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Note

Reporting off-target effects of recombinant engineering using the pORTMAGE system

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ABSTRACT

pORTMAGE recombineering is a simple technique for incorporation of novel point mutations into bacterial genomes that eliminates off-target effects. Here we inserted point mutations into the *cusS* gene from *Escherichia coli*, then, using Illumina sequencing, report genetic variants in all mutant strains. Several off-site mutations were found at high frequency. Low frequency mutations also show high heterogeneity. This means that it is essential for studies to report all off-target effects and acknowledge the effect that this may have on resultant phenotypes.

Genome editing has advanced significantly over the past few decades by providing a faster and more cost-efficient way to genetically modify bacterial genomes at specific target sites. Genome editing was largely based on inducing genetic variation and screening/selecting for a desired phenotype (Pines et al., 2015). It is now possible to target specific genomic sites using indirect techniques such as programmable nucleases (CRISPR /Cas9, Zinc Finger Nucleases, and Transcription Activator-Like Effector Nucleases (TALENS)) (Esvelt and Wang, 2013) and more direct methods such as multiplex automated genome engineering (MAGE) (Court et al., 2002; Wang et al., 2009; Wang et al., 2012; Wannier et al., 2021). Specifically, MAGE uses single stranded oligonucleotides carrying desired mutations that are recombined into the genome and rely on successful inactivation of the methyl-directed mismatch repair system. This ultimately leads to an increase in background mutation rate by two-orders of magnitude and the accumulation of off-target mutations impacting future phenotypic studies (Csörgő et al., 2020). Nyerges et al. (Nyerges et al., 2016) then modified this method (pORTMAGE) to overcome the limitations of MAGE by creating a plasmid harboring a temperature controlled dominant negative *mutL* allele which only limits DNA repair during oligonucleotide integration along with a λ Red recombinase enzyme. This reduces the time in which bacteria are susceptible to increased mutation rate thereby decreasing off-target effects. Some have even claimed that the use of this system can essentially eliminate off-target effects (Nyerges et al., 2016; Csörgő et al., 2020). Many have now used these methods to associate novel phenotypes with specific nucleotide changes, albeit with no report of off-target mutations (Russ et al., 2020; Tiz et al., 2019; Moura de Sousa et al., 2017; Sato et al., 2018; Spohn et al., 2019). Here we used pORTMAGE recombineering in *Escherichia coli* K12 MG1655 to insert chromosomal mutations into the histidine kinase *cusS* shown through experimental evolution to be involved in silver resistance (Graves Jr et al., 2015; Randall et al., 2015; Tajkarimi et al., 2017). As a control, we also inserted a chromosomal mutation (D516G) in *rpoB* (Wannier et al., 2020). We then carried out whole genome Illumina sequencing to evaluate off-target mutations and heterogeneity in our resultant populations.

To design ssDNA oligonucleotides to insert both *cusS* and *rpoB* mutations, we used the MAGE Oligo Design Tool (MODEST) (Bonde et al., 2014) (Table 1). We then followed a standard protocol (Sawitzke et al., 2013) for recombineering. In short, wild-type (WT) *E. coli* K12 MG1655 harboring the pORTMAGE-4 plasmid were cultured overnight in Luria Broth (LB) supplemented with 25 μg/mL chloramphenicol at 32 °C. Then 1 mL was subcultured into 2 × 70 mL of LB and incubated at 32 °C to an OD_{600} of 0.5. One culture was then grown at 32 °C uninduced (control) and the other at 42 °C for 15 min to induce the λ Red recombinase and the double-negative mutator allele of MutL (Nyerges et al., 2016). After induction, the flasks were immediately put on ice, cells were washed twice in cold water then resuspended in 200 μL of cold water and kept on ice. For electroporation, 50 μL of the uninduced cells and 1 μL of ddH_2O were transferred to pre-chilled cuvettes (1 mm gap), and in a second, 50 μL of the induced cells and 1 μL (~100 ng) of the desired single stranded oligonucleotide were electroporated for 5 msecs at 1.8 kV and a capacitance of 10 μF. After electroporation, 1 mL of LB was added and placed on ice, cells were then transferred into a 15-mL conical tube and incubated for 30 min at 32 °C. As our desired *cusS* mutations had been previously shown to lead to silver resistance (R15L,

