Determining the prevalence of CRISPR off-target effects in bacteria: Can we detect if an organism has been genetically engineered?

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Abstract

Tools for genetic engineering have expanded rapidly in recent years, driven in large part by the development of CRISPR gene editing. This powerful, dual-use technology has been used to modify a variety of organisms, from bacteria to humans. While CRISPR has been touted for its simplicity, specificity, and versatility, CRISPR off-target effects—unintended modifications elsewhere in the genome—have been reported. Extensive work has gone into understanding these off-target effects in higher organisms due to the potential ramifications when applying CRISPR to human gene therapy, but this area has remained relatively unexplored in bacteria. As the barriers to genome editing in diverse bacterial species continue to drop at an accelerated rate, there is a growing need to be able to detect genetically-modified organisms, especially those created using techniques like CRISPR that lack traditional markers at the point of editing.

Here, we describe work to understand the off-target effects of CRISPR editing in bacteria as a potential method to identify signatures of engineered threats (Figure 1). We developed an algorithm to identify putative neutral insertion points in bacterial genomes, and applied this algorithm to 10 common synthetic biology chassis organisms. The sites identified in *E. coli* were experimentally validated and used to construct novel selection strains with engineered on- and off-target sites to aid in quantifying potentially rare off-target effects *in vivo*. In parallel, we applied an *in vitro* sequencing approach to assess off-target effects using purified molecular components. This knowledge of rates and identities of signatures of CRISPR editing in bacteria has the potential to enable detection of CRISPR-engineered threats and inform software tools being developed to identify genetically-modified organisms.

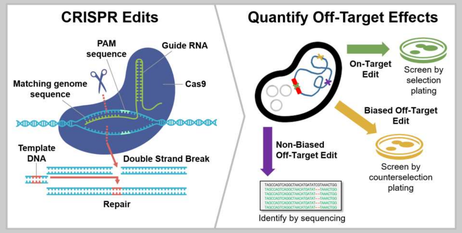


Figure 1. Overview of approach to identify CRISPR off-target effects.

Identification of Neutral Genomic Insertion Points in Bacteria

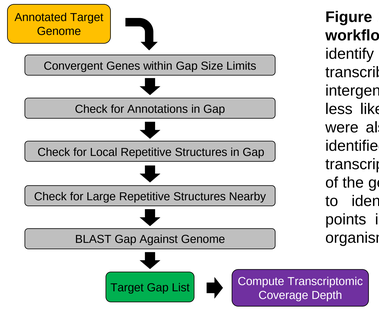


Figure 2. The targetFinder algorithm workflow. targetFinder was designed to identify gaps between convergently transcribed genes where critical intergenic regulatory sequences are less likely to occur. RNA-seq datasets were also analyzed to assess whether identified gaps had low apparent transcriptional activity relative to the rest of the genome. This approach was used to identify putative neutral insertion points in 10 synthetic biology chassis organisms, including *Escherichia coli.*

Experimental Validation of *E. coli* Insertion Sites

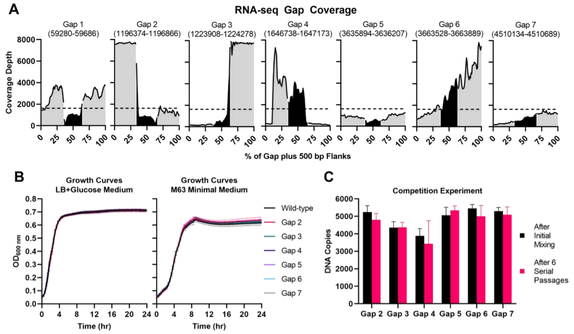


Figure 3. Identified *E. coli* insertion sites are phenotypically neutral. (A) RNA-seq coverage of each identified *E. coli* gap. Panels display the identified gap (black shading) plus the 500 bp flanking either side (gray shading). Dashed lines indicate the median coverage depth across the genome. (B–C) Sites were experimentally validated by inserting a genetic barcode[1] at each site via CRISPR-Cas[2] and assessing strain fitness. (B) Growth curves of strains (C) Abundance of each strain during repeated passages of competitive growth as assessed by qPCR. No apparent fitness differences were observed.

