Lecture 14

To understand the basic strategy of bacterial genetics experiments it is important to keep in mind the general ways that we can identify genetic changes in bacteria.

The first method is known as a **genetic screen**. A screen usually involves identifying a colony that has some altered property such as being white on an X-gal plate ($LacZ^-$ mutant) or not being able to grow on a -histidine plate (His^- mutant). Since a maximum of ~200 colonies can be plated on a plate as single colonies. A large 100 plate experiment would involve screening a maximum of about 2×10^4 colonies.

The second method is known as a **genetic selection**. A selection involves plating cells on a medium where the parent strain cant grow but the desired mutant can. An example would be to plate wild type E. coli on an antibiotic containing medium that kills wild type cells and in this way a very rare antibiotic mutant could be selected. Realistically one can plate about 10^7 cells on a single plate. Therefore on one plate one can identify mutants that are about 10^3 fold rarer than in a 100 plate screen. Overall for the same amount of work a selection is about 10^5 fold more sensitive than a screen.

Because the processes underlying genetic manipulation of bacteria are usually very rare, bacterial genetic experiments are often designed to first involve some kind of selection for type of mutant or change desired that is then followed by a screen for the exact change or mutant that one is seeking.

Suppressor mutations

A powerful mode of genetic analysis is to investigate the types of mutations that can reverse the phenotypic effects of a starting mutation. Starting with a $LacZ^-$ mutant of E. coli (white on X-gal), that was obtained by a screen, one could then select for mutants that had regained the ability to grow on lactose medium. Such mutants that have reversed the effect of a primary mutation are called **revertants**. In general, revertants can arise because the starting mutation was reversed or they could have acquired a new mutation that somehow compensates for the starting mutation.

The possibilities are:

1) back mutation - true wild type

2) intragenic suppressor - compensating mutation in same gene

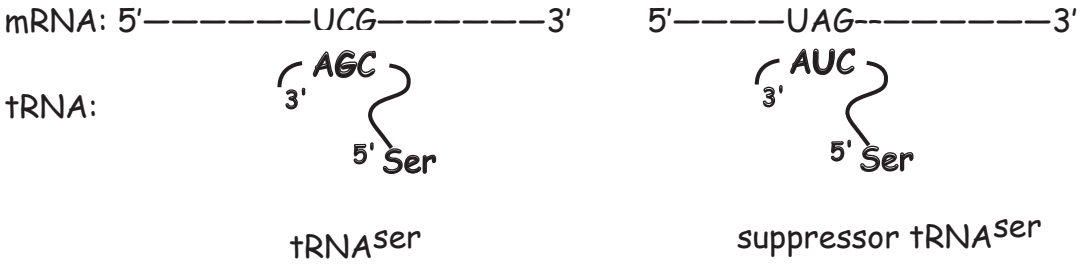
3) extragenic suppressor - compensating mutation in different gene

Nonsense suppressors.

An important class of extragenic suppressor mutations can suppress nonsense mutations by changing the ability of the cells to read a nonsense codon as a sense codon. Such extragenic revertants were originally isolated by selecting for reversion of amber (UAG) mutations in two different genes. Since simultaneous back mutations at two different sites is highly improbable the most frequent mechanism for suppression is a single mutation in the gene for a tRNA that changes the codon recognition portion of the tRNA. For example, one of several possible nonsense suppressors occurs in the gene for a serine tRNA ($tRNA^{ser}$). One of six $tRNA^{ser}$ normally contains the anticodon sequence CGA which recognizes the serine codon UCG (by convention sequences are given in the 5′ to 3′ direction). A mutation that changes the anticodon to CUA allows the mutant $tRNA^{ser}$ to recognize a UAG codon and insert serine when a UAG codon appears in a coding sequence.

Recognition of UCG (serine codon) by wild type tRNAser

Recognition of UAG(stop codon) by amber suppressor mutant tRNAser



$tRNA^{ser}$

suppressor $tRNA^{ser}$

The presence of an amber suppressing mutation is usually designated Su+ whereas a wild-type (nonsuppressing) strain would be designated Su–.

