



## Bacterial plasmids

Plasmids are defined as double stranded, extrachromosomal genetic elements that replicate independently of the host cell chromosome and are stably inherited. Plasmids capable of integration into the chromosome were earlier called episomes. Plasmids differ from chromosomes in being small and coding for genes that are non-essential for the bacterial survival. Absence of plasmids doesn't kill bacterium, but their presence provides additional benefits to the bacterial cell. Plasmids vary in size; smallest plasmid is only 846 bp long and contains only one gene. Some plasmids carry more than 500 genes. The largest plasmid known carries 1,674 genes. Like chromosomes, plasmids are also closed circular and supercoiled; although certain plasmids (Eg. *Borrelia burgdorferi*) are known to be linear. *Borrelia burgdorferi* has at least 17 plasmids, 2 are circular and 9 are linear molecules. A cell may have different plasmids existing together. Copy number refers to the number of copies of a plasmid present in a cell. Larger plasmids are present in smaller numbers (1-2) and small plasmids may be found in high copy numbers (~40). Plasmids are capable of transfer to other cells but not all plasmids are transferable. Only those plasmids that have genes for self transfer can be transferred. Plasmids have two prominent properties; the ability to replicate and to partition themselves between the daughter cells after cell division. Plasmids don't freely float in the cell cytoplasm, instead are membrane bound.

Sometimes, a plasmid is said to be "lost" when the progeny cells don't receive the plasmid. The loss of plasmids in a population is sometimes referred to as plasmid segregation. The process of obtaining plasmid-free isolates is termed 'curing'. It can be achieved a) spontaneously as in replica-plated colonies, b) following an enrichment, c) elevated temperature d) treatment with chemicals such as acridines, ethidium bromide, sodium dodecyl sulfate (SDS) and novobiocin.

Compatibility refers to the ability of two different plasmids to coexist stably in the same cell. Incompatibility refers to inability of two plasmids with similar replicon or segregation system to coexist in the same cell. Whenever two different plasmids have similar replication or partitioning system, there will be competition between the plasmids and whichever is able to utilize the system better will prevail over the other. All plasmids belong to only one of the approximately 30 compatibility groups.

### Types of plasmids:

- Naturally occurring plasmids are wild plasmid found naturally in bacteria. Recombinant plasmids are altered plasmids introduced into the bacterium for genetic studies.
- Cryptic plasmids are those that serve no known functions. They may be present for possible exclusion of plasmids that are incompatible with the resident plasmid.
- Integrative plasmids: These are plasmids that can occasionally integrate into chromosome (previously called episomes). In *Bacteroides uniformis*, certain sections of chromosome separate themselves from chromosome and become plasmids, which are capable of conjugation.
- Metabolic plasmids carry some genes that help in cells metabolism.
- Virulence plasmids carry one or several genes that confer virulence properties on the bacterial cell.
- Conjugative plasmids are those that are able to mobilize self transfer. These tend to be larger because they have to accommodate genes coding for self transfer. Conjugative plasmids in Gram-positive bacteria tend to be smaller than those in Gram-negative bacteria.

- Mobilizable plasmids are those plasmids that lack genes to initiate self transfer but do encode the functions needed specifically for transfer of their own DNA. The initiation function is provided by other conjugative plasmid present in the same cell.
- Suicide plasmids are referred to those plasmids which get transferred to another bacterial cell but does not replicate further. These are actually mobilizable plasmids.
- R (resistance) plasmids are large conjugative plasmids that carry one or more antibiotic resistance genes. Resistance plasmid can be conjugative or mobilizable. Usually, R plasmids code for their own replication, and they are known to have mobile genetic elements.
- Colicin plasmids are small plasmids which encode the genes to synthesize colicins (bacteriocines).
- F (fertility) plasmids are those that have complete gene set to mediate self transfer by conjugation. Cells possessing it (donor) are termed F<sup>+</sup> and those lacking it (recipient) are termed F<sup>-</sup>.

