Chapter 1

Introduction and review of literature

1. **Introduction**

1.1.1. ***Staphylococcus aureus***

S. aureus belongs to phylum Firmicutes, and is a gram-positive, spherical-shaped facultative anaerobe, which is positive for catalase and coagulase (Figure 1.1.). The bacteria behaves as a normal flora in the human body and is frequently found in the nose, respiratory tract, and on the skin. *S. aureus* and *S. epidermidis* are opportunistic pathogens and cause major diseases (Coates et al 2014). Today, *S. aureus* has evolved as one of the most difficult pathogens (Diekema et al 2001). In India, the rate of infections causes by *S. aureus* is much higher because of climatic conditions (warm and humid). *S. aureus* has ability to grow at a wide range of temperature (7º C to 48.5º C) and pH (4.2 to 9.3) (Devriese 1984). The cell wall peptidoglycan layer of *S. aureus*, specifically the pentaglycin linker, is sensitive to lysostaphin.

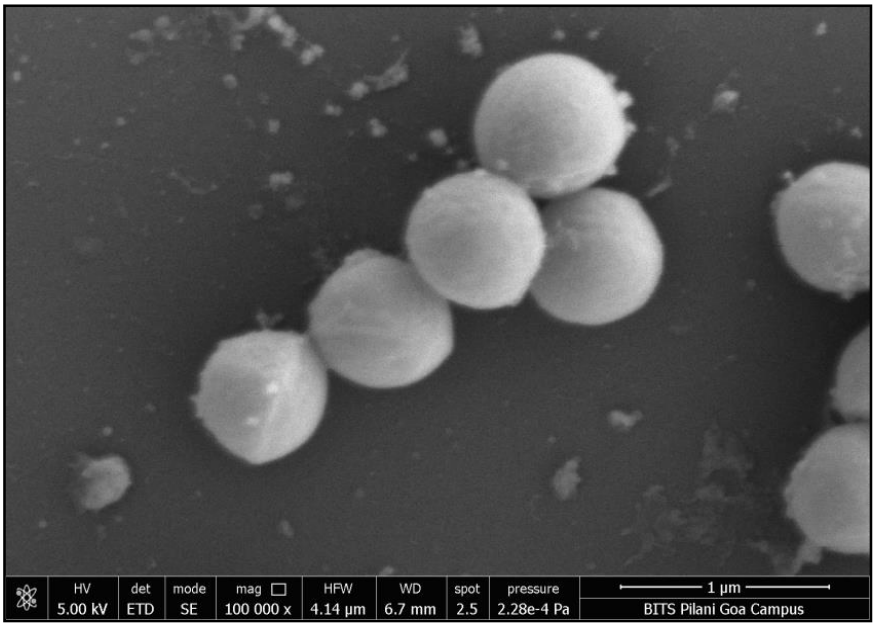

Figure 1.1. || Scanning electron micrograph of *S. aureus* RN4220, phage Phi11 host bacterium. Scale bars 1µm.

1.1.2. ***S. aureus* pathogenicity**

The pathogenicity of *S. aureus* is accentuated by several factors such as the presence of toxins, invasiveness of the bacteria and the evolution of antibiotic resistance (Cookson & Phillips 1988). In addition to multi-drug resistance, *S. aureus* also causes toxic shock syndrome (TSS), various skin diseases and food borne diseases. Some other skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses are also caused by this pathogen. In medical sciences, *S. aureus* is considered as a life-threatening pathogen in hospitalized as well as non-hospitalized patients.

In 1961, *S. aureus* was first detected to become resistant to methicillin (Dowling 1961). A global epidemic of methicillin-resistant *S. aureus* (MRSA) has been a major problem since the last four decades (Grundmann et al 2006). To tackle methicillin resistance, vancomycin was introduced as the first-line of control for MRSA. In 1997, a team of Japanese scientists reported the presence of vancomycin-resistant strains of *S. aureus* for the first time (Hiramatsu et al 1997). This was followed by an increase in the number of infections caused by both VISA (vancomycin-intermediate *S. aureus*) and VRSA (vancomycin-resistant *S. aureus*).

