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Technical Note

Fast, Simultaneous Tagging and Mutagenesis of Genes on Bacterial Chromosomes

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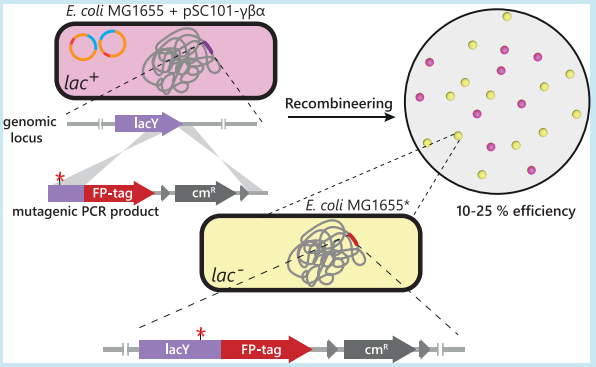
ABSTRACT: Fluorescence microscopy has become a powerful tool in molecular cell biology. Visualizing specific proteins in bacterial cells requires labeling with fluorescent or fluorogenic tags, preferentially at the native chromosomal locus to preserve expression dynamics associated with the genomic environment. Exploring protein function calls for targeted mutagenesis and observation of differential phenotypes. In the model bacterium *Escherichia coli*, protocols for tagging genes and performing targeted mutagenesis currently involve multiple steps. Here, we present an approach capable of simultaneous tagging and mutagenesis of essential and nonessential genes in a single step. We require only the insertion of a stretch of the target gene into an auxiliary plasmid together with the tag. Recombineering-based exchange with the native locus is then carried out, where the desired mutation is introduced during amplification with homology-bearing primers. Using this approach, multiple tagged mutants per gene can be derived quickly.



KEYWORDS: *protein tagging, point mutagenesis, recombineering, genome engineering, fluorescence microscopy*

Advances in high resolution, single-molecule, and live cell microscopy, as well as genetically encoded fluorescent tags have revived bacterial cell biology within the past decade and opened new avenues for synthetic biology of, *e.g.*, stochastic gene expression or regulatory networks.[1−3] Cellular position, dynamics, and copy number are valuable estimates of protein function and interactions and serve to understand bacterial organisms.[4,5] Since it is often preferred to preserve native copy numbers, expression dynamics, and genetic integrity, genes of interest are desired to be fluorescently tagged at their native loci. At the same time, studying protein function by inserting site-specific mutations in the target gene and observing the effects has long been a universal logic in molecular biology. As opposed to traditional mutagenesis tools such as UV irradiation, modern approaches call for a high specificity, efficiency, and speed. Yet, to date, tagging and mutating essential genes even in the model bacterium *Escherichia coli* require multistep procedures that replace the original gene with a selection cassette while rescuing the deletion with the same gene on a plasmid, and subsequently reintroducing the modified target gene.[6,7]

Here we present a single-step protocol for simultaneous tagging and site-specific mutagenesis of target genes. Our method is based on recombineering and represents a simple way to modify even essential genes without the need for persisting rescue plasmids. Recombineering is a powerful method that allows introducing insertions, deletions, or single mutations into large pieces of DNA. It is based on the expression of a specific set of genes from either Rec or Red operon found in Rac prophage or bacteriophage λ, respectively.[8,9] We use recombineering based on the Red operon, where three genes are provided on a temperature-sensitive plasmid to the *E. coli* K12 strain MG1655 together with a double-stranded DNA fragment containing homology arms.[10,11]

In this note we expand these previous recombineering strategies for tagging by additional introduction of site-specific mutations. We require a stretch of the target gene in an auxiliary plasmid prior to recombineering (Figure 1a). With the development of a series of methods that allow seamless plasmid construction[12] or whole gene synthesis, obtaining a preliminary plasmid carrying a stretch of the genomic sequence is not limiting. The dsDNA fragment is amplified by PCR using a primer that introduces the desired mutation together with the required homology to the genome (Figure 1b). For successful PCR, the mutagenic primer should include at least 11 bp downstream of the mutation. During subsequent recombineering, the desired mutation and the tag will be

