A highly precise and portable genome engineering method allows comparison of mutational effects across bacterial species

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Currently available tools for multiplex bacterial genome engineering are optimized for a few laboratory model strains, demand extensive prior modification of the host strain, and lead to the accumulation of numerous off-target modifications. Building on prior development of multiplex automated genome engineering (MAGE), our work addresses these problems in a single framework. Using a dominant-negative mutant protein of the methyl-directed mismatch repair (MMR) system, we achieved a transient suppression of DNA repair in *Escherichia coli*, which is necessary for efficient oligonucleotide integration. By integrating all necessary components into a broad-host vector, we developed a new workflow we term pORTMAGE. It allows efficient modification of multiple loci, without any observable off-target mutagenesis and prior modification of the host genome. Because of the conserved nature of the bacterial MMR system, pORTMAGE simultaneously allows genome editing and mutant library generation in other biotechnologically and clinically relevant bacterial species. Finally, we applied pORTMAGE to study a set of antibiotic resistance-conferring mutations in *Salmonella enterica* and *E. coli*. Despite over 100 million y of divergence between the two species, mutational effects remained generally conserved. In sum, a single transformation of a pORTMAGE plasmid allows bacterial species of interest to become an efficient host for genome engineering. These advances pave the way toward biotechnological and therapeutic applications. Finally, pORTMAGE allows systematic comparison of mutational effects and epistasis across a wide range of bacterial species.

genome engineering | synthetic biology | recombineering | off-target effects | methyl-directed mismatch repair

Recent advances in genome engineering technologies are transforming basic research and industrial biotechnology through the previously unprecedented ability to engineer biological traits. Techniques incorporating zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided nucleases have allowed efficient targeted modification of a host of model organisms (1), promising better understanding of biological processes and more efficient production of bioproducts. Although directed nucleases have widened the range of bacterial species into which individual genomic modifications can be introduced, there seems to be a technical limit when it comes to using these techniques for simultaneous modification of multiple loci (2, 3). Among others, multiplex genome editing is required for explicit genotype-phenotype mapping, as well as modification of protein complexes and biosynthetic pathways (4).

Currently, the only genome engineering method in bacteria that enables rapid, automated and high-throughput genome editing is multiplex automated genome engineering (MAGE) (5). MAGE uses recombineering (6) to simultaneously incorporate multiple single-strand DNA (ssDNA) oligonucleotides (oligos), and thereby rapidly creates desired allele combinations and combinatorial genomic libraries. From accelerated optimization of biosynthetic pathways (5, 7–9) to the construction of a so-called "genomically recoded organism" (10–12), MAGE has allowed genome-engineering endeavors of unparalleled complexity in *Escherichia coli*. Functionality of ssDNA-mediated recombineering has been described in various other species, including lactic acid bacteria (13), *Corynebacterium glutamicum* (14), and *Bacillus subtilis* (15). However, portability remains seriously limited as these efforts require prior optimization for each individual target species (Table S1).

Even in *E. coli*, for efficient and unbiased incorporation of mutations by MAGE, extensive modifications—expression of the λ Red recombinase enzymes, as well as inactivation of the native methyl-directed mismatch repair (MMR) system—need to be made to the host strain. Additionally, because of the inactivation of the MMR machinery (16) necessary for MAGE, there is a nearly two orders-of-magnitude increase in the background mutation rate during the process, leading to the accumulation of undesired, off-target mutations (17). These off-target mutations could in turn interfere with the phenotypic effects of the engineered modifications. Recently, we attempted to address this issue by replacing wild-type *mutL* and *mutS* with heat-sensitive mutants, and limiting the inactivation of MMR to a short period

Significance

Current tools for bacterial genome engineering suffer from major limitations. They have been optimized for a few laboratory model strains, lead to the accumulation of numerous undesired, off-target modifications, and demand extensive modification of the host genome prior to large-scale editing. Herein, we address these problems and present a simple, all-in-one solution. By utilizing a highly conserved mutant allele of the bacterial mismatch-repair system, we were able to gain unprecedented precision in the control over the generation of desired modifications in multiple bacterial species. These results have broad implications with regards to both biotechnological and clinical applications.