1.1.3. **Adherence and invasion of host cell by the pathogen**

S. aureus attaches to the host cells with the help of its extracellular proteins such as fibronectin binding proteins (FnBPs), a collagen binding protein, fibrinogen binding proteins (FgBP), a vitronectin binding protein and an elastin binding protein. The bacteria uses these extracellular proteins to attach to the host cells surface proteins, like laminin and fibronectin. *S. aureus* cell-surface protein, enolase plays a key role in host-cell adherence and invasion (Mölkänen et al 2002). Enolase of *S. aureus*, also identified on its cell surface is capable of binding to the host laminin, which is involved in pathogen invasion (Carneiro et al 2004). The bacterium mainly produces fibrinogen or fibrin binding protein, which helps it to invade blood and traumatized tissue.

1.1.4. **Symptoms of staphylococcal infections**

Staphylococcal infection shows some histologically abnormal characteristics in gastrointestinal tract such as neutrophils infiltration and blood accumulation in the epithelium cells. Also, blood accumulation was observed on membrane of the stomach, small intestine and jejunum. Pus is also observed in the lumen of duodenum (Bhatia & Zahoor 2007).

1.1.5. **Treatment of Staphylococcal infections**

Infections caused by methicillin registrant *S. aureus* (MRSA) require aggressive treatment. MRSA is susceptible to non-β-lactam antibiotics. Hence, several antibiotics treatment are available for countering infections caused by MRSA strains, such as clindamycin, daptomycin, doxycycline, linezolid and vancomycin (Rybak & LaPlante 2005). In 1997, *S. aureus* vancomycin resistant strain was first reported (Hiramatsu et al 1997 which makes *S. aureus* infections a leading cause of global concern today. In this regard, alternative treatment of *S. aureus* infections using bacteriophages is an attractive option today. Promising results for the treatment of *S. aureus* infections have been reported using a cocktail of phages (Chhibber et al 2017).

1.1.6. **Mechanism of Staphylococcal pathogenesis**

S. aureus invades susceptible hosts and spreads its virulence by two main steps - colonization and pathogenesis. Primarily, this bacterium colonizes on skin or mucosal surface (such as mucus surface of nose) of the human host (Liu 2009) thereby leading to nosocomial infections. However, the pathogen has to counter the host defense and other competing microorganisms which already reside within the host. To this effect, the bacteria uses an array of molecules, collectively called microbial surface components recognizing adhesive matrix molecules (MSCRAMM) to invade the host epithelial cells. In addition to MSCRAMM, two other factors, namely, wall-associated teichoic acid and clumping factor B are also reported to play an important role in colonization. Host cells upon exposure to *S. aureus* trigger upregulation of bacterial virulence genes (Novick 2003). It is interesting to know how the pathogen overcomes the host immune responses which are activated upon exposure to tissue injury or to the products of the pathogen. It is well reported that *S. aureus* can exist outside as well as inside the host epithelial cells, endothelial cells, or macrophages cells.

Outside these cells, the pathogen encounters the host complement system as well as antibodies, which leads to opsonization of the pathogen leading to its death. To avoid opsonophagocytosis, *S. aureus* expresses protein A on its surface in addition to a number of complement inhibitors, thereby destroying the host immune defences. *S. aureus* cells residing within the host epithelial cells, endothelial cells, or macrophages cells faces a tougher challenge posed by the host neutrophils. Neutrophils mainly recognize chemotactic factors and mount a response. To counter these neutrophils, the bacteria secretes a number of toxins which lyses the neutrophils (Tomita, T. & Kamio 1997). Additionally, CHIP (Chemotaxis Inhibitory Protein) and Eap (Extracellular adherence protein) are also secreted by the bacteria which play an important role in inhibiting the recognition ability of neutrophil (De Haas et al 2004).