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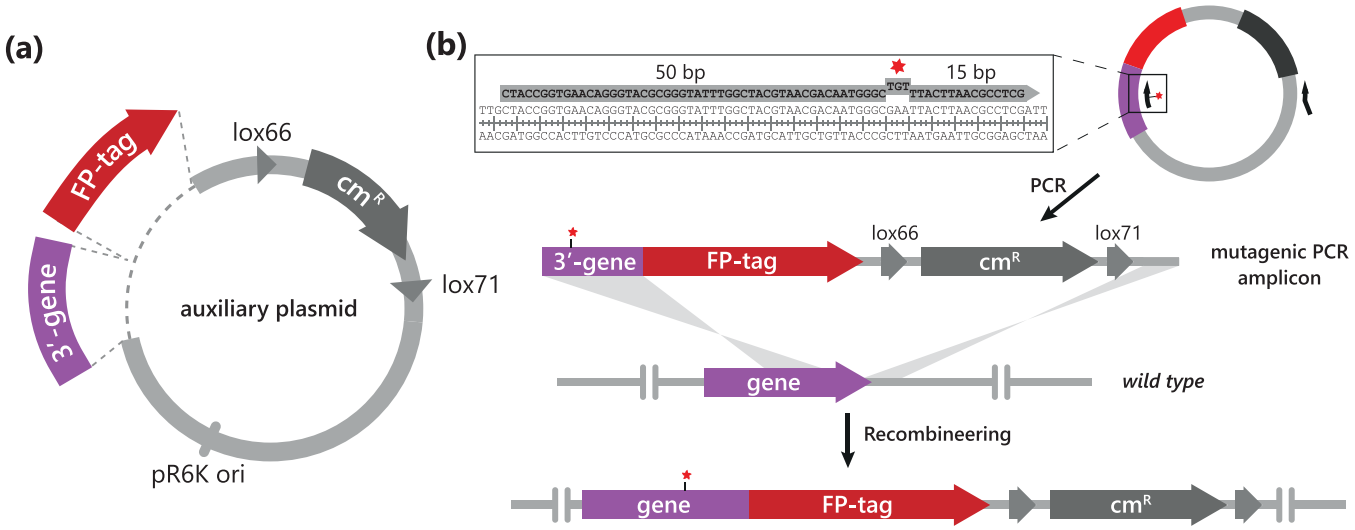

Figure 1. Plasmid and primer design for simultaneous 3′-end tagging and mutagenesis. (a) An auxiliary plasmid is assembled by cloning the desired tag together with the 3′-end of the target gene into a backbone carrying a resistance cassette (*e.g.*, chloramphenicol acetyltransferase, cm^R). (b) PCR is carried out using one primer that introduces 50 bp homology to the target region, and a mutagenic primer as displayed in the enlarged inset. Recombineering is then carried out with the resulting PCR amplicon, yielding a tagged and mutated target gene.

chromosomally integrated. We apply this protocol to *lac* operon genes (nonessential) and *lexA* (essential) to estimate its efficiency. We furthermore correlated this efficiency with the distance between the site-specific mutagenesis and the tag. Efficiencies varied between 3% and 27%, thus allowing for rapid and efficient generation of strains with tagged and mutated target genes.

RESULTS

In a first set of experiments, we chose to tag the lactose permease LacY C-terminally with mCherry and introduce mutations G46W, R144C, E269C, and K368T, which have all been reported previously to strongly reduce lactose transport efficiency[13,14] (Figure 2a, left). Next, we tested whether our approach is also suitable for N-terminal tagging on LacZ (Figure 2a, center). The *lacZ* gene is approximately 3 kb long and thus allows for extended testing of mutational efficiencies as a function of the distance between the mutation and the tag. We therefore introduced the mutations L52*, P219*, V336*, E461A, and E537A.[15,16] Finally, we tagged the essential protein LexA, encoded outside of the *lac* operon, with photoactivatable mCherry (PAmCherry) and simultaneously introduced the mutation S119A, which was reported to inhibit autoproteolytic cleavage of LexA and, by extension, suppress the SOS response[17] (Figure 2a, right). Mutation efficiencies in *lexA* were quantified by sequencing.

