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Genome Engineering Using Targeted Oligonucleotide Libraries and Functional Selection

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Abstract

The λ phage Red proteins greatly enhance homologous recombination in *Escherichia coli*. Red-mediated recombination or “recombineering” can be used to construct targeted gene deletions as well as to introduce point mutations into the genome. Here, we describe our method for scanning mutagenesis using recombineered oligonucleotide libraries. This approach entails randomization of specific codons within a target gene, followed by functional selection to isolate mutants. Oligonucleotide library mutagenesis has generated hundreds of novel antibiotic resistance mutations in genes encoding ribosomal proteins, and should be applicable to other systems for which functional selections exist.

Keywords

Antibiotic resistance; electroporation; oligonucleotide; recombineering; ribosomal proteins; spectinomycin

1. Introduction

Red-mediated recombination, or “recombineering”, exploits the bacteriophage λ Red proteins (Gam, Exo, and Beta), which promote homologous recombination in *E. coli*. The Gam protein inhibits the *E. coli* RecBCD nuclease, thereby prolonging the half-life of transformed linear duplex DNA. The 5'→3' exonuclease activity of Exo generates single-stranded DNA, which then anneals to complementary regions on the chromosome through the activity of Beta (1, 2). Red-mediated recombination is quite efficient, occurring between DNAs with as little as 50 nucleotides of homology. This technology has had a tremendous impact on the pace of molecular genetic research in *E. coli*, facilitating the construction of an ordered collection of single-gene knock-out mutants (3). Recombineering has been extended to other γ -proteobacteria (*Shigella flexneri*, *Salmonella typhimurium*, *Pseudomonas syringae*) and to *Mycobacteria* species (4, 5), and this approach could be applicable to many other systems.

Court and colleagues have shown that recombineering may also be used to introduce single-stranded oligonucleotides directly into the *E. coli* chromosome (6). This recombination requires only the Beta protein, which facilitates hybridization of oligonucleotides to single-stranded regions of DNA that are exposed during replication. Consistent with this model, oligonucleotides that anneal to the lagging strand template DNA recombine more efficiently than those complementary to the leading strand (7). Presumably, the hybridized oligonucleotide is ligated to newly replicated DNA, resulting in its incorporation into the genome. Some oligonucleotide recombination efficiencies can be quite high, allowing the

engineered mutations to be isolated by screening. Recombination efficiencies can be further enhanced by recombineering into methyl mismatch repair (MMR) deficient mutants, which are unable to correct mismatched base pairs introduced by the recombined oligonucleotide (7) (*see* Note 1). Thus, oligonucleotide recombineering is a remarkably powerful molecular genetic tool capable of introducing single nucleotide mutations into the genome without the cumbersome rounds of positive and negative selection required for standard allelic exchange.

We have exploited Red-mediated recombineering to perform scanning mutagenesis using synthetic oligonucleotide libraries. Oligonucleotide libraries containing one or more randomized codons are introduced into Red-expressing cells by electroporation, and the transformed cells are then subjected to functional selections to isolate mutants. This approach requires foreknowledge that mutagenesis of a particular target gene will result in a selectable (or screenable) phenotype, and is therefore best suited to characterized systems. We have used oligonucleotide libraries to mutagenize the genes encoding ribosomal proteins L4, L22, and S12, resulting in the identification of novel macrolide and aminoglycoside antibiotic resistance mutations (8, 9). One powerful feature of this approach is that unusual missense mutations, such as Lys (AAR) to Phe (TTY), can be isolated readily, whereas these mutations are virtually impossible to obtain using standard chemical mutagenesis.

