# Genetic Study of Bacteria and Bacteriophage

6

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## 6.1 Bacterial Genetics

Bacterial genomes are made up of a circular chromosome that possesses a single-stranded DNA (ssDNA) molecule of several million base pairs in total length. A classic example is *E. coli* genome that is widely organized in approximately 4.6 million base pairs of DNAs. No wonder that some bacteria contain multiple chromosomes, for example, *Vibrio cholerae* that causes cholera has two circular chromosomes and *Rhizobium meliloti* has three chromosomes. Apart from this composition, there are few bacteria that do not carry circular DNA and instead carry a linear chromosome. In addition to its own genome, the bacterial genome also possesses an additional structure known as "plasmid"—a small circular DNA molecule present in many numbers (usually known as copy number). Plasmids accommodate genes that are not essential at the functional point of view for bacteria but that might have been involved as an important factor for the life cycle and growth of their bacterial hosts. Some plasmids are involved in the mating process between bacteria (provide a channel for the exchange of genetic material during mating, which will be discussed in the next section). Some plasmid leads to an important role in the generation of antibiotic resistance among the population. Most plasmids are circular and of several thousand base pairs in length and, however, some of small size; about a hundred base pairs in the length have been found. Each plasmid structure carries at least an origin of replication (*ori*), a start point for DNA replication. The main function of *ori* is to let the plasmid replicate independently without involving a bacterial chromosome. Episomes are plasmids that are capable of freely replicating and integrating into the bacterial chromosomes. Episomes are categorized into several types based on importance and functionality. The F (fertility) factor of *E. coli* is one type of episome found robustly. F factor basically

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regulates the mating and gene exchange between *E. coli* cells (which will be discussed in the next section). In this part, we have focused on the bacterial mutant gene mutation process that contributes to genetic variation in the bacterial population. We begin with an overview of the chemical nature of the mutation in bacterial genetics and its effect at both molecular and organismal levels.

### 6.1.1 Bacterial Mutant Genetics

In microbiology, studying mutants is an important part. The study of mutants requires a vast knowledge of detection methods; geneticists should be able to find them out quickly even when they are rare and should be able to isolate them efficiently from wild type or least interested other mutants. What are the mutants? The bacterial population is the most important tool in genetics and biotechnology, and scientists have observed so many unusual new bacterial strains and are still observing to date. Every time new strain discovery is an outcome of occurrence of mutation ever so often. A slight variation in the genome of bacterial strains within a colony results in the rise of new features in the colonies and appears as different from its wild type and known as mutants.

Geneticist increases the likelihood of obtaining mutants by using mutagens to increase the rate of mutation from the usual one mutant per $10^7$ to $10^{11}$ cells to about one per $10^3$ to $10^6$ cells. To study bacterial mutants, we should know the selection, isolation, and detection method that is described in the following section.

#### 6.1.1.1 Mutant Detection

When it comes to detecting and collecting a mutant of a particular organism, one should know the wild-type characteristic of that organism to recognize the difference between a mutant and a wild type, and thus detection of mutants has been evolved along with the time as the necessity in the field of bacterial genetics. Mutation in prokaryotes is easy to detect even if it is a recessive mutation because it has been seen immediately and onward. A very simple example of albino mutants, which is a type of mutant of pigmented bacterium, can be visualized by observing the color differences of colonies. This mutation simply requires color detection. On the other hand, there are other detection methods available that are more complex. Replica plating method is one of them and is used for the detection of auxotrophic mutants, which has been explained in Fig. 6.1. This method distinguishes a mutant from the wild-type strain. Auxotrophic mutants are able to grow in the deficiency of particular biosynthetic end product that is necessary for an efficient growth of cells.

#### 6.1.1.2 Mutant Selection

Specific incubation conditions can be a tool for the selection of specific mutants that can only grow under such conditions in which wild type will not be able to grow. Thus, this is an effective method for mutant selection. What exactly mutants do to a wild-type strain and what makes them grow in a specific condition? A selection method will either develop a reversion mutation (a mutation that restores the wild-

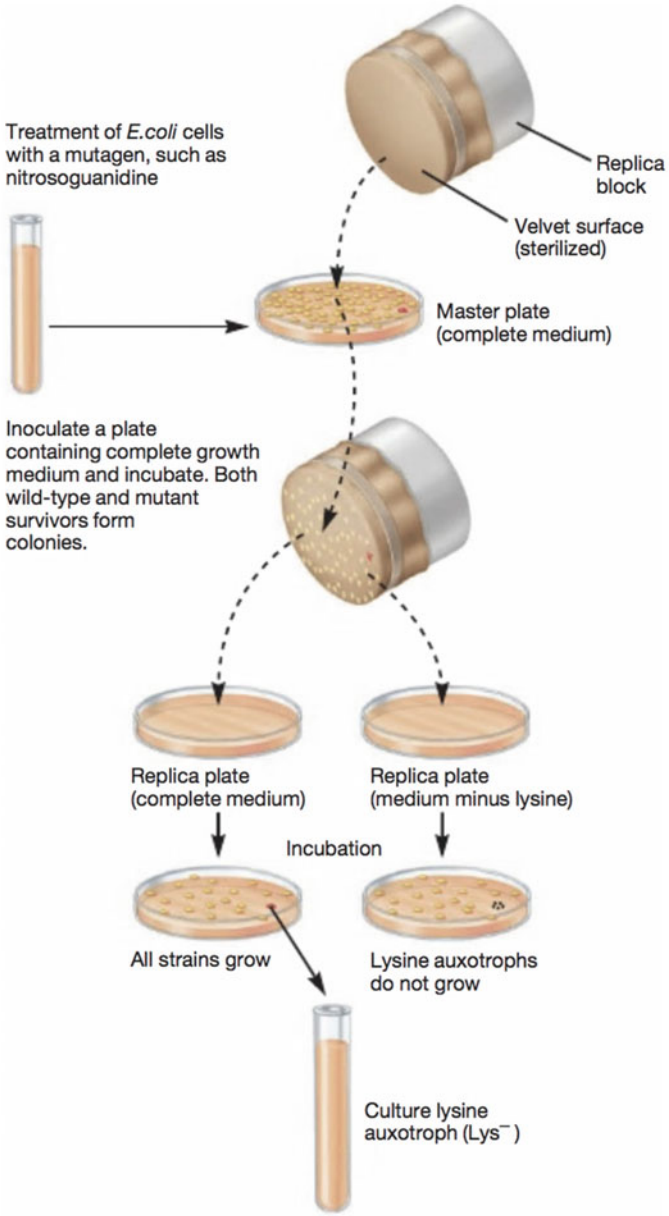


**Fig. 6.1** Isolation method for bacterial mutants from the culture through replica plating method. Replica method for separation of bacterial mutants from the culture. Replica plating is popular for the isolation and detection of lysine auxotrophs. Auxotrophic mutants can be easily generated by using a mutagen. Both wild type and auxotroph are cultured together and plated on the complete medium. The culture containing wild type and auxotroph is plated on a complete medium. Immediate to colony formation, a soft velvet cloth is pressed on the culture plate, and bacterial colonies are picked by this piece of cloth. This traced colonies are transferred to another culture plate of minimal media (lacking Lysin) and in the same orientation as the master plate is Location of

type phenomenon if a wild-type strain is already mutated in the forward direction) or develop a resistance to the environmental stress. For example, if the purpose is to isolate a revertant (a strain that regains the former capability) from lac $Z^-$ auxotroph, then the method would be simple. Let us assume that a huge population of lac Z auxotrophs is plated on the minimal media lacking the lactose, incubated, and examined for selection. In this case, only cells that have mutated which can regain the ability to grow on lactose will grow on the minimal media (Fig. 6.2). Out of this experiment, we can suggest that millions of strains can be plated, grown, and isolated on a single Petri dish, but those revertants that have the ability to manufacture the nutrient source lacking in the medium will grow selectively. Thus, this method can be a selective technique to test for mutation by a mere scanning of a single Petri dish. This method has successfully accounted for screening many environmental substances containing mutagenicity.

The selection method for mutants due to environmental stress is more likely similar to the previous approach that has been used for auxotrophs. It is known that wild-type strains may be susceptible to any environmental stress like virus (phage) attack or antibiotic treatment. Thus, plating a phage-sensitive wild-type bacterium on the minimal media produced colonies that are resistant and have become phage-mutant. This type of selection method is useful for many other environmental stress conditions, that is, virus attack, temperature, antibiotics, etc.

In addition to the abovementioned selection method, several bacterial strains utilize few specific substrates to survive. Not all bacteria use all the nutrient substrates present in the growth medium. Many bacteria just use a few primary carbon sources rather than the complete ones. In this case, bacteria can isolate by plating on the Petri dish containing an alternate carbon source. If colonies appear on the plate, this strain will be considered a mutant for that specific substrate being utilized in the culture for its survival.

The mutant selection method has eventually become useful for understanding the complexity of genes and biochemistry of a particular bacterial strain. Particularly, this technique is a significant tool for understanding the role of carcinogens in developing mutagenicity.

### 6.1.2 Spontaneous Mutation in Bacteria

Spontaneous mutation allows a DNA to be mutated without any exogenous factor or selective pressure such as UV rays or chemicals; instead, endogenous factors are the primary sources, that is, DNA damage, error in DNA replication machinery, less effective DNA repair machinery, and so on.

**Fig. 6.1** (continued) auxotroph bacteria which must not grow on second plate and can compare to the master plate to spot the lysin mutant. This bacterium can be picked from the master plate and can further grow as a single culture of lysine auxotroph

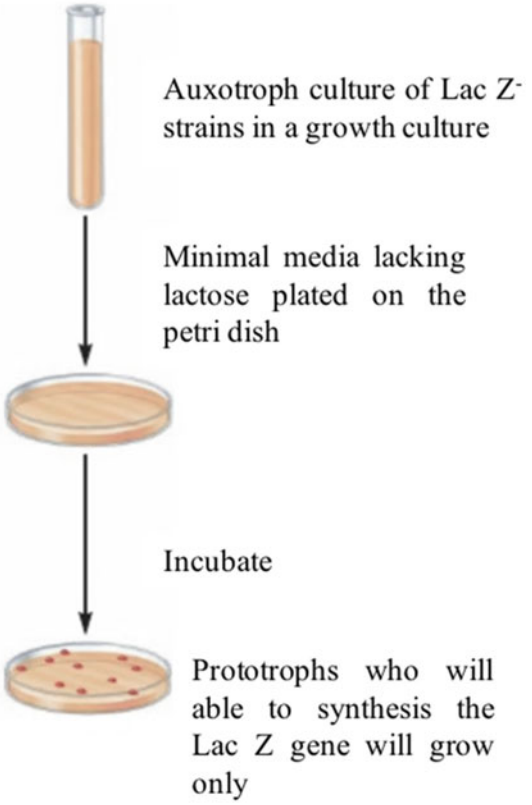


**Fig. 6.2** Production and selection method for auxotrophic revertants (mutants). In this example, lac Z revertants are selected after plating a lac $Z^-$ auxotroph because the agar contains a minimal medium that does not support auxotroph growth

*Foster* and her colleagues in 2012 have demonstrated the mutation rate in three different strains of very primitive and common bacteria *E. coli* by using the whole-genome sequencing (WGS) approach. They explained in their work that the mutation rate of 1–2 $\times$ $10^{-3}$ mutation per generation and per genome is natural for this bacterium which has not been induced by any external factors. Experimentally, they observed that at any given defined medium or growth condition, the mutation rate of any specific gene remains constant. Interestingly, if a small inoculum that may contain few mutants will be transferred to a culture medium, then the proportion of mutants in that growing culture is positively correlated with the progressively increased bacterial population. From the historical point of view, spontaneous mutation was first time spotted by "Salvador Luria and Max Delbrück" in 1943. In their experiment, they have found that when the *E. coli* was plated on the nutrient medium in the presence of T1 phage, it appears as a phage-resistant mutant before phage display on the plate. It means the *E. coli* observed on the plate were resistant to the phage attack, which might be the result of a spontaneous mutation. This discovery brought us the understanding of genetic diversity in the bacterial genome as a result of spontaneous mutation. Lately, this understanding has widened by including another piece of information based on spontaneous mutation which

suggested that the occurrence of genetic changes due to spontaneous mutations is essential for developing antibiotic-resistant strains, host evasion, and acclimatization to the new environment, thus contributing to the bacterial evolution. Interestingly, a recent study from Tomasetti, C et al. has revealed that random mutagenesis in somatic cells of some tissue causes an error in DNA replication which transformed the normal cells into cancerous cells compared to other cells from different tissue at the same time. This study has underlined the role of spontaneous mutation in cancer risk; however, this study has been the reason for debate as well.

How can mutations in bacteria occur spontaneously? What is the method to detect them? The fluctuation test is one of the experimental techniques widely used for its detection. As we have discussed the Luria and Delbrück experiments where they have explained that if a mutation occurs spontaneously, then the incidence of the mutation at a different time in a different culture would be higher which led to the variation in resistant colonies throughout the cultures. This concept is known as the "fluctuation hypothesis." In this experiment, small inocula were grown in the culture medium, and the number of phage-resistant *E. coli* from this culture was measured and compared with other grown multiple *E. coli* colonies. According to the spontaneous hypothesis, we can understand two conditions:

1. If the phage resistance mutation occurs after exposure to the phage, then the number of phage-resistant mutants and mutation rate should be similar between the colonies of both sets of conditions.
2. On the other hand, if the mutation is spontaneous and occurs before the exposure to the phage, then the variability in the mutation for all the independently grown cultures would be highest because the difference in the size of the bacterial population which received the mutation at first will contribute to the overall observed variability till the end of generations. The data indicated that the mutations to phage resistance in *E. coli* occurred spontaneously with a constant probability per cell division.

Let's think about what could be those reasons governing spontaneous mutation? Spontaneous mutation does not need any exposure to external agents besides a mere error in DNA replication, or base substitution could bring the results as a spontaneous mutation. One of the reasons being considered is a malfunction in the machinery of DNA polymerase III (an enzyme associated with replication) during DNA synthesis. Adding, mispairing, or omitting a nucleotide on a parent DNA strand will appear as a mutated granddaughter DNA strand and thus lead to mutation in the continuity. In addition, some mobile genetic materials like transposons are also considered as the source for spontaneous mutation in the bacteria where these elements are present other than the nuclear chromosome.

Replication error appears when the nitrogenous base of a template nucleotide exists in a rare form such as tautomeric form. Tautomerization is a chemical process in which natural forms of nucleotide bases are (keto(C=O) and amino ($C=NH_2$)) converted into two rare structural isomers (imino (C=NH) and enol (C=OH)). These isomers of nucleotide base pairs make nonconventional hydrogen bonding and can

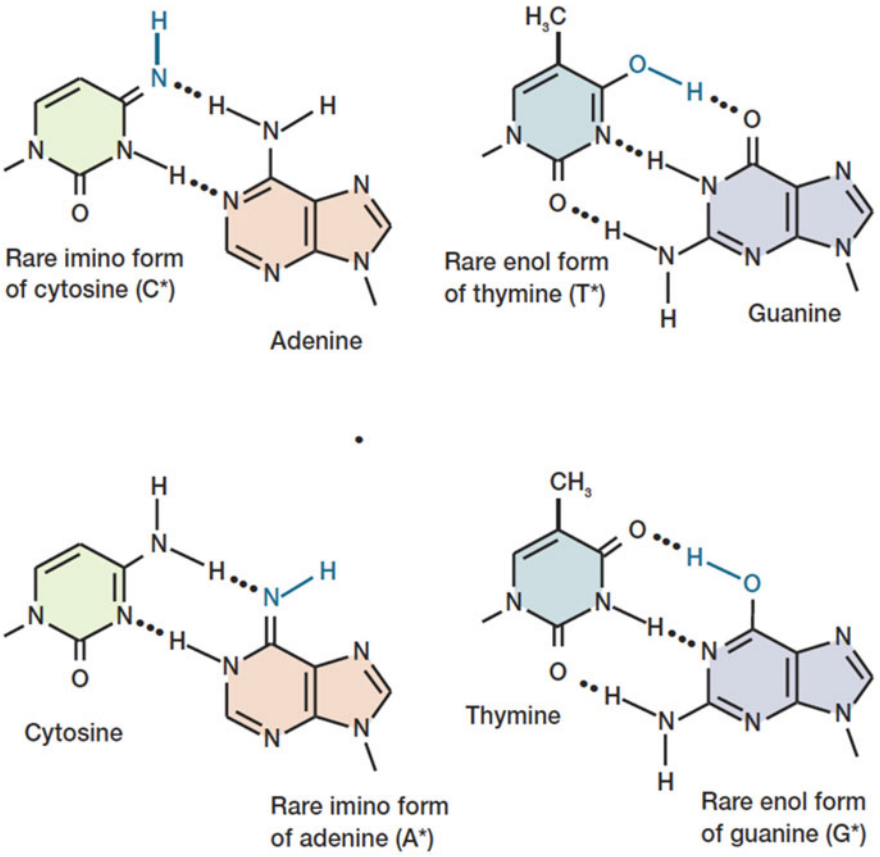


**Fig. 6.3** Schematic diagram of base pairs undergoing tautomerization. Normally the keto form of the base formed a normal hydrogen bond-like A–T and C–G, but the enol tautomer instead produces A–C and G–T base pairs. The upper lane shows the normal pattern of A–T and C–G pairing, while the lower lane shows the rare bonding between (1) imino form of adenine and cytosine and (2) enol form of guanine and thymine

readily change to each other (Fig. 6.3). Thus, tautomeric shifts change the characteristics of the hydrogen bond among the four bases. In turn, this shift allows purine to bind with purine and pyrimidine to pyrimidine instead of pyrimidine–purine binding and eventually generates an alteration in the nucleotide sequence of the daughter strand after the first round of replication.

