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Transposon-Based Strategies for the Identification of Essential Bacterial Genes

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Summary

We present a conceptual review of transposition-based strategies for determining gene essentiality on a one-by-one basis in bacteria. Many of the techniques are described in greater detail in individual chapters of this volume. The second section of this chapter deals with transposition-deletion—based strategies for determining the essentiality of blocks of genes. This latter approach has the potential to experimentally define the minimal required genome for a given organism.

Key Words: deletion; essential genes; insertion; transposon.

1. Introduction

A century of research work has been focused on the analysis of genetic determination of biological properties. With the advent of genome projects, in which the DNA sequences of the genomes for an ever-expanding group of organisms are now available, we are still faced with the daunting challenge of determining the functional importance of the various genes present in any given genome. One approach to this functional gene analysis is to determine which genes in an organism's genome are required for survival and growth in any particular environment; in other words, which genes are essential. A strategy for determining gene essentiality is to attempt an isolation of knockout mutations of the genes in question. Failure to isolate such a knockout mutation in a particular gene is taken as presumptive evidence that the gene in question is essential in the tested (all?) growth conditions. Alternatively, a gene might not be essential in one defined condition but be essential in another test circumstance. In these cases, the gene mutants can be studied for their effects on survival and growth under various test circumstances. DNA transposition, in which the transposon acts as an insertion mutagen or, in some cases, as a deletion mutagen, is a powerful approach for the generation of appropriate knockout mutations for these studies. This chapter provides an overview of transposition strategies for determining gene essentiality. The individual

From: *Methods in Molecular Biology, vol. 416: Microbial Gene Essentiality*
Edited by: A. L. Osterman and S. Y. Gerdes © Humana Press Inc., Totowa, NJ

strategies are described in more detail elsewhere in this volume and in other cited references.

There are two different types of questions that are addressed in these studies. The first most common approach is to ask whether a given particular gene is essential in an otherwise complete, intact genome. This one-by-one approach looks at particular genes but sometimes misses the particular functions encoded by the genes. This is because genomes sometimes contain more than one gene encoding products capable of performing the same function. We call such genes redundant. In this case, each such redundant gene could be individually destroyed with no impairment to the organism's survival and growth even if the function is essential. To determine that the particular function is essential, one would need to destroy all redundant genes and demonstrate survival and/or growth impairment. Thus, we must also look at strategies that can be used to define essential functions regardless of whether various functions are encoded by unique individual genes or redundant genes. For this type of inquiry, one can also use transposition-based approaches to generate large-scale deletions. These large-scale deletions not only offer an approach to identifying essential functions encoded by redundant genes but also suggest a strategy for dramatically shrinking the size of the organism's genome perhaps to the extent of defining a minimal essential genome.

This chapter shall first describe transposition systems that are used to generate individual insertion mutations. These techniques are based on the straightforward application of standard transposition mutagenesis that is schematically described in **Figure 1**. For the more global goal of shrinking the genome in order to define essential functions,

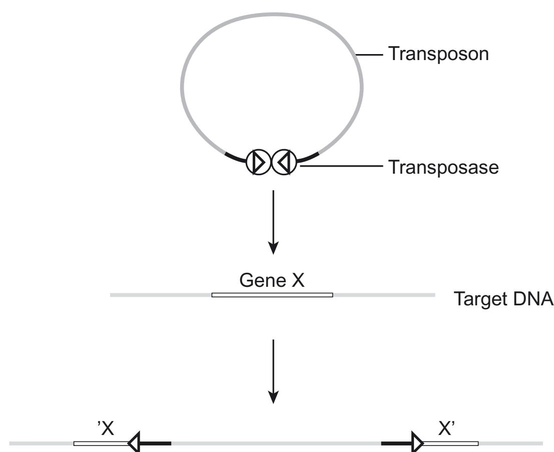

Fig. 1. Intermolecular transposition. The DNA transposons typically used for genetic analysis experiments are excised in a transposase-catalyzed fashion from their original genomic location. Pictured here are the next steps in transposition. The excised transposon complexed with transposase binds to target DNA (Gene X), and the transposase catalyzes integration of the transposon into Gene X thus generating ′X and X′ sequences. The transposase is presented as a circle. The specific end DNA sequences of the transposon are presented as open triangles.