1.2.1. **Phage therapy**

In 1928, the antibiotic Penicillin, discovered by Professor Alexander Fleming, heralded the golden age of antibiotics. However, with time, the indiscriminate use of antibiotics have rendered many of life saving compounds as ineffective against a variety of pathogenic bacteria. Now we are in a zone where pathogens like *Pseudomonas*, *Staphylococcus* and *Mycobacterium* have become antibiotic resistant. These superbugs can no longer be successfully treated with antibiotics. The Infectious Disease Society of America (IDSA) have emphasized upon the requirement for new antibiotics to counter the superbug infections. Superbug infections are fueled by overuse and misuse of antibiotics

randomly. The resistant pathogens are not limited to MRSA and vancomycin-resistant enterococci (VRE) infections; instead, the numbers are growing with the emergence of additional resistant pathogens to commonly used antibiotics. New strategies need to be developed to counter the antibiotic resistant pathogens. Antibacterial activity of bacteriophages was reported for the first time in 1896. A British bacteriologist, Ernest Hankin, reported the antibacterial activity of phages against *Vibrio cholerae*, which he observed in the Ganges and Jamuna river. Later, he stated that an unidentified substance was actually responsible for the observed antibacterial activity. Soon after this, a Russian scientist, Gameleya, noticed the same phenomenon against *Bacillus subtilis*. In 1915, British pathologist, Frederick Twort explained these observations as a virus infection of bacteria. In 1917, French-Canadian microbiologist Felix d'Herelle, first began testing his phages in human patients. In 1923, phage therapy trials were successfully carried out in the United States and it was concluded that, "the bacteriophage holds enormous possibilities as a weapon for fighting infectious disease".

1.2.2. **Advantage of phage therapy over antibiotics**

Phages, specifically bacteriophages, are very specific to their hosts. They are the perfect predators for their specific host bacteria, which gives them an advantage over antibiotics. Antibiotics target both normal host flora as well as pathogenic bacteria which results in secondary infections in the antibiotic treated patients. Some other noteworthy advantages of bacteriophages over antibiotics are as follows:

1. Phages replicate at the site of infections, whereas antibiotics spread throughout the body of the patient.
2. No side effects were observed in patients after administration of phage therapy; on the other hand, antibiotic treatment can cause major side effects like, antibiotic resistance, allergies and secondary infections.
3. Phages are environment friendly and can be rapidly and easily isolate and used against specific bacteria. On the other hand, development of new effective antibiotics needs major investment of time and money with lots of failures in clinical trials.
4. Bacteria can develop resistance to phages. However, phages can co-evolve to counter phage resistant bacteria. Moreover, phage resistance can be eradicated by the use of phage cocktail (mixture of different phages for infection).

On a positive note, the synergistic effect of phage therapy and antibiotic therapy can also help greatly to treat infections.

1.2.3. **Bacteriophages: Discovery and Significance**

"The enemy of my enemy is my friend"

---Chanakya (4th Century BCE).

"Bacteriophage" (in short phage) is a virus which infects bacteria (both beneficial and pathogenic bacteria). Phages are very helpful to us in countering bacterial infections. These enemies of bacteria infect specific bacterial cells (host cells) and multiply within them. Bacteriophages were first discovered by a British pathologist, Frederick Twort in 1915 (Figure 1.2). He described the growth of micrococci as transparent or glassy colonies (Twort 1915). After two years, a French-Canadian microbiologist Felix d'Herelle isolated an "anti-microbe" of *Shigella* and coined the term 'bacteriophage' (d'Herelle 2007). Genetic material of bacteriophges consist of either DNA or RNA and the nucleic acid of phages are protected by phage encoded protein called capsid.

Phages are well-known as obligate intracellular parasites of bacteria and have diverse life cycles- both lytic and lysogenic. To infect a host, a phage must interact with receptor proteins on the host cell membrane, followed by injection of its genome into the host cell. The subsequent steps involved in the infection process will depend upon the nature of the phage, whether it is virulent or temperate. Virulent phages are only able to replicate through lytic cycle with a strategy for the production of new progeny phages and their release from the infected host cells. Temperate phages can follow both lytic and lysogenic cycles. In lysogenic development, phage genome gets integrated within the host genome and forms a stable association with the infected host. This process is known as lysogeny and the integrated phage genome is termed as a prophage. To maintain lysogeny, the phage genome replicates with the host genome. Under unfavourable conditions (UV light or certain chemicals), prophage can exit from the host genome, produce more number of progeny phages and burst out from the infected host cell -a process known as induction. The release of progeny phages from the host cell causes lysis and death of the host cell (Young 2013; Roach & Donovan 2015).

