Metabolic Engineering of Antibiotic-Producing Actinomycetes Using In Vitro Transposon Mutagenesis

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Abstract

A program of mutation and screening, with stepwise reverse engineering or "decoding" of the improved strain, is a way to better understand the genetics and physiology of the strain improvement process. As more is learned about the genetics of strain improvement, it is hoped that more fundamental principles will emerge about the types of mutations and genetic manipulations that reliably lead to higher producing strains. This will accelerate the construction of higher producing strains by metabolic engineering in the future. In this chapter, a detailed tagged mutagenesis approach is described using in vitro transposon mutagenesis which allowed the successful identification of key genes involved in macrolide (erythromycin) antibiotic biosynthesis.

Key words: In vitro transposon mutagenesis, Reverse engineering, Actinomycete, Protoplast transformation, Erythromycin, *Aeromicrobium erythreum,* Microtiter fermentation

1. Introduction

Industrial microbiologists have been using the classical mutate- and-screen approach to strain improvement (1, 2) for over 60 years, and although the method works well for producing improved strains, very little knowledge has been gained over the years of how it works. Microbial geneticists, on the other hand, have accumu-lated a vast array of genetic tools and information over the same period of time, but have had limited success in converting their knowledge to commercial use.

Recently, the application of rapidly evolving technologies, including in vitro transposon mutagenesis (3, 4) and especially low-cost comparative genomic sequencing (5), has made it possible to determine how the classical mutate-and-screen method actually generates better strains. The idea goes beyond the reverse engi-neering of an improved strain to reverse engineering the actual process for producing improved strains. Once the process is reverse engineered over a cycle or several cycles, the principles that are subsequently revealed should allow the direct construction of improved strains in the future, rather than relying on the luck of random mutagenesis. It may even be possible that the information gained will have applications to other strain improvement processes in higher organisms. Work in this field has already begun to pro-duce interesting results (6–10).

Using the in vitro transposition approach, each improved strain generated by random mutagenesis is reverse engineered before proceeding to the next round of mutagenesis. If

comparative genomic sequencing were used instead (11, 12), then each strain in the lineage of a commercial process would be compared by sequence analysis to the strain that preceded it. By focusing on the early stages of strain improvement, where the biggest gains in pro-duction are typically seen, the most important principles should be revealed. Possibly, only one or two rounds of reverse engineering will be necessary before a useful picture emerges of what happens during a mutate-and-screen strain improvement program. This chapter outlines the materials and methods needed to perform a reverse engineering or "decoding" of the microbial strain improvement process for *Aeromicrobium erythreum* NRRL B-3381, but can be generally applied to any organism of commercial value or process of interest. An important upfront consideration is first to determine whether in vivo transposon mutagenesis works in the organism of interest. If so, then an efficient gene transfer system would need to be developed if none is currently in place. In vivo transposon mutagenesis has advantages and perhaps is preferred over in vitro transposon mutagenesis, for such reasons as reduced cost, time, and labor to get to the screening stage. However, most organisms do not have efficient in vivo transposon expression systems in place. Furthermore, the mutagenesis process has been found to be biased toward "hotspot" insertion sites and thus would not generate a useful, high-quality knockout library. In vitro trans-poson mutagenesis offers more control over the randomness of the insertions and thus more complete coverage of the genome-wide mutagenesis process. Moreover, in in vitro transposon mutagenesis the quality control process can be more easily quantified and if a reasonably efficient gene transfer system is in place, it is likely a high-quality knockout library will be achieved in the host strain.

A strain at any point in the engineering process can be a candidate for a decoded mutate-and-screen program, but more information is obtained when the procedure begins with a wild-type strain, since then the entire strain improvement process becomes known. If the process starts with an improved strain, then only the later parts of the strain improvement process can be learned, which means the potentially most important improvement steps would remain unknown. Low-cost, next generation comparative genomic sequencing can be used as an alternative approach using random mutagenesis with UV or chemicals, but these often reveal many mutations per cycle, thus making it difficult to pinpoint the strain improvement targets. This can be especially true if several rounds of random mutagenesis preceded a detailed genomic analysis. The comparative whole genome sequencing approach is most useful for programs in the very early if not the beginning stage of the strain improvement process when fewer neutral, deleterious, and beneficial mutations have accumulated.

Since an in vivo transposition method is not available for erythromycin-producing organisms, including *A. erythreum,* or for most actinomycetes in general, an in vitro transposition method is used instead. *A. erythreum* and many other filamentous actinomy-cetes have gene manipulation tools in place. The actinomycete DNA is mutated in *Escherichia coli* then transferred back into the host of choice in a later gene replacement step. In vitro transposon insertion kits are available for *E. coli* from Epicentre Technologies (Madison, WI) (EZ-Tn5 <R6Kγ/KAN-2> or EZ-Tn5 <oriV/ KAN-2>; Fig. 1a). Although the transposons can be customized with different drug resistance genes, outward reading promoters, and other features (8, Fig. 1b), they can also be used unmodified. The advantage of using them unmodified is that the available trans-posons are smaller (~2 Kb). Avoiding incorporation of promoters in the transposon simplifies the analysis of the mutant phenotypes which occurs during the reverse

engineering process, but limits the possible mutant phenotypes.

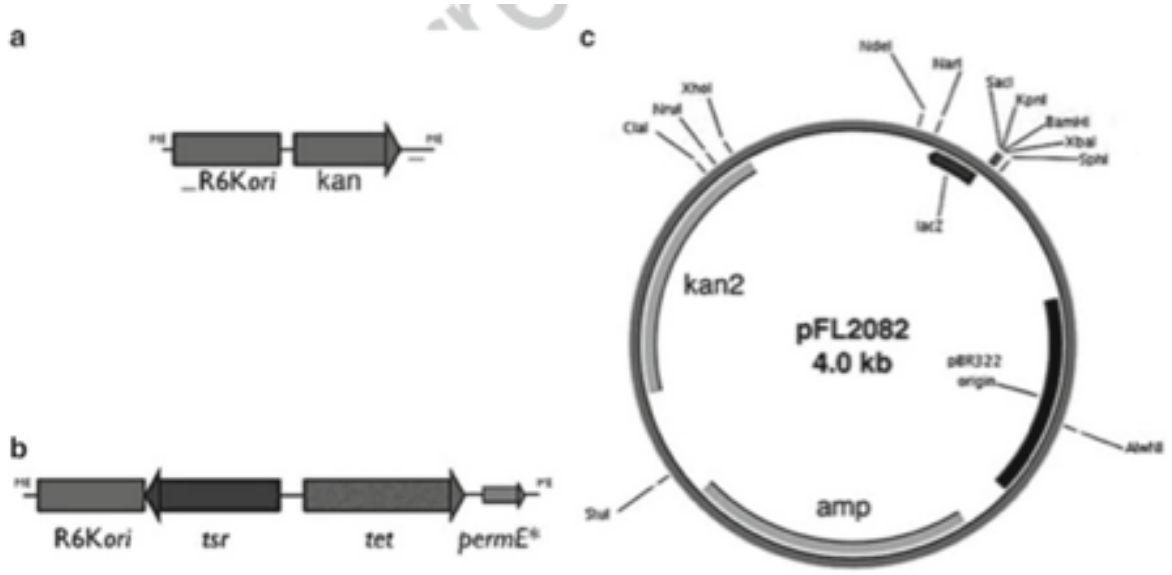

Fig. 1. (a) Unmodified EZ-Tn5 <R6Kγori/kan2> transposon from Epicentre Technologies. (b) Modified EZ-Tn5 <R6Kγori/ kan2> derivative used for in vitro transposition in *A. erythreum.* (c) Plasmid pFL2082 used in *A. erythreum.* Abbreviations: R6Kγori *E. coli* origin of replication; *kan* kanamycin resistance gene from *Tn916; tsr* thiostrepton resistance gene; *tet* tetracycline resistance gene; *permE* * modified *ermE* promoter; *amp* ampicillin resistance gene; *kan2* kanamycin resistance gene on transposon.

