goal, we focused on *E. coli* K-12 (BW25113-T7), a derivative of commensal *E. coli* that naturally inhabits the human digestive tract, and on *Salmonella enterica* (SB300A#1, a derivative of LT2), a major food pathogen. Both species are Gram-negative enterobacteria, and they share  $\sim 71\%$  sequence homology (Fig. 4A) (31). Using genomic analyses, we designed CRISPR spacers targeting a PAM-flanking sequence unique to *E. coli* (within the *arpA* gene, involved in the regulation of acetyl-coenzyme A [CoA] biosynthesis), a PAM-flanking sequence unique to *S. enterica* (within the *mviM* gene, encoding a putative virulence factor), and a shared PAM-flanking sequence (within the *groL* gene). The resulting plasmids were transformed into pure cultures harboring pCasA-E and pCas3 (see Fig. S1 in the supplemental material). As expected, targeting *arpA* removed only *E. coli*, targeting *mviM* removed only *S. enterica*, and targeting *groL* removed both strains (Fig. 4B).

**Selective and titratable removal of individual strains in mixed cultures.** We next proceeded from pure cultures to mixed





**FIG 5** Selective and titratable removal of individual bacterial strains in mixed cultures. (A) Selective removal of BW25113-T7 and BL21(DE3) in mixed cultures. Equal numbers of BW25113-T7 and BL21(DE3) cells harboring pCas3 and either pCasA-E (+ *casABCDE*) or pCasA-E' (- *casABCDE*) were mixed prior to preparation for electroporation. Mixed cultures electroporated with the indicated pCRISPR plasmid were plated on LB agar containing IPTG, X-Gal, L-arabinose, IPTG, and antibiotics. Only BL21(DE3) possesses the *lacZ* gene, which yields blue colonies (see Fig. S8 in the supplemental material). Plates are representative of three independent experiments. (B) Titratable removal of BL21(DE3) in mixed cultures. Transformations were conducted similarly to the method described for panel A except that cells were transformed with different ratios of the *E. coli* B-targeting plasmid (pCRISPR-ogr) and the nontargeting plasmid (pCRISPR) for a total of 100 ng. The ratio of blue to white colonies was normalized to the ratio of blue to white colonies for the same culture transformed with the pCRISPR plasmid. Values represent the geometric means and SEM of data from three independent experiments.

cultures in order to evaluate the selective removal of target strains. We repeated the transformation experiments with *E. coli* B [BL21(DE3)] and *E. coli* K-12 (Bw25113-T7), except that both strains were cocultured and plated on agar with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). Under these plating conditions, BL21(DE3) yields blue colonies, whereas BW25113-T7 yields white colonies (see Fig. S8 in the supplemental material). Similar to the experiments with pure cultures, targeting the PAM-flanking sequence within the *ogr* gene selectively removed BL21(DE3), targeting the PAM-flanking sequence within the *fucP* gene selectively removed Bw25113-T7, and targeting with the original pCRISPR plasmid maintained both strains (Fig. 5A). Furthermore, in the absence of the *casABCDE* operon, the two strains were maintained in similar ratios regardless of the transformed plasmid (Fig. 5A). These results demonstrate that CRISPR-Cas systems can be employed for the selective removal of bacterial strains in mixed cultures.

The above mixed-culture experiments utilized single plasmids to either remove or maintain individual strains. We hypothesized that transforming combinations of targeting and nontargeting plasmids could remove a portion of targeted cells, conferring control over the composition of the population. To test this hypothesis, we transformed different amounts of the pCRISPR plasmid and the BL21(DE3)-targeting plasmid (total of 100 ng) and then quantitated the ratio of blue and white colonies. Remarkably, the fraction of the BL21(DE3)-targeting plasmid strongly correlated with the selective removal of BL21(DE3) (Fig. 5B). The almost-perfect linear correlation ( $R^2 = 1.00$ ) further suggests that almost all transformed cells received a single plasmid that either removed

or sustained *E. coli* B. We thus conclude that CRISPR-Cas systems can be reprogrammed to quantitatively modulate the composition of a mixed population.