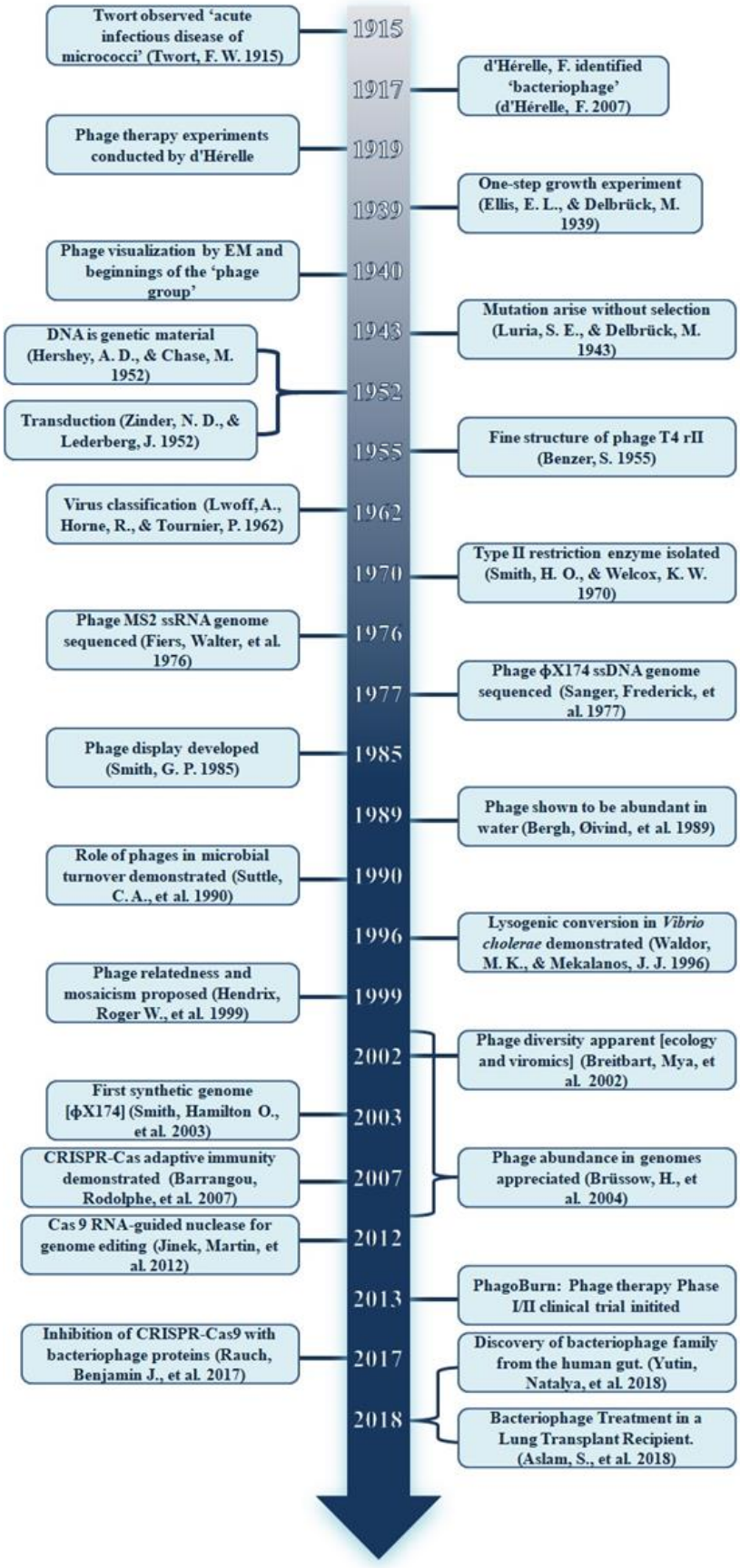

Figure 1.2. || Some major discoveries in the phage research.

1.3. **The life cycle of bacteriophages**

Based on the mode of development of bacteriophages, the life cycle can be of two types- lytic and lysogenic. Virulent phages follow only the lytic mode of development, whereas temperate phages follow both lytic and lysogenic mode of development (Figure 1.3.).