As described in this chapter, the mutagenic procedure is divided into three major steps corresponding to the formation of two plasmid libraries in *E. coli,* and the final one in the actinomycete of interest. As a guide to the quality of the libraries, an analysis of the randomness of the libraries and mutants is included at each step of the procedure.

In vitro transposon mutagenesis has been successfully used on the erythromycin-producing organism *A. erythreum,* which led to a greater understanding of the role that precursor feeding plays in strain improvement of macrolide-producing organisms. In this study, 3,049 Tn mutants were screened in duplicate fermentations and analyzed for a significant percent increase or decrease in erythromy- cin titers. Seventy of these mutants showed unusually high or low erythromycin titers compared to the parent strains in a high-throughput microtiter screening process. Twenty-six mutants showed increased titers of greater than 25% above parent controls and 44 showed either a 25% or greater decrease in production or no erythromycin production at all. Analysis of the antibiotic resistance phenotypes of the first 2,267 mutants indicated that 88% of the mutants contained a single-crossover insertion. The remaining 12% were gene-replaced strains. The high percentage of single-crossover strains led to the development of a prescreening procedure (described in Subheading 3.5, Note 28 and in Fig. 4) based on a colorimetric assay that could distinguish gene-replaced (double crossover) strains from strains with an integrated plasmid (single crossover). In total, 1,070 gene- replaced transposon mutants were screened which would represent about 27% coverage of the predicted 4 Mb *A. erythreum* chromo-some assuming random insertion events.

Further screening and retesting of selected mutants from the primary screen identified

seven high-producing mutants. These mutants consistently produced greater than a 50% increase in erythromycin titers when compared to the parent strain (8). Three of the seven mutants mapped to *mutB*, encoding the alpha subunit of methylmalonyl-CoA mutase. The remaining four mapped to *cobA*, encoding a cob(I)alamin adenosyltransferase, involved in one of the later steps of vitamin B12 biosynthesis. The metabolic connection of the *cobA* mutation is that methylmalonyl-CoA mutase is one of the few enzymes in the bacterial cell that has a requirement for vitamin B12.

In summary, an in vitro transposon mutagenesis approach was used in an erythromycin-producing organism to create improved strains. The improved strains were reverse engineered and found to have mutations in genes clearly involved in precursor feeding into the erythromycin biosynthetic pathway. In follow-up studies, *mutB* knockout strains were generated in the commercially important erythromycin-producing organism *Saccharopolyspora erythraea* and found to have similar positive strain improvement effects on erythromycin production (13, 14).

2. Materials

2.1. Preparation of 10–15 kb Chromosomal Library (Library 1)

To ensure high-quality libraries, all reagents were of molecular biology grade and only double-distilled water should be used (18 Ω at 25°C).

1. Growth medium (see Note 1): Modified Soluble complete medium (MSCM), 15.0 g Soluble Starch, 20 g Soytone, 1.5 g Yeast extract, 26.25 g MOPS buffer, 0.1 g Calcium Chloride dehydrate; distilled water to 1 L. Adjust before autoclaving to pH 7.5 with NaOH. After autoclaving add, per liter: 30.0 mL of 50% w/v D-(+)-glucose dissolved in distilled water, 2.0 mL of trace elements solution (15), 5 mg of Multivitamin.
   Trace element solution (added in this order): 0.040 g of zinc chloride (ZnCl2), 0.2 g ferric chloride hexahydrate (FeCl3·6H2O), 0.010 g cupric Chloride dihydrate (CuCl2·2H2O), 0.010 g manganese chloride tetrahydrate (MnCl2·4H2O), 0.010 g sodium borate decahydrate (Na2B4O7·10H2O), 0.010 g ammonium molybdate tetrahydrate ((NH4)6Mo7O24·4H2O), Distilled water, to 1,000 mL.

2. All molecular biology techniques use standard methods and can be adjusted as necessary to the organism of interest. All techniques should be carried out according to the manufac- turer's instructions for best results (see Note 2).

3. Lysing solution: 25 mM EDTA in 25 mM Tris pH 8.0 and lysozyme was used at a final concentration of 1 mg/mL.

4. One unit of *MboI* used per 2 μg chromosomal DNA. Incubated for 5 min at 37°C. Stop solution: 2 μL 0.5 M EDTA and 5 μL electrophoresis running dye for a 30 μL reaction. Can adjust accordingly. (The *MboI* concentration can be adjusted as needed to favor production of 10–15 kb fragments.)

5. One Weiss unit of T4 DNA ligase was used in an overnight incubation at 16–22°C.

6. *Escherichia coli* DH5α electrocompetent cells were used at 2.5 KV and recovered in SOC medium (15).

7. 2XYT medium per liter: 16.0 g Tryptone, 10.0 g Yeast extract, 5.0 g sodium chloride. Adjust to pH 7.0 with NaOH, add distilled water to 1,000 mL. Ampicillin selection was per- formed at 100 μg/mL in 2× YT agar.

8. X-gal indicator (bromo-chloro-indolylgalactopyranoside) was added to a final concentration of 32 μM. IPTG inducer solution (isopropyl-β-D-thiogalactopyranoside) was used at a final con- centration of 160 μM.

9. Single-colony isolates were grown in Luria Bertani (LB) medium-containing ampicillin sodium salt at 100 μg/mL.

2.2. Preparation of Transposon Mutagenized Library in E. coli (Library 2)

All reagents and reactions should be carried out carefully and according to manufacturer's conditions for best results. The amount of DNA can be adjusted up or down but the ratio of trans- poson to chromosomal DNA needs to be carefully determined for effective mutagenesis.

1. 0.2 μg of library 1 DNA in all in vitro transposition reactions using the EZ::Tn mutagenesis kit (Epicentre Technologies, Madison, WI; see Note 3).

2. 2×YT agar-containing ampicillin sodium salt at 100 μg/mL and kanamycin sulfate at 50 μg/mL.

3. Use PCR primers Kan-2 FP1 and Kan-2 RP1 (Epicentre Technologies, Madison, WI) to identify regions where the transposon inserted as a gauge of randomness.