For all experiments, we cloned stretches of our target genes *lacY*, *lacZ*, and *lexA* into auxiliary plasmids. Selection of successfully recombined clones was mediated by a resistance gene cassette (Figure 1a), which was flanked by target sites for the site-specific recombinase Cre to allow for excision of the antibiotic resistance gene.[11] Our plasmids were based on the pR6K origin of replication, which can only be maintained in *E. coli* strains harboring the *pir* gene,[18] thereby ensuring the resistance does not occur from residual plasmids after recombineering a wildtype strain such as MG1655. The dsDNA fragment included homology arms and was amplified using mutagenic primers and electroporated into the target strain (Figure 1b). Since addition of a resistance cassette proximal to the native promoter can affect the expression of a downstream gene, we introduced the synthetic constitutive promotor *J23116*[19] in front of the N-terminally tagged *lacZ* gene (Figure 2a, center) to allow for continuous transcription and functionality of the mutational assay.

For a systematic analysis of the efficiency of simultaneous mutagenesis and tagging, we probed for mutation efficiencies up to 1600 bp distance from the tag, which would cover most genes in *E. coli*.[20] For both LacY and LacZ, mutagenesis efficiencies can be quantified by plating *E. coli* on selective MacConkey agar after recombineering, which yields magenta colonies when the *lac* operon is fully functional and yellow colonies when LacY or LacZ are defective (Figure 2c). Color assay reliability was verified by replating (Figure 2c) and sequencing, where all sequences matched the corresponding colony color (five clones per mutant). For confirming functional tagging in addition to mutagenesis, mutant colonies were probed by colony PCR and fluorescence microscopy. PCR showed expected fragment lengths for all colonies (Figure S1), and functional fluorescence in the membrane (LacY) or cytoplasmic region (LacZ) was confirmed by imaging (Figure 2d). Tagging without introducing a mutation yielded magenta colonies exclusively (Figure 2b, 0 bp point). The efficiency of mutagenesis decreased with increasing distance from the tag (Figure 2b), possibly caused by partial annealing within the region between mutation and tag. We observed one exception for $lacZ^{L52}$*, which yielded an unexpectedly low mutagenesis efficiency. We believe this resulted from secondary structure formation within the homology arms, which is supported by a particularly low free energy[21] for the mutation-bearing homology arm (Table S1). This sequence is difficult to optimize since it is determined by the position of the mutation. We determined that our approach resulted in efficiencies between 3% and 22%, suggesting that it allows for rapid generation of tagged and mutated genes from scratch, although the optimal homology for recombineering is 100−135 bp.[10] We observed these efficiencies despite the inevitably long (in our experiments up to 1662 bp, including the 1−3 bp mismatch 50 bp from the 5′ or 3′ end) and asymmetric