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of the MAGE cycle. Although we managed to reduce the number of off-target mutations by 85% (18), modification of the parental strain was still a prerequisite. This issue was also recently addressed by the so-called transient-mutator MAGE technique (19), which allows for flexible, plasmid-based modification of bacterial chromosomes. However, transient-mutator MAGE was only demonstrated to work in *E. coli*, therefore portability across species remained a formidable challenge.

Here, we developed a generalized strategy for bacterial genome editing that overcomes these limitations of MAGE and expands multiplex recombineering to other bacterial species. Key to this process is the temperature-controlled expression of a dominant-negative mutator allele of the *E. coli* MMR protein MutL (20), enabling transient suppression of DNA repair during oligonucleotide integration. MutL is a component of the MutHLS complex responsible for methyl-directed mismatch repair, acting to recruit the MutH endonuclease to sites of DNA damage (21). Importantly, this particular mutator allele cannot be complemented by the native MutL protein (20). Therefore, in contrast to traditional MAGE, no prior disruption of the genomic copy is needed.

In this work, a set of plasmids (dubbed pORTMAGE) expressing the λ Red recombinase enzymes, as well as the dominant-negative mutator allele of MutL, all under the control of the cI857 temperature-sensitive repressor, were constructed. During each MAGE cycle, expression of the synthetic operon is induced by a single temporal temperature shift. Lowered MMR activity is only necessary during a brief period of each MAGE cycle (i.e., during allelic replacement). Because our protocol allows for rapid switching between mutator and nonmutator states, it minimizes the time the bacterial population spends susceptible to the accumulation of off-target mutations. As the standard MAGE protocol already incorporates a shift in temperature during induction of the λ Red recombinase enzymes, the temperature shift required during pORTMAGE is entirely compatible with the established procedure (5).

Additionally, because of the highly conserved nature of MutL (22), expression of this *E. coli mutL* allele can suppress mismatch repair in distant relatives of *E. coli*, diverged from it ∼100–200 million y ago (23). Thus, pORTMAGE simultaneously allows genome editing and mutant library generation in several biotechnologically and clinically relevant bacterial species. As a proof-of-concept study, we demonstrate that pORTMAGE is a valuable tool to study clinically relevant issues, such as the phenotypic impact of antibiotic resistance conferring mutations across bacterial species.

Results

Characterization of the MutL E32K Dominant-Negative Mutator. First, we characterized the effect of the previously described (20) dominant-negative mutator allele MutL E32K on the functionality of the MMR system in wild-type *E. coli* K-12 MG1655. Using standard techniques (*SI Materials and Methods*), we cloned the mutant allele into the inducible expression vector pZA31tetR (24, 25). Induced expression resulted in an over 30-fold increase in mutation rate in the wild-type as measured by a rifampicin resistance assay and subsequent fluctuation analysis (26) (Fig. S1). This approached the mutation rate of the MG1655Δ*mutS* deletion strain lacking functional MMR machinery, and showed that the wild-type copy of MutL was not able to suppress the effect of the dominant-negative mutant allele. We conclude that this construct enables controlled disruption of the MMR machinery from an extrachromosomal expression system.

pORTMAGE Allows Highly Efficient and Unbiased Allelic Replacement in *E. coli*. We rationalized that temperature-controlled expression of a dominant-negative mutator allele of MutL (18) would allow a transient switch from a nonmutator to mutator phenotype of the host cell. At nonpermissive temperatures (30–34 °C), the mutant protein is not expressed, allowing the native MMR system to function properly, thus limiting the number of background mutations to wild-type level. Moreover, as the particular E32K mutator allele cannot be complemented by the native MutL protein, no disruption of the genomic copy is needed.

For ease of use, all required genetic parts for MAGE were assembled on a single plasmid with a broad host range origin of replication (pBBR1) (27), resulting in the pORTMAGE plasmid (Fig. 1). In this arrangement, expression of the *mutL E32K* gene, as well as the three λ Red recombinase enzymes (*exo*, *bet*, and *gam*) were under the control of the cI857 temperature-sensitive λ repressor. Quantitative real-time RT-PCR (qPCR) testing showed that temperature induced transcription of the operon resulted in a 320- to 770-fold increase in *mutL* expression (Fig. S2).