Such mutation based on tautomerization, known as **transition mutation**, is relatively common to be found. On the other hand, **transversion mutation** is another type of mutation in which purine may substitute to a pyrimidine and vice versa, but this mutation is not frequent due to stearic hindrance in the pairing of purine to purine and pyrimidine to pyrimidine.

Replication error also occurs when a purine and pyrimidine base leads to apurinic (loss of purine from the nucleotide sequence) or apyrimidinic (loss of pyrimidine

base from the nucleotide sequence) sites in the sequence. Spontaneous loss of purine or pyrimidine base due to hydrolytic cleavage of n-glycosylic bonds with sugar moiety forms lesions, and thus polymerase enzyme is unable to synthesize complementary nucleotide on this site which leads to mutation.

Redox attack on guanine base results in the conversion of guanine to 8-hydroxydeoxyguanosine (8-OhdG). This modified base ultimately pairs with adenine instead of cytosine and produces G->T transversion in the end, during the replication.

Although most geneticists believe that spontaneous mutation is a random process without any induction through external agents, this theory lately in 1988 has been modified by John Cairns whose theory is an extended version of Luria and Delbrück's concept of spontaneous mutation. He stated that mutation does not happen only spontaneously in growing cells but preferably happens in nonproliferating cells by giving lethal conditions. In his experiments, he demonstrated that when mutant *E. coli* strain was subjected to grow on a complete medium, it was unable to utilize the lactose as a whole carbon source and interestingly if lactose were continuously added to the culture medium as an only carbon source, *E.coli* would more rigorously avoid the lactose to take as a carbon source. In this case, John found that lactose seemed to induce the mutation which allows the *E. coli* to choose the sugar over lactose for a carbon source. This study took us to interpret that this type of mutation is "adaptive mutation" where bacteria are supposed to choose the mutation that occurs frequently so that they can adapt to a better surrounding for their survival.

## 6.2 Viral Genetics

Every organism such as plants, animal, fungi, and bacteria are susceptible to viral infection. A virus is a simple replicating machinery that consists of a core nucleic acid and is protected by a surrounding protein coat usually known as a capsid. Virus can be categorized based on their shape and size and nucleic acid whether it is a double-stranded DNA or single-stranded DNA or sometimes a single-stranded RNA.

Virus has a particular class that infected their specific host, for example, a virus infected bacteria is called "bacteriophage," and this phage will not infect plants or animals directly but will be carried away with bacteria itself to further infect animals or plants. Phages have been involved in genetic research since the late 1940s. It has become an essential and advanced research tool in the area of genetic research since it contains a small and handy genome, reproduces rapidly, and produces a large number of progenies. The study of the phage genetic system has been employed long ago because this microorganism plays an important role in human society. In this section, we will focus on many unique aspects of phage genetics, that is, study of structure and life cycle of bacteriophage, detection methods for phage infection, application in genetic research, and so on.

The Discovery of phage included many efforts and stories that have been made by several scientists. First time, it has been spotted something vague in the river of India by Ernest Hanbury Hankin, a British bacteriologist in 1896. He simply reported that there is something in the water that had antibacterial properties and killed cholera, but he did not specify his finding. Later in 1915 another British bacteriologist Frederick Twort discovered that a very small molecule or an unknown thing kills bacteria in the bacterial culture; he had published his finding, but unfortunately, this work had been interrupted due to WWI. Later in 1917, Felix d'Herelle discovered a killing agent for bacteria at Pasteur Institute, France. He actually observed that when he added a filtrate that was collected from sewage to the bacterial culture of dysentery, in a few minutes, the culture colonies disappeared, and he named this filtrate as "invisible antimicrobial agent," and later this work was published. In the row of findings, a first-time phage study was developed in the Elvia institute, Tbilisi, Georgia, in 1923 to study this invisible thing and to develop phage therapy. In 1969, Max Delbrück, Alfred Hershey, and Salvador Luria discovered the replication of virus and virus genetics for which they were awarded the Nobel Prize in Physiology or Medicine.

### 6.2.1 Structure of Bacteriophage T4

T4 is one of the most extensively studied bacteriophages among all other phages such as T1–T7. T4 bacteriophage is specific to *E. coli* and has been demonstrated as a model for phage study by Delbruck and coworkers in 1944. In the modern time of genetic engineering, the study of phage included advanced tools and techniques particularly to understand its structure at the atomic level. Early discoveries on this bacteriophage have included the prediction of phage image using electron microscopy (EM) obtained by Brenner et al. in 1959. This work extended to an extensive study on phage head symmetry, tail, and baseplates through EM. For the first time, a complete T4 genome was sequenced in 2003. In the continuity of this work, high-resolution cryo-electron microscopy (cryo-EM) image was obtained which revealed a dome-shaped baseplate structure in the infectious virus. In subsequent years, star-shaped baseplate and prolate head structure of post-infection T4 were published. Thereafter, many other techniques like complementation assay (to study recombination in bacteriophage), cross-linking analysis (protein–protein interaction study), X-ray crystallography, and cryo-EM provide high-resolution atomic-level structure model for T4 phage. Studies from the structure model reveal the structural similarities among phage protein and bacterial protein which suggested the common evolutionary ancestry or coevolution with the bacterial host. Structural similarities among phages as well as of T4 components with bacterial proteins demonstrate common evolutionary ancestry or coevolution with bacterial hosts.

Bacteriophage T4 belongs to the family *Myoviridae* that infects *E. coli*. A basic T4 structure includes a head (capsid), tail, and baseplate. Phage has a rigid tail that is composed of many layers; the inner layer of the tail is surrounded by a contractile sheath that helps a phage during infection. Phage *Myoviridae* family like T4 contains

a massive baseplate at the end of the tail with long attached fibers that guide the phage to find the receptor in the host cell and mediate the initial contact. A contractile tail is helpful in penetrating the bacterial outer membrane before the DNA delivery during the infection. Long attached fibers are made of six short tail fibers that are folded underneath the baseplate and unfold on the recognition of the host as a host sensor. The baseplate is a puncture device for phage located at the end of the long tail and the last element located at the end of the tail.

Capsid of T4 phage is assembled with three main components: (1) gp23 (48.7 kD) forms hexagonal capsid lattice, (2) gp24 forms pentamers of the vertices, and (3) gp20 forms a unique dodecameric entrance as a portal vertex that makes a gateway for DNA packaging and exit during the infection. Genetic material of T4 is a linear dsDNA of 168kbp which has 289 open reading frame (ORF).

#### 6.2.1.1 DNA Packaging

DNA is translocated in the capsid through the central channel of portal protein on one vertex of the capsid. The portal complex at vertex works as a docking point for terminase; a viral ATPase complex that is bound to the end of the portal vortex provides an active packaging motor complex that assists the entry of viral DNA into the capsid. The packaging of DNA is evenly regulated till the threshold amount of DNA is entered into the capsid. Interestingly Smith et al. in 2001 have reported in their work that dense packaging of DNA after some point will raise the pressure inside the capsid approximately equal to 6Mpa. Termination of DNA packaging, after enough DNA has been densely packed into the capsid, is mediated by bolting of the active portal complex, which prevents the phage genome leakage. Portal complex on vertex alone is not enough in the packaging machinery; therefore, in addition, head completion proteins sometimes also called adaptor proteins are required to bind with the complex dodecameric portal and form the connecter at the end of the capsid.

If we take a closer look at the symmetry of capsid and tail, a mature or fully assembled capsid in a phage follows four to fivefold symmetry or so-called icosahedral symmetry. From the structure point of view, each multiple structural unit in the capsid has been displayed as a regular lattice with two, three, and five rotational symmetries. Portal protein shows dodecameric oligomerization pattern while tails show overall six- or threefold rotational symmetries, and protein repeats in this symmetry are shown in a helical pattern.

Brenner et al. in 1959 successfully illustrated phage image through electron microscopy (EM).

EM image of mature phage has been described in Fig. 6.4:

1. 1150 Å-long and 850 Å-wide icosahedron head encompassing genomic DNA
2. 925 Å-long and 240 Å-diameter contractile tail attached to the one end of the head through portal vertex
3. 270 Å-high and 520 Å-diameter hexagonal baseplate
4. 1450 Å-long six long tail fibers attached to baseplate

In the past decade, advanced techniques such as single-molecule tweezers and fluorescence studies revealed a few amazing facts about bacteriophage; T4 motor packages DNA at the rate of 2000 bp/s which is confirmed as the *fastest packaging motor* till date. Another fact that FRET-FCS studies on phage suggested that DNA in phage gets condensed during the translocation process.

### 6.2.2 Life Cycle of Bacteriophage T4

A phage life begins and ends during the infection period and has been characterized into two phases: lytic cycle and lysogenic cycle. Lytic cycle is a virulent phase since it infects a cell, destroys the cell, lyses the cell, and replicates and produces more phage particles. A lysogenic phage is a temperate phase because it infects the cell and incorporates dsDNA to the host cell, and no progeny will be produced. In lysogenic phase, foreign DNA such as viral DNA is incorporated into the bacterial chromosome and replicates multiple times as the host cells divide. However lysogenic phase also undergoes a lytic phase in some circumstances not capable of undergoing the lysogenic life cycle and therefore directly undergoes the lytic cycle.

Other than lytic and lysogenic, a phage also has been found in pseudolysogeny life. It is an unfavorable and unusual life cycle that occurs when a phage grows in unfavorable growth conditions that enable a phage to survive by preserving the phage genome until the host growth condition becomes advantageous again.

Lytic phase has been categorized as follows:

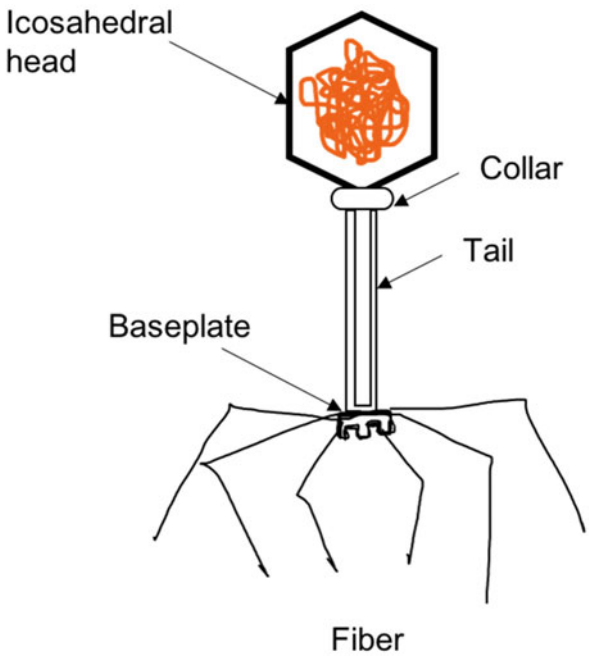


**Fig. 6.4** Assembled mature bacteriophage T4. The assembly of T4 can be divided into three independent subassemblies: the head, the tail, and the long tail fibers. The tail binds to the head followed by the attachment of the fiber in the end. Six long tail fibers are then attached to form a viable T4 virion

1. Attachment to the bacterial cell and phage infection
2. Passage of DNA through the bacterial cell wall
3. Infected bacterium converted to phage factory
4. Production of phage DNA and protein
5. Assembly of phage
6. Release of phage particle

### 6.2.3 The Plaque Assay

Plaque assay has been developed to the necessity of phage detection in several medical conditions. Plaque assay has shown the ability of host cell to transform into plaque lawns if any bacterial colonies are being infected within the phage. Spot-on lawn assay has been identified as one of the simplified versions of the plaque assay which aims to identify potential viral plaques or virus infection in the growing bacterial culture. To this aim, approximately 1 μL aliquots of virus suspension would be enough to be applied to fresh lawns of diverse microbial host strains. For example, if the suspension of a phage (i.e., T4) is applied to the susceptible bacterial host (*E. coli*), then phage infects the bacterial cell, replicates independently, and promotes the lysis of the cell due to the occurrence of lytic phase and kills the bacteria in the end. This lysed bacterial cell indicates the formation of a clearing zone on the bacterial lawn known as plaques. In some circumstances where the lytic cycle is absent, bacterial colonies grow confluently. Any single plaque determines the number of phages that have infected the single bacteria and further keep infecting bacteria in the vicinity and develop the new plaque in the vicinity which together forms a big plaque, enough to be seen with naked eyes. Notably, plaque does not continue indefinitely, and the size of plaques is totally dependent on the type of phage, host cell, and condition of grown culture. Measuring the frequency of plaque formation or detecting the number of plaques formed on the bacterial lawn can be calculated by the dilution method. If phage suspension can be diluted in a serial dilution method, thus appropriate dilution factor can be used for calculation of plaque formation unit by using the following equation:

$$\textbf{Plaque forming unit}(\mathrm{PFU}) = \frac{\text{Average Number of Plaques}}{\text{Dilution factor} \times \text{volume of diluted virus added}}$$

### 6.2.4 Lysogeny

Lysogeny is another life cycle or reproductive pathway of phage other than the lytic cycle. The very first evidence of lysogenic behavior of phage is confirmed by two subsequent experiments in 1920 and 1940 as follows:

1. In the 1920s, some remarkable results were obtained in the study of phage in the *E. coli* culture. Earlier microbial geneticists have performed an experiment in which they mixed two microbial strains: lysogenic-resistant and non-lysogenic-resistant strains. Lately, they have found that non-lysogenic-resistant strain was lysed in the culture. The geneticist explained that this phenomenon happened because resistant strain somehow causes lysis of nonresistant strain and thus resistant cell known as lysogeny or lysogens (a causative factor for cell to lysis). It is further important to understand that sometimes nonlysogenic bacteria might get infected with phage-derived lysogenic strain, and very few infected cells were not lysed but rather itself became lysogen.
2. However, in 1940, André Lwoff performed another experiment in which he studied the lysogenic bacteria *Bacillus megaterium* and he followed its behavior and cell divisions in the culture throughout. Once he established the culture, he separated each daughter progeny after each cell division. From this daughter progeny, he has put back one cell in a new culture while he followed the other cell throughout the cell division. In this experiment, he followed 19 cultures that represent ten consecutive generations. He also separated culture medium and found no phage existing in the free medium through which he confirmed that lysogenic behavior of the bacteria is followed by each cell division or each reproduction in the absence of any external phage in the medium.
   On the other hand, he spreads this separated phage-free medium, on the lawn of non-lysogenic bacteria, and astonishingly, he observed a spontaneous plaque formation in the culture. This observation he explained in his proposed hypothesis. Lysogenic behavior of phage passes from generation to generation, and this gives rise to pure noninfective strain but somehow this noninfective factor converted into infective phage although no free phages presence in the medium. This event is an exceptional case. In the term of microbiology, Lwoff named this factor as "prophage" which can change the noninfective factor to infective factor by chance or in an inductive manner.

Now we know why non-lysogenic-resistant cells have been found to be infected and lysed in the culture because prophage, which is a feature of lysogenic bacteria, eventually produced infective phage into the medium that causes the cells to lyse.

A common lysogenic cycle can be described in few steps (Fig. 6.5):

1. Linear phage DNA first injected to the host cell.
2. Initially phage mRNA will synthesize for short period, followed by synthesis of (a) repressor protein that inhibits the synthesis of the enzyme needed for lytic cycle and (b) site-specific recombination enzyme. Later, phage mRNA synthesis is turned off by repressor protein.
3. Phage DNA molecule incorporated into the bacterial chromosome.
4. As bacteria replicate and grow in the medium, phage DNA also multiplies and produces more phage progeny as part of the bacterial chromosome.

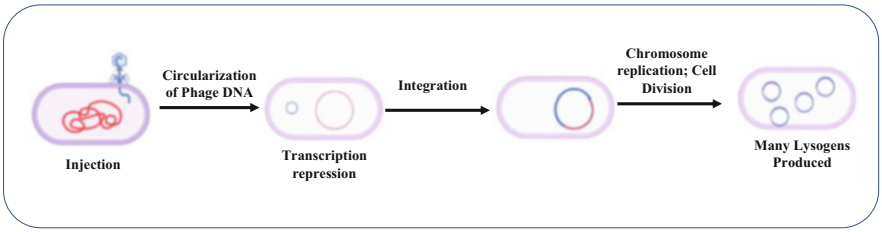


**Fig. 6.5** A lysogeny phase after immediate to virus infection in the host cell. Infection of phage initiates circularization of phage DNA at first, followed by integration with bacterial chromosome and replication. Cell division reproduces more numbers of bacterial cells containing integrated phage DNA which accomplishes the lysogenic cycle of any phage

### 6.2.5 T4 Phage Modulates Bacterial Genetics

Other than its characteristic to invade bacterial hosts, T4 phage infection has been the subject of much scientific research to how phage has an impact on the bacterial genome. Such phenomenon can take place by either generalized transduction or introduction of phage-encoded protein whose expression results in changes of their host phenotype and activity. Phages have acquired these genes from their host and continued to evolve and change within its own genome. These extra genes can be named as "accessory genes" that can govern the biology of their bacterial host and find the tune in the way in which bacteria interact with their environments. Such observation has been possible by our ability to sequence phage genomes, and this information will serve as a start point for further study to determine how phage infection can contribute to their bacterial host's physiology endurance and evaluation. In many ways, phage affects the bacterial genome or phenotype, and few examples are given below:

#### 6.2.5.1 Human Gut Microbiome Interaction

Human gut microbiome is another example of phage–bacteria interactions which consist of densely colonizing microorganisms including T4 phage and interacting with mammalian hosts. Metabolome data from several research on the role of phage in the gut microbiome suggested that T4 phage remains as a modulator of bacterial colonization in the human gut and supports the benefits out of this interaction to human health.