2.3. Preparation of Protoplasts

1. 2×PT Buffer (per liter): 200.0 g sucrose, 0.50 g potassium sulfate, 10.17 g magnesium chloride hexahydrate, 4.0 mL trace elements solution. Distilled water to 750 mL. At time of use complete by adding to 150 mL of above 2×PT solution: 10.0 mL of 1 M Calcium Chloride dehydrate, 40.0 mL of 0.25 M TES buffer, pH 7.2.

2. 1× modified P Buffer (adapted from ref. (14)): Make up the following basal solution: 103 g sucrose, 0.25 g K_2SO_4, 2.0 g $MgCl_2 \cdot 6H_2O$, 2 mL trace element solution, 800 mL distilled water. Dispense in 80 mL portions and autoclave. Before use add to each flask in order: 10 mL of 3.68% $CaCl_2 \cdot 2H_2O$, 10 mL of 5.73% TES buffer adjusted to pH 7.2 (see Note 4).

3. 50% PEG solution: 50.0 g polyethylene glycol (ave. mol.wt 10,000), Distilled water to 100 mL.

4. 10.3% sucrose solution: 10.3 g sucrose in ddH2O to 100 mL.

5. 1 mg/mL Lysozyme in distilled water.

6. 0.4% Glucose.

2.4. PEG-Mediated Transformation

1) Fresh or frozen protoplasts can be used. Thaw rapidly at 37°C and aliquot to individual eppendorf tubes.

2) R2T2 agar (per liter): 103 g Sucrose, 0.25 g Potassium sulfate, 6.5 g Yeast extract, 5 g Tryptone, 22 g Agar, 850 mL Distilled water. After autoclaving the following sterile solutions are added: 20 mL of 50% w/v D-(+)-glucose, 25 mL of 1 M Trizma Base (pH 7.0), 5.0 mL 0.5% Potassium phosphate monobasic, 2.5 mL of 1 N Sodium hydroxide, 50 mL 1 M Calcium Chloride dehydrate, 50 mL of 1 M magnesium chloride hexa- hydrate, 2 mL Trace elements. Plates should be dried to 90% of original weight (see Note 5).

3) 100 mg/mL stock Thiostrepton in 50% ethanol and 50% DMSO.

2.5. Preparation of Host Integration Library (Library 3)

1. DNA concentration greater than 0.2 μg/μL.

2. Thiostrepton at 25 mg/mL in 50% ethanol and 50% DMSO.
   Store at –20°C.

3. Kanamycin sulfate at 100 mg/mL in sterile distilled water.
   Store at –20°C.

4. Prepare selection media plates if an assay to distinguish between single-crossover and double-crossover insertions is available (see Note 6; Fig. 4).

5. Use primers KanFP-1 and Kan RP-1 to sequence from ends of the transposon into the adjacent chromosomal DNA (Epicentre Technologies, Madison, WI); (see Note 7).

2.6. Microtiter Screen

1. Alligator magnetic stirrer system (V&P Scientific, San Diego, CA; see Note 8).

2. Power setting 3 using steel magnetic stir rods.

3. 96-deep well plates with 1 mL capacity.

4. Prepare a lid to cover the 96-well microtiter dish lid by taping tightly tissue paper over the lid to create a seal between deep-well plate and lid.

5. Sterile glycerol, 80%.

2.7. Shake Flask Screen

1. Sterile MSCM broth (see Subheading 2.1).

2. Add 1/10 v/v of thawed glycerol stock to sterile medium.

3. Add 1/20 v/v of inoculum into fermentation flask.

2.8. Bioassay

1. *Bacillus subtilis* strain.

2. 1% solution of 2, 3, 5 triphenyltetrazolium chloride (tetrazolium red) in double-distilled H2O.

3. Tryptic Soy Broth (per liter distilled water): 17 g Tryptone (Pancreatic Digest of Casein), 3 g Soytone (Pancreatic Digest of Soybean Meal), 2.5 g dextrose, 5 g NaCl, and 2.5 g dipotassium phosphate.

4. Erythromycin dilution series made from a 1 M stock using pure erythromycin resuspended in pure ethanol.

5. 245-mm Bioassay dish (Corning Costar, Cambridge, Mass).

6. 1/4-in paper bioassay discs (Schleicher & Schuell, Keene, NH).

2.9. Small-Scale gDNA Prep

1. Lysozyme solution: 2 mg/mL in 0.3 M sucrose, 25 mM Tris buffer pH 8, 25 mM EDTA pH 8, and 50 μg/mL RNase A.

2. 2% SDS solution in water.

3. Phenol: Chloroform: isoamyl alcohol (25:24:1).

4. 3 M sodium acetate pH 5.2.

5. Isopropanol.

6. 70% Ethanol: contains 70 mL of 100% ethanol and 30 mL of ddH2O.

7. TE buffer: contains 10 mM of Tris base pH 8.0 and 1 mM of EDTA, pH 8.0.

2.10. Plasmid Rescue

1. DNA concentration at least 0.2 μg/μL.
2. Use a frequent cutting restriction enzyme. For high G+C% actinomycetes these could be *ApaI, StuI, BssHI, XhoI, SmaI,* and *NdeI*.

3. Ligate using T4 DNA ligase and incubate overnight at room temperature.

4. Clean up ligation reaction using QiaQuick kit (Qiagen, Valencia, CA).

5. Transform *E. coli* pir + strain EC100D cells and select with tet- racycline at 10 μg/mL.

6. Use transposon-based sequencing primers Kan-2 FP1 and Kan-2 RP1 to localize Tn insertions.

3. Methods

3.1 Preparation of 10-15 kb Chromosomal Library (Library 1)

All enzymes or temperature sensitive reagents should be kept as briefly as possible at room temperature or 4°C.

1. Prepare *A. erythreum* chromosomal DNA as follows. Resuspend 500 mg of cells wet weight from 3.0 mL of a 2-day MSCM culture in 5.0 mL of lysozyme solution and incubate at 37°C for 30 min or until cells become somewhat translucent.

2. Add 2.5 mL of a 2% SDS solution and mix on vortex for 1 min, this will reduce viscosity and clear solution noticeably.

3. Add 2.5 mL of phenol-chloroform solution (pH 8.0), mix on vortex for 30 s, centrifuge for 2 min in a microcentrifuge.

4. Remove 3.0 mL of the supernatant, leaving the white interface behind.

5. Add 0.1 volume of 3 M sodium acetate, pH 5.2 and mix on vortex, then add 0.6–1 volume of isopropanol and mix by inverting tube several times, a small clump of DNA should form and visibly fall to the bottom of the tube.

6. Centrifuge for 2 min in a microcentrifuge and a small white spot of DNA appears at the bottom of the tube.

7. Carefully pour off supernatant and wash pellet twice with 5.0 mL of 70% EtOH, drain liquid

off and air dry for 20 min.

8. Dissolve pellet in 1.0 mL of TE 8.0 or double-distilled water. Mixing and pipetting may be required to get the DNA in solution.