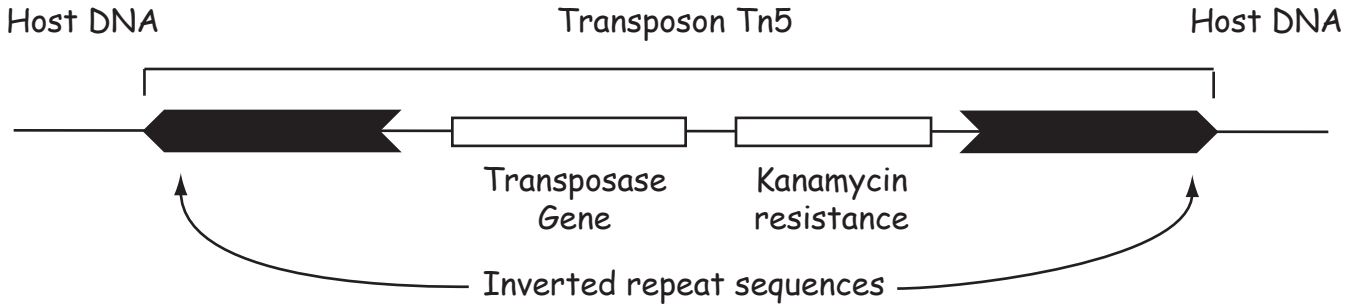
Example: LacZam designates an amber (nonsense) mutation in the LacZ gene. Whereas a LacZam E. coli strain is Lac^- and therefore is white on X-gal plates, a strain with both a LacZam mutation and an amber suppressor (Su+) mutation in a tRNA gene might be phenotypically Lac^+ because of extragenic suppression of the LacZam mutation.

The combined use of amber mutations and an amber suppressor produces a conditional mutation, ie a mutation that is expressed under some circumstances but not under others. Conditional mutants are especially useful for studying mutations in essential genes. Another kind of conditional mutation is a temperature sensitive mutation for which the mutant trait is exhibited at high temperature but not at low temperature.

Transposable elements

Transposons are usually from 10^3 to 10^4 base pairs in length, depending on the transposon type. The key property of transposons is that a copy of the entire transposon sequence can at a low frequency become inserted at a new chromosomal site. The mechanism by which transposons insert into new sites differs from one kind of transposon to another, but the details are not important to understand how transposons can be used. It is worth contrasting the recombination events that occur during transposition to the homologous recombination events that we have considered in meiosis. In homologous recombination, crossovers occur between like sequences. While this type of recombination can generate new combinations of alleles the arrangement of genes is left undisturbed. In contrast, transposition involves recombination between unrelated sequences, namely the ends of the transposon and a site in the target sequence. Transposition therefore results in a new arrangement of genes along the chromosome.

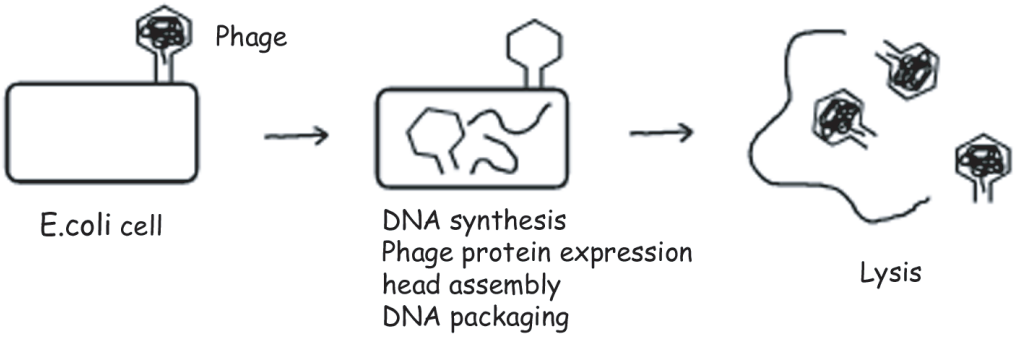
The generic structure of a transposon looks like this:



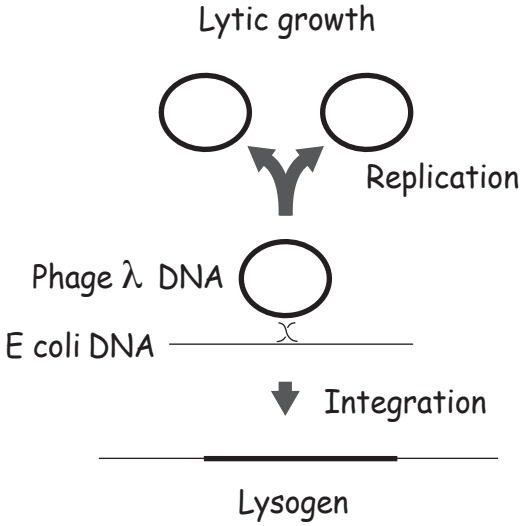
Transposon Element	Function
Transposase	An enzyme that cuts the target DNA more or less at random and splices the transposon ends to the target sequences, Other steps in transposition are performed by host enzymes.
Inverted Repeats	These sequences direct transposase to act at the ends of the transposon. Note that because the sequences are inverted, the two ends have identical sequence.
Selectable Marker(s)	Transposons are thought to have evolved by providing a selective advantage to the host cell. Many transposons carry genes that confer antibiotic resistance or some other benefit to the host.

The study of transposition mechanism and the biology of transposons is an interesting subject in genetics but for our current purposes we are going to concentrate on how transposons can be used for bacterial genetic analysis. For this purpose we will focus on the transposon Tn5 which can function in E. coli as well as a wide variety of other bacterial species. The selectable marker in Tn5 is a gene that confers resistance to the antibiotic kanamycin. Thus bacteria without Tn5 are sensitive to kanamycin (Kan^S), whereas bacteria that have Tn5 inserted into the chromosome are resistant to kanamycin (Kan^r).

One of the best ways to introduce a transposon into E. coli is by using a virus that infects bacteria known as a phage. For this purpose we will use a well studied type of phage known as λ.



Once the DNA from phage λ enters a cell it circularizes and then can undergo two possible fates.



In the lytic growth mode phage DNA is replicated in preparation for packaging into new phage particles. Among the phage genes required for replication is the **P** gene.

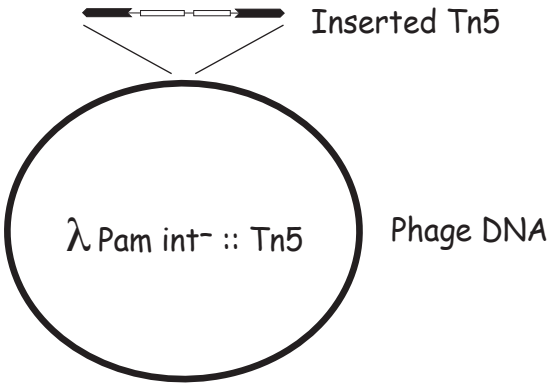
In the lysogenic mode phage DNA is integrated into the chromosome. This quiescent state the phage is replicated along with the chromosome in a state known as a lysogen. The phage gene required for integration is the **Int** gene.

To introduce random insertions of Tn5 into the E. coli chromosome we will start with Tn5 carried on a special λ phage vector: λ Pam int⁻::Tn5.

Pam designates an amber (nonsense) mutation in the λ phage P gene. When λ Pam phage infect E. coli with an amber suppressor (Su^+) the phage multiply normally, which allows propagation of these mutant phage. But when λ Pam phage infect a nonsuppressing host (Su^-) the phage DNA cannot replicate.

int– is a mutation in the λ integrase gene. Phage with this mutation can not integrate into the host chromosome to make a stable prophage.