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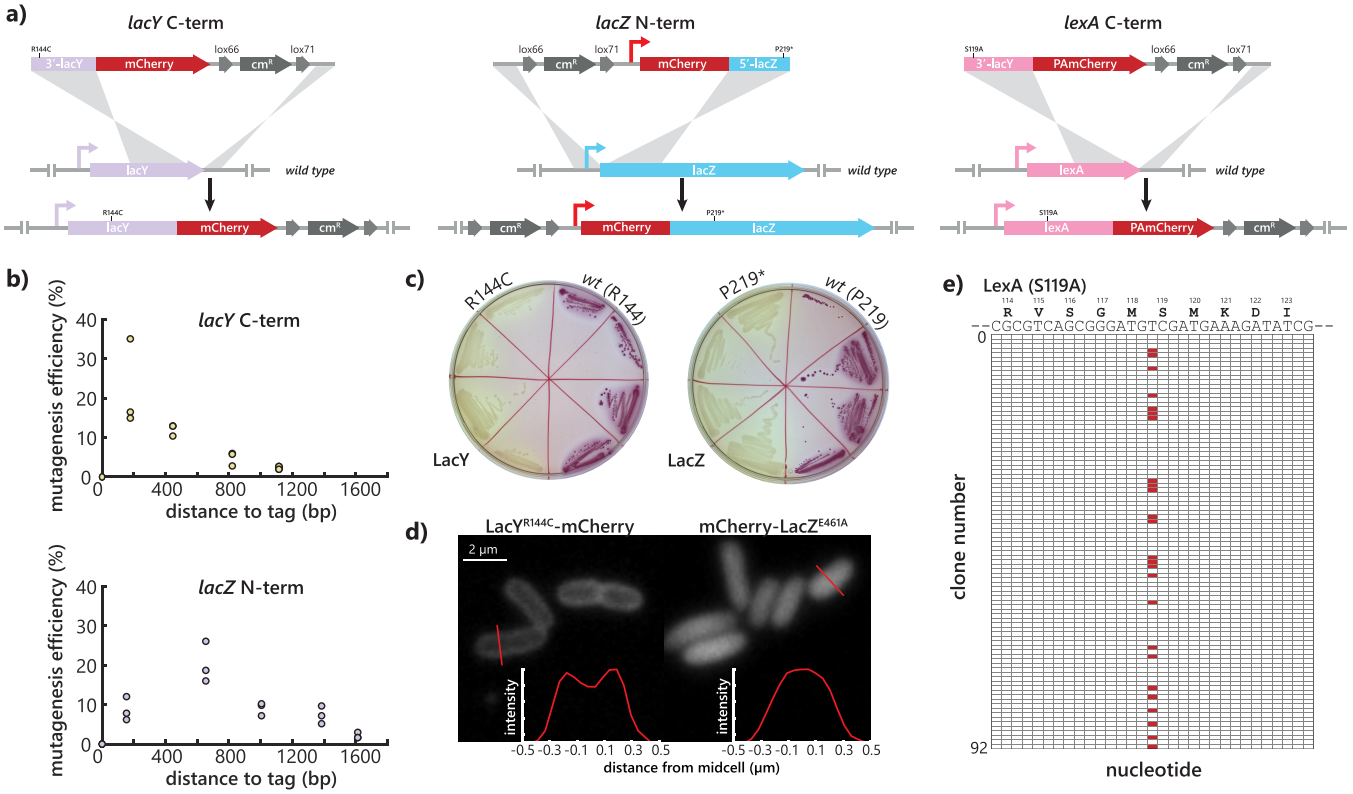

Figure 2. C- and N-terminal tagging and mutagenesis: Efficiency and verification. (a) Tagging and mutagenesis strategy for the three model genes. Regions of homology are shaded in gray. *lacY* (left) is tagged C-terminally with mCherry while introducing G46W, R144C, E269C, or K368T; *lacZ* is tagged N-terminally with mCherry while introducing L52*, P219*, V336*, E461A, or E537A; and *lexA* is tagged C-terminally with PAmCherry while introducing S119A. (b) Mutagenesis efficiencies for increasing distance between mutation and tag. (c) Stable mutagenesis is verified by replating on MacConkey agar, where mutants appear yellow and nonmutated strains appear magenta. Four yellow and magenta colonies were arbitrarily selected from each primary plate after recombineering. (d) Fluorescence micrographs of tagged and mutated LacY-mCherry and mCherry-LacZ. Line profile plots verify correct tagging by expected membrane or cytoplasmic localization of the fluorescence signal. (e) Sequencing verification of 92 clones after mutagenic tagging of the essential *lexA* gene. White boxes indicate wildtype nucleotides and red indicates T to G mutation events.

homology arms (Figure 2a). We also noted that the efficiency of mutation differs for comparable distances between LacZ N-terminal tagging and LacY C-terminal tagging (Figure 2b). This might have multiple reasons, including the order of annealing where either the long, mutation bearing arm or the shorter 50 bp arm anneals first to genomic DNA during replication. Secondary structure formation within the terminal 50 bp homology regions (Table S1) might contribute, but a strong correlation was not observed. Additionally, secondary structures within the recombineering fragment body might also influence efficiencies, despite being reduced by SSB.[22]

Finally, we aimed to tag and mutate an essential gene in the *E. coli* genome. We chose the gene coding for the transcription factor LexA, which represses the SOS response. The S119A variant renders the repressor noncleavable and the resulting strain is SOS response deficient.[17] Since there is no colony-based assay to select for successful LexA mutagenesis, we sequenced the *lexA* gene of 92 clones and determined a mutagenic recombineering efficiency of 27% (Figure 2e).

