



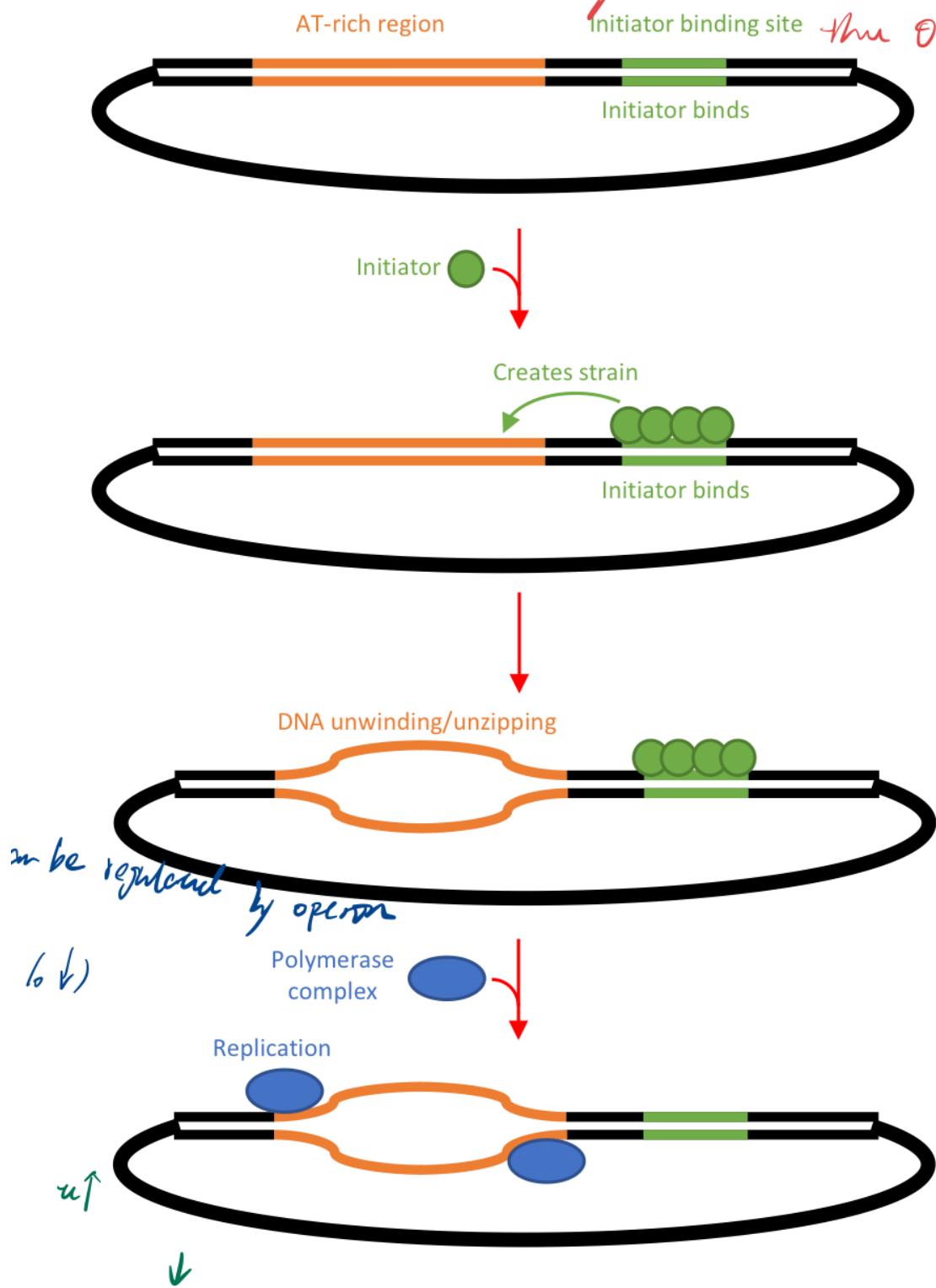
Plasmid

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Origins of Replication

- site dictates the initiation of replication
- determines how many copies can present in the cell
 - high copy number: pUC
 - Ultra low: RP4 — need special machinery to ensure both daughter cells receive a copy of the plasmid
- Some is conditional: e.g. temperature-sensitive; good for fine regulation
- Initiator needs to bind to the origins to then unwind double-stranded DNA
 - pUC origin: initiator is an RNA; in many others, it is protein
- To unwind the double strands, the threshold of the amount of initiators has to be reached

In eukaryotes, the initiator is the ORE



- Incompatibility: the plasmids of types of origins of replication **in the same category** cannot be stably maintained, one type will eventually be lost
- Toxicity of high-copy-number plasmid: if the gene products are toxic to E.coli, or place a severe metabolic burden, even this plasmid originally has a high copy number, it will be selected against.
- Plasmid incompatibility refers to the inability of two plasmids to coexist stably over a number of generations in the same bacterial cell line. Generally, closely related plasmids tend to be incompatible, while distantly related plasmids tend to be compatible. The most frequent reason for two plasmids being incompatible is that they both possess a replicon with the same specificity of Rep protein or controlling elements. However, incompatibility can also be due to other types of competition, for example, between the same or closely related partitioning systems. Incompatibility may be reciprocal, in that both plasmids have the same chance of being lost from a cell line that starts with both, or it can be unidirectional, if one of the plasmids has additional features that give it an advantage – for example, possession of a second replicon.

Plasmid Partitioning

Selective Advantage

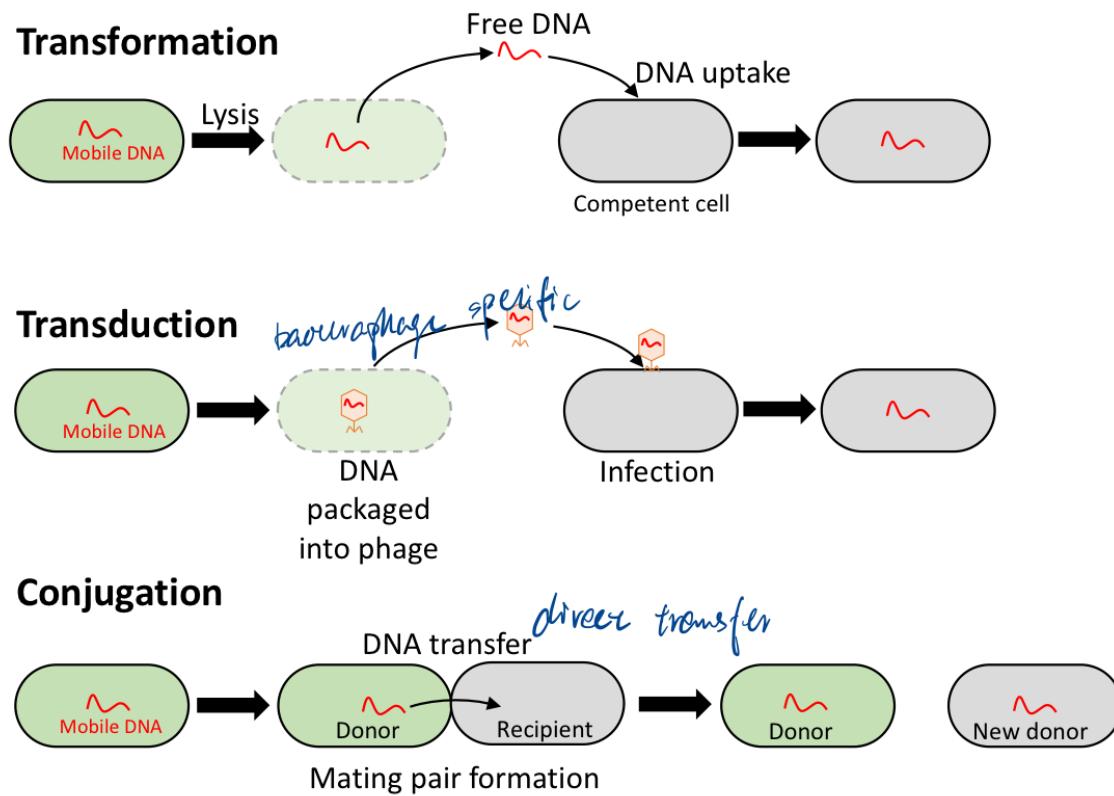
- Antibiotic resistance
- Expanded metabolic functions: PCBs slowly degraded to chlorobenzoate, which can be catabolised by plasmid **pA81**
- Virulence: contain genes (e.g. *Shigella flexneri* : plasmid contains genes for Type II secretion system & transposons & non-natural resistance marker → infect different other eukaryotic cells & survive in the hosts)
- Carry toxins that inhibit the growth of specific bacterial species: Colicin plasmids kill E.coli, while itself contains Colicin immunity protein

hok/sok

Horizontal gene transfer — three ways

- Transformation: by lysing the donor strain and extract plasmid / mobile DNA
→ heatshock / electroporation to make the recipient bacterial cell competent
→ insert donor plasmids
- Transduction: the plasmid / mobile DNA is taken by bacteriophage → infect the recipient bacterial cells
- Conjugation: **Mating pair formation** between the donor and recipient bacterial cells (**requires direct cell-cell contact**) → both donor and recipient have the sharing plasmids