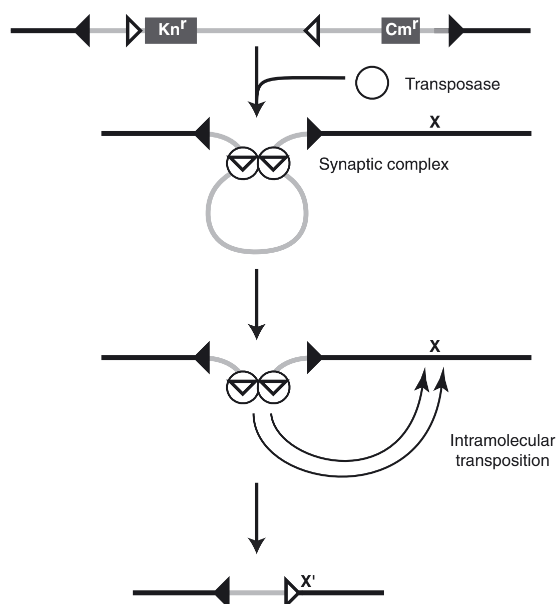

Fig. 2. Intramolecular transposition and adjacent deletion formation. A composite transposon is used for adjacent deletion formation. The composite transposon is made up of two transposable elements both defined by one open triangle and one closed triangle. Insertion events are first generated using intermolecular transposition of two closed triangles (not shown). An open triangle—specific transposase is synthesized, binds to open triangle ends, forms a synaptic complex, and then catalyzes intramolecular transposition to a site thousands of base pairs away, thus generating a deletion. This technology is described in more detail in Ref. ***12***.

we shall describe deletion strategies that either use a random, transposition-based technology in which a composite transposon catalyzes inside-out intramolecular transposition (**Fig. 2**) or a transposon-to-transposon excision methodology between two insertions generated previously by standard transposition methodology (**Fig. 3**).

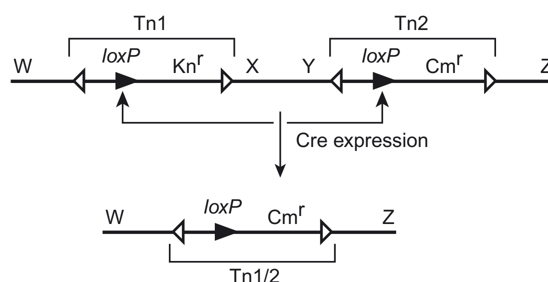

Fig. 3. Site-to-site deletion through Cre-catalyzed excision of DNA defined by two transposon inserts. Two Tn*5*-like inserts are separately generated through the electroporation of premade transposition complexes. Both transposons carry *lox*P sites (filled-in triangles), but one encodes kanamycin resistance (Kn^r) and the other encodes chloramphenicol resistance (Cm^r). Cre expression catalyzes the excision of DNA between the two *lox*P sites. See Ref. ***19*** for a more detailed description of the technology.

2. General Requirements

Hundreds of transposons have been identified and studied to some extent ***(1)*** so it would seem that a very large number of tools are possible. The transposition systems that are discussed in later chapters in this volume or related literature include Tn*3*, Tn*5*, Tn*7*, Tn*10*, Tn*4001*, and *mariner*. Although historical accidental choices certainly played a role in choosing these systems, the choice of transposon tools are restricted to ones that are well-enough studied so that we know that they can be made to fulfill the following requirements. First, the element must manifest a sufficiently high frequency of transposition through the desired protocol so that it is possible to achieve saturated mutagenesis (every gene hit at least once) in the organism's genome. Second, the targets chosen by a given transposition system should be sufficiently random so that any gene can suffer an insertion within the given procedure. It should be noted that all transposons likely manifest some degree of target sequence bias. Nonetheless, several of the transposition systems that are used as tools manifest a reasonable approximation of target randomness. Third, the transposition products should be genetically stable. This last criterion is typically achieved by not having the transposon-specific transposase synthesized in the target cells subsequent to the planned transposition event. Once these general requirements are met, the element of choice needs to be compatible with the transposition strategy used to generate the knockout libraries. We will describe below several different transposition strategies. Finally, the transposon of choice needs to contain the desired genetic markers demanded by the particular strategy. The most universal marker needed is an appropriate antibiotic resistance marker that will allow the selection of the desired transposition events in the particular host cell.