9. Partial digestion of 2 μg of chromosomal DNA into 10–15 kb DNA fragments using restriction enzyme *MboI* (see Note 9). The partial digestion is performed as follows: Transfer 90 μL of the DNA preparation from step 1 to a sterile 1.5-mL tube containing the equivalent of 10 μg chromosomal DNA (may need to dilute stock). Add 10 μL of the appropriate 10× restric-tion enzyme buffer to the 90 μL DNA solution.

10. Set up five 1.5-mL microfuge tubes (labeled A–E) on ice con-taining 30 μL of the DNA/buffer mix in tube A, 20 μL of the DNA/buffer mix in tubes B–D, and 10 μL of the DNA/buffer mix in tube E.

11. Add 10 units of restriction enzyme to tube A, mix thoroughly and transfer 10 μL of reaction mix to tube B using a fresh pipette tip. Continue serial transfers of 10 μL restriction enzyme mix from tube B to tubes C–E. All tubes should con-tain a final volume of 20 μL.

12. Incubate tubes A–E at 37°C for 15 min. At the end of the incubation period, rapidly transfer tubes to ice water bath and add 2.0 μL of 0.5 M EDTA and 5 μL of electrophoresis running dye to stop the restriction enzyme reaction.

13. Resolve chromosomally restricted fragments on a 0.6% agarose gel, excise fragments in 10–15 kb region, and purify using Qiaex kit or other suitable gel purification kit. Chromosomal fragments are concentrated and checked for degradation on a 0.6% agarose gel.

14. Perform ligation reaction using a fivefold molar excess of chromosomal fragment to vector. Ligation reactions should be performed at 16°C or at room temperature (depending on manufacturer's instructions) using T4 DNA ligase (ref. (15); see Note 10; Fig. 1b, c).

15. Transform a high electrotransformation efficiency *E. coli* (DH5 alpha, JM110, XL1) with ligated DNA, select for ampicillin-resistant transformants at 100 μg/mL.

16. Screen visually for white transformants on X-gal+IPTG supplemented agar plates.

17. Restreak white ampicillin-resistant transformants for single colonies on X-gal supplemented agar plates.

18. Pick several single colonies from streaks to inoculate 4-mL test tube cultures containing LB with ampicillin (100 μg/mL).

19. Perform high-purity DNA preparations on overnight (16 h) cultures.

20. Analyze plasmid DNA using suitable enzymes that cut outside the *BamHI* site on agarose gels to determine the size of the insert DNA and relative copy number of the plasmid (see Fig. 2a, b; Notes 11 and 12).

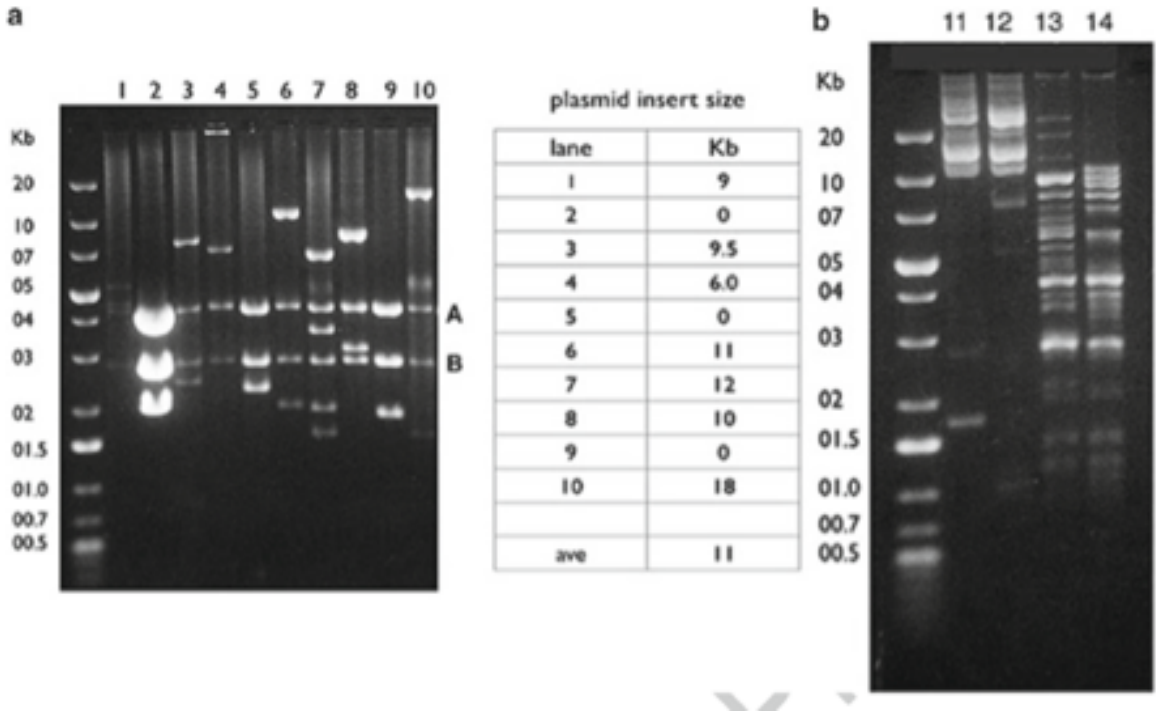

Fig. 2. (a) Representative library 1 plasmids subjected to *PstI* digestion and agarose gel electrophoresis. Bands labeled (a, **b)** and are vector-related bands; the sizes of the remaining bands in each lane are added together (minus 1.5 kb for the size of the band that was cloned) to determine the size of the cloned DNA. Only plasmids with inserts in the range of 9–15 kb were chosen for Library 2. Deleted plasmids can be seen in lanes 2, 5, and 9. A table showing the calculated size of the plasmid inserts is shown to the right. (b) Library 2 DNA, showing lanes 11 and 12 which are uncut Qiagen-kit preparations, lanes 13 and 14 are cut with *Pst* I. DNA size ladders are shown in the left-most lane of each gel.

3.2. Preparation of Tn Mutagenized Library in E. coli (Library 2)

1.Combine 45–50 plasmids (approximately 500 kb of chromo- somal DNA fragments) from the same copy number group from Library 1 (see Note 13).

2.Perform an in vitro transposition reaction on each pool of plas- mids from Library 1 combining similar copy number plasmids in each reaction.

3.Prepare highly purified target DNA for the in vitro transposon mutagenesis reaction. In this study pFL2082 containing an average insert size of 10–15 kb was used. Confirm that no chro- mosomal DNA is present since this will compete with the target DNA for Transposon insertion

sites and reduce efficiency.

4.Tn insertion mutagenesis reaction is performed using 0.2 μg of target DNA and equimolar amounts of transposon (see Note 14).

5. Add an equimolar amount of transposon, 1 μL of 10× reaction buffer, 1 μL of transposase, and ddH2O to a final volume of 10 μL.

6. Incubate at 37°C for 2 h.

7. Stop transposition reaction by adding 1 μL of 10× EZ <Tn>
stop buffer.

8. Heat at 70°C for 10 min.

9. Prepare reaction mix for electrotransformation by diluting fivefold with ddH2O to dilute salt concentration or by purifying the reaction mix with a reaction clean up kit.