To investigate the effect of the expression of the dominant-negative MutL allele on allelic replacement frequency of ssDNA oligos, we used a previously characterized test system (28). We introduced a diverse set of single-base pair mismatches (A:A, G:G, T:T, G:A, G:T, C:A, and C:T) at specific genomic locations within *lacZ*. Because these mutations introduce premature stop codons within *lacZ*, the frequency of allelic replacement could be easily measured by a colorimetric assay (*SI Materials and Methods*). Two separate oligos were used to generate a G:A mismatch at different positions to show that repair of a specific mismatch type is also context-dependent, as demonstrated previously (28). We found that in all cases, pORTMAGE allowed highly efficient and unbiased oligo incorporation, comparable to the capacity of the traditional MAGE protocol on a mismatch repair knockout background (18, 28). Indeed, allelic replacement frequency greater than 20% was observed in all instances, even in the cases of mismatches that are otherwise well recognized and almost completely corrected by the wild-type MMR system (Fig. S3).

Efficient Multiplex Genome Editing Is Coupled with Low Off-Target Mutation Rate. Using pORTMAGE, we carried out multiplex genome editing to investigate allelic replacement frequency and off-target mutagenesis simultaneously. Three strains were compared: a

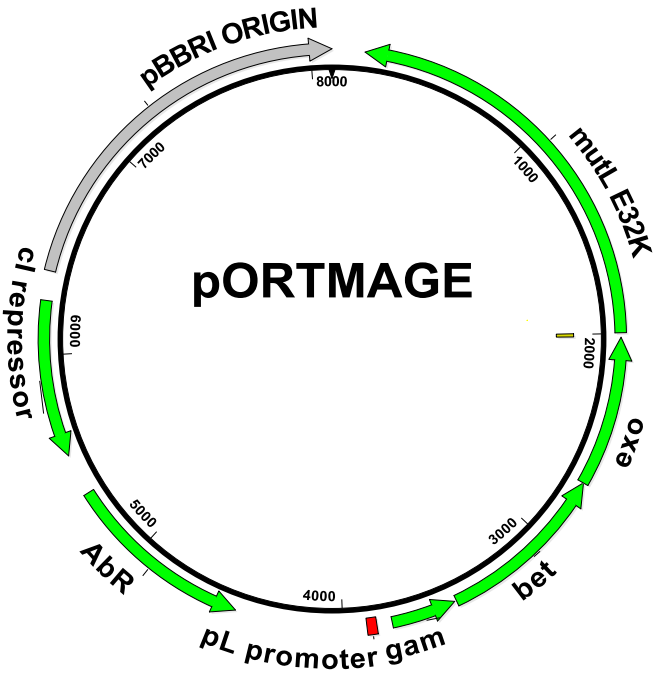

Fig. 1. General map of the pORTMAGE plasmid. Expression of the *mutL E32K* gene [along with the three λ Red recombinase enzymes (*exo*, *bet*, and *gam*)] is controlled by the cI857 temperature-sensitive λ repressor.

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mutator strain carrying the control pSIM8 plasmid (27) expressing the λ Red recombinases [Δ*mutS*(pSIM8)], the wild-type nonmutator strain carrying pORTMAGE [MG1655(pORTMAGE)], and the wild-type strain carrying the control plasmid [MG1655(pSIM8)].

In 24 cycles of MAGE, six different loci were subsequently targeted (four cycles each). These loci are widely distributed across the genome and were targeted by oligos that introduce various types of mismatches into them (see ref. 18 for details). Clones carrying a particular modification were verified by capillary sequencing, followed by MAGE cycles targeting the next locus. Allelic replacement frequencies were determined at each locus either by colorimetric assay or allele-specific PCR (*SI Materials and Methods*).

As expected, allelic replacement frequency with MG1655 (pSIM8) was very low in all cases. MG1655(pORTMAGE) generally displayed a high level of replacement, approaching the values observed with traditional MAGE using Δ*mutS*(pSIM8) (Table 1). In some cases (e.g., *araB* T50A and *cycA* AA139TG), the values observed with MG1655(pORTMAGE) were significantly lower than with Δ*mutS*(pSIM8) but still over an order-of-magnitude higher than that of MG1655(pSIM8).