### Classification

In 1971, Hedges and Datta proposed a plasmid classification scheme based on the stability of plasmids during conjugation (incompatibility). The first incompatibility (Inc) groups were IncI, IncN, IncF, and IncP. Currently, 26 Inc groups are recognized in Enterobacteriaceae including six IncF (FII to VII) and three IncI (I1, I $\gamma$ , I2) variants. Major plasmid families occurring in Enterobacteriaceae are HI2, HI1, I1- $\gamma$ , X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, K, B/O, FII, FIII, FIV. IncF plasmids are low-copy-number plasmids, often carrying more than one replicon with at least one replicon strongly conserved.

In 1988, Couturier and colleagues proposed a plasmid typing scheme based on Southern blot hybridization, using cloned replication regions (replicons) as probes. This approach successfully provided classification for both conjugative and nonconjugative plasmids. However, the low specificity of this method did not give clear picture of plasmid diversity as cross-hybridization reaction occurred among highly related replicons. Since plasmid replicon type determines Inc group, the terms Inc and Rep type were interchangeably used to describe plasmid types. Initially, Inc typing relied on introduction of a plasmid into a strain carrying another plasmid and determining whether both plasmids were stably maintained in the progeny. However, physical Inc typing is time consuming and not easily used in large-scale applications. Since 2005, plasmids are typed using PCR based replicon typing (PBRT), which target the replicons of major plasmid families. The PCR-based replicon typing protocol to detect plasmid replicons found among the Enterobacteriaceae requires several steps, including five multiplex and three simplex PCR procedures. Thus PCR too is a tedious and costly procedure to apply to large collections of isolates. PCR based typing system is useful in identifying known Inc plasmid groups, but it can not identify novel or divergent replicons. Hence, complete DNA sequencings alone can give an insight of novel plasmids.

Francia et al. and Garcillan-Barcia et al. have proposed the use of conjugative relaxases as a plasmid-typing tool. Relaxase-based typing can be applied to any plasmid (conjugative or immobilizable). Another advantage of this system is that while a plasmid can have multiple replicons, there is always only one relaxase per plasmid. Since relaxases are believed to be stable markers of plasmid evolution, they represent better evolutionary relatedness.

### Functions of plasmids:

Antibiotic resistance, heavy metal resistance, virulence, environmental adaptability & persistence, and metabolic functions that allow utilization of different nutrients are some of the known functions coded by plasmids. Another important feature includes plasmid-mediated biodegradation of a variety of toxic substances such as toluene and other organic hydrocarbons, herbicides, and pesticides.

### **Role of plasmid in virulence and antibiotic resistance:**

They code of adhesins (pili/fimbriae) of ETEC, EAEC and diffusely adherent *E. coli*; LT and ST of ETEC; invasiveness of EIEC; aggregative adherence phenotype of EAEC; toxins Pet and EAST1 of EAEC; host cell invasion of EIEC and Shigella; hemolysin of STEC and localized adherence of EAEC. The epidermolysin toxin exfoliatin that is responsible for staphylococcal scalded skin syndrome (SSSS) is either a chromosomal or a plasmid-derived toxin. Plasmid-borne genes encode tetanus neurotoxin, botulinum toxin G, and anthrax toxin (protective antigen, edema factor, lethal factor). *Enterococcus faecalis* cytolysin, *Shigella dysenteriae* enterotoxin 2, the *E. coli* RTX type enterohemorrhagic (EhxA) toxin, and both heat-labile and heat-stable enterotoxins are also plasmid encoded. Genetic determinants of *E. coli* alpha-hemolysin are generally found on large plasmids. ETEC and extra-intestinal strains of *E. coli* from animals also often carry Vir plasmid, which encodes the gene for cytotoxic necrotizing factor 2. Pathogenic strains of Yersinia (*Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*) contain a large virulence plasmid (pYV) that encodes the TTSS type III secretion systems and Yop (Yersinia outer proteins) effector proteins, which inhibit macrophage function. Shigella and enteroinvasive *E. coli* (EIEC) strains produce (Ipa) proteins that are encoded on the large invasion plasmid.