Quantification of CRISPR Editing Rates *in vivo*

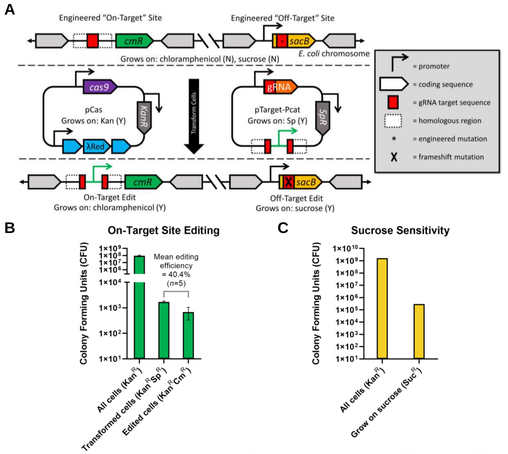


Figure 4. Engineered strains to quantify CRISPR on- and off-target effects. (A) Engineered on- and off-target sites were inserted into neutral sites in the *E. coli* genome. Both sites contain a gRNA target sequence that can be made less similar through synonymous point mutations in the gRNA region of *sacB*. Following transformation, growth on plates containing chloramphenicol or sucrose is used to indicate if an edit has occurred at the on- or off-target site. (B) Quantification of on-target editing efficiency. (C) Sucrose sensitivity of engineered strain. The *sacB* off-target site results in significant sucrose-mediated killing; however, the background of remaining sucrose-resistant cells is too high for the quantification of off-target edits.

Identification of CRISPR Off-Target Effects *in vitro*

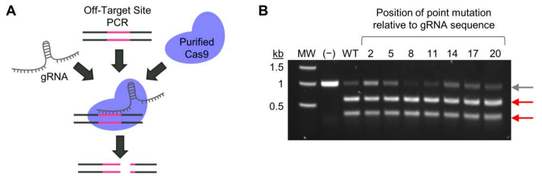
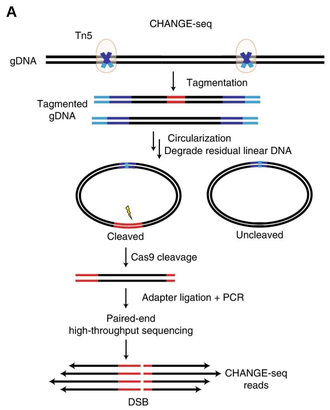


Figure 5. CRISPR cleavage of off-target site DNA *in vitro*. PCR products containing *sacB* off-target sites were tested for cleavage by CRISPR components *in vitro*. In addition to the wild-type *sacB* sequence, *sacB* variants with point mutations were also tested. (A) Diagram of the *in vitro* cleavage experiment. (B) Gel electrophoresis of resulting DNA fragments. Gray arrow indicates uncleaved DNA, and red arrows indicate DNA cleaved by CRISPR components. Despite mismatches between the target DNA and gRNA sequence, each off-target site was able to be cleaved by the Cas9/gRNA complex *in vitro*.



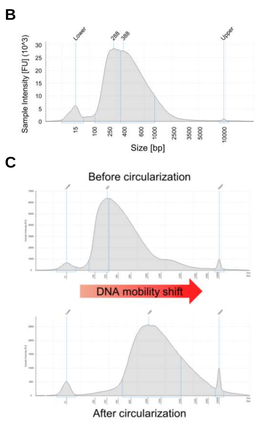


Figure 6. CHANGE-seq *in vitro* sequencing. Circularization for high-throughput analysis of nuclease genome-wide effects by sequencing has previously been used for off-target site discovery in eukaryotic organisms[3] and is being applied to *E. coli* in this study. (A) CHANGE-seq workflow.[3] (B) *E. coli* genomic DNA tagmented to an average size of ~400 bp. (C) Successful circularization of *E. coli* genomic DNA fragments indicated by a shift in DNA mobility.

Conclusions

Using neutral *E. coli* genomic insertion points identified and experimentally validated, novel strains were constructed with on- and off-target sites to aid in the quantification of CRISPR editing rates. Engineered strains were successfully used to determine on-target editing efficiency; however, we were unsuccessful in assessing off-target editing rates using this approach, highlighting the difficulty of detecting CRISPR off-target effects *in vivo*. We also demonstrated that screening of CRISPR off-target effects in bacteria can be accomplished *in vitro*, and are in the process of performing CHANGE-seq to identify off-target effects *in vitro*. Cleavage sites determined *in vitro* using purified components can additionally be utilized for targeted sequencing of *in vivo* CRISPR experiments. Knowledge of CRISPR off-target effects in bacteria will inform tools to identify signatures of genetic engineering in emerging threats.

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