Horizontal Gene Transfer

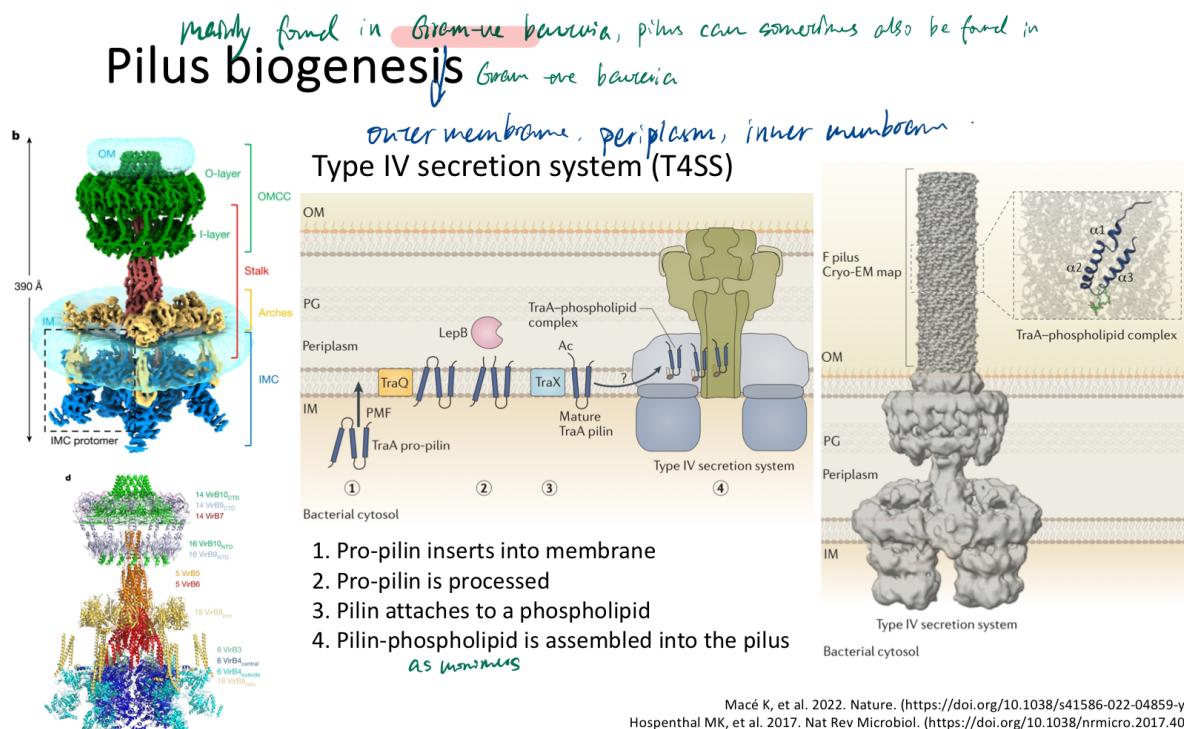


Conjugation

- bacteria and archaea
- requires direct cell-cell contact
- conjugative elements can be plasmids or sometimes parts of the chromosome
- use Type IV secretion system (not Type IV Pili)
- RP4 system is available to most **Gram-ve bacteria**
 - it has oriT: origin of transfer — essential for the **initiation of conjugative transfer**

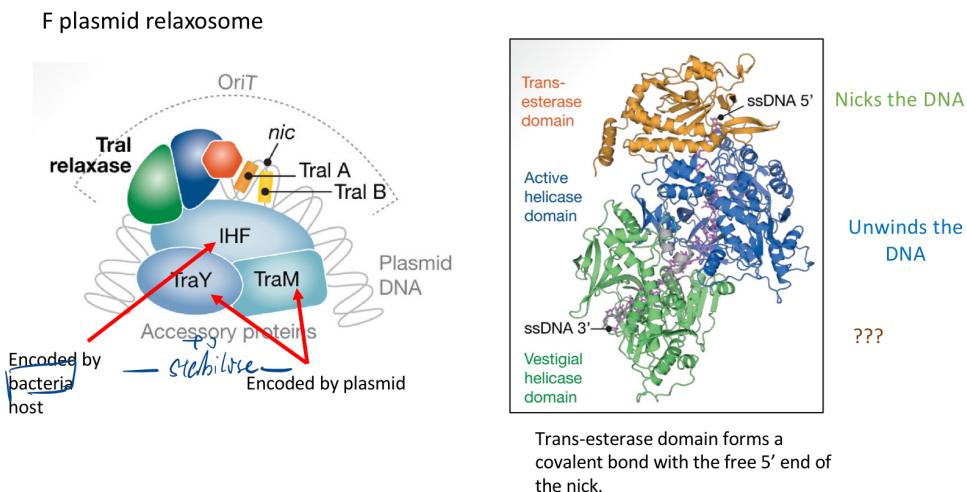
- **Steps:**
 - **Pili formation**
 - **Mating pair formation — donor and recipient form close contact with each other**
 - **Relaxosome assembly**
 - **DNA transfer**
 - **Complementary strand synthesis**
 - **Separation between donor and recipient**
- Gram positive: bacterial cell wall consists chiefly of peptidoglycan; no outer membrane (purple)
- Gram negative: cell wall consists of small amount of peptidoglycan and outer membrane (pink)

▼ Pilus formation



▼ Relaxosome

- Relaxase + oriT + accessory proteins (relaxase is only essential component that is always present, the rest vary with the system)
1. Relaxase binds to the oriT
 2. Nick DNA by one strand and unwind it
 3. accessory proteins bind to stabilise this complex / bend the DNA
 - a. RP4 only has one accessory protein encoded by the plasmid
 4. Coupling proteins bridges the relaxosome with Type IV secretion system
 5. Relaxosome and coupling proteins are not needed for mating pair formation



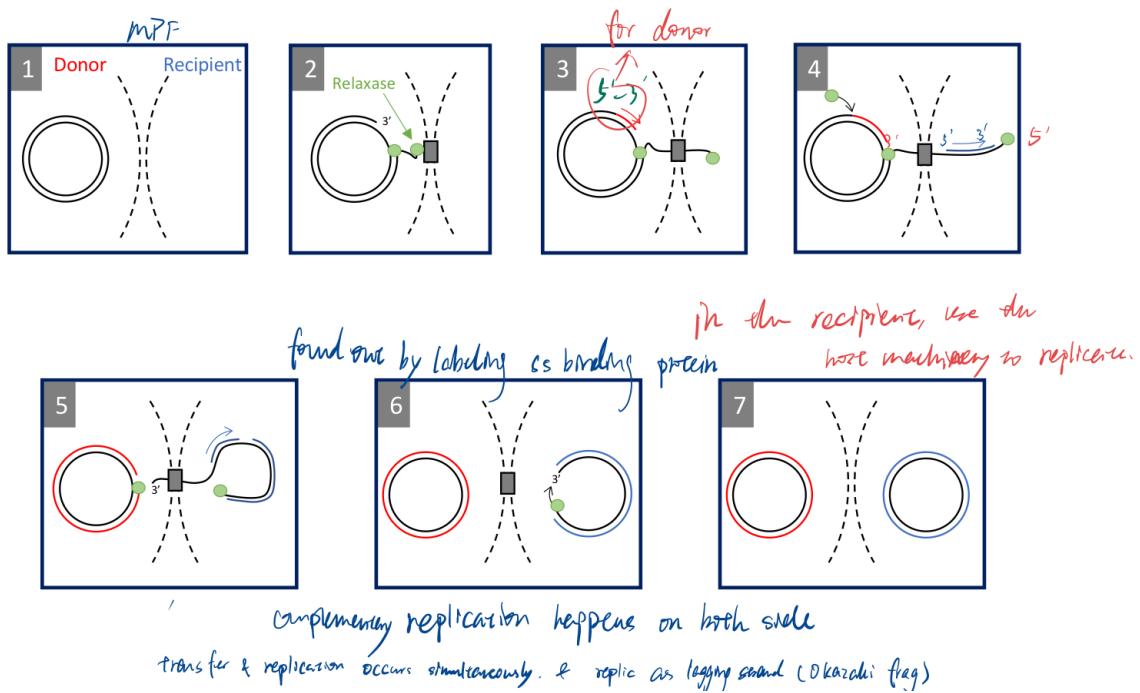
▼ DNA transfer

- When coupling protein is by itself: sits outside the T4SS structure — allow pilin subunits to integrate to pilus
- when it is bound to relaxosome, it inserts itself into the T4SS and allows the relaxosome to pass through the structure

▼ Complementary synthesis

- Happens at both donor and recipient cells; both from 5' to 3'
- In donor: replication as leading strand
- In recipient: replication as lagging strand

Complementary strand synthesis



Fertility Inhibition — plasmids are selfish

- when donor cells have multiple conjugative systems, one conjugative plasmid may contain elements to inhibit the other systems to conjugate
- turns out only a small subset of plasmids are conjugated
- another form of plasmid incompatibility