## DISCUSSION

We demonstrated the sequence-specific removal of individual strains using CRISPR-Cas systems. While the extent of removal was extremely high (>99.9999% for the  $\alpha$ -*ftsA* plasmid [Fig. 2B]), a fraction of the transformed cells consistently survived targeting. Sequencing survivors revealed consistent loss of the genome-targeting spacer in the transformed pCRISPR plasmid (see Fig. S4 in the supplemental material), likely through recombination between the identical repeats. This insight is consistent with findings of a recent study showing loss or inactivation of CRISPR elements under evolutionary pressure (28). This insight also suggests one potential countermeasure against surviving colonies: reducing the number of repeats within the CRISPR array. The array could even be reduced to a single repeat-spacer, paralleling the engineering of single-guide RNAs for use with Cas9 (32). Other potential strategies include expressing multiple CRISPR-Cas systems or eliminating CRISPR-encoding plasmids that underwent recombination. Targeting multiple sites at one time did not appear to be an effective strategy (see Fig. S7), perceivably due to rearrangement of the CRISPR-encoding plasmid (see Fig. S4).

We also demonstrated that potent removal could be achieved using type I and type II CRISPR-Cas systems. An interesting distinction between these systems is that type I systems cleave and degrade DNA through the action of a 3'-to-5' exonuclease, whereas type II systems only cleave DNA (33). The additional effect of DNA degradation by type I systems may further improve

the potency of genome targeting by preventing DNA repair, although a direct comparison between type I and type II systems would be needed to directly evaluate this potential contribution.

We found that multiple mismatches within the seed region were required to fully disrupt targeting by the type I-E system from *E. coli* (Fig. 2). This may explain the absence of mutations within the protospacer of surviving colonies (see Fig. S4 in the supplemental material). Separately, the number of required mismatches contrasts with the single mismatches that disrupted immunity to the M13 bacteriophage (9). This discrepancy is intriguing considering that the same Cas-encoding plasmids were used in these studies. One possibility is that the seed region can accommodate different numbers of mismatches when targeting genomic DNA or when targeting invader DNA. Such differences may help explain emerging reports of elevated off-target effects associated with genome editing (34–37).

Delivery arguably poses the most immediate challenge to the downstream use of CRISPR-Cas systems for the selective and titratable removal of microorganisms. However, opportunities in nanoparticle development and the engineering of bacteriophages present potential solutions. Nanoparticles have been used to deliver nucleic acids to bacteria (38), but little subsequent work has been done; the delivery of CRISPR RNAs may provide the impetus to further investigate nanoparticles as vehicles of delivery to microorganisms. Separately, bacteriophages have been widely used for heterologous protein expression, gene delivery, and the treatment of bacterial infections (39, 40). Lysogenic bacteriophages or phagemids with broad host ranges would be particularly beneficial for the delivery of CRISPR-Cas-encoding constructs (41–43). While silver nanoparticles and lytic bacteriophages also could be used to remove bacteria (44, 45), they lack the specificity or the programmability offered by genome-targeting CRISPR-Cas systems and cannot be easily dosed to quantitatively control the composition of a microbial consortium.