CONCLUSION

Editing a bacterial genome to perturb or expand original functions is central to many branches of synthetic biology. Especially fluorescent tagging of proteins allowed for significant insights into protein function, localization, and behavior. Consequently, various genome editing tools have been developed to either introduce, delete, mutate, or tag a protein of interest and study its function and mechanism in depth. *In vivo* studies often demand for genomic tagging and mutation rather than expression from an additional plasmid in order to preserve native expression patterns and genomic environment. We presented here a simple, precise, and efficient strategy to simultaneously tag and introduce a site-specific mutation into a chromosomal locus.

Various techniques for genetic manipulation have been used in the past for fluorescent tag fusion or mutational changes, such as UV irradiation, homologous recombination, use of transposons, or more recently, CRISPR-Cas9 mediated systems. Our method is based on the well-established recombineering technique, where precise modifications in the bacterial genome are made using a set of proteins from bacteriophage λ. Due to the precision of the insertion, recombineering-based methods have previously been used to edit the genomic[10,23,24] or plasmid DNA.[25] However, these methods require multiple steps or are not suitable for genomic modifications. Our method enables simultaneous mutagenesis and insertion of the fusion tag into the target gene in its native locus. With efficiencies ranging from 3 to 27%, even in unfavorable cases where mutations are far away from the tag (up to 1.6 kb), one out of 30 colonies is positive on average. With minor modifications to the design of the mutagenic primer, this method can be readily employed for short

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deletions, insertions, and frameshift mutations in lieu of the here presented point mutagenesis approach. The sequence of the tag itself is arbitrary, allowing for any gene of interest as a tag, like Halo, SNAP, CLIP,[4] or other nonfluorescent tags. In summary, the presented method can serve as an easy and rapid tool to manipulate or reveal bacterial protein function in a native genomic context.

METHODS

The gene for mCherry or PAmCherry was cloned into the plasmid pR6K-lox71-cm-lox66 using the In-Fusion HD cloning kit (Takara Bio). Stretches of *lacZ*, *lacY*, and *lexA* genes were amplified from the *E. coli* genome with colony PCR and cloned into the auxiliary plasmid using In-Fusion cloning. Amplicons of the full-length tag including the truncated original gene carrying the desired mutation, as well as a homology arm next to the resistance cassette were produced by PCR using high-fidelity Herculase II polymerase (Agilent). The mutagenic primer covers 15 bp upstream and 50 bp downstream of the mutation, and the other primer adds 50 bp homology next to the resistance cassette. Recombineering was carried out using a protocol adapted from Wang *et al.*[26] *E. coli* MG1655 was transformed with pSC101-BAD-$\gamma\beta\alpha$A,[11] where expression of Red operon genes was induced with 0.25% L-arabinose. For all constructs, 250 ng of the targeting cassette were electroporated into competent cells, and the cultures were plated on MacConkey agar containing 30 μg/mL chloramphenicol. The temperature-sensitive plasmid was removed during incubation at 37 °C overnight. Phenotypic evaluation by fluorescence microscopy was performed on a Nikon Ti-E STORM/PALM microscope (1.49 NA 100× TIRF objective (Nikon), Andor iXon Ultra 897 EMCCD camera). The genotype of five clones per mutation was verified by colony PCR and sequencing. For the LexA^{S119A} mutation, 92 colonies from a single recombineering experiment were randomly chosen for sequencing.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00202.

Figure S1: Agarose gels of colony PCRs with mutant phenotypes; Table S1: Primer sequences and secondary structure stability of homology arms. (PDF)

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Author Contributions

[⊥]L.S. and M.T. contributed equally. Conceptualization, L.S., M.T., and M.S.; Methodology, L.S. and M.T.; Investigation, L.S. and M.T.; Visualization, L.S. and M.T.; Writing (Original Draft), L.S., M.T., and M.S.; Writing (Review and Editing), L.S., M.T., and M.S.; Supervision, M.S.; Funding Acquisition, M.S.

Notes

The authors declare no competing financial interest.

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