#### 6.2.5.2 Host Communication

T4 phage further manipulates bacteria by accessing quorum sensing pathways which have effects on one or multiple behaviors of bacteria as shown in (Fig. 6.6). Genomic study of phage reveals that phage DNA sequence contains homologs of response regulator that is involved in quorum-sensing pathway. Also, it has been noticed that induction and release of temperate phages have been observed following the N-acyl homoserine lactone exposure (LuxS system of quorum-sensing pathway).

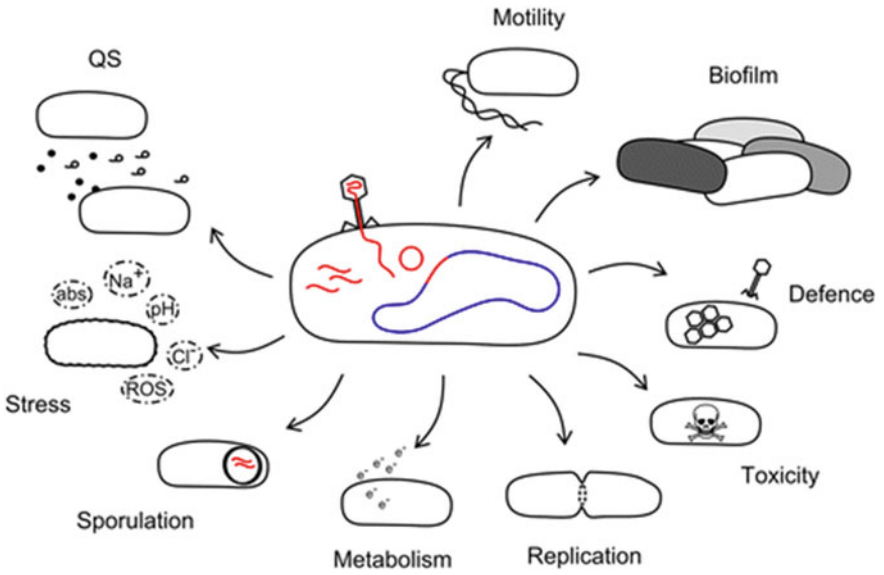


**Fig. 6.6** T4 phage modulates bacterial genetics in several ways. After insertion of phage into a bacterial DNA, it further regulates several signaling in the bacterial cell which are essential for bacterial life cycle, stress responses, replication, metabolism, and so on

#### 6.2.5.3 Host Replication

Phage uses bacterial machinery to manipulate its own replication and host replication system, and phages manipulating host replication can be seen in the dimorphic bacterium *Caulobacter crescentus*. Moreover, another example of phages influencing their host's replicative process is the phage-encoded homologs of MazG. MazG is a regulator of cell death in *E. coli*, and its expression influences bacterial replication in nutrient-limited environments. Interestingly, homologs of MazG have been found in phages infecting several diverse bacterial species, including several cyanophages, *Burkholderia cenocepacia* phages, and *Mycobacterium* phage L5.

#### 6.2.5.4 Host Metabolism and Energy

Many phages that invade bacterial cells also control its metabolic regulation, such as carbon, nitrogen, and phosphate metabolism, and its utilization. Cyanophage is one example of such kind of modulation by phage in the host cell.

### 6.2.6 CRISPR/Cas9 Bacteria in Genetic Engineering

In the past decades, an endogenous machinery of bacteria/archaea has been used as a powerful tool in genome editing (called gene editing) technology which allows genetic material to be added, removed, or altered at particular locations in the

genome. To this purpose, several techniques have been developed, and the very recent one is known as CRISPR/Cas9. CRISPR-Cas9 stands for clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9. Interestingly, CRISPR/Cas9 was adapted from a naturally occurring genome editing machinery in bacteria. Just like us, bacterial cells can be invaded by viruses, and in response to defense against the virus, the bacterial CRISPR immune system can thwart the attack by destroying the genome of the invading virus.

Interspersed between the short DNA repeats of bacterial CRISPRs are similarly short variable sequences called spacers that are derived from viral DNA that have attacked bacteria previously. However, this spacer helps bacteria to recognize the viral genome of its attacks again, and CRISPR (Fig. 6.7) defense system will cut up any viral DNA matching the spacer sequence. Thus, these spacers are termed "genetic memory."

Genetic manipulation such as gene insertion or gene overexpression is very well established; however, inhibition or abrogation of a particular gene is quite challenging until CRISPR/Cas9 came into existence in the last decade. CRISPR/Cas9 system has been categorized into types I, II, and III, of which type II is the most successful and widely used in genome editing since it requires one enzyme and one RNA to function as a DNA endonuclease. Moreover, the RNA components of the CRISPR/Cas9 system can be used separately by fusing the crRNA (mature CRISPR RNA) to

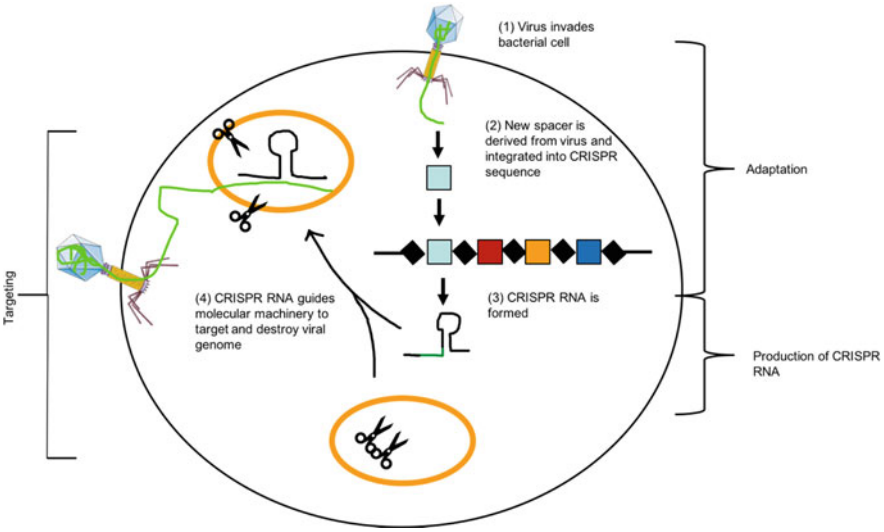


**Fig. 6.7** CRISPR-mediated gene editing: CRISPR are regions in the bacterial genome that help participate in the defense against invading viral genome. These regions are composed of short DNA repeats (black diamond) and spacer (colored boxes). When a new virus infects a bacterium, a new spacer is generated by the viral genome and incorporated among existing spacers of CRISPR. CRISPR is transcribed and processed into short CRISSPR RNA molecule. This CRISPR RNA guides bacterial molecular machinery to a matching target sequence in the invading virus. The molecular machinery cuts and destroys the invading viral genome

the tracrRNA (trans-activating CRISPR RNA) generating a single guide RNA that recruits the Cas9 nuclease to specific genomic locations via standard Watson–Crick base pairing and facilitates double-strand break. The creation of site-specific double-strand breaks by the CRISPR/Cas9 complex then triggers genome editing through two different mechanisms: (1) repair through homologous recombination and (2) nonhomologous end joining. Notably, both pathways lead to functional inactivation of targeted genes with high efficiency, and thus CRISPR/Cas9 methodology has rapidly become the state-of-the-art technique for genetic manipulation of mammalian cells and genetically modified mice and has the potential to be used in a diverse range of gene therapy approaches in the future. Generation of the knockout mouse model for many disease studies has been possible by using adenovirus (AVV)-associated CRISPR/Cas9 system in recent years.

### 6.2.7 Application of CRISPR/Cas9

CRISPR/Cas9 is a simple and rapid tool that enables the efficient modification of endogenous genes in various species and cell types. A number of clinical trials using CRISPR/Cas9 system for genome editing are underway, and the first clinical trial involving CRISPR/Cas9-mediated gene modification has started in October 2016 at West China Hospital, Chengdu. CRISPR/Cas9 complex is nowadays an easy tool for many therapeutic approaches such as for immunotherapy in lung cancer, HIV, beta-thalassemia, Duchenne muscular dystrophy, hepatitis B virus (HBV) infection, and so on. CRISPR/Cas9 system is used in the current scenario and its applications are listed in Table 6.1.

## 6.3 Conjugation

### 6.3.1 Discovery of Conjugation

For many years it was thought that bacterial reproduction is only done by simple binary fission that splits a bacterial cell into two identical daughter cells excluding the exchange or recombination of genetic material. The very first evidence of exchanging the genetic material within the bacterial population was “conjugation”—a method of DNA transfer mediated by direct cell-to-cell contact. This result became part of the knowledge from a subsequent series of experiments conducted by Joshua Lederberg and Edward Tatum in 1946 (reported in Nature and the Journal of Bacteriology (JB) in 1946 and 1947). In this experiment, two auxotrophic strains were first selected and mixed which is further followed by incubation, culturing into a nutrient medium for many long hours, and plated on the minimal medium. Later, they observed a recombinant prototrophic colony on the minimal medium which has an incorporated recombination chromosome in each cell. Thus, this experiment suggested that the chromosome of two auxotrophs can associate with each other and undergo the recombination process (Fig. 6.8).

**Table 6.1** CRISPR/Cas9 implementation and its applications in medical science

| CRISPR/Cas9 use | Application |
|---|---|
| 1. CRISPR/CAS9 delivery to edit the mutation in fumarylacetoacetate hydrolase (genetic disorder) | Gene editing in mouse type I tyrosinemia model in vivo |
| 2. Gene correction by repairing the mutation in the CFTR gene for cystic fibrosis (genetic disorder) | Therapeutic |
| 3. Duchenne muscular dystrophy (genetic disorder) | Therapeutic |
| 4. Removing one or more exons from the mutated transcript by CRISPR/Cas9 system allowed for the production of truncated but still functional dystrophin protein in a mouse model of muscular dystrophy | Therapeutic |
| 5. Generating autocatalytic mutations to generate homozygous loss-of-function mutations | Generating homozygous animal model |
| 6. Gene deletion in mouse model to generate particular disease study model | Knock mouse model |
| 7. Injection of CRISPR/Cas9 components into zygote/ early stage embryo to modify genetic structure permanently | Genome editing in human |

However, Lederberg and Tatum failed to prove the concept of "physical contact between cells" which is the major requirement for gene transfer. But in the following years, 1950, Bernard Davis has demonstrated this gene transfer in "U tube" experiment. Bernard Davis constructed a U tube that contains two pieces of curved tube fused together at the base to form a "U" shape separated with a piece of fritted glass filter fixed between halves.

This filter does not allow the passage of bacteria; rather it allows the passage of the medium. During the incubation time, the medium was pumped back and forth through the filter to make sure that the medium is thoroughly switching between halves. After 4 h of incubation, bacteria were plated on the minimal media condition. And interestingly, Davis observed that when the auxotrophs were separated and cells were not in contact, the conjugation does not occur which means gene transfer needs direct contact (Fig. 6.9).

The next question rises to know what component or factor promotes conjugation. F factor (fertility factor)-associated gene transfer is the most common type of conjugation in bacteria that will be discussed in the next topic.

### 6.3.2 Discovery of Fertility Factor (F)

After being given the experimental evidence by Lederberg and Davis for conjugation, William Hayes in 1953 came up with the idea that genetic transfer occurred only in one direction in the abovementioned crosses. This is the reason that it has never been found that gene transfer in *E. coli* could be in a reciprocal manner. Thus, one cell must act as a donor, and the other cell must act as the recipient. This unidirectional gene transfer seemed to be compared with the original sexual difference between participants, according to which donor cell should be known as "male"

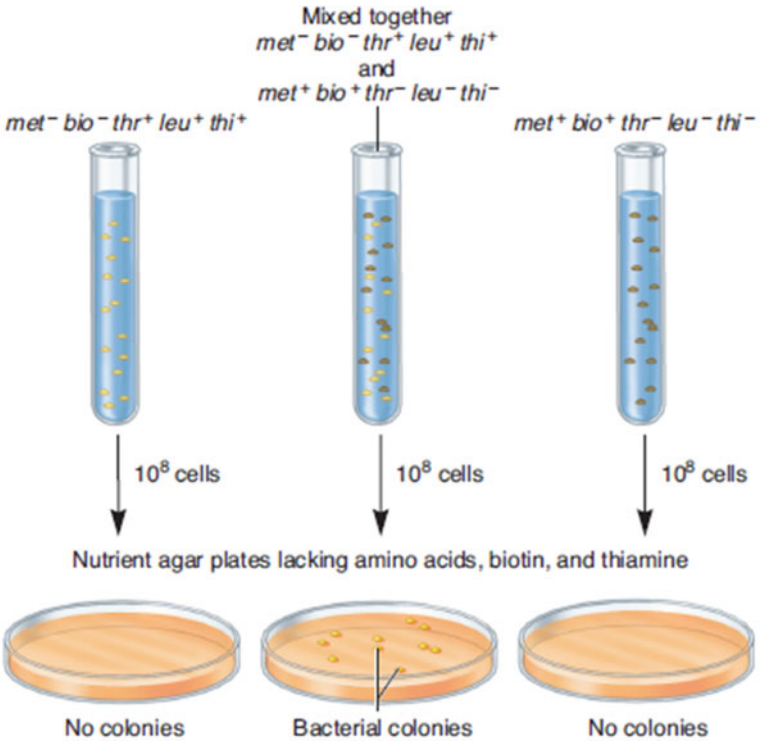


**Fig. 6.8** Experimental setup by Lederberg and Tatum. Tube 1 contains a single auxotroph population that has met− bio− thr+ leu+ thi+, meaning this bacteria contains the functional gene only for thr, leu, and thi (amino acid) while methionine and biotin genes were absent and that this bacteria cannot grow on the minimal media which is lacking all the essential amino acid and biotin needed for any bacterial growth. In tube 3, another auxotroph population was present containing met+ bio+, thi−, leu−, thi− which is an opposite composition in tube 1. In tube 2, both populations were mixed and incubated for 4 h. Later when this population was plated on minimal media from these three tubes, the population from tube 1 and tube 3 was unable to grow on minimal media, while mixed population from tube 2 was successfully grown on the minimal media, which shows that transfer of gene between these two population has occurred somehow which has given the new mutant colonies containing met+ bio+, thr+, leu+, thi+ genes. This experimental evidence has proven the concept of gene transfer in the bacterial population

and recipient cell should be known as "female." Although such gene transfer is only possible in eukaryotic organisms but not in bacteria and hence conjugation is not a type of sexual reproduction at all. In bacterial gene transfer, one cell that has to transfer the gene behaves similar to a donor, and the other cell which is supposed to receive the donor's genetic material and change its own genetic makeup behaves similar to a recipient cell, while sexual reproduction has equally contributed to donor's and recipient's genetic information. Lately, it was discovered that gene transfer in *E. coli* through conjugation is eventually driven by one of the circular DNA plasmids known as fertility factor or "F factor" which is sometimes also called

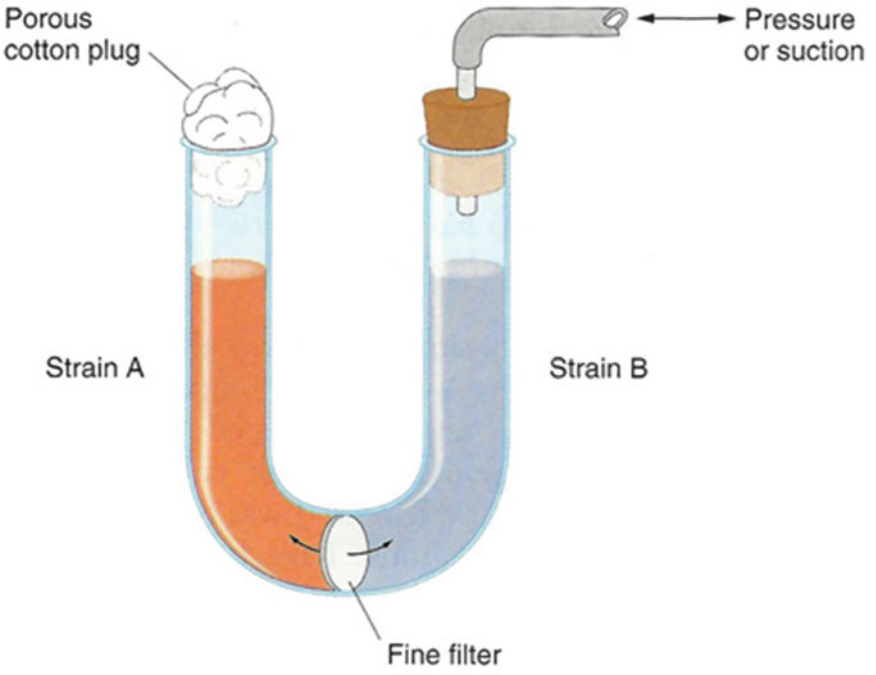


**Fig. 6.9** Bernard Davis U tube experimental setup to prove that bacterial mating or physical contact is a must for gene transfer through conjugation. When auxotrophic strain A and auxotrophic strain B were plated on the minimal media and incubated for few hours, no growth was been observed on the media which confirms that no gene was transferred between the bacteria when they were separated through the filter paper

as sex factor. F factor is found in some species but not in all bacterial cells. First, we need to understand the characteristics of the F factor prior to following its role in conjugation.