Next, we investigated the accumulation of off-target mutations. After 24 cycles of MAGE, we selected one independently edited clone each derived from MG1655(pORTMAGE), Δ*mutS* (pSIM8), and MG1655(pSIM8), respectively. To infer the number of off-target mutations, the genomes of the starting and the MAGEderived clones were sequenced using the IonTorrent PGM system. MG1655(pSIM8) accumulated only two off-target mutations. In sharp contrast, Δ*mutS*(pSIM8) carried 84 different off-target genomic mutations, a figure that is in line with previous reports (17, 18). Remarkably, we failed to find any off-target mutation in MG1655(pORTMAGE). For a complete list of all off-target mutations, see Dataset S1.

Taken together, these results demonstrate that high allelic replacement frequency in pORTMAGE is coupled with an exceptionally low off-target mutation rate.

pORTMAGE Allows Rapid Genome Editing Across a Range of Bacterial Species. We first tested the impact of the dominant *mutL* allele on mutation rates in several enterobacterial species with biotechnological or clinical relevance. We selected as models the clinically important *Salmonella enterica* serovar Typhimurium (strain LT2), the fish pathogen *Edwardsiella tarda* (strain ATCC15947), the opportunistic pathogen *Escherichia hermanii* (strain HNCMB35034), as well as the biotechnologically relevant organisms *Citrobacter freundii* (strain ATCC8090), and *E. coli* BL21(DE3). Phylogenetic comparison of MutL sequences (Fig. S4) showed that glutamic acid is conserved in all investigated species at the position of the mutation in the *E. coli* dominant-negative allele E32K (22). We therefore assumed that the mutant allele would have similar phenotypic effect in these species. In agreement with expectation, overproduction of the *E. coli* mutant MutL showed similar, at least an order-of-magnitude increase in mutation rates in all other investigated species (Fig. S5), indicating the feasibility of the pORTMAGE strategy in other species.

Next, we compared the efficacy of pORTMAGE in *E. coli* K-12 MG1655, and distant relatives *S. enterica* and *C. freundii*. To broaden the potential applications of pORTMAGE, we engineered three modified pORTMAGE plasmids with elevated MutL expression and different antibiotic markers (*SI Materials and Methods*). To characterize the performance of pORTMAGE in a uniform manner across species, we constructed a landing pad sequence integrated into the host genome, and used it as the target sequence (Fig. 2*A*; see also Dataset S2 for all bacterial strains used in the study and the corresponding genotype information, and Dataset S3 for the complete nucleotide sequence). A set of five oligos (90 nucleotides in length each), introducing all possible single-base mismatches at given positions were designed. Each oligo carried a degenerate base at one of four specific positions representing all four nucleotides, plus an additional oligo to assay positional differences of the same target nucleotide. One MAGE cycle was carried out using these oligos pooled together.

As in the previous section, we compared the performances of the wild-type strain carrying the control plasmid, the wild-type nonmutator strain carrying a pORTMAGE plasmid, and the mutator Δ*mutS* strain carrying the control pSIM8 plasmid. To measure allelic replacement frequencies within the population after one MAGE cycle, we performed deep-sequencing of the targeted landing pad sequence using an Illumina MiSeq set-up.

Replacement efficiency in the wild-type control carrying pSIM8 varied substantially across the three investigated species (Fig. 2 *B–D*), suggesting natural variation in mismatch repair. In addition, mutations introducing the same mismatch at different positions showed a difference in some cases compared with each other, indicating a context dependency of the efficiency of mismatch repair. Despite these differences, allelic replacement frequency with pORTMAGE was in several cases two orders-of-magnitude higher and largely unbiased in all three species compared with the wild-type control (Fig. 2 *B–D*). Moreover, in *E. coli* K-12 MG1655 (Fig. 2*B*) and *S. enterica* (Fig. 2*C*), allelic replacement frequencies with pORTMAGE approached the values obtained with Δ*mutS* for all incorporated mismatch types. In *C. freundii*, the pORTMAGE plasmid showed the same robust performance as in *E. coli* and *S. enterica* (Fig. 2*D*).