Once delivery challenges are overcome, we foresee CRISPR-Cas systems being exploited to control bacterial populations in diverse ecological niches and scientific fields. In biotechnology, CRISPR-Cas systems could be used to selectively clear contaminating microorganisms or to quantitatively control the composition of microbial consortia in industrial processes or in environmental samples. In medicine, CRISPR-Cas systems could be used to control the composition of the gut flora or as “smart” antibiotics that circumvent commonly transmitted modes of antibiotic resistance and distinguish between beneficial and pathogenic bacteria. For applications that require the removal of more than one strain, multiple spacers that target shared or unique sequences could be encoded in a single CRISPR array. The arrays could also be combined with a complete set of *cas* genes to instigate removal of strains lacking functional CRISPR-Cas systems (15, 46). Because of the sequence specificity of targeting, CRISPR-Cas systems could be used to distinguish strains separated by only a few base pairs. The use of CRISPR-Cas systems would require detailed knowledge of the genomic sequences of the bacterial population, although the dwindling cost and increasing speed of high-throughput sequencing along with powerful metagenomics tools would alleviate this challenge. Overall, CRISPR-Cas systems offer a unique opportunity for the selective and titratable removal of microorganisms for industrial and medical purposes, which can be added to the ever-expanding applications of this versatile immune system (11, 20, 23, 47, 48).

## MATERIALS AND METHODS

**Strains and plasmid construction.** See Table S2 in the supplemental material for a list of all strains used in this work. *E. coli* K-12 strain BW25113-T7 was generated by transferring *araB::T7-RNAP-tetA* from IY5163 to BW25113 by P1 transduction. Successful transduction was verified by PCR. BW25113-T7m2,5,7' (Fig. 2A) was generated using three rounds of oligonucleotide-mediated recombination with *ftsA*-m257-spacer.recomb and the pKD46 plasmid encoding the  $\lambda$  *red* recombination genes (49, 50). The oligonucleotide contained two phosphorothioate linkages at each end to improve the recombination efficiency (51, 52). Successful recombinants were verified by PCR and by sequencing.

See Table S2 in the supplemental material for a list of all plasmids used in this work. The origins of replication for the pCas3, pCasA-E, and pCRISPR plasmids used with *E. coli* and *S. enterica* belong to different incompatibility groups (26, 53). To generate the pCasA-E plasmid lacking the *casABCDE* operon (pCasA-E'), pCasA-E was digested with NcoI/NotI, blunt ended using *Pfu* polymerase, and ligated.

To generate the pCRISPR plasmid, the pBAD18 plasmid (53) was linearized with XbaI and amplified by PCR using primers pBAD18.fwd/pBAD18.rev. A chemically synthesized gBlock (IDT) was then inserted downstream of the  $P_{BAD}$  promoter by Gibson assembly (54). The gBlock encoded four repeats and three intervening spacers from the endogenous CRISPR locus in *E. coli* K-12 MG1655 (see Table S2 in the supplemental material), where the first spacer was modified to include a KpnI restriction site and an XhoI restriction site. These restriction sites allow the sequential insertion of engineered repeat-spacer pairs (see Fig. S2). Each pair was chemically synthesized as two oligonucleotides (IDT), phosphorylated with polynucleotide kinase, annealed, and ligated into the pCRISPR plasmid digested with KpnI and XhoI.

The pBAD18-*asd*,*msbA*,*ftsA*,*nusB* plasmid was constructed in a manner similar to that for the pCRISPR plasmid, wherein a chemically synthesized gBlock (IDT) was inserted downstream of the  $P_{BAD}$  promoter of the linearized pBAD18 plasmid by Gibson assembly (54). The gBlock encoded the first repeat-spacer sequence from the endogenous *E. coli* CRISPR locus, followed by five repeats and four intervening spacers targeting four different locations in *E. coli* Bw25113 (*asd*, *msbA*, *ftsA*, and *nusB*) (see Table S2 in the supplemental material).

To generate pORI28 (55) with engineered spacers, pORI28 and each insert generated through PCR assembly were digested with BamHI and SacI and ligated together. To generate the insert encoding the *lacZ1* spacer, template-free PCR was conducted with C1-*lacZ1*.fwd/C1-*lacZ1*.rev, followed by using the resulting product in a subsequent PCR with C1-BamHI.fwd/C1C3-SacI.rev. To generate the inserts encoding the *lacZ2* and *lacZ3* spacers, first the CRISPR3 leader region was amplified by PCR from LMD-9 genomic DNA with C3-leader.fwd/C3-leader.rev. Next, the resulting product was used as the template in a subsequent PCR with C3-leader.fwd/C3-*lacZ2*.rev or C3-leader.fwd/C3-*lacZ3*.rev. Finally, each PCR product was used as the template in a final round of PCR with C3-leader.fwd/C1C3-SacI.rev. All oligonucleotides and enzymes were purchased from IDT and NEB, respectively. All cloned plasmids were verified by sequencing.