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T14P, T17P and L329P (Graves Jr et al., 2015; Randall et al., 2015; Tajkarimi et al., 2017)), we performed a 10-fold serial dilution and plated on LB agar supplemented with 20 μg/mL of silver nitrate to screen for mutations. We then picked single colonies from the *cusS* mutant plates, grew them up overnight in LB broth alone, extracted genomic DNA using the OMEGA E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Inc., GA, USA), PCR amplified the *cusS* gene and sent for sequencing (ETON Biosciences, Durham, NC, USA). After mutations were confirmed, the plasmid was cured by serial plating on LB alone, typically it took only 1–2 overnight platings to cure the plasmids. We then extracted genomic DNA from the cured populations and performed whole genome Illumina sequencing at the Microbial Genome Sequencing Center (MiGS Center) at the University of Pittsburgh. We used four different controls, (1) recombineered *rpoB* mutants were serial diluted and plated on LB agar supplemented with 50 μg/mL rifampicin for selection, (2) WT + pORTMAGE and WT cured of pORTMAGE were plated on (3) LB agar alone (WT (cured) LB) and (4) LB agar supplemented with 20 μg/mL silver nitrate (WT (cured) Ag). Colonies were picked, grown up overnight in LB, pelleted and sent directly to MiGS Center for genomic DNA extraction and whole genome sequencing. Sequence alignment and variant calling from the samples were done using the *breseq* 0.30.0 pipeline (Deatherage and Barrick, 2014) and aligned to the *E. coli* K12 MG1655 reference sequence (NC_000913). DNA sequencing data has been deposited into the BioProject ID: PRJNA869586. The original WT strain was sequenced in one of our previous studies (Graves Jr et al., 2015).

PCR amplification of the *cusS* gene from genomic extractions showed successful insertion of all our desired point mutations with a success rate > 90% in picked clones. Whole genome resequencing (DNAseq) also confirmed successful incorporation and maintenance of our desired *cusS* mutations after curing the pORTMAGE plasmid, in addition to successful insertion of our desired *rpoB* mutation. All *cusS* and *rpoB* mutations were detected at a frequency (*f*) of 1.0 (Table 2 and S1-S4 and S8) confirming that the entire population carried the desired mutations to fixation.

DNAseq of our WT + pORTMAGE populations (Table 2 and S5) showed fixation ($f = 1.00$) in a variety of genes associated with the presence of the plasmid including a 1 bp deletion in the intergenic region between *xisD* and *exoD*, 8 different *exoD* mutations, a 1 bp deletion in *hsdS* and a non-synonomous mutation in *mutL*. Once the plasmid is cured (WT (cured) LB (Table 2 and S6) and WT (cured) Ag (Table 2 and S7)) all these mutations are gone except for the 1 bp deletion in *hsdS*. Also, through the curing process to remove the pORTMAGE plasmid, this population acquired a novel 1 bp deletion in the non-coding region of *gadY* which was maintained when plated on both LB and LB supplemented with silver. Interestingly, the WT (cured) Ag, also acquired a spontaneous mutation in *cusS* to fixation due to the selection process as it was not present in WT (cured) LB.

DNAseq also showed that, in addition to our desired mutations, all the recombineered clones carried the same 1 bp deletion in *hsdS* ($f = 1.0$) found in WT + pORTMAGE, WT (cured) LB and WT (cured) Ag. As this mutation has never been seen in any of our past sequencing studies using this exact population of *E. coli* K-12 MG1655 (Graves Jr et al., 2015; Tajkarimi et al., 2017; Thomas et al., 2021; Boyd et al., 2022) it is clear that this mutation is the result of the presence of the pORTMAGE plasmid and remains after curing of the plasmid. Along with this, each of our recombineered clones carried unique selective sweeps ($f = 1$); the R15L mutant carried a synonomous mutation in the multidrug efflux pump *mdtB*, the T14P mutant carried a synonomous mutation in *maeB* and the L329P mutant carried four mutations to fixation in *ecpC, ydcF, ptrB* and *lptF*. We also sequenced three T17P recombineered clones and found both commonalities and differences between the three. They all carried a mutation in *ycaI,* and a synonomous mutation in the intergenic regions between *ycdU* ← / ← *serX*. One population had a 34,308-bp deletion, two carried an intergenic mutation between *yfjL* ← / ← *yfjM* and one in *yqiJ*. None of these selective sweeps are within close proximity to the target gene with the closest one being almost 300,000 base pairs away (Table 2). We also detected off-target mutations in our *rpoB* mutant which carried a unique synonymous mutation in *feaB* in addition to the *hsdS* found in all populations. Again, none of these mutations have been detected in the WT population.

Finally, all the mutant populations also carried many low frequency mutations (Table S1-S8). The R15L mutant carried 9 mutations above a frequency of 0.1 and 97 mutations below a frequency of 0.1. T14P carried 6 and 102, T17P 31 and 283 and L329P 8 and 120 respectively. The *rpoB* mutant carried 3 above 0.1 and 68 below. Our control WTs also carried low frequency mutations, WT + pORTMAGE carried 3 and 45, WT (cured) LB carried 3 and 51 and WT (cured) Ag carried 4 and 55 above and below 0.1 respectively. It is important to note the heterogeneity in these populations and that these low frequency mutations have the potential for selection during subsequent phenotypic studies that commonly follow recombineering protocols and could contribute or alter observed phenotypes during assessment.