10. Transform the transposon-mutagenized plasmid DNA pool into an *E. coli* strain containing the pir+ protein which is required for expression of the R6Kγ origin of replication (see Note 15).

11. Select for ampicillin and kanamycin-resistant transformants.

12. Assess the randomness of Library 2 by performing DNA sequence analysis from the ends of the transposon (see Notes 16 and 17).

13. Harvest approximately 6,000 or more primary transformant colonies off agar plates from each pool of primary transformants.

14. Perform a high-purity and high-concentration DNA preparation on the harvested colonies (see Note 18).

15. Analyze high-purity DNA preparations by restriction digestion and gel electrophoresis to assess the diversity of DNA fragments in the DNA pool (see Note 19).

3.3. Protoplast Preparation (Adapted from Ref. (17))

1. Grow 20 μL of a dense glycerol stock of *A. erythreum* B-3381 in test tubes containing 4 mL 2XYT + glucose (0.4%) and incubate overnight at 33°C, 350 rpm (see Note 20).

2. Next day, transfer 2.5 mL of the overnight culture to 25 mL of fresh 2XYT + glucose (0.4%) in a 250-mL shake flask and incubate for an additional 2–4 h.

3. Pellet the cells at 4,270×g for 7 min.

4. Wash the pellet with 20 mL of 0.3 M sucrose, centrifuge as before, and resuspend cells in 5 mL of 1× modified P buffer+5 mg lysozyme per mL.

5. Incubate cells at 33°C for 1 h with occasional shaking.

6. After 1 h add 6 mL of 1× modified P buffer and incubate another 1 h.

7. Centrifuge at 4,270×g for 10 min.

8. Wash protoplasts with 12.5 mL of 1× modified P buffer and centrifuge again.

9. Resuspend in 750 μL of 1× modified P buffer.

10. Freeze at −80°C or use fresh (see Note 21).

3.4. PEG-Mediated Transformation

1. Mix 50 μL of protoplasts with 5 μL of highly purified and concentrated DNA and 220 μL of 25% PEG (10,000 mw) solution (see Note 22).

2. For integrative transformation use denatured DNA (see Note 23).

3. Spread the protoplast mixture over an R2T2 plate that has been dried to ~95% of its original weight. This can be per- formed in a sterile laminar flow hood. Incubate at 33°C (see Note 24).

4. Between 20 and 24 h later, overlay the regenerated cells with 1 mL of 800 μg/mL thiostrepton solution or drip proportion- ately smaller volumes onto sectors.

5. Spread the thiostrepton solution evenly over sector and place in a sterile laminar flow hood until all the liquid is absorbed.

6. Score transformants 3–4 days later (Fig. 3).

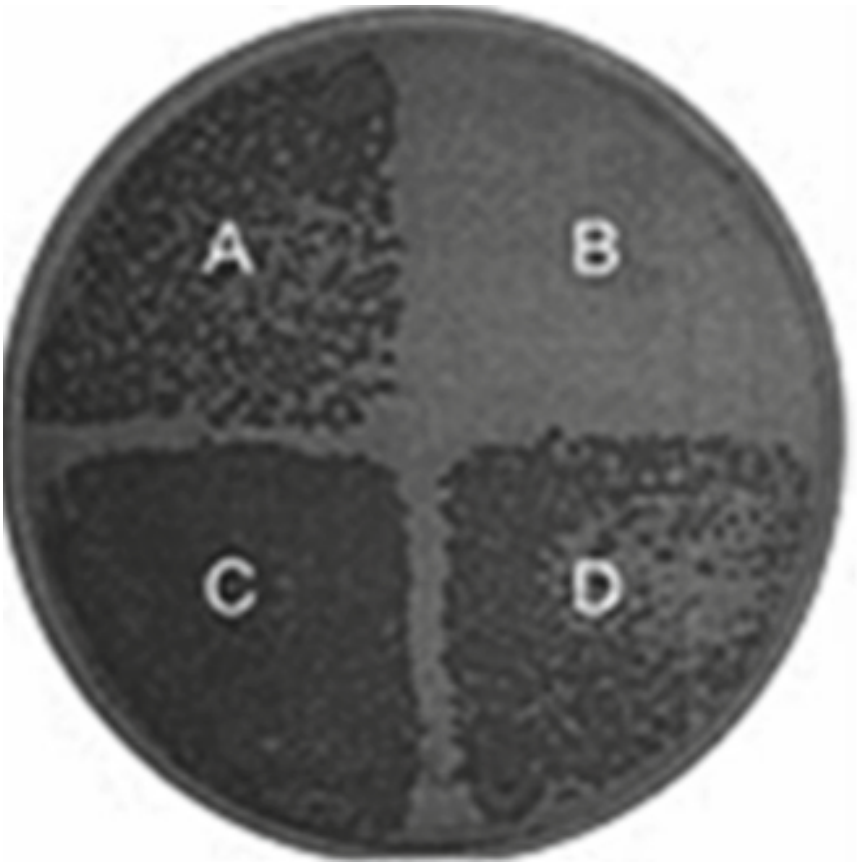

Fig. 3. Primary transformants of *A. erythreum* selected with thiostrepton using the proce- dure described in Subheading 3.4. *A. erythreum* protoplasts were transformed by the PEG-mediated method using purified, denatured DNA. (a). 8 μg denatured plasmid DNA purified by anion-exchange column; (b) no DNA added; (c) 16 μg denatured plasmid DNA isolated by a rapid alkaline lysis method; (d) 8 μg denatured plasmid DNA isolated by a rapid alkaline lysis method. All quadrants containing DNA resulted in confluent or nearly confluent lawns of transformants which were later tested for antibiotic resistance and shown to be recombinants. In this experiment, the amount of denatured plasmid DNA used had the greatest effect on the number of primary transformants obtained.

3.5. Preparation of Host Integration Library (Library 3)

1. Transform *A. erythreum* protoplasts with highly pure and concentrated DNA from each pool from Library 2 (see Note 25).

2. Select primary transformants with thiostrepton (see Subheading 3.4).

3. Plate primary transformants for single colonies on 2XYT with kanamycin (15 μg/mL) plates.

4. Replica plate single colonies to diagnostic media to determine kanamycin and

thiostrepton drug resistance phenotypes (see Note 26).

5. Choose colonies with the proper drug resistance phenotypes for –80°C storage and further analyze in fermentation screening process (see Note 27).

6. Perform visual inspection of stained colonies or other assay to distinguish single-crossover integrants from double-crossover (gene replaced) integrants (see Fig. 4; Note 28).

7. Assess randomness of the library by plasmid rescue (see Subheading 3.10; Note 29).

8. Analyze transposon insertion site gene sequences and determine how the insertion might have led to the improved strain's production phenotype. Correlate insertion mutation into existing models of strain improvement (see Note 30).

3.6. Microtiter Plate Screening

These instructions assume the use of the V&P Scientific Alligator magnetic stirrer system (see Note 31).