Plasmid-encoded antibiotic resistance encompasses most classes of antibiotics currently in clinical use therapy including cephalosporins, fluoroquinolones and aminoglycosides. Vancomycin resistance in Enterococci (VRE) is due to the presence of *vanA* gene cluster residing in Tn1546 transposon, which is carried on plasmids. Vancomycin resistant *S. aureus* (VRSA) has resulted because of transfer of plasmids from VRE to MRSA. Tetracycline resistance in *S. aureus*, erythromycin resistance in *E. faecalis*, multi-drug resistance in *S. aureus* are also plasmid mediated. Resistance to penicillins and cephalosporins in several members of Enterobacteriaceae is by plasmid mediated extended-spectrum beta-lactamases (ESBL) and AmpC beta-lactamases. The blaCTX-M-15 gene, often associated with the blaTEM-1, blaOXA-1, and *aac(6)-Ib-cr* resistance genes, has been located mainly on plasmids belonging to the IncF group. Aminoglycoside-modifying enzymes are often plasmid encoded but are also associated with transposable elements. Plasmid-encoded *K. pneumoniae* carbapenemases (KPCs), are now been found in a range of Enterobacteriaceae, including *E. coli*, *Enterobacter spp*, *Citrobacter spp*, *Salmonella spp*, *Serratia marcescens*, *P. mirabilis*, and *P. aeruginosa*. Beta-lactamase resistance to penicillin in *H. influenzae*, *N. gonorrhoeae*, metallo-beta-lactamase (MBL) production in Acinetobacter spp and Pseudomonas spp, and tetracycline resistance in gonococci are other examples of plasmid-mediated antibiotic resistance.

### **Replication:**

The set of sequences need for autonomous replication of the plasmid (or chromosome) is known as replicon. Replication of plasmid requires presence of *oriV* genes (in the plasmids) and *tra* encoded proteins. A proper partition mechanism is in place to ensure that the plasmids are successfully transferred to daughter cells. There are two general modes of replication; THETA Replication (found in most plasmids of the common gram negative bacteria) and rolling circle replication (found in some plasmids of gram positive bacteria).

For self transfer to another cell, a plasmid requires both the origin of replication (*ori*) and trans-acting replicator (Rep) protein. A plasmid may have one or more of origin of replication. Multiple replication systems allow plasmids to replicate in a variety of dissimilar hosts. This can be disadvantageous sometimes, as multiple origin of replication makes the plasmid larger in size. The Rep proteins bind and stimulate the iterons, which are the binding sites of Rep protein in origin of replication. The Rep protein not only stimulates replication but also act as negative regulators of own synthesis (repressor of transcription). Once the Rep proteins bind the repeated iterons, additional proteins are

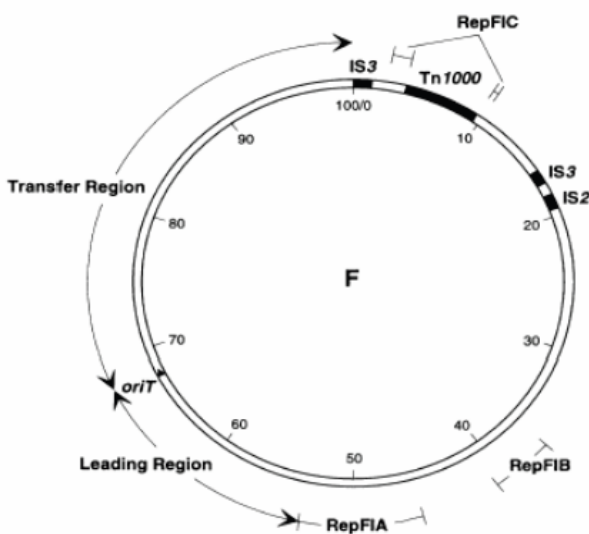
recruited to the site resulting in bending of DNA and strand separation. This facilitates binding of DNA helicase and primase, which then permit the activity of DNA polymerase. Thus, a plasmid gets replicated.