3. Transposon Structure

In general, natural transposons have the following basic structure (**Fig. 4**). They are defined by short (typically less than 50 bp), transposon-specific terminal DNA sequences. In many cases, these terminal sequences are inverted versions of the same or closely related sequences. The specific terminal inverted repeat sequences are key components of all the transposons that we shall use. Natural transposons also contain a gene encoding the transposon-specific transposase. The transposase binds specifically to the terminal inverted repeat sequences, forms a transposase-DNA synaptic complex, and, in the presence of Mg^{2+}, catalyzes the transposition events. Because of our need to generate genetically stable transposon inserts, the gene for transposase synthesis has been deleted from all of the constructs used in our studies. Instead, the transposase is encoded by a gene located outside of the transposon structure and is lost after transposition or else the transposase is provided biochemically. All transposons used in these studies encode antibiotic resistance in order to allow for the biological selection of the desired genetic events. Finally, transposons can be constructed to contain DNA sequences encoding other desired functions such as primer binding sites, T7 RNA polymerase promoters, site-specific recombination sites, genes encoding reporter functions, and so forth. In fact, transposons can carry any desired sequence as long as the

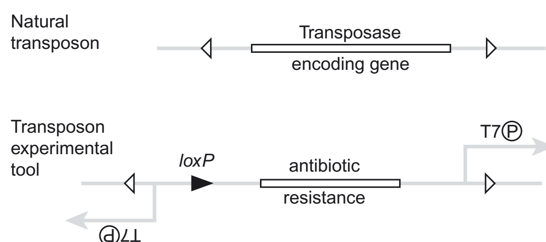

Fig. 4. Transposon structure. A natural DNA transposon has three components. The transposon ends are defined by two short (<50 bp), terminal, specific DNA sequences that typically are inverted versions of each other (open triangles). The transposon also encodes a transposase protein that catalyzes transposition of DNA sequences defined by the inverted terminal DNA sequences. Not shown are other genes that may be carried on the transposon. These other genes encode products that typically play no role in the transposition mechanism (i.e., antibiotic-resistance genes). By supplying the transposase exogenously, the transposon can be simplified as an experimental tool. In this case, the terminal transposase recognition sequences bracket DNA that contains the desired sequences. For example, the transposon can be constructed to contain an appropriate antibiotic resistance gene, outward-facing T7 promoters, and a *lox*P site.

length of DNA between the terminal inverted repeats is not so long (typically over several thousand base pairs) as to impair transposition.

4. Transposition Strategy

There is extensive literature that describes the use of transposons as genetic tools utilizing *in vivo* technologies; for instance, see the review by Berg et al. ***(2)***. These technologies utilize plasmid transformation or conjugation, or phage infection as a means for introducing the transposon into the target organism. The first adaptation was the use of plasmids or phages that were "suicide vectors." For suicide vectors, the phage genome or plasmid cannot be stably inherited by the target organism under the desired experimental conditions. The second property of suicide vectors is that the transposon-specific transposase is encoded by a gene that is contained on the phage or plasmid but outside of the transposon itself. Thus, after transposition and loss of the suicide vector, no transposase encoding sequence would be present and the transposition product would be genetically stable (**Fig. 5**). Systems that have utilized this type of *in vivo* strategy include the following examples: the signature-tagged mutagenesis (STM) Tn*5* system ***(3)***, the Tn*4001*-based individual knock-out system ***(4)***, the Tn*10*-based individual knock-out system ***(5)***, the *mariner*-based individual knock-out system ***(6–9)***, and the Tn*5*-based system for distinguishing cytoplasmic versus membrane proteins ***(10, 11)***. Finally, a modified version of a suicide vector strategy was used in the Tn*5*-based adjacent deletion technology ***(12)***.