The size of F factor varies from few kb to 100 kb in the form of duplex DNA keeping two distinct replication origin regions (Fig. 6.9). Among these two, the bigger one is denoted as *ori V* or vegetative replication region which is a point that supports the F factor to replicate autonomously in a particular situation when the plasmid is not being transferred such as cell division of F plasmid; this origin is bidirectional, whereas *ori T* is unidirectional and responsible for replication and transfer of F factor to the recipient cell. F factor shows the similar copy number of a plasmid as bacterial chromosome shows, and therefore one bacterium has one or two copies per bacterial chromosome.

The conjugation process is regulated by *sex pili* or F pili, a thin rod-like structure that appears as an extension of the cell wall. A protein subunit of pili is pilus coded by gene *tra* which polymerizes into pili. Bacteria carrying F plasmid (male or donor) attach to the recipient bacterial (female or recipient) cells for conjugative transfer. An F-positive bacterium has 23 pili on the surface and a *tra* operon encoding

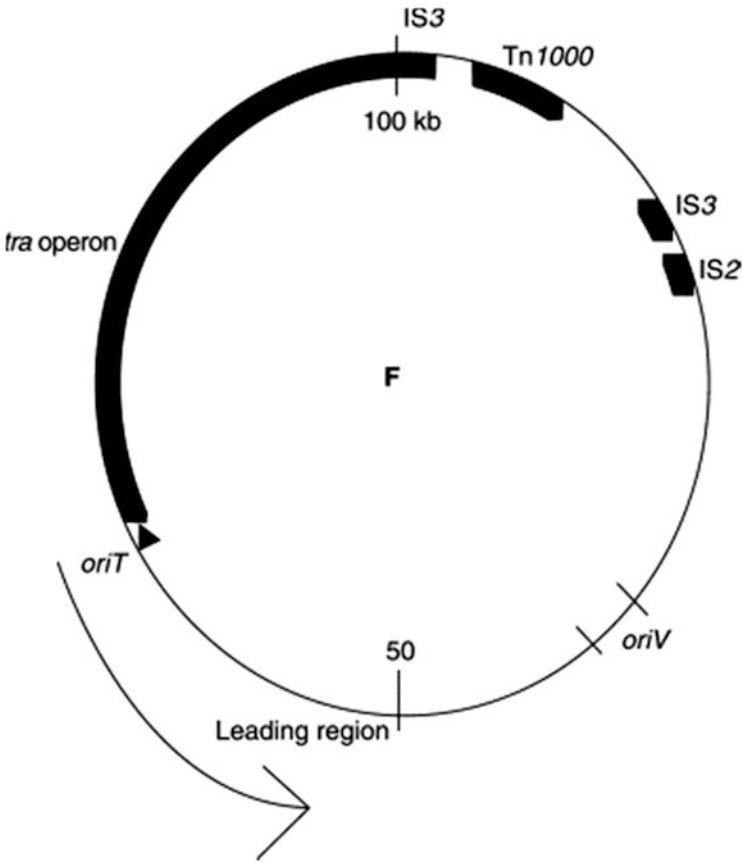


**Fig. 6.10** A physical map of F plasmid (100 kb). This circular DNA is further divided into (1) *ori* V which is responsible for autonomous replication, (2) *ori* T which is the origin of replication and transfer of F plasmid, (3) *tra* operon which encodes the functional factor required for conjugation, and (4) IS3, IS2, and Tn 1000 which are transposable elements. The thin arrow indicates the direction of replication

30 functional genes that promote the transfer of the F plasmid (Fig. 6.10). Other than this, F plasmid has three *transposable elements* incorporated in the structure, in which two copies are of insertion sequence—IS2 and IS3—and one is transposons Tn1000 (sometimes known as γδ).

During the experiment, a variant of the F factor has been discovered accidentally by Hayes. He has observed that a variant from its original donor did not reproduce a recombinant after crossing with the recipient strain. This observation reflects that this donor cell apparently had lost the ability of gene transfer and had converted into recipient-like strain known as "sterile donor."

Through this analysis, Hayes realized that fertility characteristics (ability to donate) of *E. coli* could be silent and recover easily. Thus, he suggested that the donor ability of F factor shows a hereditary phenomenon.

### 6.3.3 $F^+$ and $F^-$ Bacteria

As we have discussed in the previous section, gene transfer is done through the fertility factor (F factor) in a donor cell, designated as $F^+$ bacteria, whereas bacteria that is a recipient and lacking F factor is designated as $F^-$ bacteria.

The F factor includes an origin of replication and genes required for conjugations as discussed previously (see Fig. 6.10). $F^+$ bacteria produce sex pili (singular known as pilus) that facilitate a physical contact between $F^+$ and $F^-$ to pull them together (Fig. 6.11). The most important fact about conjugation is that this type of gene transfer can only take place cells that contain F and cells that lack the F factor. Detail mechanism is highlighted in Figs. 6.3 and 6.4.

### 6.3.4 Hfr Bacteria

Hfr (*high-frequency recombination*) bacteria simply became in existence immediately after $F^+ \times F^-$ mating. It has been suggested that this is the second type of F bacteria that maintains a higher frequency of conjugation than the $F^+$ bacteria. At first, Lederberg and Tatum have observed that conjugation between $F^+$ and $F^-$ allows the transfer of genetic material in F plasmid but does not account for the transfer of chromosomal genes. Hfr-mediated conjugation transfers the donor chromosomal gene with a great efficiency into the recipient cell but does not change the

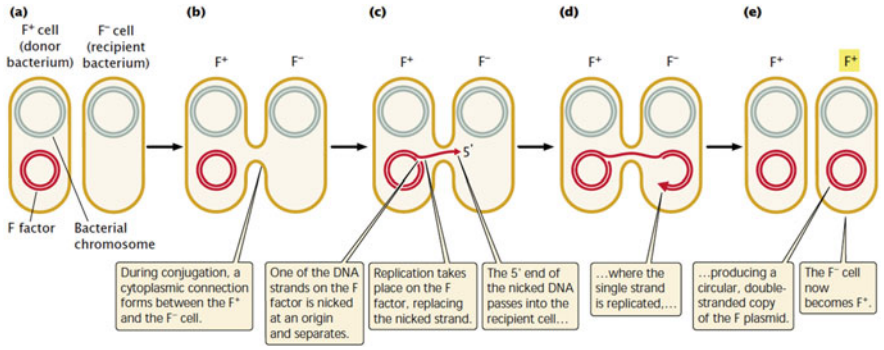


**Fig. 6.11** Gene transfer mechanism in F+ and $F^-$ cells. (**a**) $F^+$ is a donor cell that will transfer the F factor to the $F^-$ (recipient cell). (**b**) A conjugation tube or a bridge begins to happen between these two cells. (**c**) Single-stranded DNA is generated by nick at the origin and separates it from the double-stranded circular DNA. (**d**) This 5′ nicked single-stranded DNA is transferred across the cells and enters in the recipient cell where single-stranded DNA begins to replicate and convert the $F^-$ into $F^+$ cell. (Benjamin. A. Pierce., Genetics: A conceptual approach)

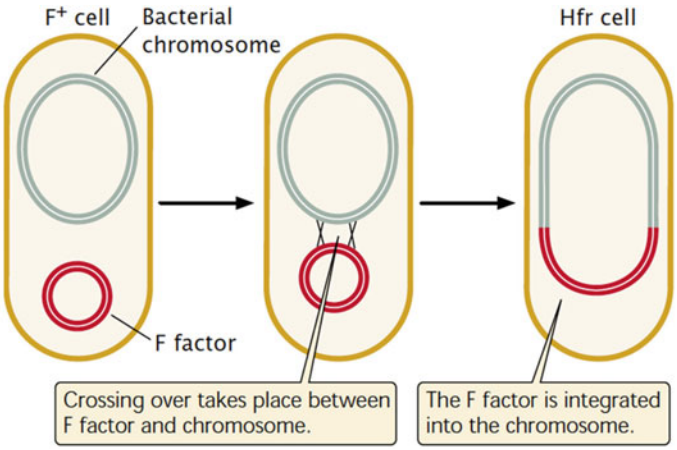


**Fig. 6.12** Construction of Hfr cell from $F^+$ cell. Integration of F factor to the bacterial chromosome in $F^+$ cells which converted the $F^+$ cell into Hfr strain containing both the features of plasmid and the bacterial chromosome. (Benjamin. A. Pierce., Genetics: A conceptual approach)

$F^-$ into $F^+$, and this is because of a partial transfer of chromosome into $F^-$ which does not change the cell into $F^+$ unless the entire chromosome has been transferred. If the F factor is integrated into the chromosome and this chromosome has to be transferred, the chromosome will require 100 min in case of *E. coli*, but unfortunately, the conjugation breaks before the process is finished. At last, the F factor is not completely transferred to the recipient cell, and it remains $F^-$.

Thus, Hfr strains contain F integrated chromosome, and $F^+$ cell further can form sex pili and conjugate with $F^-$ cell (Fig. 6.12).

In the mating between Hfr and $F^-$ cells (Fig. 6.13), integrated F first nicked at the one end on one strand. This nicked end moves toward $F^-$ cell similar to the conjugation between $F^+$ and $F^-$ cells. Since the F factor is integrated with the bacterial chromosome, nick transfer further allows the transfer of chromosomal fraction into the recipient cell. The amount of transferred chromosomes depends on the duration of conjugation between the two cells and how long they are connected.

Once the nicked and single-stranded DNA is transferred to the recipient cell ($F^-$), it starts to replicate, and sooner the crossing/recombination between the donor and the recipient chromosome will take place. When the crossing over takes place in the recipient cell, degradation of the donor chromosome occurs instantly. The recombinant recipient chromosome remains intact in the cell and starts to replicate and pass to generations. It is already mentioned in the mating between Hfr and $F^-$ cells that $F^-$ will not become $F^+$ or Hfr unless the entire F factor (F-integrated bacterial chromosome) will receive an $F^-$-recipient cell. This event seems to be a rare case or time-consuming because most of the conjugations last for only a short time and break cells apart any time before the chromosome could have been transferred.

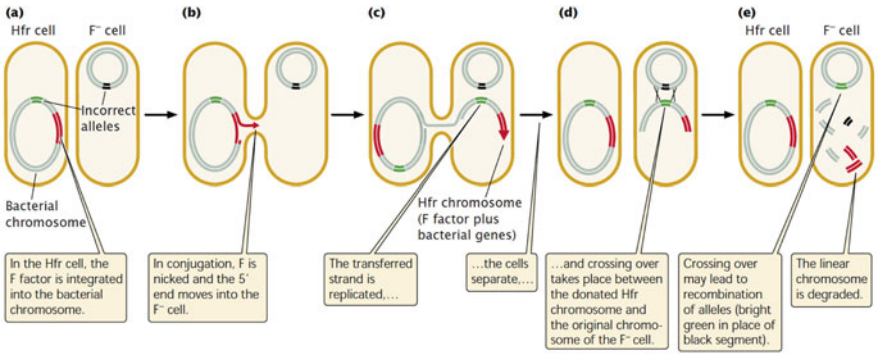


**Fig. 6.13** Mating between Hfr cell and $F^-$ cell. Mating takes place between Hfr and $F^-$ cells which have been described in steps (**a**) to (**e**). This process is time taking so it occurs rarely in the nature

### 6.3.5 Mapping of Bacterial Chromosome

Before inventing genome sequencing, microbiologists had only one genetic approach to elucidate the organization of genes in bacterial chromosomes and that is linkage analysis. Genetic mapping using linkage analysis is a tedious task for them. To date, three methods for mapping have been identified: (1) interrupted conjugation, (2) transformation, and (3) transduction. All of them have important similarities and differences which are discussed in this section.

In bacterial genetics, to map the relative location of bacterial genes, Hfr conjugation method has been used more often. In Hfr conjugation, when Hfr and $F^-$ are mixed in one culture, Hfr strain begins the chromosome transfer immediately. Transfer of chromosome from donor to recipient is not synchronous but occurs at a constant rate and over a period of minutes. The mapping is done by using an interrupted conjugation. Within an interrupted conjugation, a bridge between donor and recipient is broken, and thus matting between Hfr and $F^-$ is interrupted at various intervals. This technique is organized after some time when the conjugation begins in a mixture and is interrupted by vigorous agitation in a blender (Fig. 6.9).

Through this method, the order of gene transfer and time intervals can be interpreted because timing is a direct reflection of the gene order on the bacterial chromosome (Fig. 6.14). In the given graph, X-axis of the curve demonstrates the time point at which the gene began to enter the recipient cell. The demonstrated curve is a circular chromosome map where the distance between every gene is equals the minutes lapsed until the gene is passed to the recipient cell. In the graph, the gene that is more distant from F factor (origin of replication) shows lower plateau compared to gene that is in the vicinity of F factor. In the given example of *E. coli*, trp is the distant gene from the F factor, and thus there is even a greater chance that the conjugation bridge will beak spontaneously before the trp gene is transferred to the cell. To generate the map, it is important to know that the transfer of genes always starts within the point of origin that is the F and thus the orientation and position of F on the chromosome determine the direction of the starting point of

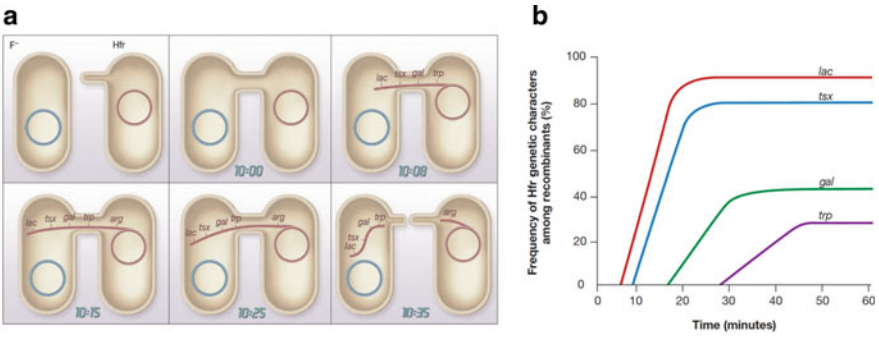


**Fig. 6.14** Mapping of the bacterial chromosome by using Hfr and $F^-$ mating system. Hfr and $F^-$ interrupted conjugation experiment at time intervals. (**a**) Schematic diagram in the left panel shows that a linear transfer of genes has been paused and a discontinued conjugation bridge is taken into the consideration of the sequence of gene transfer from donor to the recipient cell. (**b**) In the right panel, the graph shows a relationship between time intervals at which a particular gene has been transferred into the recipient cell obtained by an interrupted conjugation experiment. From the graph, we can see that the gene order is lac–tsx–gal–trp

gene transfer. In the given example, the point of origin (F factor) is just immediately before the *lac* gene in the chromosome. Since the genome of *E. coli* is relatively larger, mapping is quite lengthy through Hfr strain. Therefore, the easy way to do so is to let the several Hfr strains integrate with F plasmid at different locations, and all the fractions of the map obtained through these different locations must be superimposed to create the entire map of *E. coli*. The overall map is adjusted to 100 min in the case of *E. coli*. In this sense, the term "minutes" not literally indicates the measurement of time but the distance between the genes on the map.

Gene mapping through transformation follows a few steps including the separation of DNA from the donor strain, fragmented and integrated to the recipient strain. Loci that are widely separated on the fragmented DNA from the donor chromosome and always carried by two different fragments, then the frequency of cotransformation is different from the single transformants of per$10^3$ recipient (a normal transformation occurs at the rate of one cell per $10^3$ recipient). If the two loci are very close to each other and are carried by one fragment, the rate of cotransformation must be similar to a single transformation rate. Thus, the cotransformation will provide the information of the order of genes on the donor chromosome and will guide to map the genome.

Mapping through transduction is quite similar to transformation and also depends on the gene transfer but between two different bacterial traits. Gene transfer occurs through the bacteriophage. Similar to the transformation, small fragmented DNA will be cotransformed by phage from donor to the recipient strain. Rates of cotransformation in transduction will help to calculate the relative distance between genes and to create a genomic map.

In conclusion, all the three modes of gene transfer—interrupted conjugation, transformation, and transduction—are based on the same basic strategies that are

used for mapping. The way of DNA transfer is slightly differing such as through the physical contact between bacteria in interrupted conjugation with interrupted conjugation, small naked fragmented DNA in transformation, and fragmented DNA through bacteriophage in transduction.

Using these techniques, researchers mapped about 2200 genes of *E. coli* K12 and compared this with the actual nucleotide sequence of the genome (i.e., physical map of the genome). Genome sequencing has revealed about 4300 possible genes. Thus, genetic analysis is defined over half of the potential genes. The genetic map approximates the physical map, but they do not correspond perfectly. This is because the genetic map is derived from genetic linkage frequencies that do not correlate exactly with the number of nucleotides that separate the two genes. Roughly speaking, 1 min of the *E. coli* genetic map corresponds to 40 kilobase of DNA sequence.

### 6.3.6 $F^+ \times F^-$ Mating

It was demonstrated by William Hayes in 1952 that the gene transfer observed by Lederberg and Tatum was one-directional and had performed between polar cells. Therefore, it is predictable that there must be cells having characteristics of a donor (F+ or fertile) and the recipient ($F^-$ or infertile). This type of gene transfer is nonreversible.