At the end of partitioning, the daughter cell gets same numbers of chromosome and plasmids. Most often plasmids have a system to ensure that the each daughter cell receives the correct number. A second approach is to kill the daughter cell that does not receive the replicon. The former approach includes active plasmid localization by filaments that draw plasmids into daughter cells or by monomerization of plasmids. The latter system work by producing long-lived killing function and a short-lived kill override function. Since the daughter cells that don't receive plasmids have no kill override function, the daughter cells eventually get killed. This system is variously known as toxin-antitoxin (TA) mechanisms, and as post-segregational cell killing and addiction systems. The toxin gene encodes a stable protein, whereas the antitoxin is either a labile protein or an untranslated, antisense RNA. Antitoxin neutralizes the toxin by binding to toxin (when it is a protein) or by preventing translation of toxin translation (when it is RNA). Even if the daughter cell fails to receive the plasmid copy, it gets the TA complex in its cytoplasm. The antitoxin is rapidly degraded by the daughter cells enzymes and is not replenished at the same time due to absence of plasmid. The toxin component lives longer, interacts with cell and cause death or growth retardation.

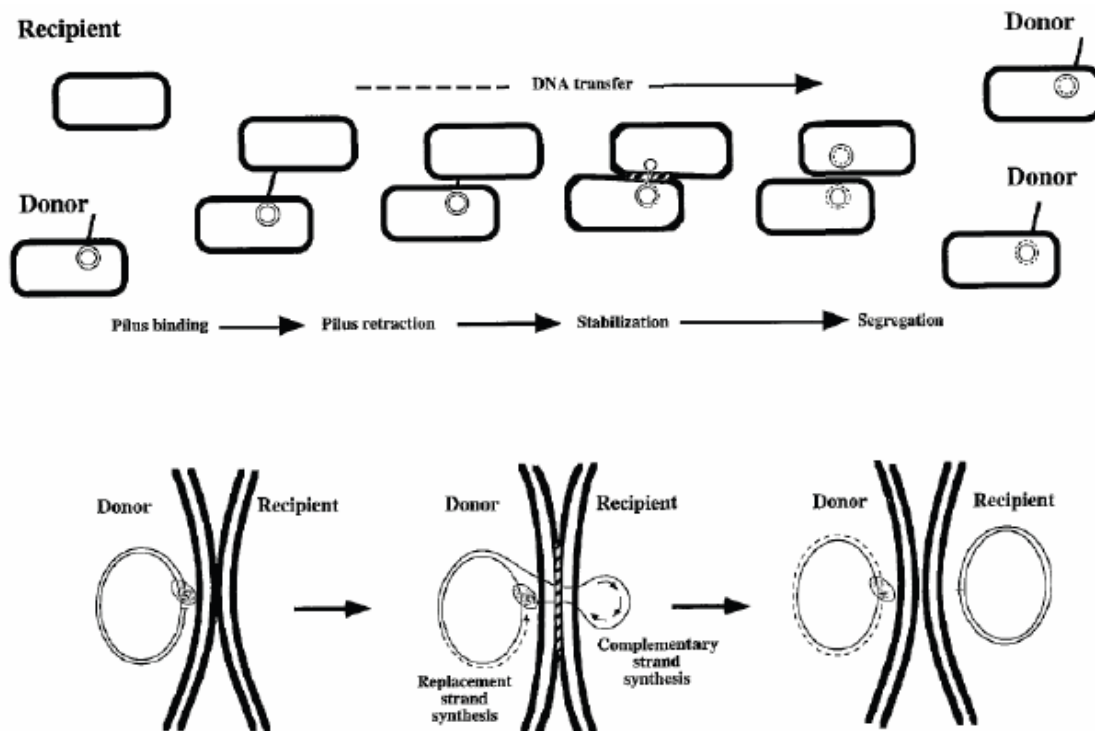
### Conjugation:

E. L. Tatum and Joshua Lederberg first described “sex in bacteria” in 1953. Conjugation is defined as the unidirectional transfer of genetic information between cells by cell-to-cell contact. It is a replicative process that leaves both donor and recipient cells with a copy of the plasmid. It is a powerful mechanism for horizontal gene transfer among bacteria. It is now widely recognized as an important contributor to the evolution of bacterial populations.