Here, we apply oligonucleotide library mutagenesis to the *E. coli rpsE* gene, which encodes ribosomal protein S5. Mutations that alter loop 2 (residues 21–31) in S5 have been shown to confer spectinomycin resistance to *E. coli*, *Bacillus subtilis*, *Streptomyces roseosporus* and *Pasteurella multocida* (10–16) (*see* Fig. 1). Based on these observations, we randomized the codons corresponding to Val25 and Lys26 using recombineered oligonucleotide libraries and selected the mutagenized cells for resistance to spectinomycin. These genetic selections resulted in the isolation of 20 distinct spectinomycin-resistance mutations that encode 14 different S5 protein variants, 13 of which have not been previously described. In principle, this methodology may be used to mutagenize any gene for which a strong functional selection exists.

2. Materials

2.1. Preparation of Electrocompetent *E. coli* Cells

1. Luria-Bertani (LB) media: 10 g NaCl, 10 g tryptone, 5 g yeast extract, 1 mL of 1N NaOH per liter. Autoclave to sterilize.
2. Antibiotic stock solutions: 75 mg/mL ampicillin in 70% ethanol; 33 mg/mL chloramphenicol in 70% ethanol; 50 mg/mL spectinomycin in water.
3. Carbohydrate stock solutions: L-arabinose, 20% in water; D-glucose, 40% in water.
4. Temperature-controlled environmental rotary shaker (set at 30 °C).
5. Temperature-controlled shaking water bath (set at 42 °C).
6. NANOpure water (Thermo-Barnstead, or equivalent 18 MΩ-cm source) sterilized by autoclave, and chilled to 0 °C in an ice-water bath.
7. Sorvall RC5B superspeed centrifuge and SS-34 rotor.

¹In principle, MMR defective strains can be used to obtain higher frequencies of oligonucleotide recombination, allowing for efficient site-directed mutagenesis directly onto the chromosome without functional selection (7). However, we have found that library oligonucleotide library recombineering in the $\Delta mutS$ MMR-deficient background can induce dozens of unintended silent mutations in the target gene. Therefore, we recommend the use of MMR proficient strains.

8. Polypropylene 50 mL Oak Ridge centrifuge tubes (Nalgene). Autoclave to sterilize.
9. Microcentrifuge chilled to 4 – 10°C.
10. Precooled microcentrifuge tubes.
11. Glass 10 mL pipettes. Autoclave to sterilize.

2.2. Electroporation and Selection

1. Mutagenic single-stranded oligonucleotide, 60 – 100 nucleotides (IDT, Coralville, IA), dissolved at 0.5 – 2.5 μ M in sterilized water. For the results presented here, we used oligonucleotides: **rpsE(V25X)**, 5' – CTG TGA AGG AGA AAA TAC GAC CAC CTT TNN NGG TTT TAG ATA CGC GGT TTA CCG CGA TCA – 3'; and **rpsE(K26X)**, 5' – GAG CTG TGA AGG AGA AAA TAC GAC CAC CNN NAA CGG TTT TAG ATA CGC GGT TTA CCG CGA – 3', where N indicates an equimolar mixture of all four bases.
2. Electroporation cuvettes, 1.0 mm (VWR). Chill on ice before use.
3. Electroporator: Bio-Rad Micropulser (Bio-Rad, Hercules, CA) or equivalent.
4. Sterile circular membranes, 82 mm diameter. Colony/Plaque Screen nylon (PALL-Perkin-Elmer), or Protran BA 85 nitrocellulose (Whatman).
5. LB-agar petri plates (100 $\times$ 15 mm): 10 g NaCl, 10 g tryptone, 5 g yeast extract, 1 mL 1N NaOH, 10 g agar-agar per 1 L.
6. LB-agar + antibiotic: Add appropriate antibiotic (25 μ g/mL spectinomycin) before pouring.
7. Stainless steel forceps. Sterilized with 95% ethanol followed by flaming with a Bunsen burner.
8. Sterile water (*see* Subheading 2.1 **step 6**).