An extra chromosome such as F factor in the $F^+$ strain encoded sex pili which is an essential need for plasmid transfer. Major role of sex pilus is to establish a physical cell–cell contact between the $F^+$ and $F^-$ mating. Figure 6.11 shows the mechanism through which bacteria can transfer its plasmids such as F to the recipient cells. Once $F^+$ and $F^-$ cells come into the vicinity, the F plasmid of $F^+$ strain directs the pili synthesis followed by its projection toward recipient cell to make contact and pull the recipient cell closer (Fig. 6.15). This protruding pilus makes a pore on the recipient cell, and thus F plasmid passes through this pore into the recipient cell. It is notable that during this transfer DNA does not transfer in a double-stranded form while it carries only one strand of F DNA, which initiates replication of the complementary strand in the conjugation tube (basically bridge like structure) which connects both the donor and the recipient cell. This replication concluded in two copies of F DNA, one remaining in donor and one appearing in the recipient cell as shown in (Fig. 6.15).

Replication of the F factor is accommodated by rolling mechanism and replication initiated by the help of a protein complex known as "relaxosome." This relaxosome first recognizes *ori T* (Fig. 6.10) site and nicks one strand from this point. Relax enzyme is a part of this relexosome and remain attached at the 5′ end of the nicked strand. During the replication of the F plasmid, replicated strand displaced and attached relax enzyme move along through the type IV secretion system to the recipient cell. Because pilus is embedded into secretion system, it has been suggested that the DNA moves through a lumen in the pilus.

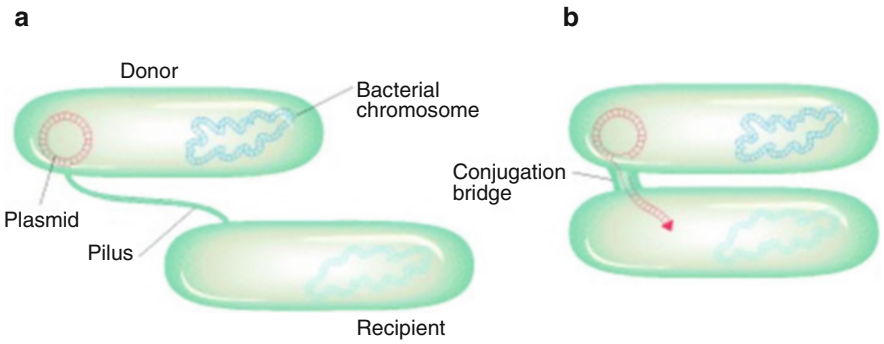


**Fig. 6.15** F+ and F− mating system. Conjugation between $F^+$ and $F^-$ initiates with pilus formation and pulls them together and during conjugation, shown in (**a**). This pilus formation and cell-to-cell contact are further followed by the formation of a bridge or a pore (essential passageway) between two cells. Single-stranded DNA passes into the recipient cell and becomes double strand by rolling replication mechanism as shown in (**b**)

### 6.3.7 F Plasmid

Plasmid has the capacity to replicate independently and to integrate into the bacterial chromosome. F plasmid is one of the important factors also known as fertility factor that integrates with bacterial chromosome to generate Hfr cells. Homologous recombination site present on either bacterial chromosome or F factor allows repairing and release of F plasmid. Occasionally integrated F plasmid exists from the bacterial chromosome by reverse recombination process. F factor is responsible for mating and gene exchange between bacteria and so the conjugation process is a totally F plasmid-dependent process. Most importantly, the F plasmid contains an origin site for replication and other genes required for conjugation and sex pili formation to make contact with the recipient cell ($F^-$ cell). DNA is always transferred from $F^+$ to $F^-$. Thus, F plasmid is essential and needed for conjugation in bacteria. One more important thing about F plasmid is the integration of F plasmid into a bacterial mutant (known as F′) that is unable to replicate known as "integrative suppression." For its own replication, F plasmid uses many *E coli* replication proteins, but it does not use the *dnaA* protein usually required for bacterial chromosome replication. In case if dna A is mutated in bacteria and temperature is elevated to the 42 (on which dna A is inactivated), initiation of replication in bacterial chromosome will not be possible, but F plasmid will replicate since it has its own origin of replication. In *dnaA* mutant strain where the F plasmid is integrated into the chromosome, the replication of the chromosome is still possible and independent at high temperatures. However, replication does not occur at the origin of the chromosome and instead occurs at *oriV*, origin site on the F plasmid. In this way, integration of F plasmid suppresses the importance of *dnaA* as a phenotype by replacing it with F plasmid-derived replication.

Note that it is also tricky to select the F integrated positive strains, and therefore integrative suppression can be a possible way to select those that have integrated F

plasmid. In a given example of mating between F′ *Lac*$^{+}$*/trp*$^{s}$ and *Lac*$^{-}$ *(dnaA)/trp*$^{r}$ would result in the colonies to be selected on high temperature and lactose/tryptophan selective medium. Colonies were successfully survived on the medium and high temperature should have contained an integrated F′ *Lac*$^{+}$.

### 6.3.8 R Plasmid

A bacterial chromosome often regulates antibiotic sensitivity or resistance. Alteration in the pathogenicity of bacteria is thought to occur due to another plasmid that allows the bacteria to grow in the host cell escaping the toxic effects of antibacterial drugs. Antibiotic resistance is a net result of a group of genes located on the R plasmid. Drug-resistant R plasmid has evolved in the past 60 years. The Discovery of antibiotic resistance came into existence after the incident in a Japanese hospital, where the first time an antibiotic resistance has been observed in *Shigella*; a bacteria causes dysentery, which later on with multiple treatments of antibiotics (penicillin, tetracycline, sulfanilamide, streptomycin, and chloramphenicol, etc.) has become resistant to drugs. Similar to the F plasmid, R plasmid contains circular small DNA that has divided into two fragments; one fragment contains a group of gene responsible for replication and copy number, transfer of gene, and sometimes resistance to the tetracycline, and another fragment includes the number of other resistance gene or factor (thus known as R factor or R plasmid). R plasmid serves several genes to act on antibiotic resistance and establish antibiotic resistance in the strains. R plasmid carrying the marker for resistance like chloramphenicol (Cam), streptomycin (Str). Stone, A. B. has mentioned in his study that R plasmid is a major factor that is responsible for the epidemic spread of multiple drug resistance throughout the bacterial population. For example, research shows that plasmid carrying genes for resistance to multiple antibiotics might have been transmitted from a cow udder infected with a hand towel. The practice of milking an infected cow followed by a farmer cleaning his hand with a towel was the reason for the antibiotic resistance passage from bovine to the human–microbe reservoir. This incident shows a severe problem in antibiotic therapy since plasmid transmission is not restricted to particular species or genera, and it may invade the unrelated species or population.

## 6.4 Transformation

Transformation is another method for gene transfer in bacteria that facilitates a DNA transfer without any donor bacteria or physical contact, rather a naked foreign DNA exogenously being uptakes by the recipient cell. This method suggested that bacterial genetic information is somehow transferable within the bacterial population where physical contact unlike conjugation is not needed. This discovery belongs to the time before when DNA structure was discovered.

### 6.4.1 Discovery of Transformation

Transformation is the second most abundant mode of gene transfer after conjugation. In the transformation, a naked DNA or partial DNA fragments are uptake by the recipient cell from the environment and incorporated into the recipient's chromosome as a result of recombination.

Transformation introduced a gene transfer method that allows the transfer of one genotype into another by the exchange of exogenous DNA. First-time transformation was confirmed in *Streptococcus pneumoniae* in 1928 by Frederick Griffith. Following this, in 1944, Oswald T. Avery, Colin M. MacLeod, and Maclyn McCarty explained that the "transforming principle" was none other than bacterial DNA in this method. This experiment was the first explanation that reveals the phenomenon like DNA can be transferrable.

Fredrik Griffith's experiment is shown in Fig. 6.16, where he used two distinguished *Streptococcus pneumoniae* strain; one is virulent and has lethal effect on most laboratory animals, and other one is nonvirulent and is not lethal for animals. Virulent strain designated as (S) and enclosed by polysaccharide cover (capsule like structure) and gives a smooth appearance when it grows on the medium so easily can be detectable on culture. Nonvirulent strain designated as (R) strain which is lacking any cover or capsule shows rough appearance when it grows on medium and thus can be detectable on medium. Griffith first boiled some virulent strains to kill them and injected these heat-killed strains into the mice, and he observed that these mice survived, and the cells do not show any lethal effect on the mice. In the next round, a mixture of heat-killed virulent cells and live nonvirulent cells were injected into the mice and mice did die. He isolated the live cells from the dead cells and grew them on the medium, gave the smooth colonies, and showed virulent characteristics on subsequent injection to the other mice. These results made him realize that heat-killed virulent (S) cells somehow converted the nonvirulent live cells (R) into live virulent cells (S) which was the reason for the death of mice (Fig. 6.16).

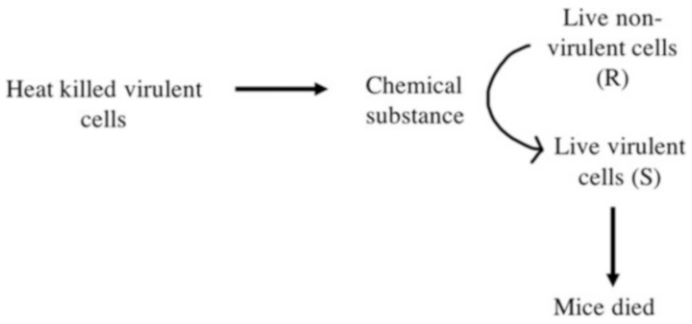


**Fig. 6.16** Transformation of the nonvirulent live cell (R) into the live virulent cell (S). An experimental setup explains that chemical substances produced from heat-killed cells someway transferred into live nonvirulent cells and transformed it into live virulent cells (S). This substance can be any biomolecule whose competency is to be transformed is chemically unknown

But unfortunately, Griffith was not able to reason why this had happened and what made the nonvirulent live cells behave like virulent live cells?

Next to this achievement, a question was awaiting that what was the chemical composition of dead donor cells had caused this transformation? Was this protein or any other element? Since it was clear that this substance that had changed the genotype of the recipient cell must be something transmissible or heritable. A group of Oswald T. Avery, Colin M. MacLeod, and Maclyn McCarty solved this doubt when they proposed a study to destroy all the chemical substances of the dead cells and see the transforming capacity of this dead extract from the cell. They found that this mixture still could develop the ability of transformation. However, heat-killed virulent cell contains polysaccharide coat while live nonvirulent does not contain any coating; hence, it is assumed that the transforming agent could be this polysaccharide coat. Destroying polysaccharide coats does not diminish the transforming activity. Proteins, fats, and RNAs show similar results not to be transforming agents.

An interesting result obtained when the mixture was treated with DNase, it lost its transforming ability which strongly suggested that DNA was the genetic material that has transformed from one bacteria (dead virulent) to other bacteria (live nonvirulent), named transformation (Fig. 6.17).

It is noteworthy that natural transformation is a random process between two bacterium and DNA transfers from the donor bacterium to Hfr + bacterium. Any portion of the entire genome may be able to transfer.

### 6.4.2 Transformation Process

In the previous section, studies on transformation in *S. pneumoniae* have been intensively explained and now it has to be understood how this process takes place? An entire transformation involves (1) the uptake of foreign DNA from the surrounding and (2) its incorporation in the recipient bacterial chromosome or plasmid. Naturally occurring transformation takes DNA from the dead bacterium which might have been released to the environment after fragmentation of the DNA due to the death of the bacterium. Thus, the marine and soil microbiome utilizes this mode of transfer of genetic material at a massive level. A cell that uptakes the DNA is known as a competent cell that is the target for exogenous double-stranded DNA. This DNA was later cleaved and processed by endonuclease into double-stranded chunks of 5–15 kb size. The entire process of transformation is energy dependent. During the DNA uptake process, one strand of DNA is hydrolyzed by an envelope associated with exonuclease while the other strand is coupled with small proteins and move along with the plasma membrane. Thus, only one strand of DNA is available for interaction with recipient DNA. Fragmented single-stranded DNA aligns with the homologous region on the recipient genome and is integrated successfully.

However, transformation in *Hemophilus influenzae*, a gram-negative bacteria, unlike *S. pneumoniae* is different in many aspects of the transformation process. It

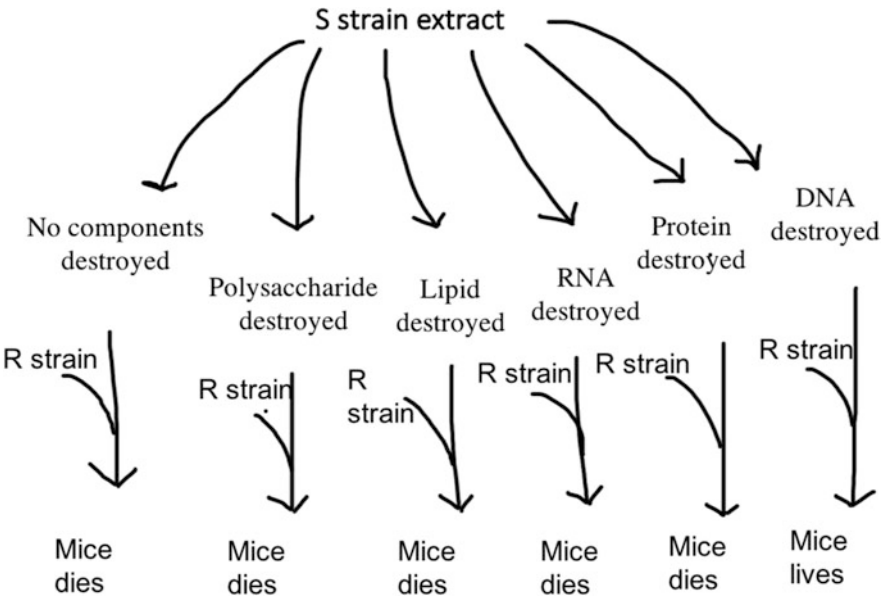


**Fig. 6.17** Experimental evidence for the transformation of DNA in mice between different strains. S strain was recovered from the live cells, all the chemical components were destroyed step wise, and the extract mixture was reinjected with the R strain into the mice. As the result, (1) R strain converted into S strain when no component destroyed, (2) R strain converted to S stain when polysaccharide destroyed, (3) R strain converted to the S strain when lipid destroyed, (4) R strain converted into S strain when protein destroyed, and (5) R strain does not convert into S strain while DNA destroyed. Thus, these results confirm that when all the other components were destroyed but not DNA, R strain was not able to be transformed into virulent death causing lethal S strain, and destroying the DNA makes the R strain incapable to transformed into s strain and mice left alive

does not produce competence factor to make cells competent and it takes up DNA from closely related *S. pneumoniae*; thus, the case of *H. influenzae* transformation is a selective method while in the case of *S. pneumoniae* is less particular about the source of DNA.

It has been demonstrated that the specificity of *Hemophilus influenzae* transformation came from the 11 base pair sequence, 5′AAGTGCG-GTCA3′, which exists in a repeat over 1400 times in DNA, and this repeat must bind with competent cells. Nonetheless, transformation is not restricted to only a few selective bacterium DNA rather a DNA source for a competent call can be anything in an appropriate condition (Fig. 6.18).

However, recombination or crossover is common in bacteria but efficiently DNA uptake is limited. Even it has been seen that a species is capable of transformation, but a very small fragment of DNA is transformed in a growing population of bacteria. For a long time, bacterial geneticists were in the search of developing a novel technique to increase the transformation frequency that may enhance the DNA uptake into the cell. Hence, transformation supplemented with an artificial add-on

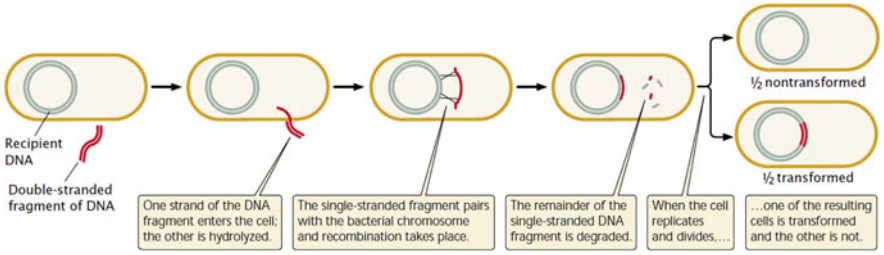


**Fig. 6.18** Gene can be transferred between bacteria through transformation. Transformation in the bacterial cell initiates with DNA uptake and its integration to the bacterial chromosome that procures a recombinant DNA that contains both bacterial and exogenous DNA intact in the daughter bacterial cell

such as calcium chloride in the medium, heat shock, or electric field makes the cell membrane more porous and permeable to uptake DNA more efficiently. Increasing the DNA concentration will also work as an enhancer for transformation efficiency. These enriched techniques for the transformation of foreign DNA into any cell are vastly in practice for molecular biology studies in laboratories.

### 6.4.3 Transformation Linked Genes

Likewise, conjugation transformation has been used to map the bacterial gene, especially for those species that do not undergo conjugation or transduction. Mapping in such strains is only possible when they are entirely different genetic strains or traits that have to mate through the transformation. For example, a recipient strain might be auxotrophic for three nutrients $p^-$ $q^-$ $r^-$ (in the figure), mate with donor cell, and prototrophic for alleles $p^+$ $q^+$ $r^+$ (in the figure). DNA from the donor strain is treated and fragmented to increase competency. Fragmented donor DNA is added to the medium of the culture of recipient strain (competent cells). Eventually, fragmented donor DNA enters the recipient cell and immediately undergoes recombination. The recombination process must be followed by a homologous sequence on the recipient bacterial chromosome where the donor DNA is attached and intact throughout the procedure. Recipient cells that positively have received the genetic material from the donor cells through transformation are called "transformed."