**Growth conditions.** All *E. coli* and *Salmonella* strains were cultured in LB medium (10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter sodium chloride) at 37°C and 250 rpm with appropriate antibiotics. The same strains were plated on LB agar (LB medium with 1.5% agar) supplemented with appropriate inducers and incubated at 37°C. *S. thermophilus* LMD-9 was cultured in Elliker broth (Elliker medium [Difco] supplemented with 1% beef extract) and plated on Elliker agar (Elliker broth with 1.5% agar) (56). Both culturing and plating of LMD-9 were conducted at 37°C. Antibiotics were administered at the following final concentrations: 50  $\mu$ g/ml streptomycin, 50  $\mu$ g/ml kanamycin, 50  $\mu$ g/ml ampicillin, 2  $\mu$ g/ml chloramphenicol, and 2  $\mu$ g/ml erythromycin. Inducers were administered at the following final concentrations: 0.1 mM IPTG and 0.02% L-arabinose.

**Design of native CRISPR RNAs.** An overview of the approach to design and insert spacer sequences into the CRISPR array within the pCRISPR plasmid is shown in Fig. S2 in the supplemental material. The spacers were designed by identifying one of the known PAMs for the type I-E CRISPR-Cas system in *E. coli* (AAG, GAG, GAG, and ATG). The downstream 32 nucleotides (nt) were then used as the spacer within the engineered repeat-spacer pair. Note that the last two nucleotides of the spacer are fixed as TC because of the adopted cloning strategy (see Fig. S2). However, these nucleotides fall well outside the seed region and therefore are expected to have a negligible effect on targeting.

The spacers for *S. thermophilus* were designed by identifying a known PAM for CRISPR1 (NNAGAAW) or for CRISPR3 (NGGNG) (10). The sequence of the 31 nt upstream of each PAM was integrated into oligonucleotides that were used to generate a leader region followed by a single repeat-spacer-repeat that was subsequently cloned into pORI28. This construct relies on processing through the native tracrRNA and RNase III.

**Transformation assay.** Freezer stocks of *E. coli* and *Salmonella* strains harboring pCas3 and pCasA-E (or pCasA-E') were streaked to isolation on LB agar. Individual colonies were inoculated into 3 ml of LB medium and shaken overnight at 37°C. The cultures then were back diluted into 25 ml of LB medium and grown to an  $A_{600}$  of 0.6 to 0.8, which was measured on a Nanodrop 2000c spectrophotometer (Thermo Scientific). The cells were then pelleted and washed with ice-cold 10% glycerol 2 times before being resuspended in 150 to 350  $\mu$ l of 10% glycerol. The resuspended cells (50  $\mu$ l) were transformed with 50 ng of pCRISPR or pCRISPR encoding the indicated spacer, using a MicroPulser electroporator (Bio-Rad), and recovered in 300  $\mu$ l of SOC medium (Quality Biological) for 1 h (*E. coli*) or for 2 h (*Salmonella*). After the recovery period, 200  $\mu$ l of different dilutions of the cells were plated on LB agar with inducers. The transformation efficiency was calculated by dividing the number of transformants for the tested plasmid by the number of transformants for the original pCRISPR plasmid. To normalize for experimental variability in transformation efficiency, the same batch of cells prepared for electroporation was transformed with each tested plasmid and the original pCRISPR plasmid.