1. For master stock generation, fill a 96-deep well microtiter plate with 500 μL of MSCM medium.

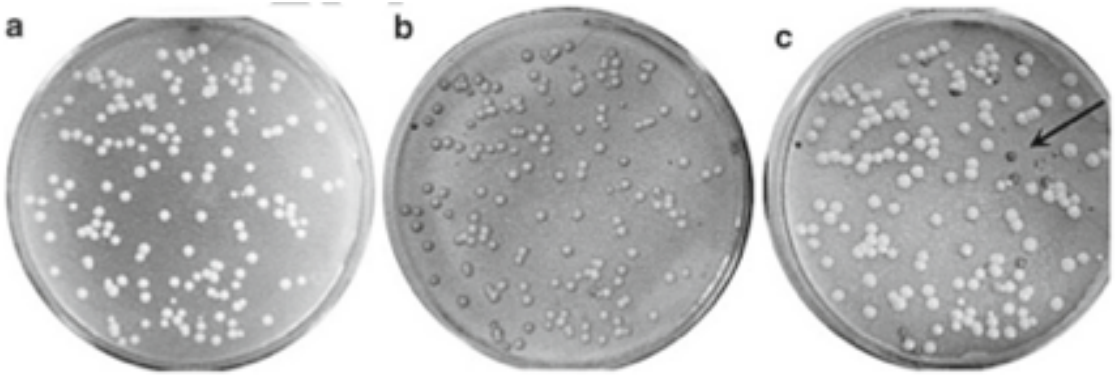

Fig. 4. Colorimetric plate assay to distinguish single-crossover recombinant strains from double-crossover strains using antibiotic selections. (a) Dilutions of primary transformants of *A. erythreum* were plated on agar-containing thiostrepton and grown for 4 days at 30°C. In this experiment, the thiostrepton-resistance marker (tsr)was carried on the transposon (Fig. 1b), and the kanamycin-resistance marker (kan2) was carried on the plasmid (Fig. 1c). (b) Same plate 24 h later after being underlayed with an agar slab containing kanamycin (20 μg/mL) and a tetrazolium dye (tetra red, 10 μg/mL). At 24 h incuba- tion, all colonies turned an orange color, indicating uptake of the vital dye. (c) The same plate 72 h post underlay. Two differ- ent colored colonies appear. The majority of the colonies remain light orange; these are kanamycin-resistant colonies. A smaller percentage of the colonies become dark red. These are the desired double-crossover kanamycin-sensitive colo- nies (see *arrow*). These colonies can be recovered alive and analyzed in the fermentation screening program.

2.Inoculate wells by dabbing a sterile magnetic stir bar to a single colony from library 3.

3.Place the 96-deep well on the magnetic stirrer and incubate for 72 h at 33°C with lids to prevent evaporation. Maintain rela- tive humidity above 50% to limit evaporation.

4.Add glycerol to 20% and freeze at –80°C until needed for screening.

5.For screening mutants, use 10 µL of the frozen master stock to inoculate 500 mL MSCM medium using a multichannel pipettor.

6.Incubate at 33°C on magnetic stirrer at appropriate power set- ting for 72 h. Relative humidity should be maintained above 50% to minimize evaporation.

3.7. Shake Flask Screening Method

1. Inoculate seed cultures in unbaffled 250-mL shake flasks with filter closures (0.2 µm pore size), containing 25 mL of MSCM broth, using 250 µL of thawed glycerol stock of culture (see Note 32).

2. Incubate seed cultures at 33°C and 65% humidity on a humid- ity-controlled incubator-shaker having a 1-in. circular displace- ment at 380 rpm for 24 h.

3. Use 1.25 mL of the seed culture to inoculate fermentations in 250-mL shake flasks containing 25 mL of MSCM medium.

4. Incubate the fermentations for 3–5 days. At harvest, correct for the loss of volume through the addition of distilled water, if necessary.

5. Perform shake flask screening method on double-crossover recombinants (see Note 33).

3.8. Bioassay of Antibiotic Activity (see Note 34)

1. Pour 100 mL of molten tryptic soy broth agar into the bottom of a large plastic bioassay dish and let harden for 30 min.

2. Pour another 100 mL of molten TSB agar, containing 250 µL of 1% triphenyl-tetrazolium red and a sufficient quantity of *Bacillus subtilis* spores to produce a confluent lawn of growth, onto the first solidified agar layer. Let agar solidify for 1 h.

3. Spot broth samples directly after 1:10 dilution with TE pH 8.0 onto 1/4-in paper bioassay discs, and let dry for 30 min.

4. Place dried bioassay discs onto the surface of the agar plate.

5. Incubate the plate overnight at 37°C.

6. Measure the diameters of the inhibition zones and convert zone diameters to erythromycin concentration using standard curves produced from zone diameters of reference standards on each plate.

3.9. Small-Scale Chromosomal DNA Preparation

1. Resuspend 50 mg of cells wet weight from 300 µL of a 2-day MSCM culture in 500 µL of lysozyme solution and incubate at 37°C for 5 min or until cells become somewhat translucent.

2. Add 250 µL of a 2% SDS and mix on vortex for 1 min; this will reduce viscosity and clear solution noticeably.

3. Add 250 µL of phenol-chloroform solution (pH 8.0), mix on vortex for 30 s, centrifuge for 2 min in a microcentrifuge. Remove 300 µL of the supernatant, leaving the white interface behind.

4. Add 0.1 volume of 3 M sodium acetate, pH 5.2 and mix on vortex, then add 0.6–1 volume of isopropanol, and mix by inverting tube several times; a small clump of DNA should form and visibly fall to the bottom of the tube.

5. Centrifuge for 2 min in a microcentrifuge and a small white spot of DNA appears at the bottom of the tube.

6. Carefully pour off supernatant and wash pellet twice with 500 µL of 70% EtOH, drain liquid off, and air dry for 10 min. Dissolve pellet in 100 µL of TE 8.0 or water. Mixing and pipetting may be required to get the DNA in solution.

3.10. Plasmid Rescue Procedure (see Note 35)

1. Prepare chromosomal DNA from improved strains containing an inserted transposon mutation (see Subheadings 3.1 and 3.9 for method details).

2. Digest the DNA with *NarI, SalI, ApaI, BssHII, StuI,* or *NdeI* (or other frequent cutter enzyme that does not cut within the transposon), using 1 µg of chromosomal DNA (see above pro-cedure) in a 20 µL reaction volume.

3. At end of digestion add 70 µL water, heat at 70°C, 20 min, cool on ice 5 min, return solution to room temperature.

4. Add 10 µL of 10× ligase buffer and 1 µL ligase.

5. Incubate at room temperature for 1 h.

6. Add 1 µL glycogen (20 mg/mL), 10 µl of 3 M sodium acetate (pH 5.2), and 250 µL of cold 100% ethanol.

7. Set on ice for 20 min.

8. Centrifuge at 15,871×g for 15 min at room temperature.

9. Pour off ethanol, revealing small white pellet at bottom of tube.

10. Wash inside of tube with 1 mL of room temperature 70% ethanol. Repeat wash once.

11. Pour off ethanol, let tubes stand inverted for 5–10 min to remove all traces of liquid.

12. Resuspend small white pellets in 25 μL of double distilled water or TE buffer pH 8.0.

13. Add 5 μL of DNA to 20–35 μL of cold electrocompetent EC100D cells in a chilled Eppendorf microcentrifuge tube.

14. Transfer the cell DNA mixture to a cold 0.2 cm cuvette and let sit on ice for 5 min.

15. Electroporate the cells at 2.5 kv, 600 Ω, and 25 microFaradays.

16. Add 1 mL of S.O.C. broth containing the 2× *oriV* inducing agent (Epicentre Biotechnologies; if transposon has the R6Kγori no inducing agent is needed).