A major accomplishment in transposition research was the development of *in vitro* transposition systems for a select group of transposons. The goal of this biochemical work was to enable research into the molecular basis of transposition, but the resulting

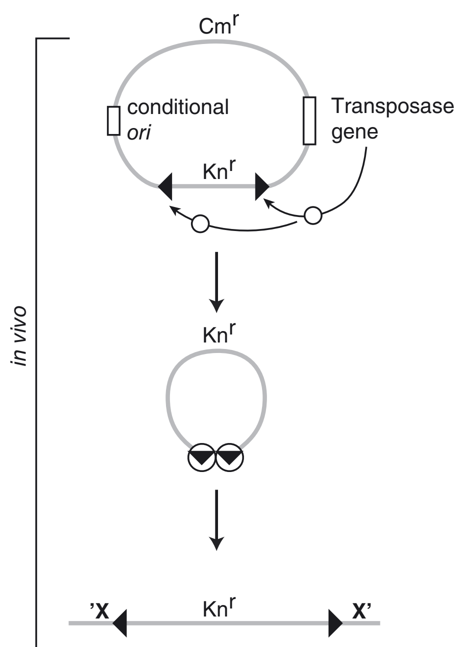

Fig. 5. Transposition mediated by a suicide vector system. The purpose of suicide vector systems is to allow *in vivo* transposition that results in genetically stable products; no subsequent transposition occurs after the initial insertion because no transposase is available. In this case, a suicide plasmid is utilized. The plasmid carries the transposon of choice (defined by solid triangles on either side of a Kn^r gene), an origin of replication (ori) that is unable to function in the chosen conditions, and a gene encoding the transposase that is located outside of the transposon. After plasmid introduction into the cell, the transposase (shown as open circles) is synthesized, and the transposase catalyzes transposition into the chromosome DNA and destruction of the plasmid (by formation of double-strand breaks). Because the plasmid is destroyed, no further synthesis of transposase occurs and no further transposition can occur. Similar phage-based suicide transposition systems have also been used.

technologies were soon adopted by investigators interested in applied uses of transposons such as the identification of essential genes. The general protocol involves *in vitro* transposition into target DNA followed by transformation of the DNA products into the target cells selecting for the presence of the transposon (**Fig. 6**). By this means, the transposon knockout strategy could be extended to organisms lacking a suitable *in vivo* suicide vector system (or allowed such a requirement to simply be bypassed). The critical requirement is that an efficient DNA transformation system must exist. Examples of the use of this *in vitro* technology can be found in the work by Akerley et al. ***(13)*** and Wong and Akerley ***(14)*** using the *mariner* transposition system, Kumar et al. ***(15)*** and Kumar ***(16)*** utilizing both the Tn*3* and Tn*7* systems, and Kang et al. ***(17)*** utilizing the Tn*5* system.

A system that combines both *in vitro* and *in vivo* manipulations involves the formation of transposon DNA—transposase complexes *in vitro* followed by electroporation of the transposition complexes (sometimes referred to as transposome or transpososome complexes) into the target cells ***(18)*** (**Fig. 7**). The *in vitro*—generated transposition complexes are catalytically activated when they encounter the intracellular Mg^{2+} leading to the random incorporation of the transposon into the cell's genome. This technology also bypasses any need for *in vivo* suicide vector strategies. The studies that have used this technology are described in Refs. ***12*** and ***19–22***.

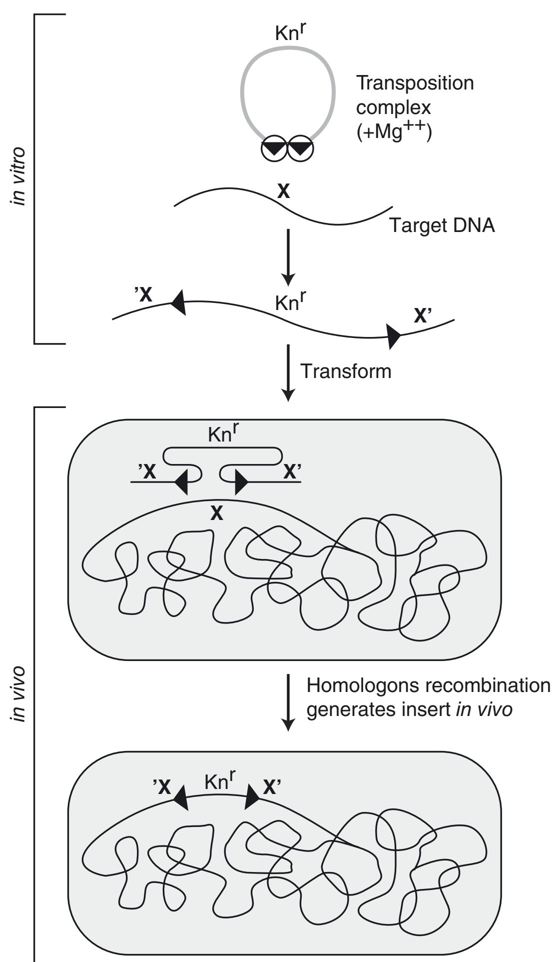

Fig. 6. Use of *in vitro* transposition systems. *In vitro* transposition systems have been developed for some transposons. These *in vitro* systems allow the pictured transposition technology in which *in vitro* transposition is performed using purified target DNA and then the resulting transposition products are introduced into cells and incorporated into the cell's genome through homologous recombination.