Conjugation occurs naturally in bacterial populations both within and between bacterial species. Transfer of plasmid DNA from bacterium to plant (*Agrobacterium*) and from *E. coli* to *Saccharomyces cerevisiae* has been demonstrated. Plasmids can also serve as vehicles for transposons and integrons. Only those cells that posses plasmid containing genes encoding conjugative transfer functions can function as donors. Plasmids that confer the ability to self-transfer are called F plasmid. Typically, it is a circular DNA molecule, 100 Kb in size and contains about 100 genes.



The DNA sequences located between the origin of conjugal transfer (*oriT*) and *RepFIA* are presumed to be the first to enter the recipient cell during conjugation, hence the region is known as leading region. Its gene products are believed to assist in establishment of F DNA in the recipient cell. The typical replication of F plasmid is maintained in *E. coli* by the *RepFIA* region. *RepFIB* is the secondary replication region, which can function independently of *RepFIA*. The F plasmid also contains a single copy of *Tn1000* and *IS2* and two copies of *IS3*. *finO*, the transfer region regulatory gene is often inactivated by insertion by transposon *IS3*, resulting in constitutively high levels of conjugative transfer seen in F plasmids. The *oriT* site and the transfer (*tra*) region encodes all genes that are required for F plasmid's self transfer.



Conjugation is a specialized form of the Type IV secretion system. Conjugation involves two dissimilar functions; the first one has to do with a site on plasmid called *oriT* or *mob*, which refers to origin of transfer and the second one involves functions of proteins (coded by *tra* genes) that are necessary for mobilization to occur. The *mob* gene also codes for membrane-associated mating pore formation (MPF) that provides a cell envelope-spanning transport channel. Conjugative plasmids code for their own set of MPF genes whereas mobilizable plasmids use MPF genes of another genetic element.

Conjugation begins with production of sex pili in the donor cells. The first *tra* function is to produce pilus that makes contact with recipient cells, drawing the two cells together. Conjugating cells are typically aggregated in close, stable wall-wall association. Contact of the pilus tip with the recipient cells triggers depolymerization of pilus, which draws donor and recipient cell together. It is now hypothesized that DNA does not move along the pilus from the donor to the recipient, instead the pilus serves to bring the cells together and to punch a hole in the recipient to allow the DNA transfer.

Following effective contact between the donor and the recipient cell, a *traI* product (relaxase) is produced. Multiple proteins assemble on the plasmid origin of transfer (*oriT*) to form the relaxosome. Relaxase is the key protein that recognizes the origin of transfer (*oriT*) in both conjugative and mobilizable plasmids. While mobilizable plasmids contain only the relaxosome component (*oriT*, relaxase and nicking auxiliary proteins), the conjugative plasmids additionally have type IV coupling protein and components of mating channel. The type IV coupling protein is involved in the connection between the relaxosome and the transport channel and is thought to energize the process of DNA transport. Mating channel is basically a protein secretion channel, through which DNA strand bound to relaxase is transported to the recipient cell.