Finally, we investigated the impact of pORTMAGE on replacement efficiency using an established protocol (cos-MAGE) that includes enrichment of the desired genetic modifications using a selectable marker gene (7). Such a coselection procedure is biotechnologically relevant, as it aids incorporation of hard-to-engineer genomic modifications. The landing pad sequence was designed to contain an inactivated *cat* gene. Accordingly, a single MAGE cycle was carried out using an oligo repairing the inactivated *cat* gene, followed by selection for the appropriate genetic marker (chloramphenicol resistance). We found that pORTMAGE substantially increased allelic replacement frequencies in all investigated species (Fig. S6).

pORTMAGE Allows Efficient Generation of Mutant Libraries in Various Bacterial Species. Next, using pORTMAGE, we introduced sequence diversity at specific genetic loci. We randomized six individual bases within the *asnA* gene in three species (*E. coli*, *S. enterica*, and *C. freundii*). Using organism-specific 90-base oligos carrying six randomized positions, we carried out five cycles of MAGE.

Table 1. Allelic replacement frequency of all six used oligos in MG1655(pSIM8), Δ*mutS*(pSIM8), and MG1655(pORTMAGE) after four genome editing cycles each

	Allelic replacement frequency (%)					
Strain	*lacZ* A652T	*malK* C252G	*araB* T50A	*hisB* C166T	*rpsL* A128G	*cycA* AA139TG
MG1655(pORTMAGE)	54.58	61.56	39.76	22.92	33.76	22.92
Δ*mutS*(pSIM8)	51.32	60.23	62.87	39.58	38.91	41.67
MG1655(pSIM8)	45.31	51.82	1.56	<0.1	0.72	1.04

Each oligo name consists of the targeted gene and the introduced nucleotide modification.