17. Incubate the broth-containing cells and the *oriV* inducer at 37°C for 1 h.

18. Plate 20 μL of the cells from the broth directly on LB plates supplemented with kanamycin (40 μg/mL) and the 2× oriV inducer. (This is the "dilute" plating).

19. Centrifuge the broth and cells at 3,220 × *g* for 5 min to gently pellet cells. Decant supernatant and resuspend cells in 50 μL of 20% glycerol.

20. Plate 20 μL of cells in at least a one-quarter to one-eighth sec- tor of a LB plate supplemented with kanamycin (40 μg/mL) and 2× oriV inducer (this is the high-density plating).

21. Incubate at 37°C overnight, colonies should appear by morning. Note that the cells will often appear on the high-density plate at high density and in the dilute plating no colonies may appear.

22. Analyze target site (see Notes 7 and 36).

4. Notes

1. The media used in this method are commonly used for actino- mycetes, but any rich medium specifically adapted for growing the host strain can be used. The trace element solution can be made up as a 1,000× stock. Add components in order and mix thoroughly

while adding individual components. Protect from light and store at 4°C

2. Standard molecular biology techniques and reagents were used according to ref. (15).

3. The ratio of target DNA to transposon is important for obtain- ing a good percentage of mutated plasmids of library 1. When purifying the transposon from an agarose gel limit exposure to UV light as this can damage the 19-bp mosaic ends and signifi- cantly reduce efficiency of the in vitro mutagenesis reaction.

4. Potassium phosphate, while important for PEG-mediated transformations of filamentous actinomycetes, was found to have a negative effect with the unicellular *A. erythreum*.

5. R2T2 agar plates need to be dried before PEG-mediated transfer is performed. The degree of dryness for efficient transformation will need to be determined and optimized. For *A. erythreum* 90–95% of the original weight gave the best results, whereas with filamentous actinomycetes plates need to be significantly dry, between 82 and 85%.

6. In this study, we developed a growth and colorimetric assay to distinguish between single- and double-crossover insertion strains since we only wanted to screen strains that had undergone allelic exchange to ensure genetic stability and not use antibiotics for plasmid maintenance which could impact antibiotic titers. The use of this type of assay, which relies on inhibition of growth using a bacteriocidal reagent, kanamycin, requires frequent observation so that gene-replaced colonies can be transferred to fresh nonantibiotic-containing medium shortly after the accumulation of color and cessation of growth to ensure survival.

7. Primers can be purchased directly from Epicentre Technologies or as part of the EZ:Tn in vitro transposon mutagenesis kit. The primers are designed to read off the ends of the Tn5-based transposon to identify the transposon insertion site. They can be used to sequence directly off the transposons from chromosomal templates, or more preferably with rescued plasmid DNA.

8. V&P Scientific has a wide variety of magnetic stirring systems to choose from with different output capacities. A large variety of stirring rods, bars, and discs are available to choose from. We found that the hollow stainless steel stir bars worked best for our application. A series of microfermentation tests was performed and erythromycin titers, well-to-well splashing, and evaporation were checked over a 3-, 4-, and 5-day period. The most consistent data was obtained with the hollow stainless-steel stir rods during a 3-day fermentation period. Longer fer mentations worked well but a decrease in culture volume was observed overtime.

9. For library 1 the chromosome of the actinomycete of choice, *A. erythreum,* is cut into 10–15 Kb DNA fragments and cloned into a bifunctional plasmid vector pFL2082 (Fig. 1c). The DNA fragments can be isolated from low percentage agarose gels or by pulsed-field gel electrophoresis (PFGE) to obtain better band resolution.

10. An important consideration for library 1 is the design of the cloning vector. The cloning

vector carrying a large insert must replicate stably in *E. coli* and be easily transformed back into the actinomycete of interest for a gene replacement reaction. For *A. erythreum,* plasmid pFL2082 was chosen because it could replicate in high copy number in *E. coli* using the pBR322 replicon from pBS(+) and allows this plasmid to integrate and evict from the chromosome of *A. erythreum* by homologous recombination. The thiostrepton-resistance gene (tsr) allows for selection of transformants in *A. erythreum*. The type of vector to be used in Library 1 for each actinomycete will be different and depends on the characteristics of the species of interest. Ideally, the vector itself should be small so that it pres- ents the smallest possible target for the transposon during the in vitro transposition reaction. In the analysis of Library 1 for *A. erythreum,* we found that 10–15 Kb chromosomal inserts could be stably cloned and maintained in pFL2082.

11. Twenty clones from Library 1 were randomly chosen and DNA sequence analysis of the clones showed that they were ran- domly distributed across the genome. The library 1 clones were visually identified by blue-white screening on X-gal-containing agar plates. Analysis of restriction enzyme digested plasmids on agarose gels revealed that about one-third of the white colonies did not contain inserts but were deleted plasmids (Fig. 2a).

12. It is also possible to observe that the copy number of the plas- mids in Library 1 can vary from very low to very high. If that is the case then combine Library 1 plasmids of similar copy number before being used in the in vitro transposition reactions to form Library 2. This helps to maintain a high degree of randomness in the transformants obtained in Library 2.

13. Library 2 is formed by combining plasmids from Library 1 of similar copy number, into pools of approximately 50 plasmids each, representing a combined total of about 500 Kb of chro- mosomal DNA per pool. The plasmid pool is subjected to an in vitro transposition mutagenesis reaction, and the resulting mutated plasmids are transformed into an *E. coli* strain con- taining the *pir* protein for generation of individual colonies containing transposon-mutagenized plasmids.

14. Molar amounts of target and transposon DNA are calculated using the following equation: μg target DNA/[target DNA (in bp) × 660]. The average target size of pFL2082 is 19,000 bp, so the amount of target DNA is 0.016 pmoles when starting with 0.2 μg. In this case an equimolar amount of transposon would require only 0.1 μL, since the stock concentration is 0.15 pmoles/μL.

15. In order to select for the transposon insertion and *E. coli* strain containing the pir+ protein is required. Epicentre Technologies has two pir+ *E. coli* strains: EC100D and EC100D pir-116.

16. Each mutated plasmid from Library 2 can be purified and sequenced using the primers that read off the ends of the trans- poson DNA. In our study, we found that 75% of the plasmids contained transposon inserts in the cloned chromosomal DNA and the rest in the vector backbone outside of essential functions such as antibiotic markers and replication regions.

17. The reason there were more Tn insertions in the cloned DNA was probably due to the fact that insertions into the vector portion of the plasmid could be non-growers due to knockouts of the antibiotic resistance gene or the origin of replication.