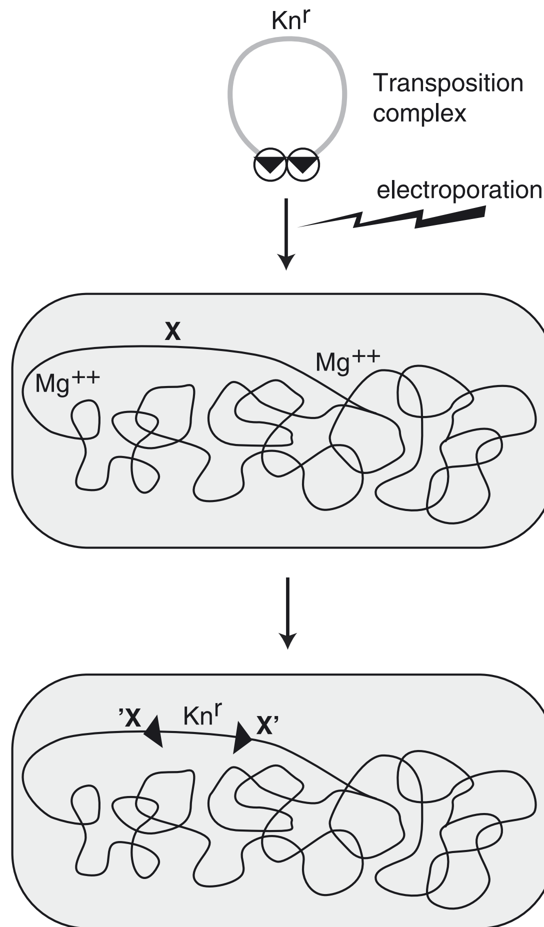

Fig. 7. Electroporation of preformed transposition complexes. Tn*5* transposase-transposon complexes give rise to transposition events after electroporation into a wide variety of target cells ***(18)***.

5. Mapping/Detection Strategies

All of the one-by-one insertion mutation strategies described in this text are based on the proposal that pools (or libraries) of insertion mutants can be followed by various high-throughput techniques to determine how the individual mutants fare in competition with their peers found in the pool. This at first seems like a formidable challenge, but it has been achieved using a variety of technologies as described below.

As a first approach, a number of investigators have addressed the above challenge by first isolating individual transposon insertion mutants as colonies and then utilizing DNA sequence analysis of polymerase chain reaction (PCR)-amplified transposon-target junctions to define the gene location of each insert ***(4, 8–11, 15, 16)*** (**Fig. 8**). The sequenced inserts define nonessential genes. Once the PCR-amplified junction sequences are available, the ability of specific mutants to grow in pooled cultures under defined

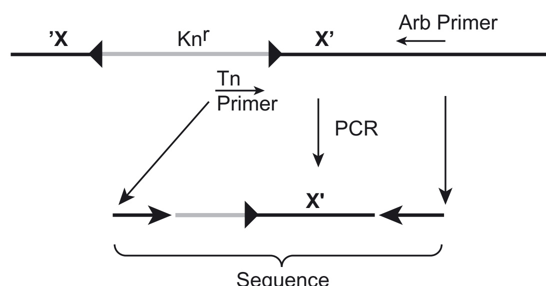

Fig. 8. Defining transposon inserts by sequence analysis of transposon—target DNA boundary sequences. A uniquely oriented transposon-specific primer (Tn primer) is coupled with an arbitrary (Arb) primer to PCR-amplify one of the transposon-target boundaries, which is subsequently sequenced in order to identify target DNA immediately adjacent to the transposon end sequence (filled triangle).

suboptimal conditions can be ascertained by nucleic acid hybridization analysis of the transposon-target joints.