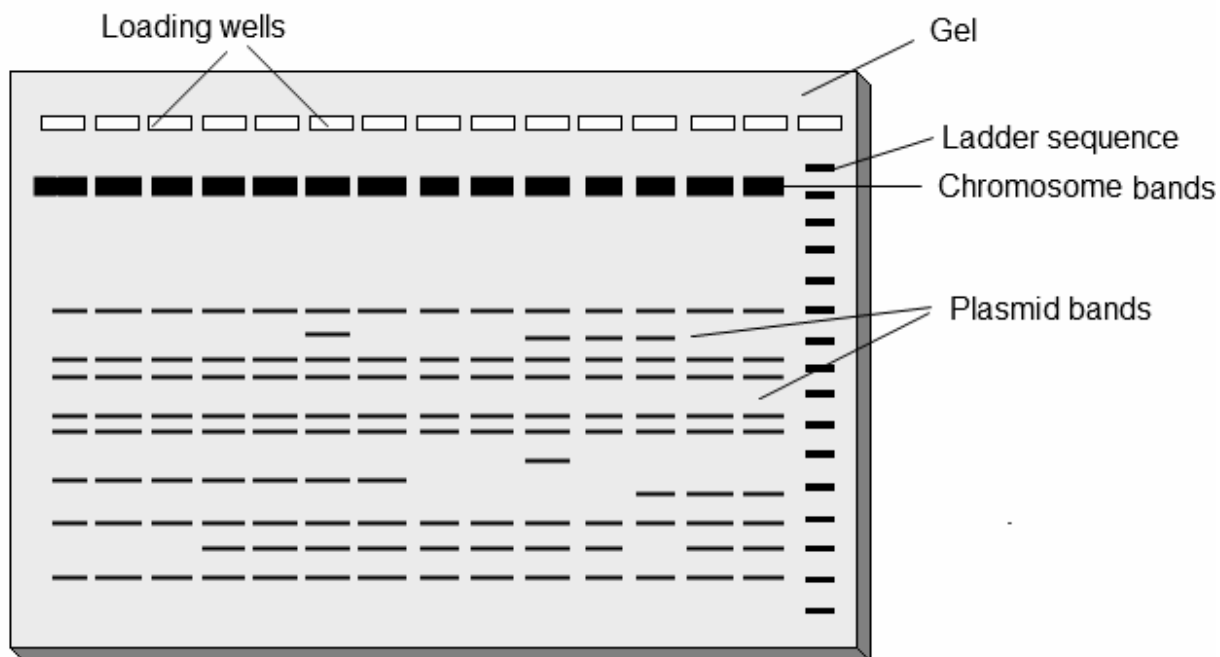
One DNA strand of the F plasmid is "nicked" at a specific position, *nic*, within *oriT* by the relaxase, which also catalyzes its covalent attachment with the 5' end of the nicked strand DNA. The nicked strand is unwound in the 5' to 3' direction. The relaxase-linked DNA is probably actively pumped through the transport apparatus into the recipient cell. In the recipient the nicking reaction is reversed,

yielding the original circular plasmid molecule and freeing the relaxase. The complementary DNA strand is then made in both recipient and donor cells by rolling-circle mechanism.

Conjugative plasmids can exhibit broad or narrow host range. In case of narrow host range, transfer is restricted generally to and between a small numbers of similar bacterial species. In case of broad host range, the plasmid is able to transfer between widely different bacterial species. Some broad host range plasmids from Gram-negative bacteria seem to have no host limitation at all. Eg., RP1 first identified in a clinical strain of *P. aeruginosa* appears to be able to transfer productively to most Gram-negative bacteria

### Plasmid profiling:

Plasmid profiling is a technique of isolation of plasmids present in a bacterial cell followed by electrophoresis to obtain plasmid counts and sizes. Isolates are grown in Luria broth at 37°C to an optical density of 0.8 at 600nm. The suspension is centrifuged at 6000 rpm for 7 minutes and the pellet is suspended in 1ml of TE buffer (Tris with glacial acetic acid pH7.9). The cells are lysed using a lysing solution containing 3% SDS and 50 mM Tris (pH 12.6). The suspension is heated to 55°C for 20 minutes in a water bath and treated with 2 volumes of phenol-chloroform mixture. Heating serves to destroy RNA and linear chromosomal DNA fragments. This solution is emulsified by shaking and then centrifuged at 6000 rpm at 4°C for 15 minutes. Supernatant is collected from the tube. A 5 mm thick 0.7-1% agarose gel is cast. 35 µl of sample is mixed with 10 µl of 0.25% tracking dye and transferred to the well in the gel for electrophoresis. Samples are electrophoresed for duration of 2 hours and then stained with ethidium bromide and observed under UV illumination. Plasmid size is determined by comparison to a supercoiled DNA marker. Regression analysis is performed to determine the linear relationship between the molecular size base 10 logarithm and the plasmid mobility. Plasmids, being smaller than chromosomes migrate ahead of chromosomes. With selective plasmid isolation techniques, chromosomal bands are not seen. Only the bright and stable bands are considered for profiling. Profiles are made by taking into account the number and sizes of plasmids. If required, the plasmid band can be dissected from the gel and eluted to obtain it in pure form.