2.3. Isolation of antibiotic resistant clones and MAMA-PCR screening

1. LB-agar plates (150 $\times$ 15 mm) containing appropriate antibiotic (25 μ g/mL spectinomycin).
2. Polymerase chain reaction (PCR) buffer (10 $\times$): 100 mM KCl, 60 mM (NH₄)SO₄, 20 mM MgSO₄, 200 mM Tris-HCl (pH 8.9), 1% Triton X-100.
3. Deoxynucleotide solution (dATP, dCTP, dGTP, and dTTP all at 2 mM) (New England Biolabs, Beverly, MA).
4. Thermocycler and appropriate PCR tubes. We use an old Perkin Elmer 480 thermocycler with 0.65 mL microfuge tubes (Thermo-Fisher Scientific).
5. Gel loading buffer (3 $\times$): 50% glycerol, 0.01% xylene cyanol. 0.01% bromophenol blue.
6. TAE gel running buffer (50 $\times$): 2 M Tris-base, 0.1 M glacial acetic acid, 50 mM EDTA.
7. DNA grade agarose (EMD, Gibbstown, NJ).
8. Ethidium bromide (100 $\times$): 5 mg/mL in water. Ethidium bromide is a potent mutagen and is light sensitive. Store in a dark container or wrap container in aluminum foil.

3. Methods

The following protocols use Red protein expression plasmids generated in the laboratories of Don Court (pSIM5 and pSIM6) and Barry Wanner (pKD46) (17, 18). Plasmids pSIM5 and pSIM6 express the Red proteins under control of the native λ phage *pL* promoter, which is induced by heat-shock using the temperature-sensitive cI857 repressor (2, 18). Plasmid pKD46 allows L-arabinose-inducible Red protein expression under control of the *araBAD* promoter (17). In brief, the procedure involves induction of Red protein expression, preparation of electrocompetent cells, transformation with single-stranded oligonucleotide, and selection for recombinants. Mutagenic oligonucleotides should be between 60 and 100 nucleotides in length, and designed to anneal to the lagging strand DNA template during replication (2, 6). Ideally, 30 – 40 nucleotides of perfect homology should flank the mismatched nucleotides. However, we typically use 60-mer oligonucleotide libraries in which a single codon is randomized, reducing the homologous regions to only 28 – 29 nucleotides on either side of the randomized positions.

We have applied the mismatch amplification mutation assay-polymerase chain reaction (MAMA-PCR) to screen antibiotic resistant transformants for mutations in the target codon (19). This screen exploits the inability of Taq polymerase to extend a primer whose 3' end does not anneal to template DNA. Primers are designed that will support efficient PCR from the wild-type gene, but not from genes containing mutations in the target codon (*see* Fig. 2). Conversely, primers can be designed to recognize specific mutations, thereby producing PCR product from mutant but not wild-type cells. In principle, MAMA-PCR can also be used to screen for targeted mutations in the absence of a functional selection.

3.1. Preparation of Electrocompetent *E. coli* Cells

1. Grow *E. coli* carrying plasmid pSIM5 (with 33 μ g/mL chloramphenicol) or pSIM6 (with 150 μ g/mL ampicillin) in LB medium overnight at 30 °C. Dilute the overnight culture to OD₆₀₀ = 0.05 in 35 mL of fresh LB media containing the appropriate antibiotic. If using plasmid pKD46, resuspend cells into two separate 17 mL LB-ampicillin cultures, one supplemented with 0.2% L-arabinose to induce Red protein expression, and the other supplemented with 0.2% D-glucose as an uninduced control. Grow cells at 30° C with aeration for approximately 2 hr.
2. Once the cell density reaches OD₆₀₀ ~ 0.5, transfer 17 mL of the culture to a 125 mL baffled Ehrlenmeyer flask and incubate in a shaking water bath at 42 °C for 15 min (this heat shock will induce Red protein expression from plasmids pSIM5 and pSIM6). The remainder of the culture should be left as an uninduced control at 30 °C. If using cells carrying plasmid pKD46, proceed to step 3 without heat shock.
3. Immediately plunge culture flasks into an ice-water bath and incubate with steady shaking for 5 min. Keep cells on ice for the remainder of the electroporation procedure.
4. Transfer each culture to a pre-cooled Oak Ridge tube and collect cells by centrifugation at 6,000 $\times$ g for 7 min at 4 °C in a Sorvall RC4 centrifuge using an SS-34 rotor.
5. Decant supernatant and carefully resuspend the cell pellet in 1 mL of ice-cold, sterile water. After resuspension add 35 mL of ice-cold water. Mix gently and centrifuge at 6,000 $\times$ g for 7 min at 4 °C to collect cells.
6. After centrifugation, carefully remove the supernatant with a sterile glass pipette. Water-washed cells are loose and it is difficult to decant the supernatant without disturbing the pellet.