A second approach is called "footprinting" (**Fig. 9**). The chromosome is divided into *in silico* segments whose lengths can be easily amplified by PCR. Primers are designed for each segment's ends. The inserts (in a much larger pool) are found within the defined segments by using a number of PCR reactions. Each PCR reaction is defined by a segment-specific primer and a transposon-specific primer. Typically, this experiment is used to define the end result of transposition plus outgrowth, but in some cases two PCR reactions are performed; one prior to growth (thereby defining the distribution of inserts in the inoculum) and one after outgrowth (thereby defining the viable mutants) (S. Gerdes, personal communication). This latter approach has the advantage of ruling

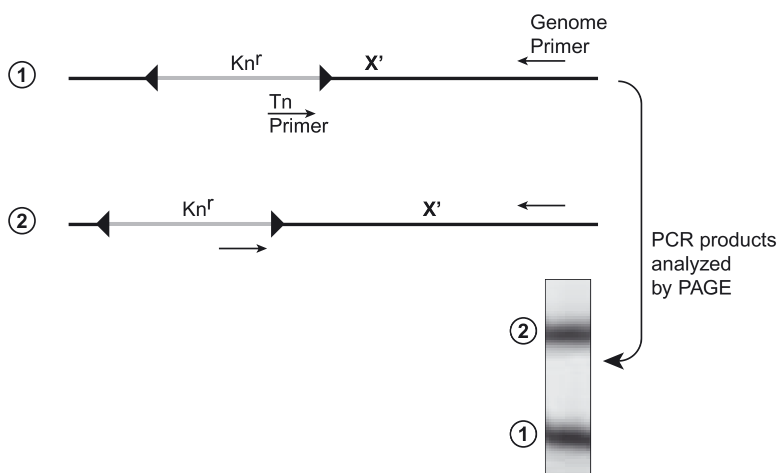

Fig. 9. Transposon footprinting. A large collection of transposon inserts are generated and pooled. The inserts in a general region are identified by performing a PCR reaction using two primers: a uniquely oriented transposon (Tn)-based primer and a primer corresponding with a given genomic site. All the transposon inserts in a given region can be identified by polyacrylamide gel electrophoresis (PAGE) of pooled PCR products.

out false-negatives; that is, the incorrect identification of genes as essential that merely do not serve as transposition targets for trivial reasons. Examples of footprinting can be found in Refs. ***13*, *14*, *20*,** and ***21***.

Another technique that allows the analysis of mutant pools (and the mapping of mutant locations) involves microarray analyses ***(5–7, 22)*** (**Fig. 10**). The transposon used for generating the inserts carries T7 promoters facing out from both transposon ends. The DNA from a pool of inserts is extracted, cleaved with a restriction enzyme, and then used to program the synthesis of labeled RNA that is interrogated by hybridization to a microarray. In some applications ***(5–7)*** of this technology, the promoter-containing fragments are amplified by PCR and a cDNA copy of the RNA is generated.

Winterberg et al. ***(22)*** used high-density, whole-genome, custom-made oligonucleotide arrays from NimbleGen Systems, Inc. (Madison, WI). They were able to track the growth of individual inserts without resorting to promoter fragment amplification,