We also plated WT cured of pORTMAGE on LB supplemented with silver nitrate (WT (cured) Ag) to determine if the secondary mutations acquired in the *cusS* recombineered mutations above were due to our selection process and as we see no mutations in common between this and our *cusS* populations, we believe that they are coming from the recombineering process as the only spontaneous mutations acquired was in *cusS* itself.

pORTMAGE recombineering is a simple and accessible technique for incorporation of novel point mutations into bacterial genomes (Csörgő et al., 2020; Nyerges et al., 2016). Albeit the claims of little to no off-target mutations, we showed here that all populations that have harbored pORTMAGE carry a 1-bp deletion in *hsdS*, and our mutant populations all carried additional novel mutations to fixation, several in common between mutants, others in common among mutants and others completely unique to each population. Due to the large positional difference of the detected mutations, it is possible that chromatin structure may influence this positional targeting as opposed to simple genomic location, but this remains to be elucidated. It is also likely that

Table 1
MODEST designed single stranded oligonucleotides used for insertion of *cusS* mutations using pORTMAGE recombineering.

Gene	Amino Acid Change	Nucleotide Change	Position	Sequence
cusS	R15L	CGC- > CTC	44 G- > T	GTTACATGCTTGAGGTGCCGGATGGTCAGTAAGCCATTTCAGCGCCCGTTTTCGCTGGCAACCC**t**CCTGACCTTTTTTATCAGCCTGGCC
cusS	T14P	ACC- > CCC	40 A- > C	ATGGTCAGTAAGCCATTTCAGCGCCCGTTTTCGCTGGCA**c**CCCGCCTGACCTTTTTTATCAGCCTGGCCA
cusS	T17P	ACC- > CCC	49 A- > C	GTAAGCCATTTCAGCGCCCGTTTTCGCTGGCAACCCGCCTG**c**CCTTTTTTATCAGCCTGGCCACCATCGC
cusS	N279H	AAT- > CAT	835A- > C	ATCGCTCACGAAATTCGCACACCAATTACG**c**ATCTCATAACGCAAACCGAAATCGCCCTCAGCCAGTCG
rpoB	D516G	GAC- > GGC	1547A- > G	AGCGGGTTGTTGTTCTGGTACATAAAC**c**GAGACAGCTGGCTGGAACCGAAGAACTCTTTC

Table 2
Selective sweeps resulting from pORTMAGE recombineering of the *cusS* gene.