Exclusion

- Because it is wasteful to conjugate into cells the elements that are already exist in recipient cells
- conjugative elements have exclusion factors
 - surface exclusion — prevent mating pair formation
 - entry exclusion — prevent DNA transfer
- RP4 only has entry exclusion
- F has both types

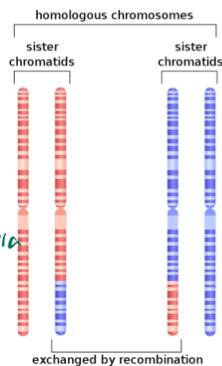
Integration of plasmid into bacterial chromosome

- **Homologous recombination**
- **Site-specific integration**
- **Transposition**

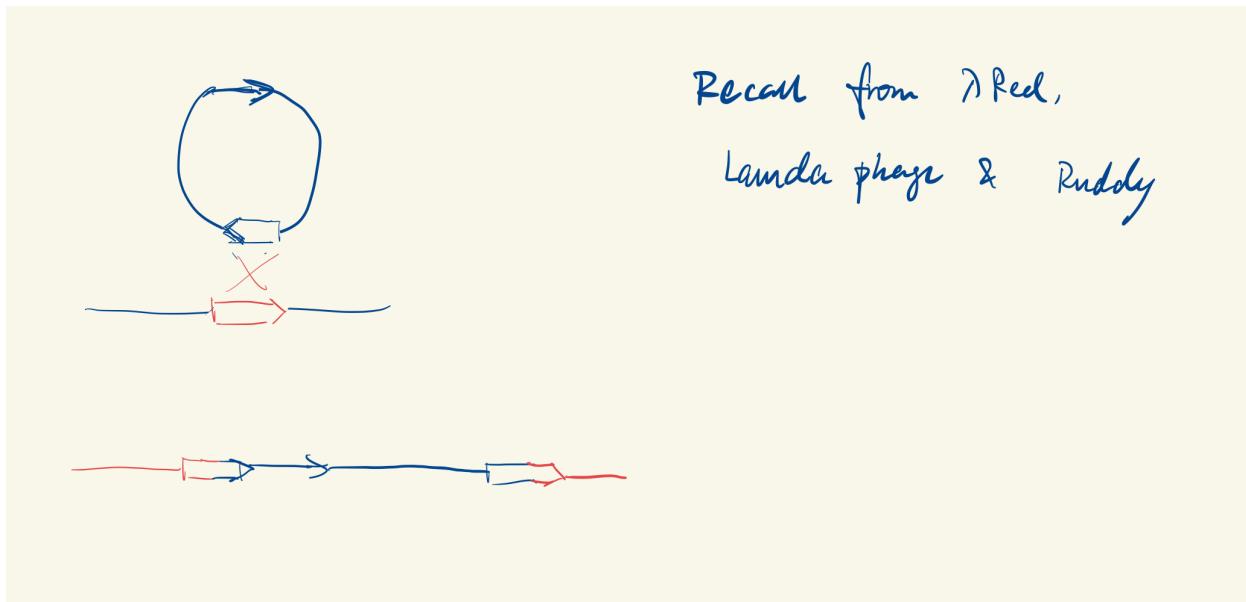
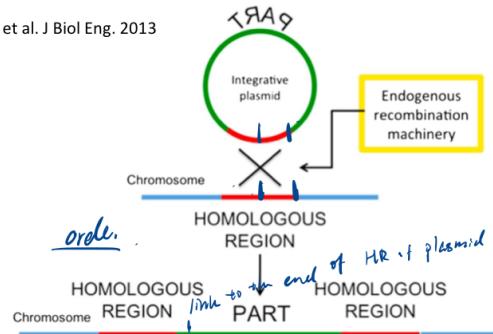
Homologous Recombination — reversible integration!

Homologous recombination

- Key process in DNA repair - you'll hear more about how this works in a later lecture (Dr. Ruddy's lectures)
- Process is conserved in all domains of life *decreed in some experimental use bacteria*
- In bacteria, this is initiated by a protein called **RecA**
- Sequences of very similar (i.e. homologous) sequences are recognized by enzymes which facilitate strand swapping.
 - Usually needs at least 20-30 bp of homologous sequence to be recognized. **The longer the stretch of homologous sequence, the higher the frequency of spontaneous recombination events occurring between these sequences.**
- Homologous recombination can allow plasmids to reversibly integrate into the bacterial chromosome



Zucca S, et al. J Biol Eng. 2013



▼ Suicide Vector

▼ Key components

- oriR6K: origins of replication — require π , encoded by *pir*
- oriT: low copy number replication origin — allow the plasmid hard to proliferate and to die if not integrated
- Homologous sequence: 20-30 bp; The longer the stretch, the higher frequency for spontaneous recombination
 - Reversible integration
- Antibiotics resistance gene e.g. Ampicillin

▼ Steps

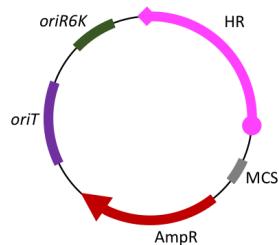
1. In an empty cell (without chromosomes), amplify the suicide vector
 - a. to amplify, the cell contains *pir*, to allow plasmid replication
 2. transform it to the target cell
 3. In the target cell without the Ampicillin resistance and *pir*
 4. Therefore, the suicide vector cannot replicate in the recipient cell
 - a. If HR happens, the bacterial chromosome contains Ampicillin resistance — can form colonies under Ampicillin
 - b. If HR does not happen, the suicide vector will eventually be lost over generations — suicide & The cells cannot form colonies on Ampicillin
- Counter-selection: kill bacteria contain the suicide vector — select for the loss of the vector
 - *sacB* — encodes **levansucrase**; converts sucrose into **levans**, which is toxic
 - *rpsL*
 - *tetA*
 - *ccdB*

Suicide Vectors

vectors X retain. X reproduce

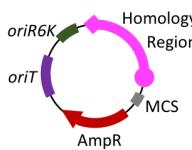
∴ if maintain, can assume integration

- Vectors that are designed to enter but not "stay" in a target cell
- Conditional (e.g. temperature-sensitive) or host-specific replication origin or replication is dependent on a specific initiator protein

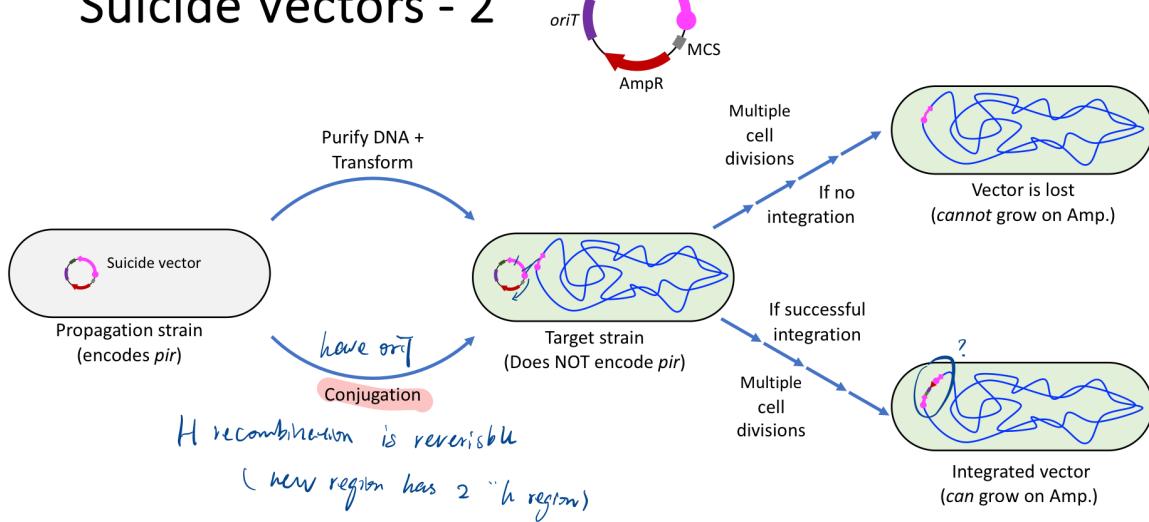


- OriR6K requires initiator protein π , encoded by *pir*
- To facilitate integration, a region homologous to the chromosome is cloned into the MCS, along with any additional genes of interest
- *Conjugation*: Transform into specialized donor strain, mate with target strain lacking *pir* – all Ampicillin resistant cells will have the suicide vector integrated
donor (pir^+) \rightarrow target ($\times \text{pir}$)
- Some suicide vectors also encode a counter-selection marker to enable selection for the loss of the vector
 - sacB* – encodes levansucrase; converts sucrose to levans, which is toxic
 - rpsL* – encodes ribosomal subunit; confers sensitivity to streptomycin
 - tetA* – encodes resistance to tetracycline; but sensitivity to fusaric acid
 - ccdB* – toxin from a toxin/anti-toxin system

kill bacteria contain suicide vector, conditional selection



Suicide Vectors - 2

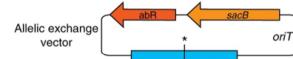


▼ 2-step allelic exchange — one step extra than HR

2-step allelic exchange

① usually use conjugation instead of transformation - ↑ possibility

Step 1: generate suicide vector with desired mutation (*), flanked by sequence homologous to chromosome



since HR low possibility

Chromosome

//

First crossover

Step 4: Grow cells in absence of abR marker to allow second crossover; grow in presence of sucrose to ensure elimination of suicide vector backbone