7. Resuspend the cell pellet in 10 mL ice-cold water, swirl gently to resuspend cells, and centrifuge $6,000 \times g$ for 7 min at 4 °C to collect cells.
8. Remove water wash with a 10 mL sterile glass pipette, taking care not to disturb the pellet. The cell pellet will be very loose now and decanting the supernatant will result in cell loss! Add 1 mL of ice-cold, sterile water to the pellet and swirl gently to resuspend cells.
9. Transfer washed cells to a pre-cooled 1.5 mL microfuge tube on ice and centrifuge at $14,000 \times g$ for 1 min at 4 °C to collect cells.
10. Carefully remove the supernatant with a micropipette and resuspend cells in 200 μ L of ice-cold, sterile water. Avoid excessive pipetting, which may damage cells and decrease transformation efficiency. Store cells on ice until electroporation. Coordinate the procedure such that electrocompetent cells are ready just prior to electroporation. Extensive incubation reduces Red protein levels, resulting in decreased recombination efficiency. Alternatively, electrocompetent cells may be resuspended in ice-cold 15% glycerol and stored at -80 °C for later use. Frozen electrocompetent cells have much lower transformation efficiency than freshly prepared cells (2).

3.2. Electroporation and Selection

1. Four separate transformations should be performed: i) Red-induced cells plus oligo, ii) Red-induced cells without oligo, iii) uninduced cells plus oligo, and iv) uninduced cells without oligo. Note that electroporation of uninduced cells with DNA may yield transformants, because oligonucleotides can recombine independent of Beta expression (4).
2. Aliquot 10 μ L of oligonucleotide solution (0.5 – 2.5 μ M) into a sterile microfuge tubes and place on ice. Aliquot 10 μ L of sterile water to the other tubes as negative controls.
3. Add 50 – 100 μ L of electrocompetent cells to the aliquoted DNA (or water) immediately before electroporation. Tap gently to mix and pipet into a pre-chilled, 1 mm electroporation cuvette. Ensure that the cell suspension is free of air bubbles, and remove all condensation from the cuvette electrode surface using a Kimwipe.
4. Pulse cells at 1.80 kV in an electroporator and immediately add 1.0 mL of sterile LB to the cuvette with a pipetman. Transfer the cell suspension to a sterile 1.5 mL microfuge tube. Expected time constants range from 4.5 to 5.5 ms for successful electroporations. If the sample arcs during the pulse (usually accompanied by an “Arc” error message on the pulser), discard the sample. Although it is possible to re-pulse after arcing, the number of viable cells decreases significantly after arcing. If successive arcs occur, it is likely that the cells have not been properly washed to remove salts. Electroporation cuvettes may be washed, sterilized, and re-used several times (*see* Note 2).
5. Spread 100 μ L of electroporated cell suspension onto an LB agar plate (no antibiotic) that has been overlaid with a nylon (or nitrocellulose) filter (*see* Note 3). Agar plates should be pre-warmed and filters should be applied prior to plating the cell suspension. Incubate plates up to 3 hr at 30 °C (or 37 °C) to allow recovery