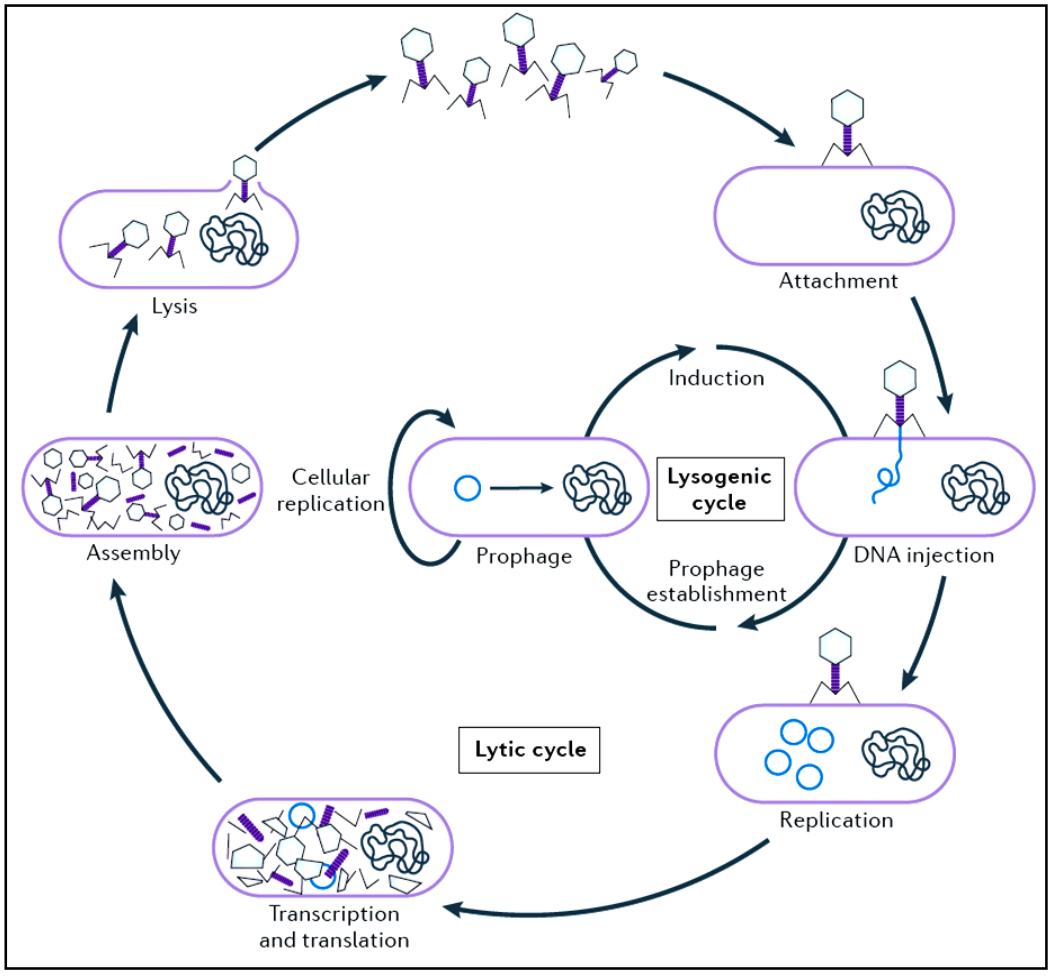

Figure 1.3. || Phage life cycles (lytic and lysogenic mode of development). Figure courtesy: Salmond & Fineran 2015.

1.3.1. **Lytic cycle of phages**

The lytic cycle or "reproductive cycle" of a bacteriophage is split into a number of stages. The phage infection starts with (i) **attachment** of bacteriophages to host cells wall, (ii) **penetration** of the host cell wall to insert the genomic content into the host, (iii) **transcription** and **biosynthesis** of phage component, (iv) **maturation** of phage particles, and finally (v) **lysis** of the host bacterium.

1.3.1.1. **Adsorption of the phages to the host cells receptors**

Adsorption to the host cell wall is the first step in the infection process and this interaction is very specific. Mostly, the interactions between phages and host cells involve protein-protein interactions. At the molecular level, the phage attachment protein will interact with the host cell surface receptor.