Antibiotic selection

partial

Merodiploid

(2 copies)

oriT

sacB

abR

//

Second crossover

oriT

//

Wild type

Step 2: Conjugate into cells; homologous recombination will result in first crossover event

Step 3: Grow cells on media selecting for recipient and selecting for abR marker

Antibiotic selection

oriT

sacB

abR

//

Second crossover

oriT

//

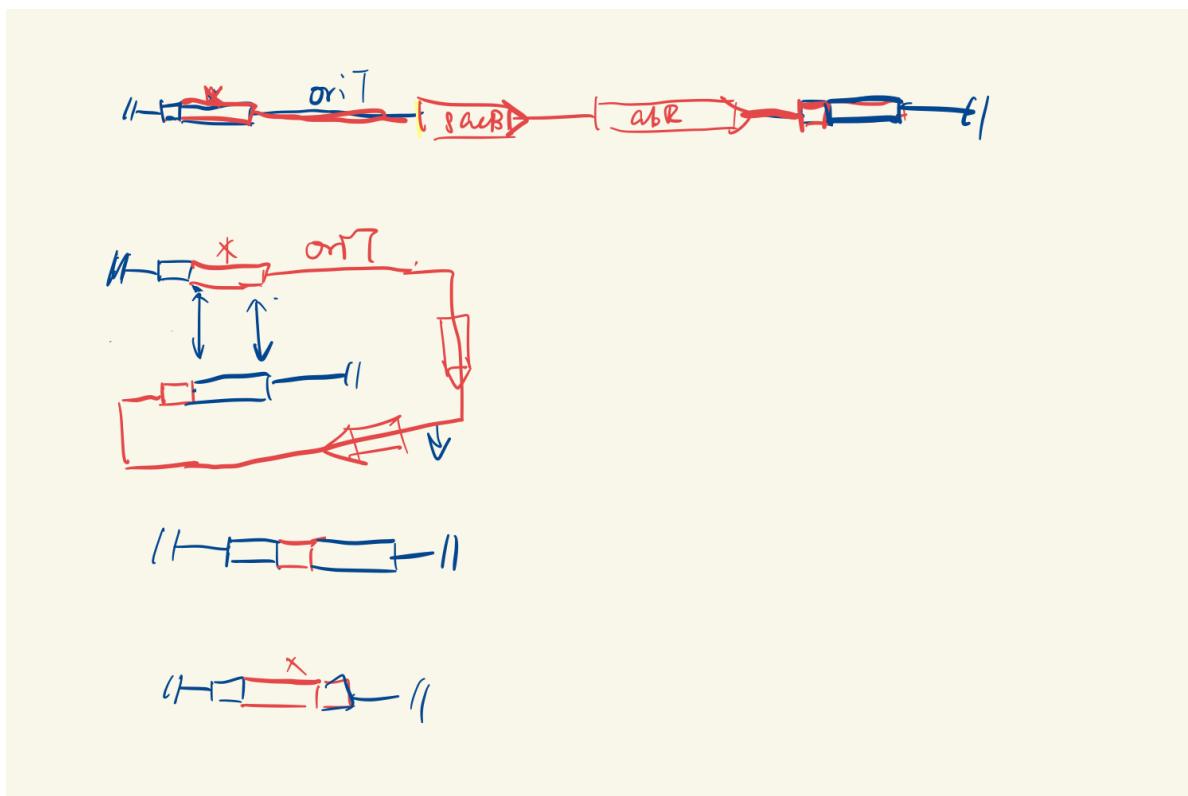
Mutant

but if there are extra HR, thus could be 0%

50/50 chance of getting mutant or regenerating original wild type

Hmelo et al., 2015 Nature Protocols

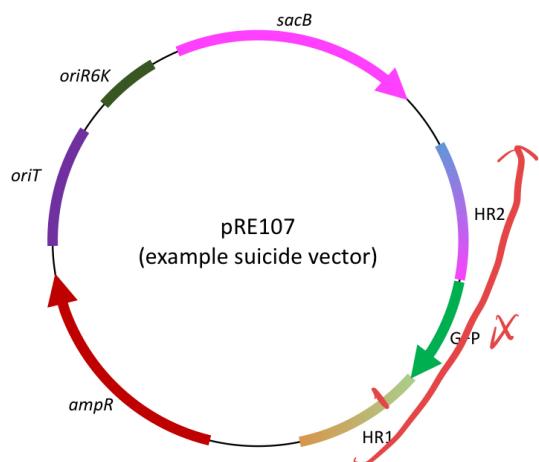
Note: the intermediate state after the first recombination is called **Merodiploid!!**



1. Generate suicide vector with desired mutation — flanked by homologous sequence
 2. Conjugate into cells
 3. Allow the first homologous cross over
 4. Grow cells on media selecting for abR marker
 5. Change media without abR marker + **sucrose instead**, allow second cross over (because in the presence of antibiotics & **add sucrose for counterselection**
 - a. grow in the presence of sucrose to make sure the second crossover and the backbone is cleaved
- To insert a new gene (**by replacement**) into the chromosome can apply the principle of two-step allelic exchange
 - Require two homologous sequence
 - For maximum efficiency, HR1 and HR2 should be the same length, just as the mutation is at the middle of the HR
 - This can also be used to insert a gene at a “neutral” intergenic region (completely insertion, not by exchanging = introducing a mutation)

Inserting a new gene into the chromosome

How?



just as the mutagenesis is at the centre of HR regions need to be 500-1000 bp long. the HR. For maximum efficiency, HR1 and HR2 (previous slide) should be same length.

Identify a “neutral” gene in target organism (e.g. *lacZ*) to be replaced



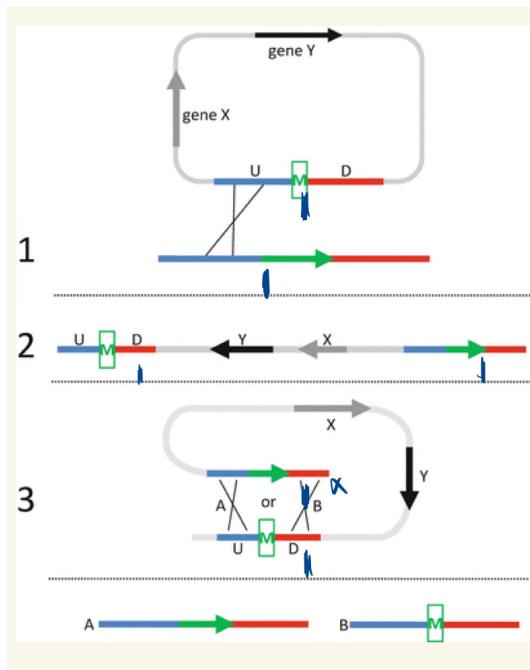
2 step allelic exchange

in reality: gene expression: promoter etc required.

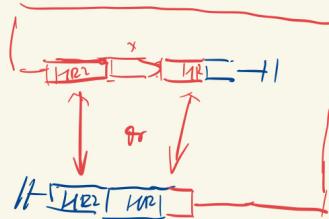
Alternatively, can find a “neutral” intergenic region to introduce the gene into



2 step allelic exchange



H—[HR1] < GFP, HR2] —+ — [HR1 | HR2 —+ //



(H—[HR2] T GFP —[HR1] —+// or

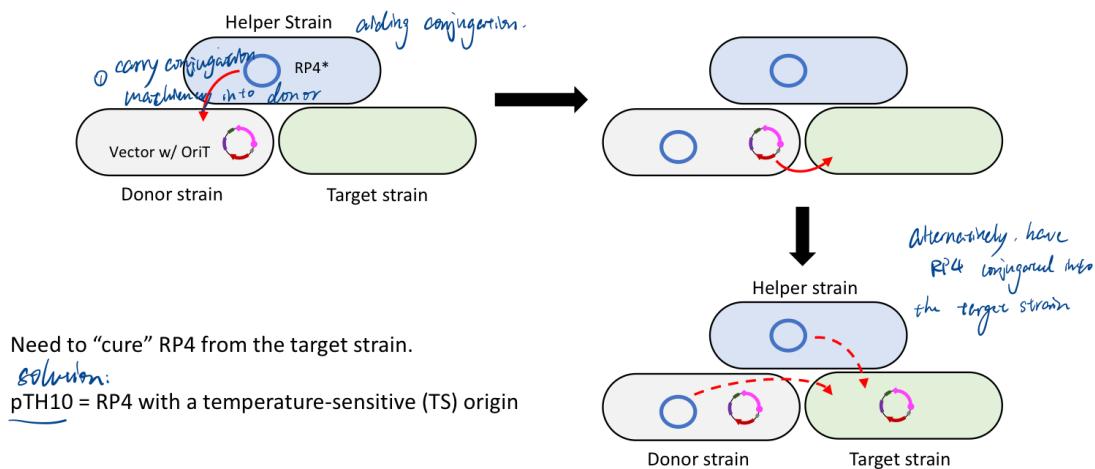
//—[HR2]—[HR1]—+// ✓

▼ Tri-parental mating — conjugation (instead of transformation, advantages explained below)