²Used electroporation cuvettes should be washed extensively (>10 times) with water, then rinsed three times with 95% ethanol and stored in a closed sterile container. Depending on the manufacturer, electroporation cuvettes can be reused several times. However, cuvettes deteriorate with multiple uses and if arcing becomes frequent, then the cuvettes should be discarded. We have found that autoclaving and bleach treatment both significantly reduce the lifespan of electroporation cuvettes.

of electroporated cells prior to antibiotic selection. Immobilization of transformed cells allows for reasonable quantification of allele frequencies, because each antibiotic resistant colony represents an individual recombination event within a single cell (*see* Fig. 3 for an example). The recovery step may also be conducted in liquid media, but fast-growing mutants will multiply during this incubation and therefore will likely be over-represented on the antibiotic selection plate.

6. After recovery, use sterilized forceps to transfer the filters to LB agar plates containing 25 µg/mL spectinomycin (or other appropriate antibiotic), and incubate for 24 – 48 hr. Recovery for up to 72 – 96 hr may be required to isolate slow growing mutants. After growth at 37 °C, the selected mutants should have lost the Red protein expression plasmid and will be sensitive to ampicillin (or chloramphenicol) (*see* Note 4).

3.3. Isolation of antibiotic resistant colonies and MAMA-PCR screening

1. Pick single colonies and streak onto fresh selective agar plates to confirm antibiotic resistance. We use large petri plates (150 × 15 mm) with a 5 × 5 grid for this secondary selection step. Incubate the secondary selection plate overnight at 37 °C.
2. The isolated mutants may then be screened for targeted mutations using mismatch amplification mutation assay-PCR (MAMA-PCR) on whole cells. Remove a small portion of a bacterial colony from the secondary selection plate and resuspend the cells in 20 µL of sterile water.
3. Set up PCR reactions as follows: 17.5 µL water, 2.5 µL of 10× PCR buffer, 2.5 µL of 2 mM dNTPs, 0.25 µL each of forward and reverse primer, 1 – 2 µL of mutant cell suspension, and 0.5 µL Taq DNA polymerase (*see* Note 5). Overlay with mineral oil.
4. The following MAMA-PCR cycling program works well for 25 µL reactions using a Perkin-Elmer 480 thermocycler:
 - 94 °C for 2 min, 30 s – 1 cycle
 - 94 °C for 1 min, 65 °C 1 min, 72 °C 1 min – 25 cycles
 - 72 °C for 10 min – 1 cycle
5. Mix 10 µL of PCR product with 5 µL of gel loading buffer and load onto a 1% agarose gel prepared in 1× TAE buffer. Run gel at 100 V and stain with ethidium bromide. Always include a wild-type control for comparison to mutant PCR products. Typical results are as shown in Fig. 2.

³For many selections nylon and nitrocellulose membranes perform equally well. However, nitrocellulose filters bind hydrophobic antibiotics, such as macrolides, and can therefore interfere with some selections. It is critical that the membranes lie flat on the agar surface, with no underlying air bubbles. Use only sterile forceps to manipulate filters. Rinse forceps with 95% ethanol and flame between uses. If plating results in a confluent lawn of cells, serially dilute the transformed cells until well-isolated colonies are obtained.

⁴After isolation of mutants, it may be necessary to remove the Red protein expression plasmid for downstream applications. Plasmids pSIM5, pSIM6 and pKD46 all contain the *repA101* temperature-sensitive replication origin (17, 18), so growth at 37 °C without antibiotic selection should result in plasmid loss. If the recombinering plasmid is still retained after growth at 37 °C, streak the cells onto an antibiotic-free LB agar plate and incubate overnight at 42 °C. Isolate individual colonies and screen for sensitivity to ampicillin (pSIM6, pKD46) or chloramphenicol (pSIM5) to identify plasmid-free mutants.

