DIRECTED GENOME EVOLUTION VIA HOMOLOGOUS RECOMBINATION

Sean A. Lynch, Andrew Garst, Tom Mansell and Ryan T. Gill; Department of Chemical and Biological Engineering, University of Colorado at Boulder; Boulder, CO 80026; rtg@colorado.edu

Key words: Recombineering, Directed evolution, Genome editing

Introduction. The engineering of bacterial genomes via homologous recombination offers a range of potential applications within the fields of synthetic biology and industrial microbiology. The development of techniques such as TRMR (1) and MAGE (2) enable one to introduce thousands of targeted mutations throughout the genome that affect cellular gene expression at the transcriptional and translational levels. The design and development of the new biological systems sought by synthetic biology will require simple methods for introducing targeted changes to the cell at the amino acid level as well. To effectively harness the power of directed evolution at either the genome or protein scale, researchers must first introduce diversity within a population with mutations giving rise to new or improved phenotypes identified following appropriate screens or selections. As recent advancements in the analysis of protein-sequence activity relationships (ProSAR) (3) of proteins isolated following a screen or selection significantly enhance the capacity to rationally combine single mutations to further improve function, there is a significant demand for techniques that create comprehensive libraries of single point mutations and incorporate features that facilitate the rapid mapping of genotype to phenotype. With existing techniques there is often a tradeoff between the amount of diversity created and the ability to direct the location of mutations within a gene (e.g Error-prone PCR vs. site saturation mutagenesis). Furthermore, these efforts are typically restricted to the study of a single protein often expressed from a plasmid. Here we present a technique that makes use of parallel DNA synthesis and homologous recombination to create diverse, predictable libraries of proteins with mutations securely integrated onto the chromosome. Using the same oligonucleotides we also demonstrate how one can incorporate a selectable marker as well as a unique molecular barcode to select for and trace the presence of mutations throughout a screen or selection.

Methods. Our approach relies heavily on the Lambda red phage-enabled recombination techniques developed over the past two decades (4), (5). Using simple molecular biology and a series of oligonucleotides we can rapidly and specifically modify the *Escherichia coli* genome and plasmids in multiplex via the integration of DNA cassettes including everything from whole genes to promoter variants and SNPs. Comprehensive libraries of targeted genes and proteins can be verified and monitored following selections using next generation sequencing technologies.

Results. Our short cassettes for mutagenesis via recombination incorporates mutations at 1-2% efficiency which is more than enough to ensure full coverage of most libraries; however, a large portion of the cells remaining after recombination still contain an unmodified sequence gene. In the absence of a strong selection pressure against wild type genes or in situations where only a screen for production is available a strategy that minimizes wt background would have great utility. To address this we incorporated design features into our oligo design that would enable the incorporation of a selectable marker into our cassettes. Here, upon recombination, cells incorporating the designed mutation would also incorporate a selectable marker thus facilitating the isolation of mutated cells.

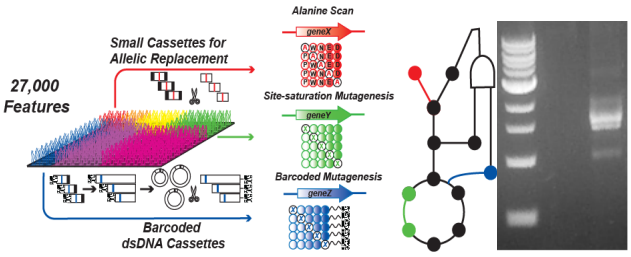

Fig.1 Strategy to create both short DNA cassettes as well as DNA cassettes incorporating a selectable marker. The gel on the right shows the ability of this to be carried out in multiplex targeting many nucleotides simultaneously.

Conclusions. Using oligonucleotides synthesized in parallel, one can integrate targeted mutations throughout the bacterial genome using homologous recombination. The incorporations of molecular barcodes and/or selectable markers within the DNA cassettes used for recombination enable the rapid recovery of modified genomes while facilitating the analysis of mutations via deep sequencing.

Acknowledgements. Work is supported by funding from the US Department of Energy and DARPA

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