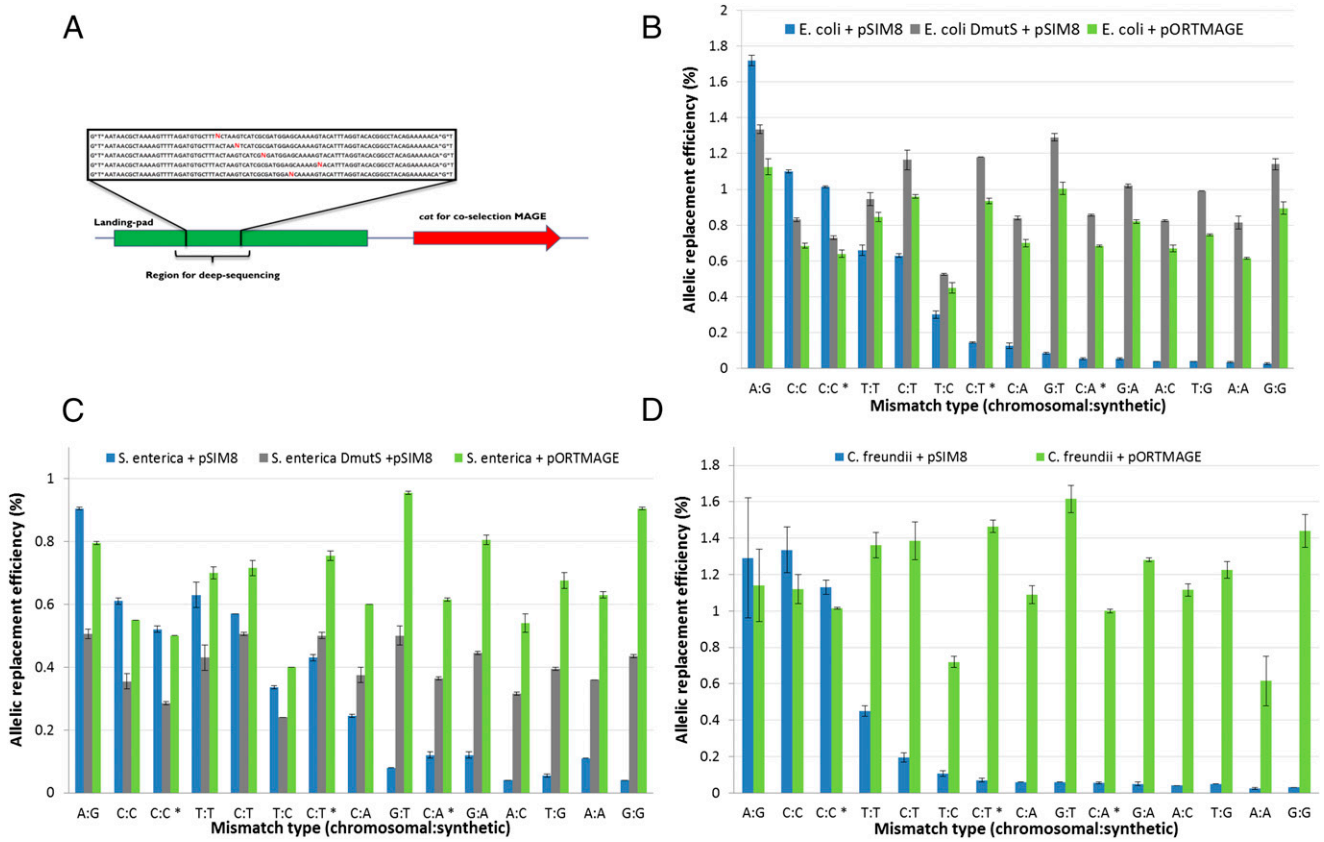

Fig. 2. (*A*) General map of the landing pad sequence inserted into the various strains. The green region represents a target sequence for allelic replacement by a set of five oligos shown in the targeting box. Degenerate bases are shown for each oligo in red. The *cat* gene conferring resistance to chloramphenicol was included in the landing pad to allow for cos-MAGE to be performed (see *SI Materials and Methods* for details). Allelic replacement frequency of the various test oligonucleotides targeting the *tetR* landing pad sequence in (*B*) *E. coli* K-12 MG1655, (*C*) TS616 derivative of *S. enterica* serovar Typhimurium LT2, and (*D*) *C. freundii* (strain ATCC8090). The values are the means of two independent measurements using Illumina deep-sequencing; error bars represent SEMs. An asterisk denotes oligos generating the same mismatch as another oligo to demonstrate context dependency of allelic replacement. DmutS denotes deletion of the mutS gene.

Genomic DNA was isolated, the target region was amplified by PCR, and finally, the fragments were subjected to Illumina deep-sequencing. In all three species (Fig. S7), allelic replacement frequencies with pORTMAGE were at least an order-of-magnitude higher at all six positions, compared with the frequencies obtained with the control pSIM8 plasmid. Additionally, pORTMAGE substantially reduced the biases in the incorporation frequency of certain nucleotides, allowing for a more uniform distribution of the mutants in the population. Using pORTMAGE, we obtained a bias-free mutant library at the target locus for each species (Fig. S7), suggesting that all possible ∼4,000 variants were represented at a reasonable quantity.

Application of pORTMAGE to Study Antibiotic Resistance in *S. enterica*. It has long been suggested that because of the prevalence of epistatic interactions, mutations beneficial in one genetic background are frequently neutral or even deleterious in another (29). This issue is especially relevant in the context of antibiotic resistance, as this phenomenon could contribute to the observed differences in the molecular mechanisms underlying antibiotic resistance in related microbial species. Better understanding of this problem demands genome editing applicable in a range of species. Here, we demonstrate that pORTMAGE is an exceptionally effective tool for studying the phenotypic effects of individual mutations.

We introduced 10 mutations individually into the genomes of *S. enterica* and *E. coli* (for the list of mutations, see Table 2). These mutations have previously been detected in an experimental evolution study (30) and confer resistance to one or multiple antibiotics. The corresponding genes are not only clinically relevant, but also show 79–99% sequence identities between the two species. The nucleotide sequences subjected to editing by pORTMAGE were fully conserved in most cases and were mutated to the same residue in all cases.

The workflow consisted of the introduction of each mutation into the receiver strain by a single MAGE cycle, selection of the desired mutation by high-resolution melting (HRM) analysis, and subsequent confirmation of the mutations by capillary-sequencing. The complete workflow took as few as 4 d for completion. As a measure of the effect of each individual mutation, the sensitivity of each mutant was measured against the antibiotic that it arose against during the course of laboratory evolution (30). Using a well-established protocol (31), the minimum inhibitory concentration (MIC) value of each antibiotic against the given mutant was determined compared with the wild-type strain (relative MIC value = MIC of the mutant/MIC of the wild-type). Finally, we compared the relative MIC changes conferred by each individual mutation in *S. enterica* and *E. coli* (Table 2 and Fig. S8).