- donor strain - also called mobilising strains: carry the GOI and transfer genes & vector oriT + antibiotics resistance
- Helper Strain — also called self-transmissible strain: conjugative plasmid & carrying genes required for conjugation and DNA transfer; e.g. RP4 plasmid
- Target strain: recipient of the plasmid from the mo
- Thus: when put three strains together:
 - if the RP4 in helper strain conjugates in donor strain — assists the plasmid in donor strain to conjugate to target strain
 - However, RP4 can also conjugate into target strain, which is not preferred —
 - Solution: Use a special RP4 strain: pTH10 — temperature-sensitive origin
 - plasmid has been engineered to replicate efficiently at a permissive temperature but fails to replicate or replicates poorly at a restrictive temperature
 - **from strains carrying pTH10 showing resistance to the antibiotics kanamycin, tetracycline, and ampicillin at 30 degrees C but not at 42 degrees C, clones are isolated resistant to kanamycin at 42 degrees C.**
- How to select for the conjugated target strain:
 - add antibiotics to the medium, + incubate at the temperature that RP4 cannot replicate — only the target strain without RP4 conjugation can form significant colonies

Tri-parental Mating

How conjugation was originally done (40+ years ago)...

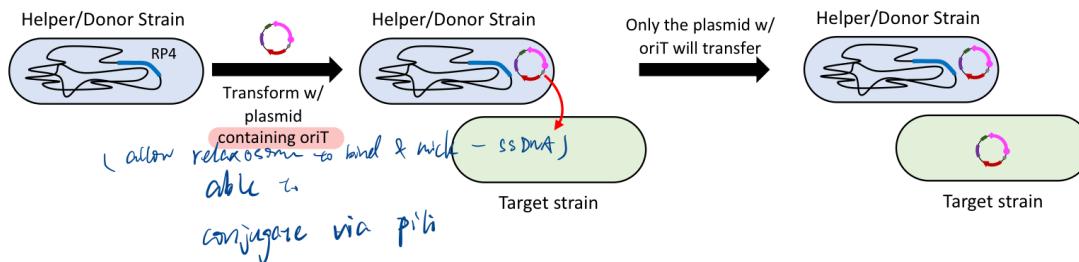


▼ Bi-parental mating

- Of note, the self-transmissible plasmid
- If one strain both contains the self-transmissible plasmid and the mobilisable plasmid — Contrast this to a mating involving only two strains, one of which contains both the self-transmissible plasmid and the mobilizable plasmid. **Only a small fraction of the cells are fertile and can mobilize the mobilizable plasmid into the recipient strain.** [because the oriT is a low copy number plasmid?] Also in a triparental mating, even if the two plasmids are members of the same **replication incompatibility group**, they coexist long enough for the mobilization to occur
- However: a novel bi-parental mating have the chromosome integrated with the conjugation machinery: RP4
- Then transform the cells with the plasmid containing GOI and antibiotics
- Conjugation happens, plasmid is conjugated into the target strain
- Selection:

- The donor strain carries an auxotrophy for diaminopimelic acid (cell wall component) — so not long after transformation and conjugation the donor cell dies
- So there is no need for typically select for the target plasmid except using the antibiotics

Bi-parental Mating



To select recipient from donor,
donor needs to be killed

"Modern" version of this system uses a donor strain that carries an auxotrophy for diaminopimelic acid (a cell wall component), so the target strain does not even need to have an additional selective marker

Ferrières L, et al. J Bacteriol. 2010

So w/o the plasmid → donor dies

▼ Hfr strains of E.coli

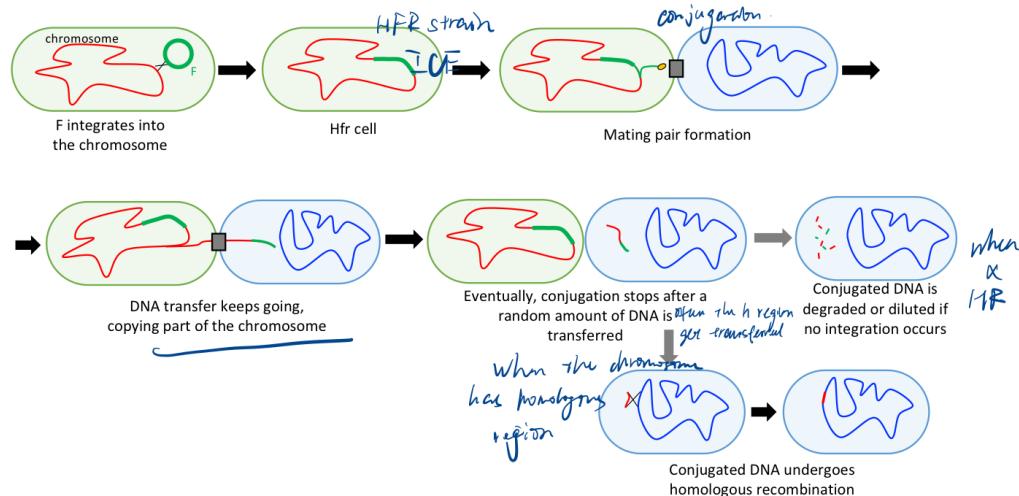
- High-frequency recombination
- When the plasmids that integrate into the chromosome, when they transfer (conjugate), they then bring small parts of chromosome

High fl. of recombination Hfr strains of *E. coli*

F^- bacteria lack the F plasmid completely (recipients)

F^+ bacteria carry the F plasmid independent of the chromosome

Hfr (High Frequency of Recombination) bacteria have the F plasmid integrated into the chromosome



Non-homologous recombination: Site specific recombination

- Only occurs between specific sequences or sites of DNA
- promoted by enzymes called **site-specific recombinases**, which recognize two

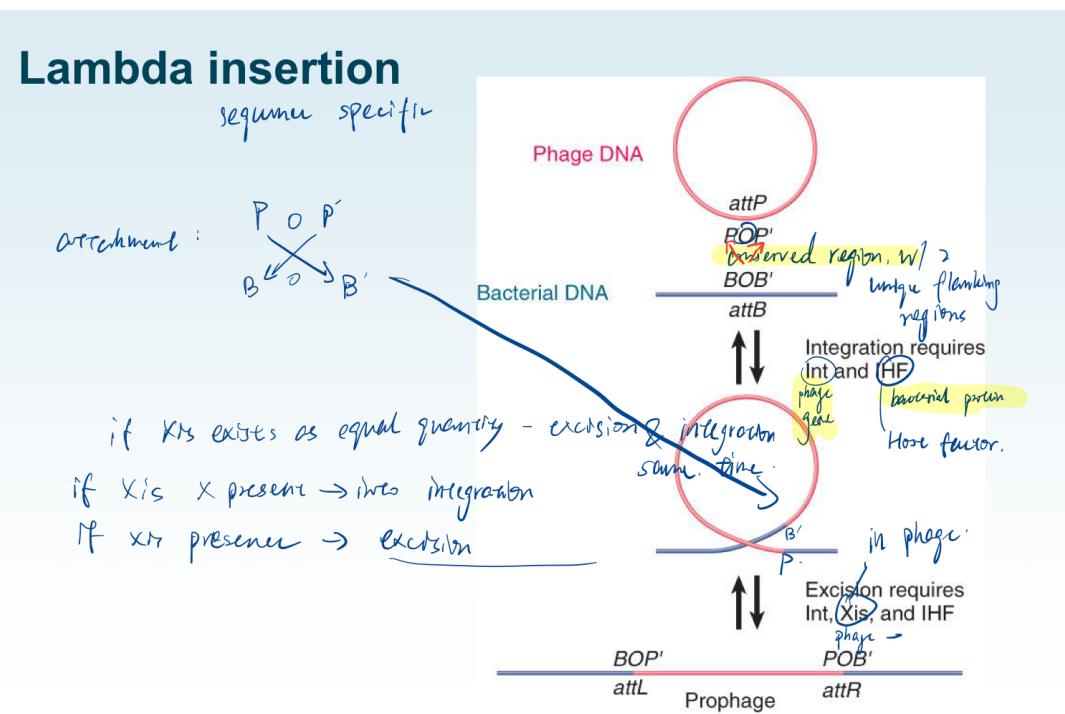
specific sites in DNA and promote recombination between them. Even though the two sites generally have short sequences in common, the regions of homology are