How to understand that how many genes and what frequency they have transformed? To this end, we first need to observe the rate at which two or more genes are transferred together (usually termed as cotransformed). The recombination rate of these genes is the basic need for the measurement of transformation frequency. Gene can be mapped by observing the rate at which two or more genes are transferred together (cotransformed) in transformation. We assume that genes that are physically close to each other on the same DNA after fragmentation are more likely to be transformed contiguously into the competent cell. For example, in Fig. 6.19, gene p and q on the DNA of donor strain are physically linked so that

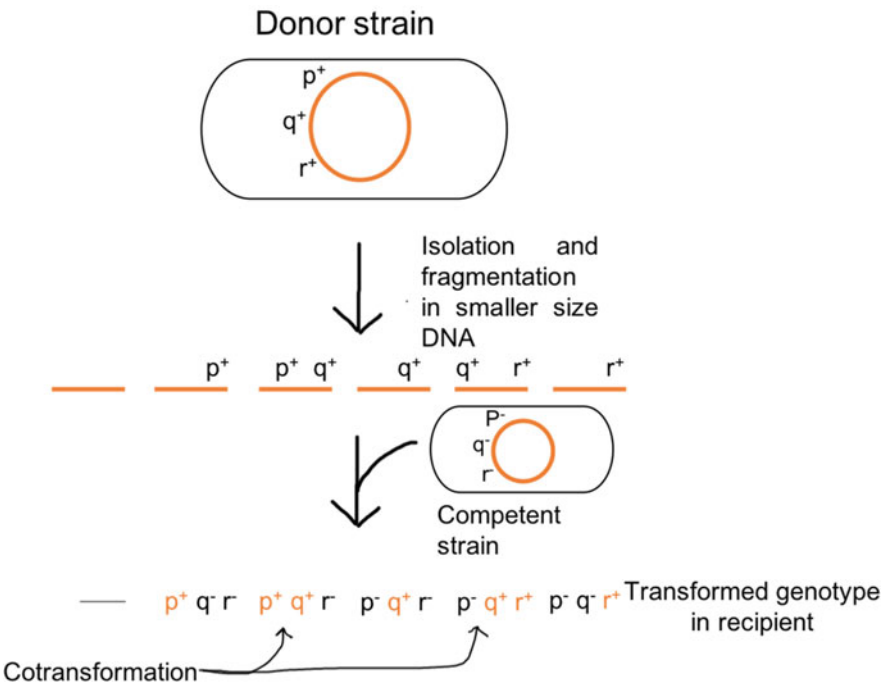


**Fig. 6.19** Transformation and linkage for mapping the bacterial genome. Gene p and q are close enough to be transformed together, gene q and r are also close enough to transform together and therefore genotype observed as (1) $p^+ q^+ r^-$ and (2) $p^- q^+ r^+$ are cotransformed. Note that $p^+ q^+ r^+$ and $p^+ q^- r^+$ are rare genotypes because $p^+$ and $r^+$ are two distant genes transformed together, and thus, the rate of cotransformation is inversely proportional to the distance between the genes

they would preferably transform together. However, genes that are far apart are unlikely to be present on the same DNA fragment and rarely will be transferred together. In Fig. 6.19, we can observe that gene p and r are separated from each other and no fragments are produced containing $p^+ r^+$ on the same DNA and therefore we have not observed cotransformed for $p^+ q^- r^+$ which is the rarest event.

Thus, after performing the transformation, transformed colonies must have been obtained on selective media and performed the genotyping of each strain. Let us assume that if gene p and q are frequently cotransformed and gene q and r frequently cotransformed, then gene q must be in between p and r and the gene order on the DNA must be *p q r*.

## 6.5 Transduction

We have already spotted a light on bacterial gene transfer in the previous section. Transduction is the third type of gene transfer after conjugation and transformation. So, let us take a closer look at the third way of gene transfer in which gene is transferred between bacteria through the phage/bacteriophage. Transduction is a type of horizontal gene transfer that occurs naturally via phage infection.

Based on structure, the virus is simple, often composed of just a nucleic acid, and the genome is always protected by a protein coat known as a capsid. Phage does not replicate autonomously; instead, they first infect the cell, take control of host machinery, use it, and force the host cell to produce multiple copies of phage particles. Most phages initiate their replication immediately after infection. When a phage begins the replication, it reaches a certain number of copies or let us say a threshold number of copies that cause bursting of cells and produces many new phage progenies to further infect new bacterial host, such phages known as a virulent and the process is called "Lytic cycle." Few bacteriophages do not kill the bacteria immediately after infection and instead insert their genome into the bacterial genome without affecting it. The inserted phage called **prophage**. These bacteriophages passively replicated as the host cells genome does and thus this bacteriophage is known as **temperate bacteriophage** and the relationship between phage and its host cell is called **lysogeny** (Fig. 6.20). Temperate phages can remain inactive as an insert for many generations in their hosts. However, they are prone to some conditions like UV irradiation which can induce lytic cycle temperate phages.

Transduction is categorized into two: (1) generalized transduction in which any gene may transferred and (2) specialized transduction where only few genes are possibly transferred. How this transduction has been identified by researchers? It is discussed in the next section.

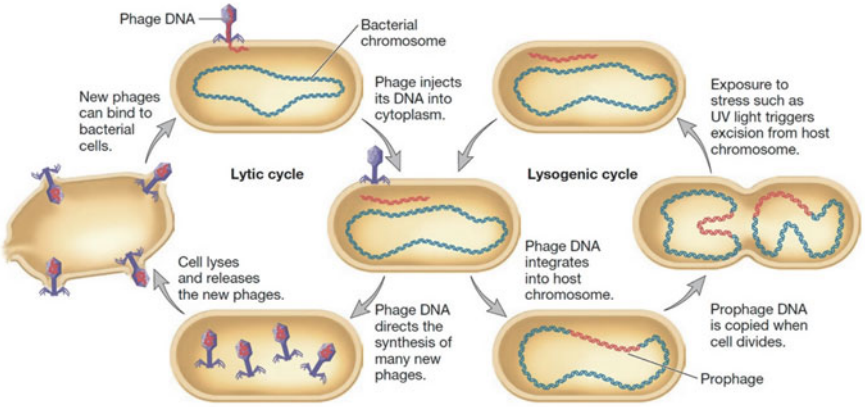


**Fig. 6.20** Transduction comprised of lytic cycle and lysogenic cycle. A typical transduction process has both lytic and lysogenic cycles which either follows one each after or remains separated as shown in the image

### 6.5.1 Discovery of Transduction

Joshua Lederberg and Norton Zinder in 1951 were testing the recombination in *Salmonella typhimurim* by using the same techniques as Lederberg had found in *E. coli.* For their experiment, they used two distinguished strains of *Salmonella*; one was auxotroph for $phe^-$, $trp^-$, $tyr^-$ and the other was auxotroph for $met^-$, $his^-$. They mixed these two strains in one culture and plated on the minimal media, then similar to the E.coli experiment, they did not observe any wild-type strain When these two strains were plated on minimal medium, no wild-type cells were observed; however, at a low frequency of about $1/10^5$, recombination was observed. In the discovery of transduction, researchers referred to the U tube experiment with few modifications. They put the porous filter instead of the fritted filter paper in the conjugation experiment to prevent cell–cell contact. Later they observed that the agent responsible for the recombination is the size of the phage of P22 which is a known temperate phage of *Salmonella*. Furthermore, many studies together suggest that the vector for recombination is a P22 phage. But there was uncertainty among the researchers whether this filterable recombination agent is a phage or something else. Therefore, a comparison of the properties of this agent with phage, where it shows the similarity in the size, sensitivity to the antiserum, immunity to the hydrolytic enzyme, and so on has confirmed its virus alike characteristics.

As a result, Lederberg and Zinder have confirmed this new type of gene transfer through the virus and named it transduction instead of conjugation.

However, in the lytic cycle, sometimes phages interact with host DNA and integrate their own DNA that is then transferred to another host bacteria and insert its contents into bacterial DNA. Both temperate and virulent phages follow the transduction method to transfer the genes.

### 6.5.2 Generalized Transduction

Besides understanding the transduction further, it came with the next question that how transducing phages are reproduced after infection? To address this question, in 1965, K. Ikeda and J. Tomizawa had discussed the experiment on temperate phage P1 in *E. coli*. They highlighted in their experiment that when P1 infected the *E. coli* and lysed the donor cell where bacterial chromosome was broken up into small fragments; however, some of these pieces were captured mistakenly by phage particles and assembled in the head instead of phage DNA. Eventually, this has become the source of transducing phage.

During the infection, phage capsid (coat proteins) determines phage's ability to recognize and attack the host bacteria and transfer its content to the host cell. But now in the case of transducing phage, the transferrable material is the donor's chromosome which the phage had assembled during the transduction. Interestingly, transduction through the transducing phage could rise the situation of merodiploid (a partial diploid bacteria) since it transferred the donor's chromosome which recombined with the recipient chromosome and now the recipient cell will have

two bacterial chromosomes which is a merodiploid situation (Fig. 6.21). This type of transduction allows the passage of any kind of host (bacterial) markers to other bacteria and is thus known as generalized transduction where any gene can possibly be transferred to another bacteria.

Phages P1 and P22 belong to the group that shows features of generalized transduction. Looking at their cycle, P1 phage is usually integrated to the host chromosome while P22 remains free in the cytoplasm.

### 6.5.3 Specialized Transduction

Unlike generalized transduction, specialized transduction will allow a few particular genes (few host markers) to be transduced as shown in Fig. 6.22.

λ phage is very well known for specialized transduction. λ prophage (shown as the red circle in the figure) integrated between gal and bio region of host chromosome (shown blue linear strand in figure) as shown in Fig. 6.23. Hence, λ can transduce only the gal and bio genes. Let us understand the mechanism of λ transduction.

Recombination between λ phage and host chromosome is catalyzed by a specific enzyme system. This enzyme guides λ phage to integrate at the same site as on chromosome which is denoted in the diagram with *att (*λ attachment site). This site also decides the lytic cycle and ensures that prophage excise at the correct point to produce a normal circular λ chromosome (see the figure, top left—a normal excision). However, errors in excision can occur as abnormal excision (see the figure top right abnormal excision). Under this type of excision, the prophage gene during the lytic cycle becomes circular but contains a nearby gene of the host chromosome and leaves behind some phage gene intact to the host chromosome (Fig. 6.23).

If we see the figure, we can assume that λ has a nearby gene gal on one side while bio on the other side. Resulting phage λ is now referred to as defective particle because it left gene behind on the host chromosome and carry either partial gal known as λdgal (λ-defective gal) or partial bio or λdbio. The resulting particles are defective due to the genes left behind and others transferred from the chromosome are referred to as λdgal (λ-defective gal) or λ dbio. This defective phage gene can be packaged into head and infect another host bacterium. In the second round of infection, λdgal gene will integrate to the *λ* attachment site on the second host chromosome (Fig. 6.23). This way, gal gene from the first host will transduce to the second host. It should be noticed that this type of transduction carries a few limited genes very near the original integrated prophage, so very few genes are able to transfer and are called specialized transduction.

### 6.5.4 Lambda (λ) Genetics

Lambda phage is the most extensively studied bacteriophage among all the other phages. Lambda phage is an important model system for latent infection of

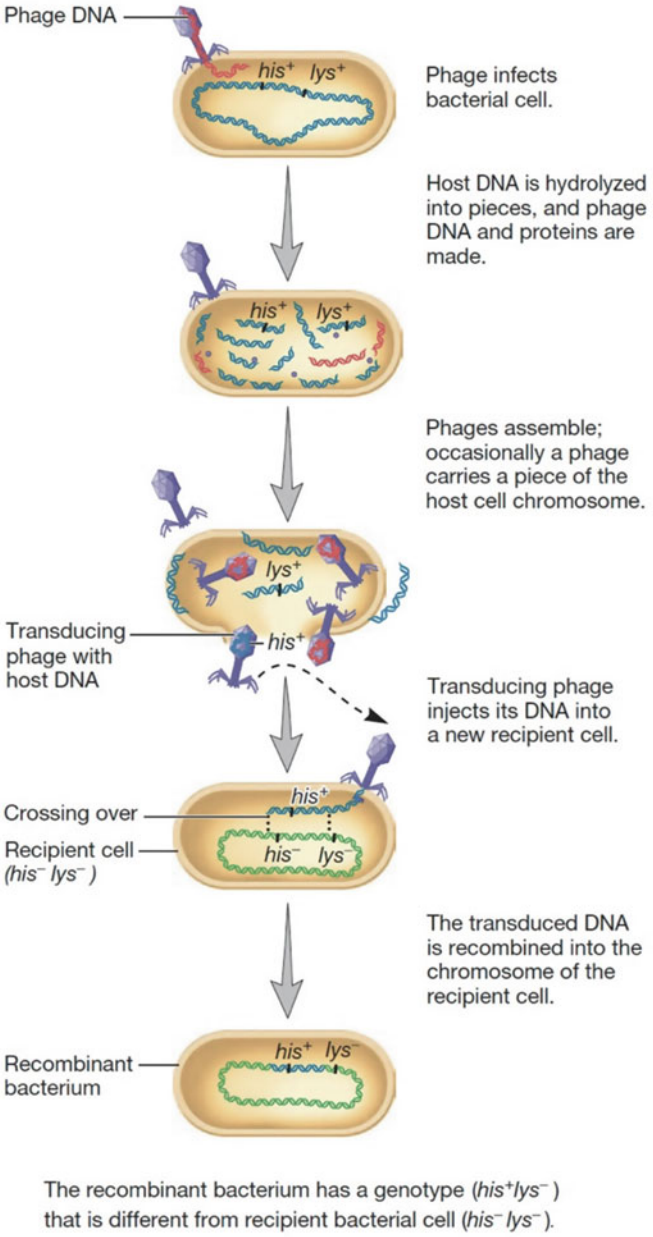


**Fig. 6.21** Generalized transduction in bacteria. Generalized transduction is processed with infection of phage to the host cell and releases its genetic material into the cytoplasm. Once the phage infects the host cell, simultaneously host DNA hydrolyzed and synthesized partial phage DNA and proteins. Assemble phage contains a small fraction of the host bacterial chromosome. This phage further transduces and infects other host cells where the crossing over between phage DNA and bacterial chromosome takes place. In the above example, crossing over between bacterial

mammalian cells by a retrovirus, and this model system has been widely used for cloning purposes.

Lambda is the prototype of a group of phages that is a well-characterized virus with both lytic and lysogenic alternatives to its life cycle.

DNA inside the phage is linear but it circularizes on the infection to *E. Coli* chromosome (Fig. 6.24). At each end are complementary 12 bp long overhangs known as cos sequences (cohesive ends). Once inside the *E. coli* host cell, these pair up and the cohesive ends are ligated together by host enzymes forming the circular version of the lambda genome. Lambda can only be compatible for packaging of genome size of 37–52 Kb and also small fragments of extra DNA can be packaged into lambda genome without hindrance. Although accommodate longer insert, some of the lambda genomes must be removed. In the lambda genome, the left-hand region has essential genes for structural proteins while the right-hand region consists of genes responsible for replication and lysis. Cro has been believed to play an active role in switching lysogenic cells to the lytic state following induction. However such lambda replacement vectors cannot integrate to host genome and form lysogeny by themselves. The Middle region is necessary for integration and recombination (Fig. 6.22).

This lambda phage has made many undiscovered questions easy for a scientist to address and to develop advanced techniques such as how to sequence DNA and discovered essential enzyme for RNA synthesis. Also, studies on Lambda phage led to the discovery of (1) basic molecular biology principles of how gene transcription is halted with rho-dependent termination manner, (2) first transcription factor, and (3) gene regulation including "Operon" concept.