We found that despite over 100 million y of divergence between the two species, mutational effects remained generally conserved. In most cases, the investigated mutations resulted in a small, but significant decline in antibiotic susceptibilities in both species (Table 2).

There were a few notable exceptions to this trend. For example, the S83L amino acid substitution in the major target protein (GyrA) confers resistance to nalidixic acid and other gyrase inhibitor drugs (32). The very same mutation is regularly observed in

Table 2. Relative MIC values of mutant strains of *E. coli* and *S. enterica*

Gene	Mutation	Antibiotic	*E. coli* relative MIC	*S. enterica* relative MIC
mprA	Arg110Leu	NIT	1.20	1.73
marR	Val84Glu	AMP	2.20	1.15
soxR	Leu139*	ERY	2.31	1.73
phoQ	Gly384Cys	NIT	1.20	0.83
trkH	Thr350Lys	STR	3.18	1.59
gyrA	Ser83Leu	CPR	16.00	16.00
gyrA	Ser83Leu	NAL	97.66	610.35
fis	Thr70Pro	ERY	1.44	1.44
acrR	Gln78*	ERY	1.20	1.00
ompC	Met1	NIT	1.20	1.20
ycbZ	Ser438Arg	ERY	1.23	1.51

The sensitivity of each mutant strain was measured against the antibiotic against which the specific mutation formed during laboratory adaptation (30). The measured MIC for each strain was then compared with the MIC of the wild-type strain, resulting in the relative MIC value. The antibiotic abbreviations are as follows: AMP, ampicillin; CPR, ciprofloxacin; ERY, erythromycin; NAL, nalidixic acid; NIT, nitrofurantoin; STR, streptomycin.

antibiotic-resistant laboratory and clinical *E. coli* strains (30, 33). Surprisingly, we found that the level of resistance conferred by S83L is at least six-times higher in *S. enterica* than in *E. coli* (Table 2). Another interesting case is MarR, a central regulator of the Mar (multiple anbiotic resistance) regulon that coordinates the expression of a global network of at least 80 chromosomal genes (34). The Val84Glu mutation in this gene confers a twofold decrease in ampicillin susceptibility in *E. coli*, whereas mutating to the same residue barely had any phenotypic effect in *S. enterica* (Table 2). Clearly, the genetic and molecular mechanisms underlying these differences in mutational effects between species deserve future investigations. Our main aim here was to demonstrate how unique pORTMAGE is to study these clinically important issues.

Discussion

Among the currently available bacterial genome-editing tools, oligonucleotide-mediated recombineering is probably the most cost-effective and versatile choice (Table S1). Bacterial recombineering has been optimized toward multiplexing and automation, resulting in MAGE (5). Additionally, oligonucleotides can now be designed (35) and synthesized (36) with great ease at large quantities, allowing for genome-engineering undertakings of unprecedented complexity (10–12). However, multiplex bacterial genome engineering has been optimized for a few laboratory model strains, demands extensive prior modification of the host strain, and leads to the accumulation of numerous off-target modifications (Table S1). Accumulation of unwanted mutations in the targeted cells can mask the effect of the intentionally introduced modifications. Furthermore, for inactivation of the MMR system, the host has to be modified beforehand, greatly limiting the ease of use and narrowing the applicable range of organisms. Although a method expanding multiplex genome editing has recently been described [MuGENT (37)], it is applicable only to naturally transformable bacteria (for other potential limitations, see Table S1). Building on prior works, our study addresses the above mentioned three major problems—ease of use, off-target mutagenesis, and portability across species—in a single framework.

We developed pORTMAGE, an all-in-one plasmid set easily applicable to a range of Gram-negative bacterial species. The main results are as follows. First, transient expression of a dominant mutator allele of the MutL MMR protein allows for a controllable switch between mutator and nonmutator phenotype in a range of clinically and biotechnologically relevant species. Second, by implementing this advance, highly efficient and precise allelic replacement was achieved in selected enterobacterial species. Third, efficient multiplex genome editing was coupled with low off-target mutation rate. Fourth, pORTMAGE allowed rapid generation of large, unbiased sequence libraries at desired positions in these species.