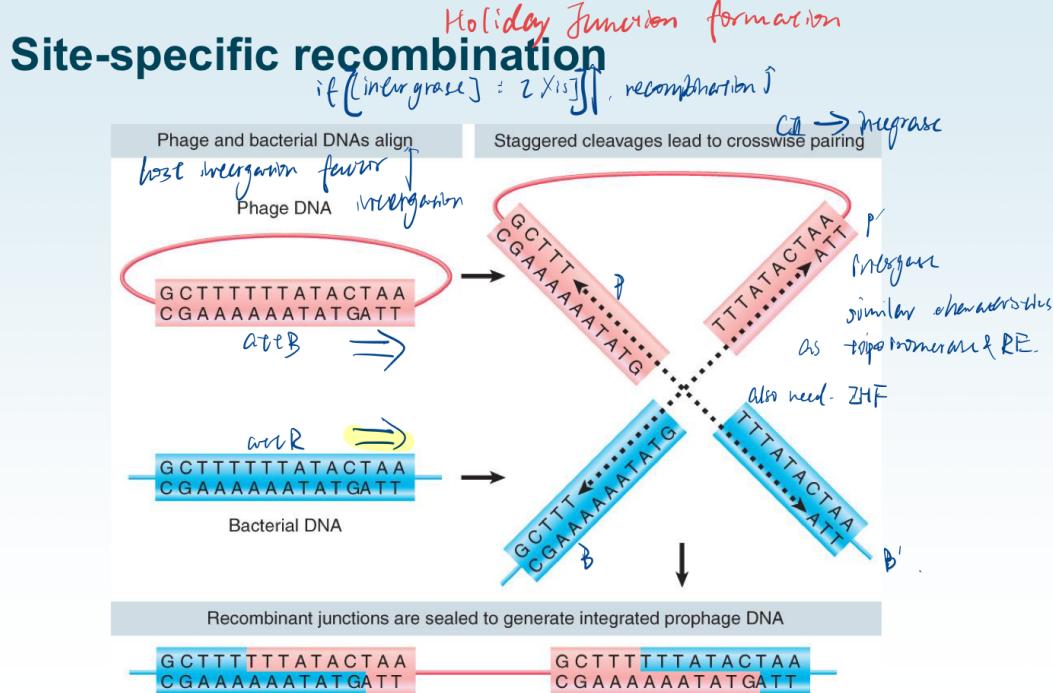
usually too short for normal homologous recombination to occur efficiently.

- Common types of recombinases
 - Integrase** in Lamda phage — recognise *attP* and *attB* sites
 - P1 Phage recombinase — **Cre**: recognise *loxP* sites
 - Fip** recombinase: recognises *FRT* sites

▼ Integrase

- specifically recognises **attP** site in **phage** and **attB** site in **bacterial chromosome** to promote recombination between them

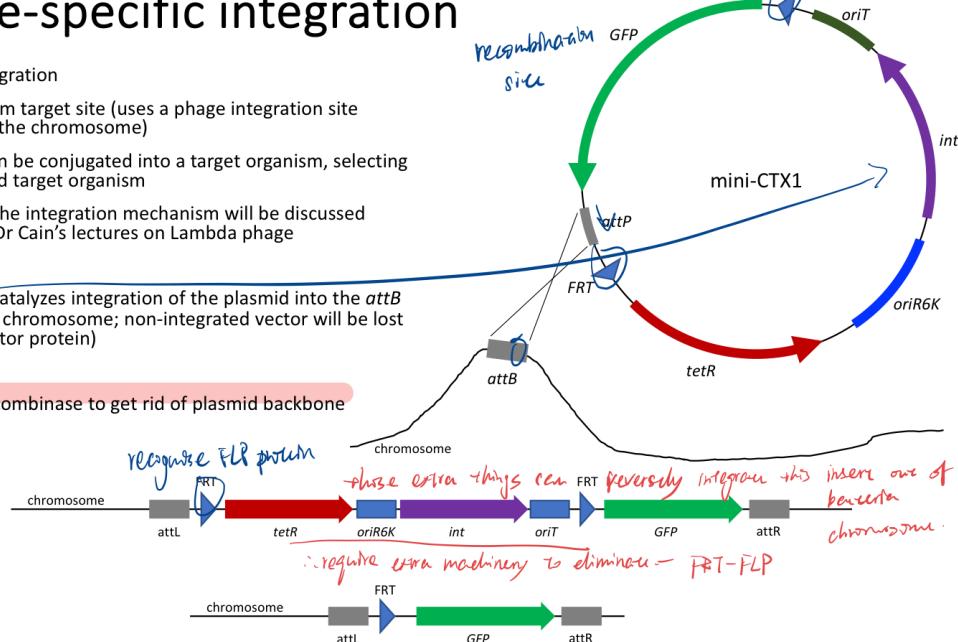


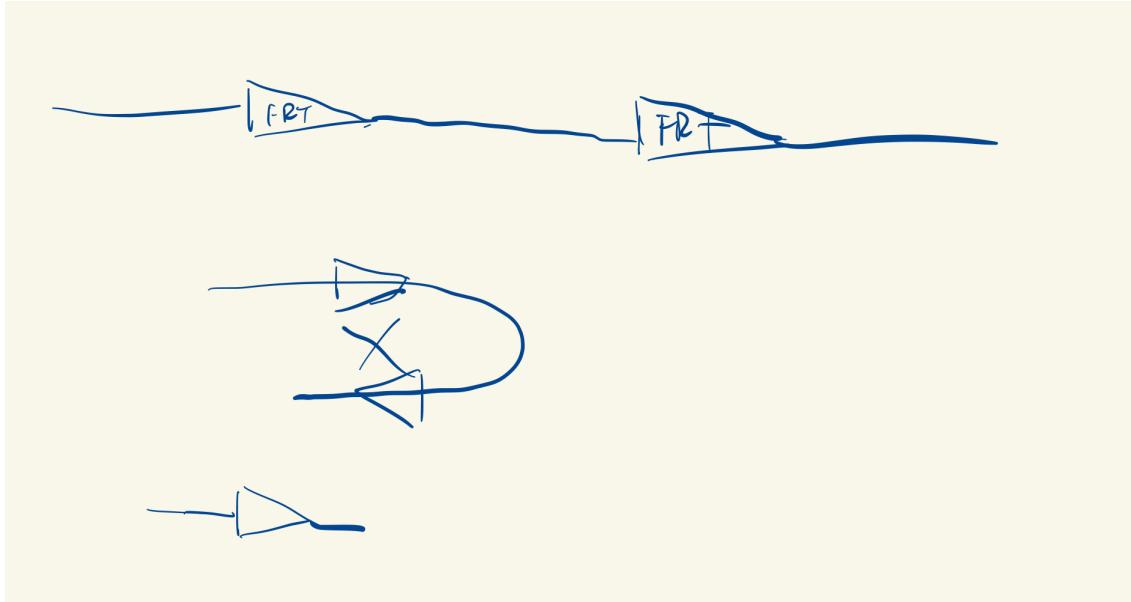


carefully construct GFP & attP & FRT (attP outside of FRT - eventually drop one GFP)

Site-specific integration

- 1-step integration
- Non-random target site (uses a phage integration site present in the chromosome)
- Plasmid can be conjugated into a target organism, selecting for *tetR* and target organism
- Details of the integration mechanism will be discussed further in Dr Cain's lectures on Lambda phage
- Integrase catalyzes integration of the plasmid into the *attB* site on the chromosome; non-integrated vector will be lost (no π initiator protein)
- Use Flp recombinase to get rid of plasmid backbone



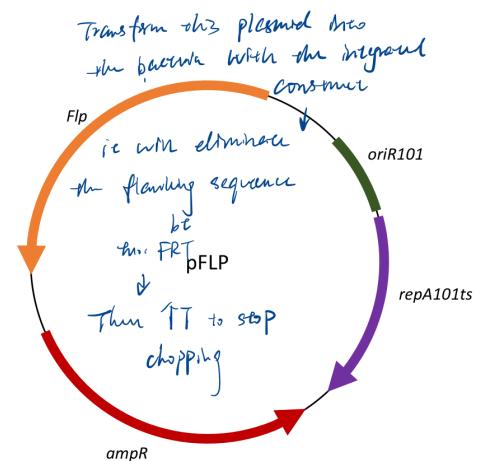


- How to integrate a GFP into the chromosome — **SUICIDE VECTOR (die after integration)**
 - ▼ Site-specific recombination
 1. insert GFP , and also : oriR6K, oriT, attP, and int — integrase; two FRT sites; selection marker: tetR
 - a. attP needs to be in the middle of the whole backbone
 - b. FRT sites need to flank the GFP (GOI)
 - c. tetR (similar to tetA?) — selection marker: The tetR gene encodes a **transcriptional repressor protein, TetR**, which binds to specific DNA sequences known as Tet operator (tetO) sites — When TetR binds to tetO sites, it **blocks the transcription of nearby genes, typically those involved in resistance to antibiotics such as tetracycline** — those with unintegrated plasmids will be killed
 2. transform, and amplify this plasmid in the cell containing ***pir*** & **RP4** with conjugation machinery; — Donor cell as **Bi-parental mating**
 3. In contact with target cell — chromosome contains attB site
 4. Conjugation occurs, plasmid is transferred to the target cell