### 6.5.5 Nature of Transduction

Nature of transduction reflects here the question to understand the nature of prophage or prophage–host interaction. As sooner as the prophage is induced immediately after the infection, as more it will produce the prophage and the genome will be restored in the prophage. Before its discovery, it is necessary to understand whether a phage is a mere small invisible particle or a plasmid that lives in the bacterial cytoplasm or a part of the bacterial chromosome. From the past, it has been observed that a temperate phage lambda (λ) promotes the lysogenic cycle in its particular host bacterium *E.coli* used by Lederberg and Tatum (mentioned earlier). So far, the studies on lysogenic cycle of phage λ have introduced the λ phage as a first preference to refer for lysogenic and well-characterized known phage. If we look at the crosses between $F^+$and $F^-$ cells obtain interesting results such as $F^- \times F^+$ (λ) gives recombinant lysogenic recipient while $F^+$ (λ) × $F^-$ results in nonlysogenic

**Fig. 6.21** (continued) chromosome ($his^-$ $Lys^-$) and phage DNA ($his^+$) results in $his^+$ $Lys^+$ positive recipient host cell. They again cross over

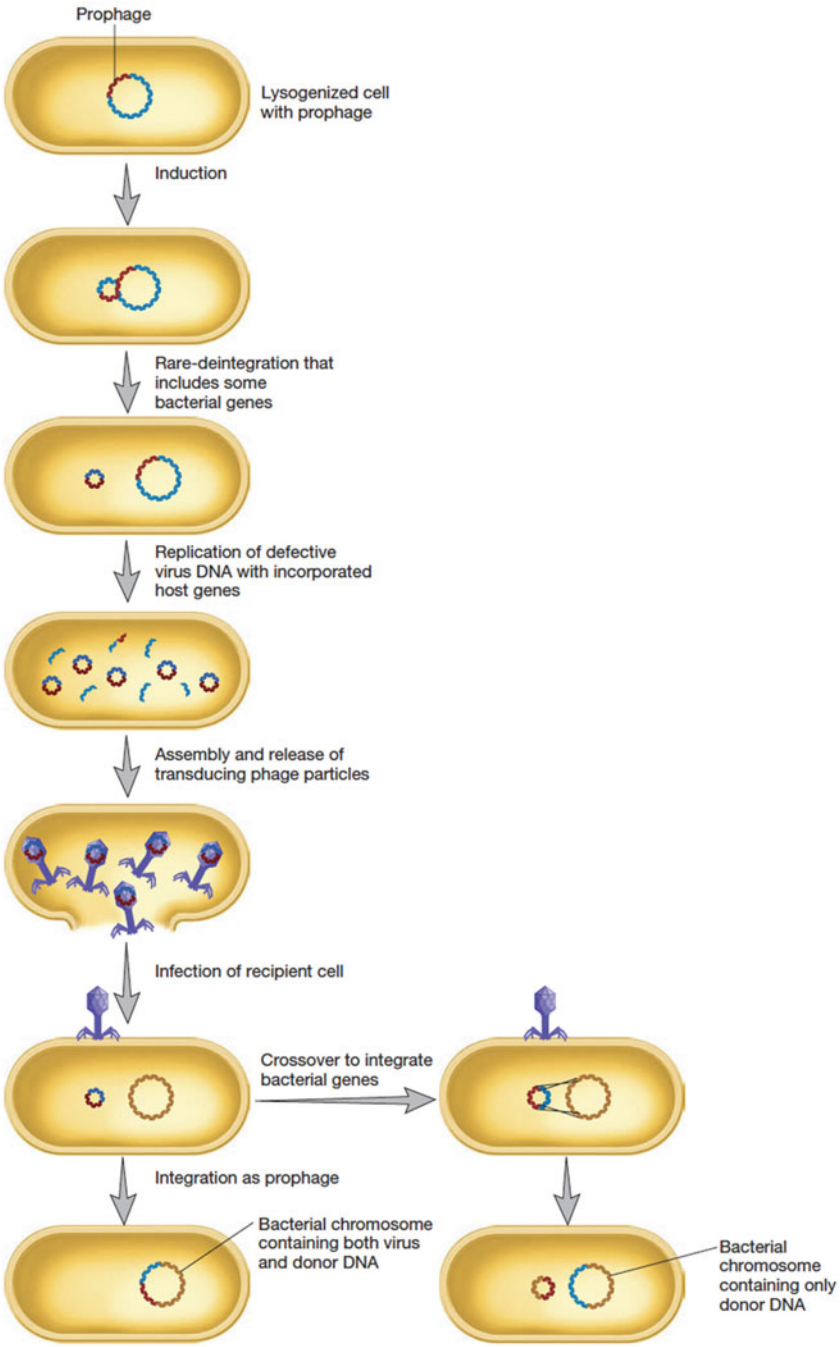


**Fig. 6.22** Specialized transduction in host bacteria. In specialized transduction, prophage that contains some bacterial gene disintegrates on the specific induction. The disintegration of prophage produces a new circular chromosome in the same host cell. Replication in the host cells took place immediately and was followed by the assembly of new phage that released and infected other host

recombinants. These results were of more importance when Hfr strain had discovered and was used for crosses. If crosses happened between Hfr × $F^{-}$ (λ), lysogenic F− exconjugants readily recovered with Hfr genes.

If a cross occurs between Hfr (lysogenic or lambda containing strain) with $F^{-}$ (non-lysogenic or nonimmune recipient), entry of lambda prophage into non-lysogenic cell will immediately trigger the prophage into the lytic cycle. This process is known as Zygotic induction. On the other hand, if the cross between Hfr (λ) × F−(λ) occurs, the resultant recombinants are readily recovered, and no prophage lytic cycle occurs. From this observation we can say that cytoplasm of F− cell must have interchanged between two states (depends on whether recipient contains λ prophage). So when the recipient cell is a nonimmune cell, the entry of prophage will induce the lytic cycle. This cytoplasmic state is specifying the fact that prophage represses the multiplication of the virus and therefore when lambda infects the nonlysogenic cell, these prophage repressing factors are diluted immediately after the infection and thus virus will multiply and reproduce the progenies. But what if a virus specifies the repressing factor and why the virus does not shut off the replication of itself?

The answer is, it does because a fraction of infected cells can become lysogenic (prophage). But there is a race between lambda gene signal for reproduction and repressor specify signal to shut down the replication. In this way, a phage-directed cytoplasmic repressor model explains the immunity of lysogenic bacteria. In conclusion, a superinfected phage will immediately encounter the repressor and become inactivated.

## 6.6 Infectious (Bacterial/Viral) Disease

Coronavirus (CoVs) is a large family of single-stranded RNA virus that can infect a wide variety of animals including humans, causing respiratory, enteric fever, hepatic, and neurological disease. In human coronavirus mainly causes respiratory tract infection. Till date, six coronaviruses have been identified including (1) alpha-CoVs HCoV-NL63, (2) HCoV-229E, (3) beta-CoVs HCoV-OC43, (4) HCoV-HKU1, (5) severe acute respiratory syndrome-CoV (SARS-CoV), and (6) Middle East respiratory syndrome-CoV (MERS-CoV). However, a novel coronavirus, SARS-CoV-2 (COVID-19) lately in 2019, has been added to the list of existing coronavirus. Although human coronavirus has been identified decades ago, their clinical and epidemic importance was not recognized until the outbreak of SARS (2002) and MERS (2012–2017). In the next section, SARS, MERS, and COVID19 will be discussed in detail.

**Fig. 6.22** (continued) cells. Multiple crossing overs between prophage and bacterial chromosome result in (1) bacterial chromosome containing only donor DNA and (2) bacterial chromosome containing both viral DNA and donor DNA

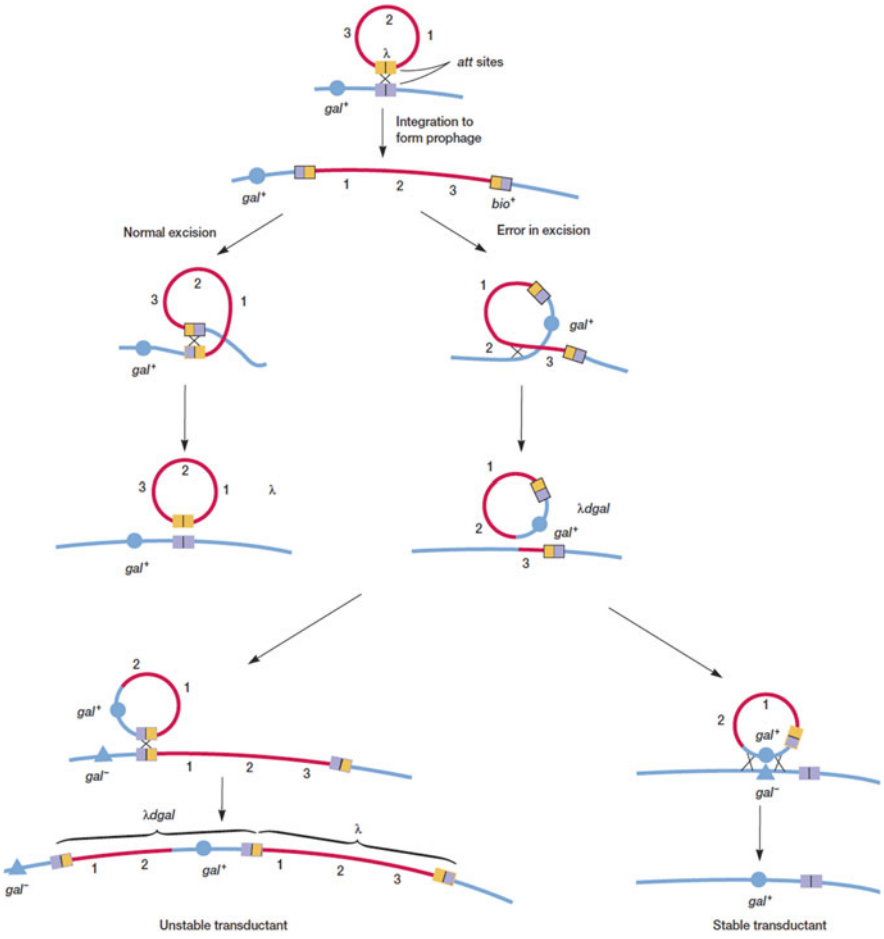


**Fig. 6.23** The mechanism of transduction for phage lambda and *E. coli*. Integrated phage lies between *gal* and *bio* genes. When a normal excise occurs (top left) new phage is complete and does not contain any bacterial gene. While rare excise occurs (top right), either the *gal* or the *bio* genes are picked up by bacterial genes and some are lost. As a result, a defective lambda phage that contains a bacterial gene can transfer to the new host cell

### 6.6.1 SARS-CoV

SARS CoV virus was identified in 2003. SARS-CoV is a zoonotic disease and was thought to be an animal virus from an uncertain animal reservoir, like bats and civet cats, and found to be the first to infect humans in the Guangdong province of south China in 2002 (Fig. 6.25). However, these animals were only incidental hosts, as there was no evidence for the circulation of SARS-CoV-like viruses in palm civets in the wild or breeding facilities. Studies reported that bats are the reservoir of a wide variety of coronavirus including SARS-CoV-like and MERS-CoV-like virus.

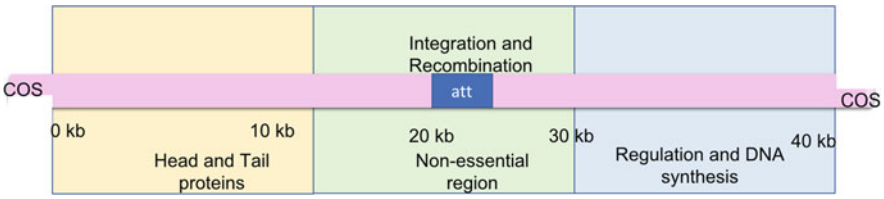


**Fig. 6.24** Lambda replacement cloning vector. Lambda phage is easy to grow and therefore it has been modified to accept foreign DNA inserts. Both left and right ends are overhangs with cohesive regions known as COS and regulate the circularization of the DNA. The green region has genes that are nonessential for lambda growth and packaging but can be replaced by the foreign DNA insert (up to 23 kb) during the cloning. The yellow region codes for proteins essential for head and tail packaging

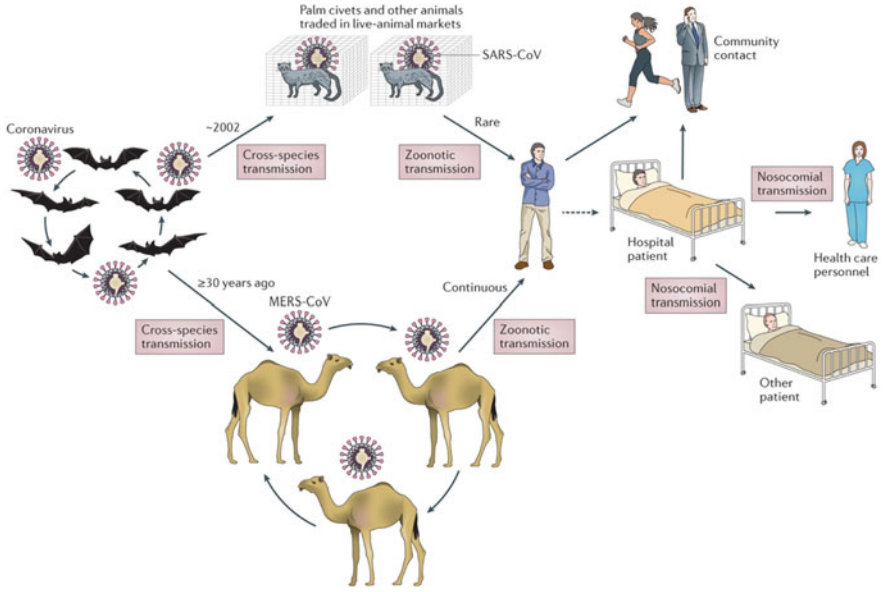


**Fig. 6.25** Insights into SARS and MERS infection Cycle: SARS-CoV crossed the species barrier into masked palm civets and other animals in live animal market in China responsible for SARS-CoV infection occurred in late 2002. Later in 2012, the cross-infection in dromedary Camel was identified as MERS-CoV infection in the Middle East. SARS-CoV and MERS-CoV spread between humans mainly through nosocomial transmission, which results in the infection of healthcare workers and patients at a higher frequency than infection of their relatives

Transmission of this disease was primarily spread from person to person and appeared to occur in the second week of illness where excretion of virus in respiratory secretion and stool was on the peak. Lately, most of the cases of human-to-human transmission were due to negligence in the healthcare setting, absence of adequate infection control, and precautions. Symptoms of SARS include influenza-like fever, malaise, myalgia, headache, diarrhea, and shivering. Cough was initially

dry, and shortness of breath and diarrhea are most prominent in first or second week of infection. Mostly severe cases developed rapidly, progressing to respiratory distress and required intensive care. Unlike COVID-19, SARS transmission counted as an epidemic since the geographic distribution was limited such as Toronto in Canada, Hong Kong, China, Chinese Taipei, Singapore, and Hanoi in Viet Nam, thus SARS infection.

### 6.6.2 MERS-CoV

Ten years after the emergence of SARS, in June 2012 a man in Saudi Arabia died with acute pneumonia and renal failure, which lately had identified infected with a novel coronavirus named Middle East respiratory syndrome coronavirus (MERS-CoV). MERS was also identified outside of Arabian Peninsula such as Jordan and United Kingdom, as a result of traveling; often, these imported MERS cases resulted in nosocomial transmission (Transmission usually occurs via healthcare workers, patients, hospital equipment, or interventional procedures). In the case of MERS, serological tests from dromedary camels from Oman and Qatar camel farms confirmed its transmission from camel to human first in Arab peninsula and later in the Middle East, Eastern Africa, and Northern Africa. Like SARS, MERSS causes acute respiratory syndrome as well which is associated with the upregulation of proinflammatory cytokines and chemokines. Immune response to SARS and MERS during the infection plays a key role in its spread since SARS-CoV and MERS-CoV use several strategies to avoid the innate immune response.

### 6.6.3 COVID-19

The 2019 novel coronavirus, also known as COVID-19 or SARS-CoV-2, is a novel human coronavirus that has emerged at the end of December 2019 in Wuhan, China. It is currently spreading all over the world in the form of a pandemic. Coronavirus disease (COVID 19) is a clinical syndrome associated with SARS-CoV-2 infection and is characterized by severe respiratory syndrome.

SARS-CoV-2 belongs to the same *Betacoronavirus genus* as the other coronaviruses that are responsible for severe acute respiratory syndrome (SARS-CoV) and the Middle East respiratory syndrome (MERS-CoV). Phylogenic analysis of novel coronavirus suggested that SARS-CoV-2 belongs together with SARS-CoV and Bat SARS-like coronavirus which is a different clade from MERS-CoV but more phylogenetically related to Bat SARS-like coronavirus (isolated from horseshoe Bats between 2015 and 2018) than SARS-CoV. Genomic comparison between SAARS-CoV-2 and SARS has shown that there are almost 380 amino acid substitutions and mainly contained in structural protein, and 27 mutations were found in viral spike protein (S) which is responsible for receptor binding and cell entry. It is assumed that due to this mutation, SARS-CoV-2 is less pathogenic than

**Table 6.2** Overview of SARS-CoV-2, SARS-CoV, and MERS-CoV

| Strain | Phylogenetic origin | Animal reservoir | Intermediate host | Receptor | Case fatality rate |
|---|---|---|---|---|---|
| SARS-CoV-2 | Clade I, cluster IIa | Bats | Controversial | Angiotensin-converting enzyme 2 (ACE2) | 2.3% |
| SARS-CoV | Clade I, cluster IIb | Bats | Palm civets | Angiotensin-converting enzyme 2 (ACE2) | 9.5% |
| MERS-CoV | Clade II | Bats | Camels | Dipeptidyl peptidase 4 (DPP4) | 34.4% |

the SARS-CoV; however, further studies are still going on to understand its pathology and its contribution to other diseases like cancer (Table 6.2).

Similar to SARS-CoV, a mutation in the receptor-binding domain (RBD) of S protein in SARS-CoV-2, which directly interacts with human cell receptor—angiotensin converting enzyme 2 (ACE-2)—is thought to be the cause for its pathogenicity. Interestingly data on affinity analysis confirms that SARS-CoV-2 binds to ACE-2 more efficiently than SARS-CoV strain from 2003 although less efficiently than the 2002 strain.

ACE-2 is an ectoenzyme (an enzyme that has the catalytic site outside the plasma membrane and is mostly found in the endothelial cell) occurring in many tissues including, the lower respiratory tract, kidney, heart, and gastrointestinal tract. In vitro studies on SARS-CoV-2 show that inoculation of 2019nCoV (SARS-CoV-2) on the surface layer of human airway epithelial cells causes cytopathy effects and cessation of cilia movement. SARS-CoV induces downregulation of ACE-2 in lung epithelium, but SARS-CoV-2 shows higher affinity to ACE-2 and results in more severe lung infection than the SARS-CoV.