Gene	Position	Mutation	Annotation **	Gene *	Description
WT (cured) - LB	3,664,962	Δ1 bp	noncoding (99/105 nt)	*gadY* →	sRNA antisense regulator of gadAB transcriptional activator GadX mRNA, Hfq-dependent
	4,580,448	Δ1 bp	coding (1015/1395 nt)	*hsdS* ←	specificity determinant for HsdM and HsdR
WT (cured) - Ag	3,664,962	Δ1 bp	noncoding (99/105 nt)	*gadY* →	sRNA antisense regulator of gadAB transcriptional activator GadX mRNA, Hfq-dependent
	4,580,448	Δ1 bp	coding (1015/1395 nt)	*hsdS* ←	specificity determinant for HsdM and HsdR
	593,467	T → G	D435A (GAC → GCC)	*cusS* ←	sensory histidine kinase in two-component regulatory system with CusR, senses copper ions
cusS R15L	594,727	C → A	R15L (CGC → CTC)	*cusS* ←	sensory histidine kinase in two-component regulatory system with CusR, senses copper ions
	2,157,170	G → A	R636R (CGG → CGA)	*mdtB* →	multidrug efflux system, subunit B
	4,580,448	Δ1 bp	coding (1015/1395 nt)	*hsdS* ←	specificity determinant for HsdM and HsdR
cusS T14P	594,731	T → G	T14P (ACC → CCC)	*cusS* ←	sensory histidine kinase in two-component regulatory system with CusR, senses copper ions
	2,577,211	G → A	I389I (ATC → ATT)	*maeB* ←	malic enzyme: putative oxidoreductase/putative phosphotransacetylase
	4,580,448	Δ1 bp	coding (1015/1395 nt)	*hsdS* ←	specificity determinant for HsdM and HsdR
cusS T17P	594,722	T → G	T17P (ACC → CCC)	*cusS* ←	sensory histidine kinase in two-component regulatory system with CusR, senses copper ions
	964,458	G → A	V47I (GTC → ATC)	*ycaI* →	ComEC family inner membrane protein (*All three populations*)
	262,958	Δ34,308 bp		*ykfN–ptwF*	46 genes (*Population 3*)
	1,097,184	A → G	intergenic (+355/+381)	*ycdU* → / ← *serX*	putative inner membrane protein/tRNA-Ser (*All three populations*)
	2,765,411	+GCACTATG	intergenic (−258/+102)	*yfjL* ← / ← *yfjM*	CP4–57 putative defective prophage, DUF4297/DUF1837 polymorphic toxin family protein/CP4–57 prophage; uncharacterized protein (*Population 1 and 2*)
	4,580,448	Δ1 bp	coding (1015/1395 nt)	*hsdS* ←	specificity determinant for HsdM and HsdR (*All three populations*)
	3,192,437:1	+T	coding (230/630 nt)	*yqiJ* →	DUF1449 family inner membrane protein (*Population 1*)
cusS L329P	593,785	A → G	L329P (CTA → CCA)	*cusS* ←	sensory histidine kinase in two-component regulatory system with CusR, senses copper ions
	1,487,689	A → G	D152G (GAC → GGC)	*ydcF* →	DUF218 superfamily protein, SAM-binding
	308,268	Δ1 bp	coding (1065/2526 nt)	*ecpC* ←	ECP production outer membrane protein
	1,927,235:1	+C	coding (1605/2061 nt)	*ptrB* ←	protease II
	4,487,071	C → T	P285L (CCG → CTG)	*lptF* →	lipopolysaccharide export ABC permease of the LptBFGC export complex
	4,580,448	Δ1 bp	coding (1015/1395 nt)	*hsdS* ←	specificity determinant for HsdM and HsdR
rpoB D516G	4,182,791	A → G	D516G (GAC → GGC)	*rpoB* →	RNA polymerase, beta subunit
	1,447,818	G → A	E100E (GAG→GAA)	*feaB* →	phenylacetaldehyde dehydrogenase
	4,580,448	Δ1 bp	coding (1015/1395 nt)	*hsdS* ←	specificity determinant for HsdM and HsdR
WT + pORTMAGE	566,097	Δ1 bp	intergenic (−18/+1)	*xisD* ← / ← *exoD*	pseudogene, exisionase in defective prophage DLP12;Phage or Prophage Related/pseudogene, DLP12 prophage; phage-type exonuclease family;Phage or Prophage Related
	566,173	C → G	pseudogene (204/279 nt)	*exoD* ←	pseudogene, DLP12 prophage; phage-type exonuclease family;Phage or Prophage Related
	566,205	T → C	pseudogene (172/279 nt)	*exoD* ←	
	566,245	G → A	pseudogene (132/279 nt)	*exoD* ←	
	566,277	C → T	pseudogene (100/279 nt)	*exoD* ←	
	566,323	C → T	pseudogene (54/279 nt)	*exoD* ←	
	566,326	T → C	pseudogene (51/279 nt)	*exoD* ←	
	566,332	T → G	pseudogene (45/279 nt)	*exoD* ←	
	566,356	T → C	pseudogene (21/279 nt)	*exoD* ←	
	4,580,448	Δ1 bp	coding (1015/1395 nt)	*hsdS* ←	specificity determinant for HsdM and HsdR
	4,397,505	G → A	E32K (GAA → AAA)	*mutL* →	methyl-directed mismatch repair protein

* Arrows represent the direction of transcription.
** For mutations in intergenic regions, gives two relative positions (e.g., +150/−119) where the numbers are the distances from the mutation to the nearest neighboring genes before and after it in the genome, and the +/− signs indicate whether the mutation is oriented upstream or downstream with respect to each of these genes.

selection is playing some role during the plating of recombineered populations to find successful clones. Although, if this playing a dominant role, we would expect a decrease in low frequency mutations as compared to the controls and we see the opposite indicating an element of randomness associated with off-target mutations.

Moving forward, it is important that future phenotypic studies using genomic engineering techniques acknowledge the heterogeneity when reporting novel phenotypes and that the genetic background could be contributing to their observations. Therefore, studies that employ genome engineering should consistently verify and report the changes that result from whole genome resequencing rather than only confirming the presence of intended mutations.

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Author contributions

B.R.S., M.F. and S.T. carried out and optimized the recombinant engineering protocols described. J.L.G. processed all DNA sequencing data sets, M.D.T. designed and supervised over the project and along with B.R.S wrote the initial draft of the manuscript. All authors revised and contributed to the final manuscript.

Declaration of Competing Interest

The authors have no competing interests to be disclosed.

Data availability

The datasets generated and analyzed in this study can be found through the NCBI BioProject database (http://www.ncbi.nlm.gov/bioproject/) under Bioproject number PRJNA869586, SRA (and will be made available upon publication).

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