Analysis of plasmid DNA is obviously limited to plasmid-containing strains. Plasmid profiles are not always discriminating enough for an adequate strain characterization since different strains may carry

non-identical plasmids of the same size. To improve selectivity, plasmids can be subjected to restriction enzyme digestion before comparison by electrophoresis. Similar plasmids may be found in different strains from the same bacterial species or even in strains from different bacterial species. Many plasmids, particularly R plasmids, are readily lost or acquired. Analysis of chromosomal DNA is a useful alternative to plasmid analysis.

### **Plasmid isolation by alkali lysis method**

1. Inoculate bacteria into 2 ml of trypticase soy broth and grow overnight at 37°C in the shaker.
2. Transfer 1.5 ml of overnight culture to 1.5 ml microcentrifuge tube. Centrifuge at 10,000 rpm for 5 minutes.
3. Discard the supernatant and drain the liquid by inverting on the blotting paper. Keep the tube on ice.
4. Resuspend the cell pellet in 100 µl of ice cold solution I (50mM Glucose, 25mM Tris; pH: 8.0, 10mM EDTA) and mix by vortexing gently. Keep at room temperature for 5 minutes.
5. Add 200 µl of solution II (0.2M NaOH, 1% SDS) and gently mix the contents by inverting the tube 5 times. (DO NOT VORTEX). Solution II addition should be done at room temperature. Keep at room temperature for 5 minutes.
6. Add 150 µl of solution III (3M potassium acetate; pH: 5.5) and gently mix the contents by inverting the tube. Place the tube on ice for 5 minutes.
7. Centrifuge at 10,000 rpm for 10 minutes.
8. Transfer the supernatant into a fresh tube and add 450 µl of isopropanol. Mix the contents by inverting the tube. Keep the tube at room temperature for 5-10 minutes.
9. Centrifuge at 10,000 rpm for 10 minutes. Remove the supernatant carefully and discard. DNA will be seen as white precipitate sticking to the wall of the tube.
10. Add 1 ml of 70% ethanol to wash the pellet. Mix the contents by inverting and centrifuge at 10,000 rpm for 10 minutes. Remove the supernatant completely and dry the pellet by keeping the cap of the tube open.
11. Add 30 µl of autoclaved double distilled water and gently mix by tapping the sides of the tube to dissolve the DNA. Let it stand at room temperature with intermittent mixing for 15-20 minutes.
12. Check the DNA by 0.8% agarose gel electrophoresis by loading 5 µl of DNA sample.

### **Conjugation (mating) experiment:**

The recipient strain (*E. coli* J53 Azr+) is resistant to sodium azide but is susceptible to cefotaxime, but the plasmid mediated-beta-lactamase producing test strain is susceptible to sodium azide but resistant to ceftazidime. When the resistant plasmid is successfully transferred by conjugation, the recipient strain (transconjugant) becomes resistant to cefotaxime and sodium azide. When small volume of this suspension is transferred to a plate containing 2 µg/ml ceftazidime and 100 µg/ml sodium azide, donor cells are killed by sodium azide, recipient cells are killed by cefotaxime but only the transconjugants that are resistant to both sodium azide and cefotaxime survive and grow as visible colonies.

- Both the test strains and standard recipient strain are subcultured separately in 4 ml Luria-Bertani or Trypticase soy broth and incubated at 37°C with mild shaking.
- The recipient and donor strains are mixed at a ratio of 10 (recipient) to 1 (donor) and incubated without any agitation at 37°C for 20 hours.
- 50 µl of transconjugants are transferred to Mueller Hinton agar containing 2µg/ml ceftazidime and 200 µg/ml sodium azide and incubated up to 48 hours.
- Samples from donors and recipients are used as controls.

- The colonies are tested for the expression of conjugative plasmid by any of the several means (plasmid extraction followed by PCR, phenotypic expression of ESBL, isoelectric focusing, DNA hybridization etc).

Conjugation experiments are not always successful. Some plasmids are readily transferable than others. In some cases several attempts may be required for successful conjugation. If conjugation (mating) experiments fail, transformation of E. coli TOP10 strain by electroporation of whole-plasmid DNA can also be done. The transformants can be selected on Drigalski agar supplemented with 1 µg/ml cefotaxime.

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