::Tn5 designates that the λ phage carries an inserted copy of Tn5.



When λ Pam int–::Tn5 infects a wild type (Su^- $Kan^{s)}$ E. coli host, the phage DNA cannot replicate (Pam) nor can it integrate (int^-) thus the only way for the E. coli to become Kan^r is for Tn5 to transpose from the λ DNA to some location on the E. coli chromosome. This type of transposition is an inherently rare process and will occur in about one out of 10^5 phage-infected E. coli cells.

This is how a transposon mutagenesis can be done:

1) Infect $2x10^9$ wild-type E. coli cells with λ Pam int^-::Tn5 so that each cell receives at least one phage chromosome.

2) Select for Kanr by plating on medium that contains kanamycin. There should be a total of about $2x10^4$ Kan^r colonies. Each of these should have Tn5 inserted into a different site on the E. coli chromosome.

The genes of E. coli are densely spaced along the chromosome and about half of the Tn5 insertions will lie in one gene or another. There are 4,200 genes in E. coli so our collection of $2x10^4$ random Tn5 insertions will likely contain at least one insertion in each gene. (Note that insertions in genes that are essential for E. coli growth such as the genes for RNA polymerase or ribosomal subunits will not be recovered because these insertion mutants will not form colonies on the kanamycin plates).

Let's say that we are interested in the E. coli genes that are involved in synthesis of histidine. To find insertion mutants that can not synthesize histidine (His^-) we could screen amongst our collection of $2x10^4$ random Tn5 insertions to find those that are His^-. The easiest way to do this would be to plate out the collection of insertions at a density of 200 colonies per plate (100 plates total). Each of these master plates would then be replica plated (first by transfer to a sterile piece of velvet) to a plate that contains histidine and also to a plate that lacks histidine. His^- insertion mutants would be identified as colonies that can not grow on the plates that lack histidine. Note that the same collection of random Tn5 insertions can be screened multiple times to find interesting mutations with different phenotypes.

3) Identify His^- Tn5 insertion mutants by replica plating to find colonies that specifically can not grow on plates that don't contain histidine.

Once we have a set of His^- insertion mutations (in the present example, one might expect to find 10-20 different His^- mutants), the affected gene(s) can be identified by the simple fact that they will be "tagged" by the inserted Tn5 sequences. The easiest way to identify the site of insertion is by performing a special PCR amplification of the DNA fragment that corresponds to the novel junction between Tn5 and the bacterial chromosomal sequences. Ordinarily PCR reactions are carried out using two DNA primers, each of which corresponding to an end of the sequence to be amplified. When we want to amplify a junction fragment we can use as one of the primers a sequence that lies near the end of Tn5 but we won't yet know the relevant chromosomal sequence to allow the other primer to be designed. There are several tricks that can be used to circumvent this problem, which are too complicated to describe here. Suffice it to say that there are ways that the junction fragment can be amplified by PCR using only sequences defined by the Tn5 portion of the junction fragment.

4) Use the known sequence of the end of Tn5 to PCR amplify a fragment that spans the junction between the end of Tn5 and the E. coli chromosomal site that was the target for insertion. DNA sequencing of the amplified junction fragments will give the identity of the target sequences. Since we know the DNA sequence of the entire E. coli chromosome, the gene that was the target for Tn5 insertion can be identified unambiguously.

5) The DNA sequence of the junction fragments will identify all of the genes that have been inactivated to give the His^- phenotype.

The procedure just outlined can be used to isolate and characterize a wide variety of useful mutations. A major limitation of this method is that as stated earlier, transposon mutations usually completely disrupt the target gene and therefore lead to a complete inactivation of the gene product. Often we will want to work with point mutations (such as temperature sensitive mutations or nonsense mutations). In the next lecture we will see how transposons can also be used to facilitate analysis and manipulation of point mutations.