Studies suggest that these macromolecular interactions are complementary to each other and lead to specificity in adsorption (Watson 1970). Phages infect the host cells through the surface receptors of the latter. The host cell surface components vary greatly ranging from flagella to pili or lipopolysaccharides present on the outer membrane of the bacteria, which facilitate the phages to make attachments (Bradley 1967). For example, the adsorption process in the bacteriophage λ is mediated by protein-protein interaction. The *Escherichia coli* e maltose transport protein LamB interacts specifically with λ phage tail protein J (Randall-Hazelbauer & Schwartz 1973).

1.3.1.2. **Penetration of the phage nucleic acid into the host cell**

Penetration of phage nucleic acid into the host bacterium involves disruption of host bacterium cell surface by a variety of mechanisms. This disruption is facilitated, in most cases, by activation of phage enzymes (endolysin). In case of the T-even phages, the tail fiber is used to attach to host cell surface. The contraction of tail sheath facilitates the tail core to insert the tail through the host cell wall. Finally, the phage DNA enters the bacterial cytoplasm while the empty phage capsid, tail and tail fiber components remain outside (Anderson & Notani 1971).

1.3.1.3. **Transcription and biosynthesis of the bacteriophage genes within the host cell**

After the release of its nucleic acid into the host cytoplasm, the phage takes over the host metabolic machinery and uses it to produce phage components. This hijack of the host machinery is the function of the phage early genes. Finally, the host machinery transcribes and synthesizes the phage components in large quantities. In a step wise manner, the phage delayed early and late genes are then transcribed.

1.3.1.4. **Maturation of phage particles and lysis of the host cell to release progeny phages**

To form a complete virion, the synthesized phage components are spontaneously assembled. The phage nucleic acid is packed inside the capsid and finally the tail and tail fiber are attached thereby generating a complete infective virus particle (virion). With an increase in the number of mature virion particles, the phage number increases greatly. Now, some special phage proteins like holin, leads to dissolution of the bacterial cell wall which makes the host cell membrane extremely porous. The bacteria cannot sustain the osmotic sock generated due to the porosity of its membrane. This results in lysis of the host cells and release of mature virions, which infect new specific hosts.

1.3.2. **Lysogenic cycle of phages**

The lysogenic mode of development in temperate bacteriophages involve the integration of the phage the nucleic acid into the host genome, thereby generating a prophage. Depending upon the environmental conditions (spontaneous, physical and chemical agents) the prophage may switch to lytic developmental pathway, in a process known as induction (Figure 1.3.). The host carrying the prophage is known as a "lysogen". The induction of prophage is deeply connected to DNA damage or SOS response (Fornelos et al 2016) in the host. Most importantly, the interplay between host regulatory genes (*lexA* and *recA*) are involved in this process (Fornelos et al 2016).

1.4. **The Model phage: Lambda (λ)**

In 1950, American microbiologist Esther Lederberg discovered the bacteriophage λ (Lederberg 1950). This phage belongs to Siphoviridae family and infects *E. coli*. The double stranded DNA genome of λ phage is 48.5kb. So far, the λ phage is the most well-studied temperate phage (a model bacterial virus). The complete DNA sequence information of λ phage (Sanger et al 1982) gives us detailed information about its gene regulation and gene organization. The genetic map of lambda DNA is shown in Figure 1.4.

1.4.1. **Life cycle of λ phage**

Being a temperate phage, λ phage can follow both the lytic and lysogenic cycle. These two developmental pathways are presented in Figure 1.3. As soon as the phage injects its genome into the host *E. coli*, the expression of early genes and delayed early genes take place which decides the developmental pathway to be followed. The success or failure of the establishing control over host system will allow the phage to follow either lysogenic or lytic development (Hershey 1971).

Gene regulations can be grouped into four major operons, namely:

- Promoter P_L, which regulates early left operon
- Promoter P_R, which regulates early right operon
- Promoter P_{RE} (promoter for repressor establishment) and P_{RM} (promoter for repressor maintenance), which regulate *cI-rex* operon; Promoters P_{RE} and P_{RM} are collectively referred to as P_M
- Late operon regulated by $P_{R'}$

The P_L and P_R promoters are involved in synthesis of 'immediate early' genes, specifically the *N* and *cro* genes. The P_M promoters are solely involved in the synthesis of repressor gene.

