Establishing and optimizing RecT-mediated homologous recombination in bacteria beyond *E. coli*

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Project Goals: The Lambda phage encoded Red recombination system (λ-Red) is among the most widely used genome editing tools in *E. coli* due to its ability to perform scarless mutagenesis using ssDNA oligonucleotides (oligos), gene knockouts and integrations, and multiplexed editing. In many bacteria, alternative methods of genome editing have significant limitations. Site-specific nucleases alone are lethal, site-specific recombinases leave genomic scars and cannot be flexibly programmed, base editors are imprecise, and native homologous recombination proceeds through inefficient random crossover. λ-Red is species-specific however, and does not function in most bacteria beyond *E. coli*, severely limiting the applicability of this technology. Here we aim to provide a framework for establishing and improving oligo-mediated editing in bacteria beyond *E. coli*.

The Lambda phage RecT-family protein Beta improves homologous recombination and facilitates multiplexed genome editing in *E. coli*. However this technology is host-restricted and has low activity outside *E. coli*. We find that host bacterial single-stranded binding proteins (SSB) affect the species-restriction of RecT proteins, and through co-expression we improve activity in foreign hosts by up to 3 orders of magnitude. We identify the SSB C-terminal 7 amino acids as a major recognition domain, and reprogram RecT-SSB compatibility by swapping this domain. We demonstrate the utility of this approach by establishing oligo editing for the first time in *C. crescentus*. Then in *L. lactis*, we explore other variables limiting editing efficiency

including mismatch repair and protein expression, improving rates of single-nucleotide mutations 500-fold to 23.8% at one site, and performing gene integrations. We optimized editing to generate millions of combinatorial variants within a ribosomal antibiotic target in *L. lactis*, finding that the majority of resistant mutants cannot be predicted by resistant single mutants alone.

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