Finally, we used pORTMAGE to introduce 10 antibiotic-resistance mutations into the genomes of *S. enterica* and *E. coli*, two species that diverged from each other more than 100 million y ago. The complete workflow took 4 d for completion. To the best of our knowledge, this is a significant improvement over traditional gene-editing protocols applicable to *S. enterica*, as they are either slow, have relatively low replacement efficiency, or are prone to off-target mutagenesis. These advances allowed us to address the extent of conservation of the molecular mechanisms underlying antibiotic resistance. We found that despite over 100 million y of divergence between the two species, mutational effects remained generally conserved. In most cases, the investigated mutations resulted in a small, but significant decline in antibiotic susceptibilities in both species. However, the phenotypic effects of certain canonical mutations varied extensively between the two species. For example, the level of resistance conferred by the canonical S83L mutation in gyrase A is at least six-times higher in *S. enterica* than in *E. coli* (Table 2). One possible interpretation of this result is that epistatic interactions within and across genes shape the antibiotic resistance profile. Clearly, pORTMAGE facilitates future studies in this direction.

In all, by combining efficient allelic replacement with low background mutation rate and portability, pORTMAGE offers a convenient tool to refactor complex cellular traits and systematically engineer metabolic pathways in a diverse set of enterobacterial species. pORTMAGE also paves the way toward exploring mutational effects and epistasis, which could potentially be exploited for the development of novel antimicrobial strategies.

However, pORTMAGE has two potential limitations (Table S1). First, an important unresolved issue is the extent to which the applicability of pORTMAGE demands sequence similarity of MutL between the host organism and *E. coli*. We suspect that pORTMAGE does not require high sequence conservation, as long as the functional role of MutL in the mismatch-repair system remains unchanged. Indeed, despite substantial differences in MutL sequences between *Pseudomonas aeruginosa* and *E. coli*, the *P. aeruginosa* copy is able to complement that of *E. coli* (38). In fact, even the human mismatch-repair protein MutL homolog 1 (hMLH1) functionally interacts with the *E. coli* MMR machinery and was able to induce a dominant mutator state in *E. coli* (39). Because inactivation of the MMR machinery greatly improves allele replacement efficiency in organisms ranging from yeast (40) to human cell lines (41, 42), pORTMAGE could inspire future development of general genome editing tools.

Another potential problem is that, similarly to other recombinase-based genome-editing tools, pORTMAGE heavily relies on the use of specific enzymes and expression vectors. The expanding repertoire of characterized recombinases and expression systems (15, 43–46) will presumably allow for broad use in the near future.

We anticipate that our work will have implications for clinical and biotechnological problems as well. For example, pORTMAGE may be used in engineering of attenuated bacterial pathogens for vaccine development (47). It may be also useful for metabolic engineering attempts in previously untapped species. For example, *C. freundii* is an efficient host for the production of valuable bioproducts (48), but optimization of metabolic pathways remained challenging in this species. Because *C. freundii* is amenable to pORTMAGE, we expect rapid future development in this area. Other species, such as environmental *Pseudomonas* strains, have great potential to serve as chassis for industrial biotechnology (49) but are thus far lacking in robust techniques allowing for efficient multiplex genome editing. Finally, pORTMAGE could also open a new avenue of research in diverse fields such as functional

genomics and evolutionary biology (50). To our knowledge, for the first time, pORTMAGE allows systematic comparison of mutational effects and epistasis across a wide range of bacterial species.

Materials and Methods

Detailed descriptions of the methodology, including (*i*) strains and reagents, (*ii*) oligonucleotides, (*iii*) plasmid construction, (*iv*) pORTMAGE cycling protocol, (*v*) whole-genome resequencing, (*vi*) high-throughput sequencing for allelic replacement frequency measurement, (*vii*) integration of landing pad sequence into host strains, (*viii*) construction of antibiotic resistance associated mutants, (*ix*) MIC measurement, (*x*) qPCR measurement, and (*xi*) mutation rate measurement can be found in *SI Materials and Methods*.

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