⁵Mismatch screening primers are typically short (17 – 18 nt) with GC-content of 50 – 70% and T_m of approximately 50 – 55 °C. The 3'-terminal three nucleotides of the screening primer should anneal to the wild-type sequence of the target codon. MAMA-PCR should be conducted with Taq DNA polymerase, or other thermostable DNA polymerases that lack 3'→5' exonucleolytic activity. This activity will repair the mismatched nucleotide residues at the 3' end of the MAMA-PCR primer and yield high levels of PCR product.

6. Mutants that have passed the secondary selection and the MAMA-PCR screen are then sequenced to identify mutations (*see* Note 6). Table 1 shows the predicted ribosomal protein S5 variants encoded by the spectinomycin-resistant mutants isolated in this procedure. Note that for some randomized positions, complex mutations affecting adjacent codons will be isolated (*see* Note 7).
7. To confirm that the recombineered mutations are responsible for the selected phenotype, we transfer all mutations into the wild-type genetic background using recombineering or bacteriophage P1 mediated transduction. For a detailed protocol on bacteriophage P1-mediated transduction, *see* Section 1, Chapter 11 of this volume.

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⁶We typically amplify the target gene from isolated mutants using whole-cell PCR. The resulting PCR products are sequenced using an additional primer that is nested within the amplification primers. The use of nested sequencing primers greatly reduces the background signals that typically plague PCR product sequencing reads.

⁷We suspect that untargeted mutations in adjacent codons are generated at low frequency in most randomization experiments. Though rare, these complex mutations will dominate the mutant pool if the more abundant simple missense mutations fail to confer antibiotic resistance. These unintended mutations significantly increase the complexity of the mutant pool, which may be informative in some instances.

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Figure 1.

Ribosomal protein S5 and spectinomycin resistance mutations. The structure of spectinomycin bound to the *E. coli* 30S ribosomal subunit is shown. The subunit is viewed toward the shoulder region with inter-subunit interface positioned on the right. All ribosomal proteins except S5 have been omitted for clarity. The right panel shows loop 2 of S5 and its proximity to ribosome-bound spectinomycin. Missense changes associated with spectinomycin resistance (Spc^R) in *E. coli* are shown by arrows in the S5 sequence and labeled in black on the loop 2 structure. Codons corresponding to Val25 and Lys26 (labeled in red) were randomized in this study. Structures were rendered with PyMol using PDB 2QOU (20).

Figure 2.

Mismatch amplification mutation assay-polymerase chain reaction (MAMA-PCR). **A)** The MAMA-PCR strategy. Short primers are designed whose 3'-ends anneal to the wild-type sequence of the target codon. PCR of the wild-type gene will result in efficient amplification. In contrast, Taq DNA polymerase will not extend the primer from the mutated codon due to the 3' mismatched nucleotides. **B)** MAMA-PCR screen of erythromycin-resistant *E. coli* mutants. Wild-type and erythromycin-resistant cells containing mutations in the *rplD* gene (encoding ribosomal protein L4) were subjected to whole-cell MAMA-PCR.

Figure 3. Isolated spectinomycin resistance mutations from the *rpsE*-K26X oligonucleotide library. The *rpsE* gene was sequenced from 51 spectinomycin resistant mutants isolated from a single library recombineering experiment. All mutants contained changes of Lys26 to hydrophobic residues. Percentages of the identified Lys26 missense mutations are presented.

Table 1Predicted S5 proteins from spectinomycin-resistant *E. coli* mutants.^a

Val25	Lys26
V25F	K26F
V25P	K26I
V25W	K26V
V25D , ΔG27	K26Y
V25R , ΔG27	
Δ (S22–K23), V25L	
ΔV25	
S22A, Δ (K23–V25)	
K23T, T24N, ΔV25	
T24K, Δ (V25–K26)	

^a Codons corresponding to S5 residues Val25 and Lys26 were randomized using separate oligonucleotide libraries. Transformed cells were selected on media containing 25 μg/mL spectinomycin. Boldfaced alleles indicate a missense mutation within the target codon. One letter amino acid code is used throughout.