5. At the target cell: site-specific recombination is carried out by integrase — the plasmid is integrated into bacterial chromosome
6. Add Flp recombinase — recombination at the FRT sites
 - a. Done by transform a pFLP plasmid that encodes Flp!
 - b. By temperature-sensitive initiator to control the replication
7. Finally the construct does not contain the original integration suicide vector (no pir) and also no this transient gene expression vector (temperature sensitive)

Transient gene expression

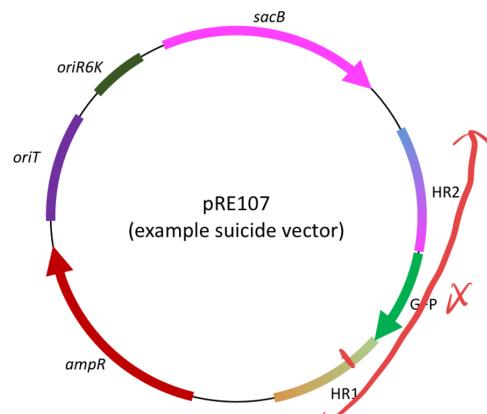
- oriR101 requires initiator protein RepA101, which is encoded on the plasmid
- RepA101ts is a temperature sensitive mutant *temperature-regulated transcription*
- When grown at 37°C, RepA101ts does not work, so the plasmid cannot replicate



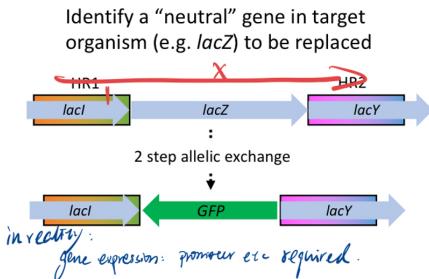
▼ Homologous recombination

Inserting a new gene into the chromosome

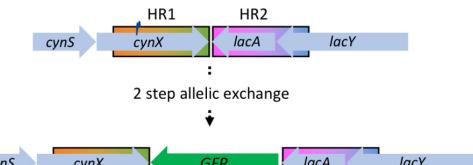
How?



just as the mutagenesis is at the centre of HR regions need to be 500-1000 bp long. HR. For maximum efficiency, HR1 and HR2 (previous slide) should be same length.



Alternatively, can find a "neutral" intergenic region to introduce the gene into



!! To apply this integration mechanism, the HR should be of 500-1000bp long!; AND HR1 and HR2 should be same length

▼ Site specific mutagenesis

1. a piece of DNA of an organism is manipulated to change the sequence in desired way
2. The mutated DNA (linear) is reintroduced into the cells, the recombination systems caused the altered sequence of the reintroduced DNA to replace the normal sequence of the corresponding DNA in the chromosome
3. The reintroduced DNA should be homologous to the sequence to be replaced:
 - a. homology does not need to be complete: base pair changes to introduce site-specific mutagenesis

Integration way: transposition (the way transposons move)

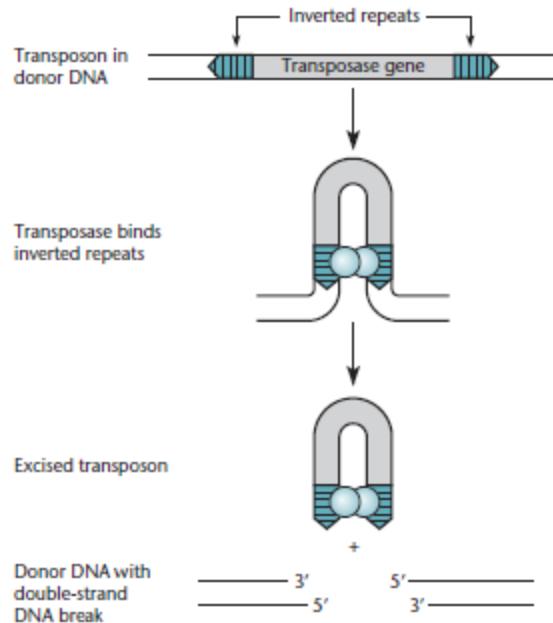
- Transposons are elements that can hop from one place in DNA to another

- Transposons exist in all organisms on Earth
 - In human, almost half of our DNA may be transposons
- Require transposase, the transposon itself usually encodes its own transposases
 - so it carries with its ability to hop each time when it moves
- The net result of transposition is that the transposon now resides at a new place in the genome where it was not originally found
- donor DNA — the DNA from which the transposon originated; recipient DNA — the DNA which transposons hop into
- Transposition must be tightly regulated and occur only rarely; so elaborate mechanisms have evolved to regulate

▼ Components:

- Terminal inverted repeats:
 - The two DNA regions are inverted repeats if the sequence of nucleotides on one strand in one region, from 5' to 3' direction, is the same or almost the same as the sequence on the other strand, read from 5' to 3' direction, in the other region
- Transposase, forming synapse
 - Two molecules of transposase bind to each of the iF sequence, and also bind to one another to form a synapse
- Target site duplication
 - Same sequence of nucleotides flanking the transposon
 - The by-product of transposons joining the target DNA:
 - The target DNA originally contains one copy of the target sequence at the place where a transposon inserts
 - The 5' ends of the transposons join the 3' ends of the target site, creating two single-stranded gaps which are later repaired by complementary base pairing, thus forming the two duplicated target sites

Figure 9.2 Steps in transposon excision. IRs (shown in blue) that are recognized by the element-encoded transposase characterize the ends of the transposon. Genes carried by the transposon are not shown but reside in the region shaded grey. A synapse is formed when the transposase binds the IRs and the ends are paired with the transposase. This signals the transposase to carry out the cleavage and joining events that underlie the process of transposition. A completely excised element is not found for all transposition processes, and not all transposons leave a double-strand break in the donor DNA.
doi:10.1128/9781555817169.ch9.f9.2



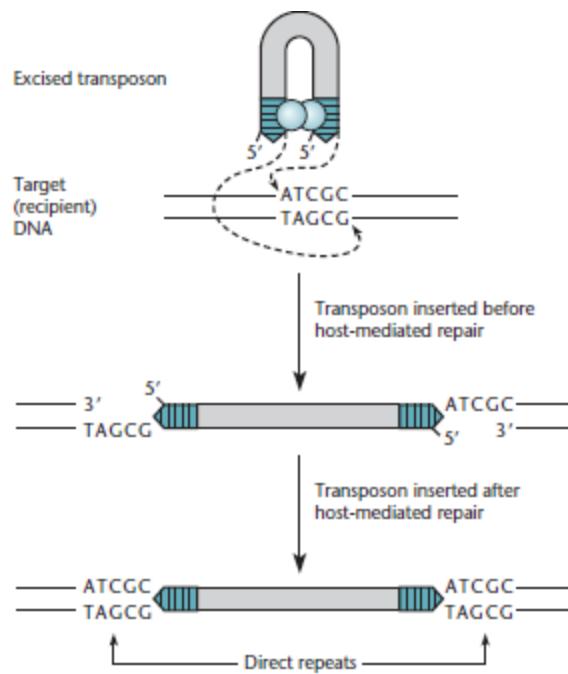


Figure 9.3 Steps in transposon insertion. The transposon inserts into a target DNA using staggered joins to the top and bottom strands (an excised transposon species found with many elements is shown). The process of inserting with staggered joins into the target DNA leaves gaps flanking the transposon that must be filled by a host DNA polymerase. Often, a few bases of sequence from the donor DNA also remain bound to the 5' ends of the element, which must also be processed by host enzymes. The effect of staggered joining events and the subsequent host repair leads to the direct repeats that are indicative of transposition.
doi:10.1128/9781555817169.ch9.f9.3

Lamda Red R

The Phage λ Red System

Phage λ also encodes recombination functions. The best characterized is the Red system, which requires the products of the adjacent λ genes *exo* and *bet*. The product of the *exo* gene is an exonuclease that degrades one strand of a double-stranded DNA from the 5' end to leave a 3' single-stranded tail. The *bet* gene product is known to help the renaturation of denatured DNA and to bind to the λ exonuclease. Unlike many of the other recombination systems that we have discussed, the λ Red recombination pathway does not require the RecA protein, since it has its own synapse-forming protein, Bet. The λ Red system was first used for recombineering (see below). Besides the Red system, phage λ encodes another recombination function that can substitute for components of the *E. coli* RecF pathway (see Sawitzke and Stahl, Suggested Reading, and Table 10.2). Apparently, phages can carry components of more than one recombination pathway.

Recombineering: Gene Replacements in *E. coli* with Phage λ Recombination Functions

One of the major advantages of using bacteria and other simple organisms for molecular genetic studies is the relative ease of doing gene replacements with some of these organisms (see the discussion of gene replacements in chapter 3). To perform a gene replacement, a piece of the DNA of an organism is manipulated in the test tube to change its sequence in some desired way. The DNA is then reintroduced into the cell, and the recombination systems of the cell cause the altered sequence of the reintroduced DNA to replace the normal sequence of the corresponding DNA in the chromosome. Because it depends on homologous recombination, gene replacement requires that the sequence of the reintroduced DNA be homologous to the sequence of the DNA it replaces. However, the homology need not be complete, and minor changes, such as base pair changes, can be introduced into the chromosome in this way as a type of site-specific mutagenesis. Also, the reintroduced DNA need not be homologous over its entire length; homology

is needed only where the recombination occurs. This makes it possible to use gene replacement to make large alterations, such as construction of an in-frame deletion to avoid polarity effects (and insertion of an antibiotic resistance gene cassette into the chromosome [see Box 12.3]). If the sequences on both sides of the alteration (the flanking sequences) are homologous to sequences in the chromosome, recombination between these flanking sequences and the chromosome will insert the alteration. Methods for gene replacement in *E. coli* have usually relied on the RecBCD-RecA recombination pathway, since this is the major pathway for recombination in *E. coli*. We mention some of these methods in this chapter and chapter 3.