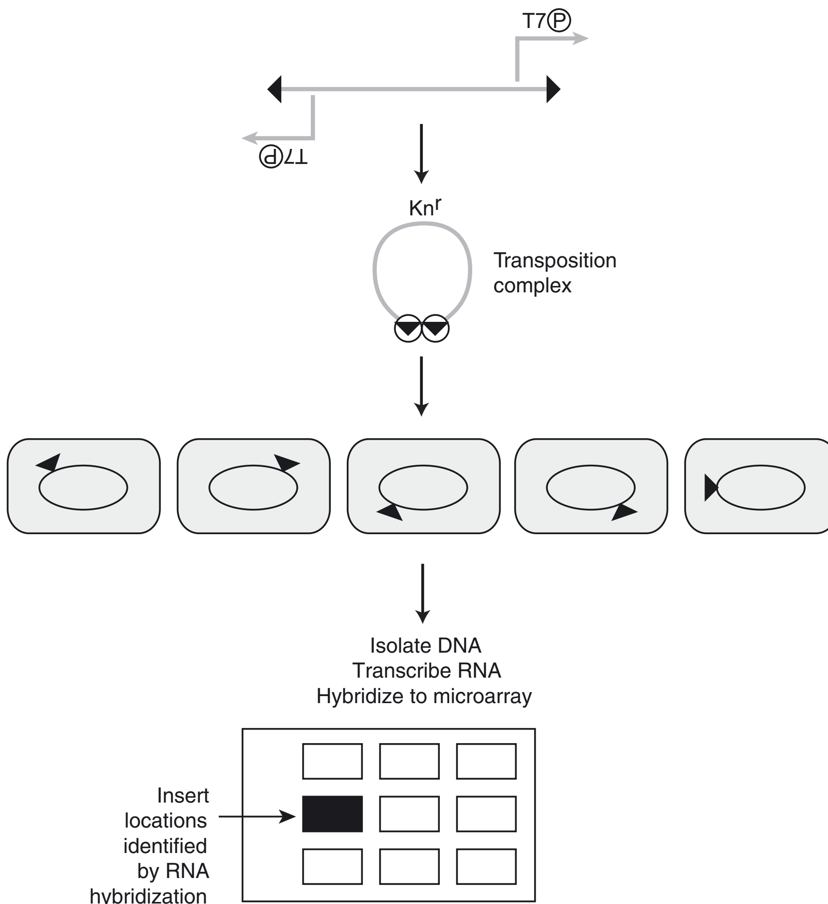

Fig. 10. Identification of pooled transposon insert locations through microarray analysis of transposon boundary transcripts. A transposon containing two outward-facing T7 promoters is used to generate a pool of inserts. The DNA from the transposition products is isolated, cleaved with a restriction enzyme, and used as templates for T7 RNA polymerase-catalyzed RNA synthesis. The location of the inserts is determined by microarray hybridization of the resulting RNA.

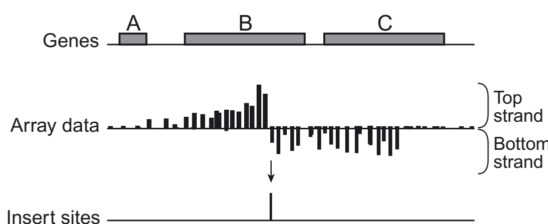

Fig. 11. Mapping inserts to within 50 bp with high-density microarrays. The experiment described in **Figure 10** is performed with high-density microarrays from NimbleGen Systems, Inc., in which 24 nt oligonucleotides correspond with each strand of DNA and are spaced at approximately 50-nt intervals throughout the entire genome. The resulting data can be analyzed to identify the site of insertion for each of ~100 inserts to within about 50 nt. This figure is similar to a figure presented in Ref. ***22***.

they were not limited to studying inserts within known open reading frames (ORFs), and they were also able to map the insert locations to within ~50 bp. The method for mapping inserts is diagrammatically presented in **Figure 11**.

The last technique that we shall review is the oldest: STM ***(3)*** (**Fig. 12**). In this technology, each transposon in the mutagenesis collection is constructed to contain one

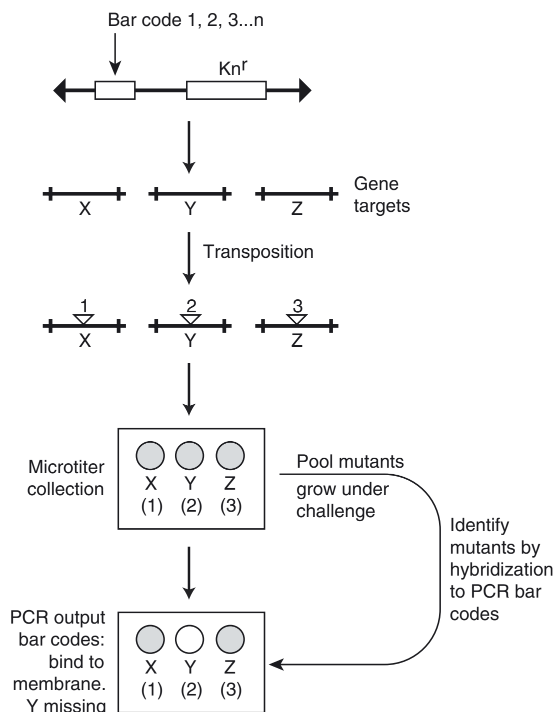

Fig. 12. Tracking inserts using signature-tagged mutagenesis (STM). Inserts are generated using transposons that carry a variety of 20-bp random sequences (bar codes). The resulting insert mutants are individually distributed into microtiter wells. The mutants are mixed to form a pool that is challenged to grow under some defined condition. The input and output pool DNAs are subjected to PCR amplification of the bar codes, which are hybridized to membrane representations of the individual microtiter arrays of the mutants leading to the discovery of which mutants did and did not grow under the challenging conditions.