18. The colonies from library 2 are harvested together from primary transformation plates and the plasmid DNA from the mixture is purified using a Qiagen or other suitable plasmid preparation kit. The plasmid preparation is analyzed by restriction enzyme digestion and agarose gel electrophoresis for the presence of a multitude of different sized DNA fragments to indicate randomness of the plasmids in the preparation (Fig. 2b).

19. The plasmids having transposons that missed the insert DNA are still kept with the other plasmids of Library 2 since these plasmids do not affect the generation or quality of Librar y 3 mutants.

20. Following the *A. erythreum* protoplast transformation procedure carefully leads to reproducible integration of suicide plasmids. Other actinomycetes, for example *Saccharopolyspora erythraea,* are more difficult to transform. In many strains, the age of the cells used for making protoplasts is critical for achieving a high rate of transformation efficiency.

21. Protoplasts suspended in 1×PT buffer can be frozen and thawed multiple times; therefore, once a high-efficiency protoplast preparation is found, it can be useful for several subsequent reactions.

22. If frozen protoplasts are used, spin for 7 min at 845×g in a microcentrifuge to pellet cells and rid supernatant of nucleases. After spin is over, quickly mix in PEG solution and concentrated DNA. Another important factor for obtaining high-efficiency transformations is the use of highly purified and concentrated DNA for the transformation reaction. Qiagen kit (or similar) DNA preparation plasmid kits are recommended. Gentle handling of protoplasts may also be an important factor in achieving high transformation efficiencies which is why lowspeed centrifugation is used and pipetting is kept to a minimum. No vortexing of protoplasts is performed.

23. Alkaline denaturation procedure as described in ref. (18). Add 1–5 μg DNA in a small volume into a 1.5-mL microcentrifuge tube. Bring volume up to 9 μL with double-distilled water. Add 2 μL of 1 N NaOH and incubate at 37°C for 10 min. After incubation, quickly place on ice and add 2 μL of 1 N concentrated HCL to neutralize. Use denatured DNA in transformations.

24. The speed of protoplast/PEG puddle absorption into the agar may be an important factor affecting transformation efficiency. To avoid long puddling times, dried regeneration plates are used with level surfaces (created by rotating the plates as they dry in the bio-hood). Also, incubators are kept at low humidity to speed absorption of the protoplast/PEG puddles.

25. Once a lawn of primary transformants is obtained using thiostrepton or kanamycin

selection (Fig. 3), the formation of Library 3 gene replacement mutants can begin. Cells from the confluent lawns of primary transformants are passed through one or two rounds of nonselective growth to allow for the spontaneous eviction of the plasmid.

26. The loss of the plasmid can be detected through replica plating of single colonies to diagnostic drug-supplemented agar plates. Single colonies that show loss of the drug resistance marker associated with the plasmid, and the maintenance of the drug resistance marker associated with the transposon, are selected for the fermentation screening process.

27. These double-crossover mutants represent only approximately 5–10% of the population of single colonies when one round of nonselective growth and sporulation is used. These double- crossover mutants will carry the desired transposon insertion without the plasmid sequences, creating essentially the same type of mutation as if the transposon had inserted via an endog- enous in vivo transposon event. With two rounds of nonselec- tive sporulation, the frequency of the desired double-crossover mutants is increased significantly.

28. In *A. erythreum* a helpful color reaction was developed to dis- tinguish double-crossover mutants from single-crossover inte- grants (Fig. 4), and a similar selection can be developed in other systems as appropriate. This visual screening process is significantly easier to perform than the replica-plating process and speeds up the identification of the Library 3 mutants.

29. Using the procedure described here it should be possible to generate thousands of random mutations in the genomes of these erythromycin-producing bacteria. However, it might be expected that the plasmid library approach may not allow satu- ration mutagenesis of the entire genome because some parts of the chromosome may not be cloned on a high copy plasmid vector in *E. coli*.

30. The transposon could insert into a gene that encodes a hypothetical protein, in which case a new, nonobvious strain improvement target is revealed. Nonobvious targets are the types of mutations that would not normally be created based on our current knowledge of how metabolism can be manipu- lated to make better strains. Nonobvious targets are therefore a valuable resource for obtaining new information to broaden our understanding of metabolism.

31. This is the method that was used to find the original *mutB* and *cobA* mutants in the *A. erythreum* screen (8). Because *A. erythreum* is unicellular and not as prone to high-viscosity fermentations, it can be cultured in liquid microtiter fermentations. A rotary tumble stirring apparatus (V&P Scientific Inc., San Diego, CA) can be used. Mycelial organisms are prone to high-viscosity liquid fermentations and are better suited to test tube fermentations at 380 rpm with 1 in. circular displacement. Microtiter fermentations may be a workable alternative, with the disadvantage that there is a larger variation in production level for a particular strain, more false positive hits are generated, and as with any screening program improved strains do not necessarily scale-up to shake flasks or larger scale-up systems without a careful medium design.

32. It is helpful to analyze the new mutant trait under a wide variety of fermentation conditions. And even more can be learned if the mutation is created and studied in different erythromycin-producing organisms. This helps to confirm the significance and validity of the mutant phenotypes found. Many different factors affect antibiotic production levels, and establishing a reliable connection between a mutation and a strain improvement effect takes a comprehensive analysis to be meaningful.

33. Screening procedures for the decoded mutate-and-screen programs are no different than classical programs (2). Our *A. erythreum* shake flask fermentation method has been published (19). Shake flask screening, though reliable, is a laborious and time-consuming process and may not be practical for most small laboratories.

34. One method for measuring erythromycin production is the bioassay (see Subheading 3.8), although colorimetric assays have also been described (18). TLC (8) and HPLC methods (20) are useful for determining the concentration of contaminating coproducts such as erythromycin B and C as well as erythronolide B and mycarosyl-erythronolide B.

35. When the circular DNA is transformed into a pir+ *E. coli* strain, the DNA propagates as a plasmid using the origin of replication (R6Kγori or *oriV)* contained within the transposon. The plasmid DNA is recovered from *E. coli* and the sequence of the plasmid DNA flanking the transposon is determined using primers that read off both ends of the transposon. Since the procedure is relatively simple and rapid, many mutant hits can be analyzed this way, including mutants that have a decrease in production. Yield-lowering mutants can be just as helpful as yield-enhancing mutants for the insight they may provide into the secondary metabolic process.

36. Once a genetic target is identified through the location of the transposon insertion site, the reverse engineering is not neces- sarily complete. If the transposon inserted into an ORF at the end of an operon (i.e., the end of a transcription unit), then the mutagenic effect is most likely due to the loss of function of that gene. However, if the transposon inserted into an ORF that is not at the end of a transcription unit, then the mutant phenotype could be due completely, or in part, to polar effects on downstream genes. A significant amount of follow-up work may be necessary to identify the gene or genes responsible for the mutant phenotype.

Acknowledgments

This work was supported by National Institutes of Health, Small Business Innovation Research (SBIR) awards R44GM58943 and R44GM063278.

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