From the clinical aspects, virus loads are higher at the time of symptoms onset and are higher in the nose than the throat specimens which is why it is suggested to collect the patient specimens from the nose. In patients affected by COVID-19, viral loads progressively decrease within days following a different pattern from SARS, in which the highest shedding is recorded after 10 days of symptoms onset. Therefore, it has been suggested that SARS-CoV-2 can easily spread within the community than SARS even when mild or no symptoms (asymptomatic) are present. To date, there are no vaccines and drugs developed which can directly target COVID-19, but many clinical trials for vaccines are underway in different parts of the world, namely, Serum India technology (India), Oxford University (UK), Moscow's Gamaleya Institute (Russia), and AstraZeneca (USA).

Antiviral drugs including ribavirin, lopinavir, ritonavir, and remdesivir in combination with other drugs like chloroquine, cyclophilin chlorpromazine, loperamide, and cyclosporine A has been reported effective in some cases. In addition, antibody therapies and plasma therapies have been the leading proposed treatment in the case of MERS. Recently, plasma therapy has shown a potential advantage in COVID-19 treatment. Many countries such as India, United Kingdom, and United States are one

of those who are successfully running a trial for plasma therapy and antibody therapies for COVID-19.

**Box 6.1 Scientific Concept: Genetic Exchange Between Escherichia coli Strains in the Mouse Intestine—Jones R T et al.**

Bacterial genome usually possesses the phenomenon of gene flow between species. Gene flow largely contributes to the evolution of bacterial species; however, the significance of gene flow is still unknown. In vitro conjugal transfer of genes in *E. coli* K-12 and some other species has been widely studied and also revealed that plasmids harbored by bacteria from the natural environment are continuously increasing. Such plasmids may introduce the determination of colicin production, multiple drug resistance, surface antigen, enterotoxin production, suppressor and mutagenic factors, hemolysin production, and so on. Some of these plasmids are enabled to conjugally transfer to the recipient cells and some of these has been identified as a fertility factor that supports the transfer of chromosomal gene at low frequency. Considering the abovementioned prospective gene flow among bacterial species in nature or in ecological niches, it has been observed that the study of gene transfer in some particular niche has become a difficult task due to the microbial complexity of the niche where these species are likely to occur. One of the common niches is the mammalian intestinal tract where enormous microorganism species live together and are associated within the tract facilitating the genetic transfer. In the given niche, *Enterobacteriaceae* is the common inhabitants where they possess the genetic transfer very often. So far, many studies have been focused to understand the in vivo transfer of genetic material between the *E. coli* K12 (Fig. 6.26) and the formation of recombinants. K12 strain is genetically compatible to investigate the in vivo gene transfer and to avoid the microbial contaminant, and germ-free mice have been used.

Recombination frequencies between three donor strains from in vitro liquid mating, which have been selected further for in vivo studies and the $F^-$ recipient ×820 are shown in Table 6.3.Cross between $F^+ \times F^-$ gave the recombination frequencies of $5 \times 10^{-5}$ for any maker while the cross between Hfr × $F^-$ gives high frequencies for proximally transferred markers and decreased for markers situated at increasing distance from the origin of the chromosome transfer.

Recombination frequency for *pur E* marker ($1.2 \times 10^{-1}$) is slightly lower than that for the more distantly transferred *pro C* ($2.4 \times 10^{-1}$). It is because of integration of markers located next to the origin of chromosome transfer (i.e., *pur E* locus in Hfr strain OR74) is always lower than more distantly transferred markers.

Thus, proximally transferred markers showed high frequencies of inheritance, which further decreased as the distance from the origin of the

(continued)

**Box 6.1** (continued)
chromosome transfer increased. Thus, these data show that the donor or recipient strain for in vivo experiments behaved in the predictable manner as they do in in vitro mating.

Therefore, gene transfer between microbial species is quite difficult to understand since the ecosystem is more complex. By creating a simplified ecosystem of microbial species through contaminating the intestine tract of mice with genetically well-characterized bacterial strains is one of the easy ways to study the gene transfer between two species. In this study, Hfr strain OR74 was chosen and maintained in the intestine of mice as its in vitro stable. Forty days after inoculation, $F^-$ and $F^+$ strain/clone was isolated in vivo. The transition of Hfr $\Leftrightarrow$ $F^+$ $\Leftrightarrow$ $F^-$ is unpredictable in in vivo and in vitro and will not remain the same during the gene transfer. The reason might be the influence of chemical, selection, and environmental factors. The study of recombinants in the natural environment based on selection and survival rate plays an important role to understand the relationship between its in vivo host and gene transfer pattern. It would be interesting to set up the experiments in vivo to compare the mechanism of gene transfer and formation of recombinants in animals similar to its natural habitats. The complexity of gene transfer among the microbial ecosystem in nature can be possible to study by performing in vivo experiments on well-characterized bacteria and produce new germ-free host animals.

**Box 6.2 Scientific Concept: Role of Pili in Bacterial Conjugation—Ou, J. T et al.**
Bacterial conjugation has been discovered in 1946 by Laderberg and Tatum where they have proved that cell–cell contact is required for the transfer of bacterial chromosomes from a donor to a recipient. Later, in 1958 Anderson et al. have provided the electron micrograph images of conjugation bridge formation between mating pairs which confirmed the hypothesis of Laderberg and Tatum. To understand the role of F-pili to F-factor and male-specific phages, Brinton proposed that F-pili not only serves in pair formation but is also important for tube formation through which bacterial chromosomes are transferred. Evidence from various studies and experiments suggested that F-pili connect the male and female and then bring them a close wall to wall contact by retracting the F-pili into male or female cells. To obtain clear evidence, microbiologists further studied the role of F-pili by isolating mating pairs by using a micromanipulator (Fig. 6.27). They compared the production of recombinants from loosely connected pairs with the ones produced by

(continued)

**Box 6.2** (continued)
mates who were in intimate connect. Results from this study suggest that F-pili accelerates the transfer of bacterial chromosome, but close contact is one of the strong phenomena that facilitates an efficient transfer of bacterial chromosomes.

Micromanipulator is associated with the micropipette that separates microdroplets of medium containing single bacteria to be studied under high magnification. This microdroplet is larger enough to facilitate the bacterial cell division, and further this daughter bacterial cell can be taken from the drop and transferred to the other microdroplets.

During the experimental setup, the primary goal was to identify the mating pairs in the oil chamber of the micromanipulator where two mixed cultures were seeded. Morphological features observed under the microscope such as short and round denotes the male mate and long and thin denotes the female mate. Motility factor also supports the evidence to their identification such as male could be motile and could be seen pulling the nonmotile female through the medium. Mating pairs that have been chosen for this study have been described in Table 6.4.

From the given sources earlier, mating between $F^+$ (lac+, pro+ leu+ $str^r$ øII$^r$, MS2$^s$) and $F^-$p678 (lac− pro+ leu− $str^r$ øII$^s$, MS2$^r$) were done by mixing these two strains and placed them on an oil chamber and both loose and close contacted mating pairs were identified with the help of visualization under a microscope. The clones after transfer and cell division were tested whether they are resistant or sensitive to the phage øII or MS2. If they are resistant to øII and sensitive to mS2, this indicates that they were received the F factor. To analyze the close contact and loose contact between $F^+$and $F^-$ p678, Table 6.3 further indicates that close mating pairs generate more viable $F^+$p678 clones with high frequency than the loose mating pairs. However, four viable $F^+$p678 clones from loose mate pairs suggest that F-pili might indeed aid as the means for transfer (Table 6.5).

During the experimental set up, it takes at least 6 min to isolate the first mating pairs after mixing male and female cells. Previous studies suggested that chromosomal bacterial transfer takes maximum 3–5 min and if we assume that the F factor takes a similar time like chromosome ($3 \times 10^4$ nucleotide pairs/min) and it gives $7.5 \times 10^4$ Nucleotide pairs so it is predictable that time for transfer the F factor would be approximately 2 min. Therefore, one thing is clear that the F factor consumes a minimum 5 min to complete the transfer. However, it is difficult to exclude the fact that the F factor had already been transferred during unobserved close contacts before mating pair were isolated. To this end, experiments were further extended to mating between Hfr X $F^-$ where bacterial chromosomal transfer could not take place at least 8 m in after the mixing the cultures.

(continued)

**Box 6.2** (continued)

Evidence together suggest that initial contact between mating pair of cells appears to be a thin thread that is difficult to observe under light microscope and the simplest assumption is that this thin thread is F-pili that can be seen under electron microscope as a connecting bridge between male and female bacteria. It has also been proved that the mating pairs that were separated during mating from each other produce F-exconjugants that lately forms recombinant clones.

In the conclusion, such studies provide us with three important information;

F pili play a significant role in the mating between two *E. coli* bacteria by providing an external organelle for attachment of mating pairs.

F-pili is a medium for the transfer of bacterial chromosomes and F factor.

F-pili beings two mating pairs in proximity to create close contact between them and help to build the conjugation bridge more efficiently.

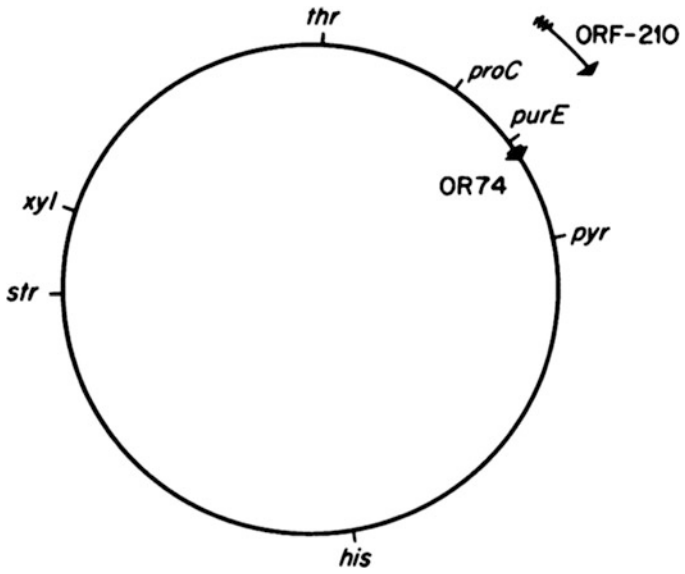


**Fig. 6.26** Image shows Linkage map of E. coli K-12. The arrow indicates the origin, direction, and gradient of chromosome transfer of Hfr strain OR74. The F′ strain ORF-210 used in these studies had the same origin and direction of chromosome transfer as Hfr OR74 when F′ was integrated into the chromosome. The relevant fermentation and auxotrophic markers of F-recipient strain ×820 are shown (Jones and Curtiss (1970))

**Table 6.3** Recombination frequencies obtained from in vitro liquid mating between the Hfr, F′ and F+ strains chosen for further in vivo studies and $F^-$ recipient $\times 820^a$ strain

| Cross | Recombinant class selected | Recombination frequenices |
|---|---|---|
| χ209 $F^+$ × χ820 $F^-$ | *purE*$^+$, *str*$^r$ | $6.0 \times 10^{-5}$ |
| | *proC*$^+$, *str*$^r$ | $3.0 \times 10^{-5}$ |
| | *thr*$^+$, *str*$^r$ | $6.0 \times 10^{-5}$ |
| | *his*$^+$, *str*$^r$ | $3.5 \times 10^{-5}$ |
| Hfr OR74 × χ820 $F^-$ | *purE*$^+$, *str*$^r$ | $1.2 \times 10^{-1}$ |
| | *proC*$^+$, *str*$^r$ | $2.4 \times 10^{-1}$ |
| | *thr*$^+$, *str*$^r$ | $5.0 \times 10^{-2}$ |
| | *his*$^+$, *str*$^r$ | $3.0 \times 10^{-5}$ |
| F′ ORF-210 × χ820 $F^-$ | *purE*$^+$, *str*$^r$ | 3.5 |
| | proC$^+$, str$^r$ | $8.0 \times 10^{-2}$ |
| | *thr*$^+$, *str*$^r$ | $2.4 \times 10^{-2}$ |
| | *his*$^+$, *str*$^r$ | $6.4 \times 10^{-5}$ |

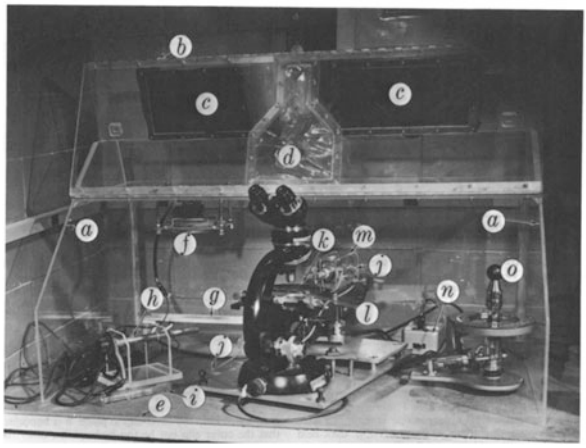


**Fig. 6.27** Image shows the arrangement of the equipment for micromanipulation. (a) Glove box, (b) hinged opening for glove box, (c) opening for arm insertion, (d) opening for microscope, (e) leveling platform, (f) fan, (g) heat strip, (h) thermoregulator, (i) 10-mL hypodermic syringe, (j) rubber tubing, (k) micropipette, (l) oil chamber, (m) micropipette receiver, (n) transformer, (o) controlling level

## 6.7 Summary

- Bacteria and virus genomes show a potential scope for genetic studies. Bacterial and virus genome is small and haploid in nature. Bacterial genome is a simple circular ds DNA, while virus genome varies as ssDNA, dsDNA or ssRNA.
- Mutation is a very common feature in bacterial DNA and mutant selection; detection has been developed through various techniques including, replica

**Table 6.4** *Escherichia coli* K-12 strains were used for recombination experiments

| Strain | Mating type | Genetic character | Morphology | Source |
|---|---|---|---|---|
| Hayes | $F^+$ | $thi^-$, derived from HfrH, motile | Fat and round | Anderson |
| Hayes | Hfr | $thi^-$, motile | Fat and round | |
| W3011 (Cavalli) | Hfr | $met^-$, motile | Slim and long | E. Lederberg |
| P678 | $F^-$ | $azi^r$, $lac^-$, $gal^-$, $mal^-$, $xyl^-$, $leu^-$, $tsx^r$, $tonA^r$, $str^r$, nonmotile | Slim and long | Anderson |
| W1-3 | $F^-$ | $lac^-$, $leu^-$, $tsx^r$, $str^r$, nonmotile | Fat and round | Tomizawa |
| W1-4 | $F^-$ | $lac^-$, $pro^-$, $tsx^r$, $str^r$, nonmotile | Fat and round | Tomizawa |

**Table 6.5** Transfer of F factor between loose and close pair mate in the cross $F^+$ X $F^-$ p678

| Types of contact between mating pairs | Close | Loose | $P^a$ |
|---|---|---|---|
| No. of $str^r$ exconjugants isolated | 21 | 20 | |
| No. of nonviable exconjugants | 1 | 1 | |
| No. of exconjugants that produced one or more clones of $F^+$ P678 | 16 | 4 | 0.01 |
| Efficiency of formation of clones of $F^+$ P678 | 0.76 | 0.20 | |

[a] Value of $P$ ($\chi^2$ test) gives the probability that there is no difference between close- and loose-mating pairs in the production of recombinants

plate method, phage display, and plaque assay. The spontaneous mutation is one of the potential sources and have been reported as the first experimental evidence for microbial evolution which is a random process.

- Plasmids are extrachromosomal nongenetic DNA molecules that coexist with bacterial chromosomes. They rapidly and independently replicate in the bacterial cell. Episome is a plasmid that can either live freely or integrate into the bacterial chromosome.
- Plasmids are involved in DNA transfer in the bacterial population by conjugation, transformation, and transduction.
- Conjugation referred a physical interaction between two bacterial cells to exchange the DNA through the bridge, known as a conjugation tube. F factor (fertility factor) is responsible for DNA transfer. F plasmid containing cell only can transfer the DNA, known as F+cell while cells lacking F factor meant to

receive the DNA, know F− cell. Hfr cell contains F factor integrated with bacterial chromosome and shows the high frequency for DNA transfer.

- Mating between F+ and F− or Hfr and F− determines the rate of gene transfer from Hfr to F− in terms of the time unit. The rate of gene transfer represents the order of the gene on the chromosome.
- In transformation, bacteria uptake exogenous DNA from the environment without any physical contact between cells. Cotransformation is a rate at which linked genes transferred and the frequency of cotransformation defines the physical distance between a gene on the chromosome.
- The virus is auto replicating machinery with DNA or RNA presence in either circular or linear forms. Most common virus being studied in genetics is bacteriophage—a virus that infects the bacterial cell. Bacterial genome can be transferred through a phage known as transduction. Likewise, conjugation transformation, transduction also provides the information to map the gene order on the bacterial chromosome about the rate of cotransduction reveals the gene order on the bacterial chromosome.
- CRISPR/Cas9 is an advanced technique using strong bacterial adaptive immune machinery to adapt in genetic engineering to edit genes of interest in any kind of eukaryotic or prokaryotic cellular system.
- In generalized transduction, any gene can be transferred from one bacterium to another bacteria. In specialized transduction, a gene linked to the site of phage integration can only transfer from one bacterium to another bacteria.
- A phage life has been differentiated in the lysogeny phase and lytic phase. In the lytic phase, phage causes bacterial cell lysis over the infection and does not integrate with bacterial chromosomes. In lysogeny phase, phage DNA integrates with bacterial chromosome and remains dormant for generations.
- Coronavirus is single-stranded RNA virus that mainly causes acute respiratory syndrome in humans. Corona to date has been identified as SARS (2002), MERS (2012), and novel coronavirus (COVID-19) in 2019.

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