A newer and more useful method for manipulating DNA in *E. coli* is called recombineering. The term recombineering is used to describe various applications with bacteriophage recombination proteins that have been optimized for performing site-specific mutagenesis and gene replacements in *E. coli* (Table 10.2). One system in particular, the λ Red system, has many advantages over the RecBCD-RecA pathway for such manipulations. This method makes it possible to use single-stranded DNA oligonucleotides, possibly as short as 30 bases, although those 60 bases long or longer work better. This is important because the synthesis of single-stranded DNAs of these lengths has become routine for making PCR primers, and oligonucleotides with any desired sequence can be purchased for a reasonable cost. Other methods of site-specific mutagenesis for making specific changes in a sequence are more tedious and require a certain amount of technical skill. Probably most important, recombineering is very efficient. Minor changes, such as single-amino-acid changes in a protein, usually offer no positive selection, and most methods require the screening of thousands of individuals to find one with the replacement.

Figure 10.10 outlines the original procedure for using the λ Red system for gene replacements. Figure 10.10A shows the structure of the *E. coli* strain required. It carries a defective prophage in which most of the λ genes have been deleted, except the recombination (Red) genes *gam*-*bet*-*exo* (Table 10.2; see also Figure 8.2). Figure 10.10B shows the replacement of a sequence in the plasmid by the corresponding region on another plasmid, in which the sequence has been disrupted by introduction of an antibiotic resistance (Ab^r) cassette. This region of the plasmid has been amplified by PCR to produce a double-stranded DNA fragment carrying the antibiotic resistance cassette and some of the flanking sequences. First, the cells are briefly incubated at 42°C to inactivate the mutant λ repressor, inducing transcription of the Red genes of the prophage. Then, cells are made competent for electroporation, and the PCR fragment is

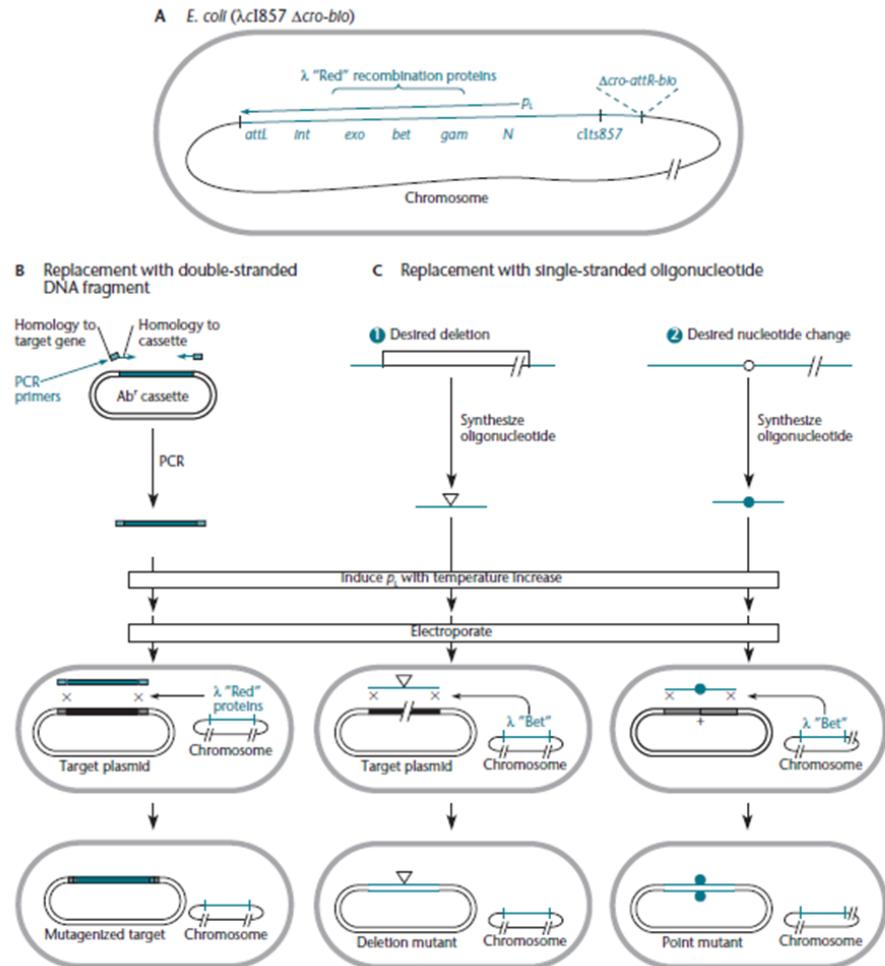


Figure 10.10 Recombineering: *in vivo* DNA modification in *E. coli* using λ phage-encoded proteins. **(A)** A deletion derivative of a λ lysogen with a temperature-sensitive repressor can be used to induce the λ Red recombination functions. **(B)** Double-stranded DNA cassettes can be amplified using primers with homology to the regions flanking the region to be replaced. Double-stranded DNA can be processed by the λ recombination proteins encoded by the *red* genes. **(C)** Oligonucleotides that contain a deletion compared to the target plasmid (indicated with a triangle), where only the Bet protein is needed, can be synthesized. **(D)** Oligonucleotides that contain a point mutation compared to the target plasmid (indicated with a small circle), where only the Bet protein is needed, can be synthesized.

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electroporated into them. The *gam* gene product, Gam, inhibits the RecBCD complex so that the linear DNA fragment is not degraded as soon as it enters the cell. The *exo* gene product, Exo, then processes the fragment for recombination. Exo is an exonuclease that plays the role of RecBCD, degrading one strand of a double-stranded DNA from the 5' end, thereby exposing a 3' overhang single strand for strand invasion. The *bet* gene product, Bet, then plays the role of RecA, binding to the single-stranded DNA exposed by Exo and promoting synapse formation and strand exchange with a complementary DNA in the cell. The cells in which the PCR fragment has recombined with the cellular DNA so that the sequence containing the antibiotic resistance gene has replaced the corresponding sequence in the cellular DNA are then selected on plates containing the antibiotic.

To determine the effect on the cell of inactivating a gene product, it is best to delete the entire gene and replace it with an antibiotic resistance cassette. This can be accomplished by using PCR to amplify the cassette with primers whose 5' sequences are complementary to sequences flanking the gene to be deleted. Recall that the 5' sequences on a PCR primer need not be complementary to the sequences being amplified. When this amplified fragment is electroporated into the cells, the antibiotic resistance cassette replaces the entire gene.

Introducing an antibiotic resistance cassette into a gene simplifies the task of selecting the gene replacement and inactivating the gene. A variation of this procedure is to include sites recognized by a site-specific recombinase flanking the antibiotic resistance cassette. In this adaptation of the procedure, the recombinase can be expressed later to remove the cassette and leave an in-frame deletion, which is important to reduce the chance of polar effects (see chapter 2 and Datsenko and Wanner, Suggested Reading).

Sometimes, we want to introduce a small change into the gene for which there is no direct selection, for example, a specific change in one amino acid that we think may play an important role in the protein product of the gene. A variation on this method allows the selection of recombinants that have a single defined base pair change or some other small change. It depends on having a cassette that has both a gene that can be selected by positive selection and a gene whose product is toxic under some conditions. This allows us to select both for acquisition of the cassette and, later, for its loss. An example of such a system could involve insertion of the recognition site for the I-SceI homing endonuclease, which is long enough to not be found naturally in bacterial genomes. The I-SceI recognition site is toxic only when the I-SceI endonuclease is expressed in the same strain. First, a DNA cassette carrying both an antibiotic resistance gene and the cleavage site for the I-SceI homing endonuclease,

flanked by sequences for the region of the gene to be replaced, is introduced into the cell by electroporation, selecting for the antibiotic resistance as described above. Then, another DNA fragment (or oligonucleotide), identical to the targeted region of the DNA but carrying the desired base pair change, is introduced. At the same time, a plasmid expressing the I-SceI endonuclease is also introduced by transformation and selected, using a plasmid-borne antibiotic resistance gene. Any DNA retaining the I-SceI recognition site will be destroyed by cleavage by the endonuclease, and only recombinants in which the corresponding region is replaced by sequence derived from the second DNA fragment will survive. Most of the surviving bacteria, therefore, have the sequence with the base pair change replacing the original sequence in the gene, and this can be verified by DNA sequencing. Various other techniques have been developed to make so-called “markerless” manipulations of the chromosome (see Box 12.3).