*S. thermophilus* strain LMD-9 harboring pTRK669 was grown in 50 ml of Elliker broth and prepared for electroporation as described previously, which concentrated the culture 100-fold (57). The resuspended cells (50  $\mu$ l) were transformed with 1  $\mu$ g of the pORI28 control plasmid or pORI28 containing the indicated spacer. Transformed cells were recovered in 950  $\mu$ l of Elliker broth overnight and plated on Elliker agar. Plates were then incubated for 48 h in a Coy anaerobic chamber with a gas mixture of 10% hydrogen, 5% carbon dioxide, and 85% nitrogen before the colonies were counted. The transformation efficiency was calculated by dividing the number of transformants for the tested plasmid by the number of transformants for the pORI28 control plasmid.

The average limit of detection of the killing assay, calculated as  $1/(\text{no. of transformants for the control plasmid})$  was  $7 \times 10^{-7}$  for *E. coli*,  $4 \times 10^{-7}$  for *Salmonella*, and  $2 \times 10^{-3}$  for *S. thermophilus*. The high transformation efficiency for *Salmonella* was achieved by purifying the pCRISPR plasmids, the pCas3 plasmid, and the pCasA-E plasmid individually from SB300A#1.

**Mixed-culture transformation assay.** The transformation assay for mixed cultures resembled that for the pure culture with a few notable differences. Cultures of *E. coli* K-12 and *E. coli* B strains harboring pCas3 and pCasA-E (or pCasA-E') were grown separately to an  $A_{600}$  of ~0.8, and then equal numbers of cells were mixed from the back dilutions prior to preparing the culture for electroporation. An aliquot of the resuspended cell mixture (50  $\mu$ l) was then transformed with the pCRISPR plasmid, pCRISPR encoding the indicated spacer, or a defined mixture of both plasmids for a total of 100 ng. The transformed cells were recovered in 300  $\mu$ l of SOC medium for 90 min. After the recovery period, 200  $\mu$ l of different dilutions of the cells were plated on LB agar with inducers and appropriate antibiotics. The ratio of blue (*E. coli* B) to white (*E. coli* K-12) colonies on the sample plate was divided by the same ratio on the

pCRISPR plate, yielding the normalized ratio. To normalize for experimental variability in transformation efficiency, the same batches of cell mixtures prepared for electroporation were transformed with each tested plasmid mixture and the pCRISPR control plasmid.

**Analysis of escape mutants.** Colonies from the transformation assay with the  $\alpha$ -*ftsA* plasmid (pCB304) were inoculated into 5 ml of LB medium with appropriate antibiotics and inducers. Growth was assessed based on the  $A_{600}$  after 13.5 h of growth. Cultures exhibiting measurable growth ( $A_{600} > 0.01$ ) were stored as glycerol stocks. Plasmids were then isolated from each escape mutant, and equal amounts of DNA were resolved by agarose gel electrophoresis. Each isolated set of plasmids was also transformed into *E. coli* K-12 and plated on LB agar containing one of the three antibiotics. Finally, the plasmid mixture from each escape mutant was sequenced using primers that specifically bind within the  $P_{BAD}$  promoter or the double terminator of the  $\alpha$ -*ftsA* plasmid. To analyze the protospacers, approximately 400 bp surrounding the protospacer within the *ftsA* gene of the escape mutant was PCR amplified and subjected to sequencing.

**Statistical analyses.** All *P* values were calculated using the Student *t* test, assuming log-normal distributions, two tails, and unequal variances.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00928-13/-/DCSupplemental>.

Figure S1, EPS file, 0.7 MB.  
Figure S2, EPS file, 0.6 MB.  
Figure S3, EPS file, 0.5 MB.  
Figure S4, EPS file, 0.6 MB.  
Figure S5, EPS file, 0.5 MB.  
Figure S6, EPS file, 0.5 MB.  
Figure S7, EPS file, 0.5 MB.  
Figure S8, EPS file, 3.4 MB.  
Table S1, DOC file, 0.1 MB.  
Table S2, DOC file, 0.2 MB.

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