of a multiplicity of 20-mer random sequences. Thus, upon mutagenesis, each mutant is uniquely defined by the specific 20-mer. Ninety-six separate mutant DNAs (and thus 96 different 20-mers) are arrayed on a hybridization detection membrane. Mutants are pooled and interrogated before (input) and after (output) challenging outgrowth (or colonization) by labeling PCR-amplified identifying 20-mers found in the mixed culture DNAs and hybridizing them to membranes imprinted with 96 mutant DNAs each. Missing mutants are readily identified by a failure to detect a hybridization signal between input and output samples. The transposon insertion contained in the missing mutant can be characterized by PCR and sequencing of the transposon DNA junctions.

6. Identifying Essential Functions: Serial Deletion Generation

It should be possible to define a minimal genome sequence, that is, a gene complement that encodes all essential functions, by attempting to shrink the genome size through repetitive deletion generation. Transposons have been used in this process through two entirely different technologies: one is random in nature and the other is semidirected. Both technologies represent work in progress because neither is likely near defining the minimal required genome.

The random transposon-mediated deletion approach is depicted in **Figure 2** ***(12)***. This technology utilizes well-known aspects of transposon biology. A composite transposon is used in which the transposon is constructed from two transposable elements each defined by the same two different terminal DNA sequences and each inverted relative to each other. Thus, the composite transposon contains two “inside” terminal DNA sequences and two “outside” terminal sequences. A second property of the technology is that it uses both intermolecular transposition and intramolecular transposition. The intermolecular transposition, involving the “outside” terminal sequences, is accomplished via electroporation of preformed transposase-transposon complexes to generate random inserts into the bacterial chromosome. Intramolecular transposition from the “inside” terminal sequences utilizes *in vivo* transposition catalyzed by “inside”-specific transposases to generate adjacent deletions that extend out from the transposon insert to a site on the adjacent chromosome (typically ~10,000 bp away). This random transposon-mediated deletion approach has been repeated 47 times to generate a MG1655 derivative lacking ~14% of its original genome, obviously a long way from achieving a minimal genome (J. Apodaca, personal communication). Already, however, 55 genes that were previously reported as essential have been deleted with limited physiologic effects (an elongated lag time in rich medium).

The above technology can be modified to allow the deleted material at each cycle to be maintained as a plasmid. This would allow a conditional assessment of the role of deleted material by analyzing the cells before and after loss of the deletion-generated plasmid ***(12)***.

A semidirected deletion approach, using transposons as tools, has also been reported ***(19, 23)*** (**Fig. 3**). Tn*5* transposons modified to contain one of two different antibiotic-resistance genes and a *lox*P site were randomly inserted into the *Escherichia coli* genome utilizing electroporation of premade transposition complexes. Strains were generated with two different inserts and then Cre site-specific recombinase was used

to excise the genomic material between the two sites. Survival and growth of the resulting mutants indicated that no essential genes were removed. Some individual deletions were combined to achieve a reduction of more than 300 kbp. Again, this work is a long way from achieving a minimalized chromosome but the technology is available and in use.

6. Conclusion

Transposons are important genetic tools for performing genetic analyses. The current chapter and many of the chapters in this text describe how transposons can be used to define essential genes. These technologies are based on two strategies: a one-by-one knockout strategy that identifies which genes can be interrupted and still allow growth (and by subtraction, which genes are not found to suffer mutations and are thus likely essential), and deletion-based approaches that remove several genes at once. The deletion approach could lead to an identification of the minimal required bacterial genome for growth under specified conditions.

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http://www.springer.com/978-1-58829-378-7

Microbial Gene Essentiality: Protocols and
Bioinformatics
Osterman, A.L.; Gerdes, S.Y. (Eds.)
2008, XIV, 486 p., Hardcover
ISBN: 978-1-58829-378-7
A product of Humana Press