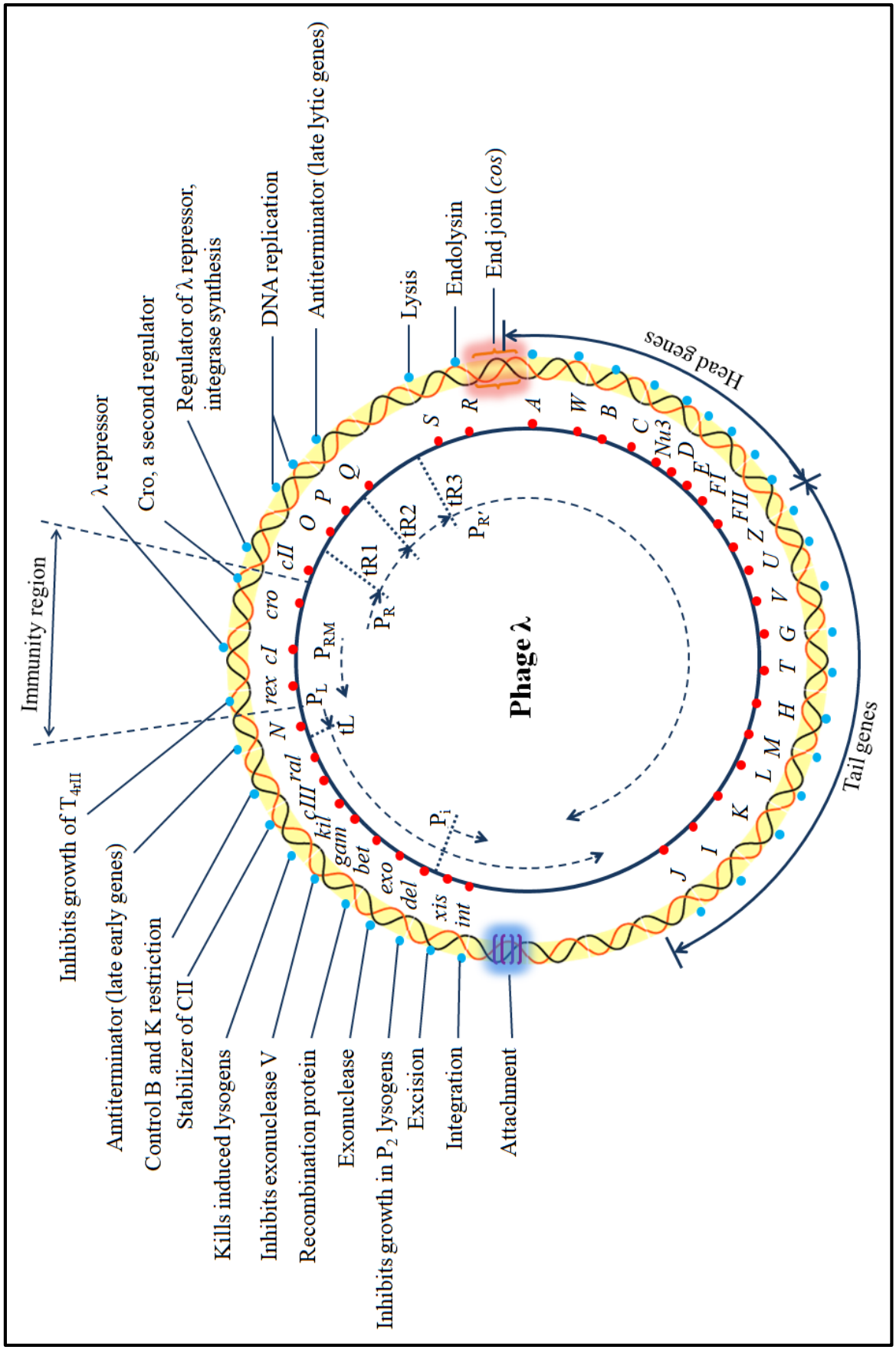

Figure 1.4. || The genetic map of model lambda (λ) phage.

1.4.2. **The genetic switch in phage λ**

Soon after the infection of *E. coli* by λ phage, the early genes of λ get transcribed from the P_L and P_R promoters with the support of the host machinery. The decision to follow the developmental pathway takes place based on the expression level of two proteins, namely, CI (λ repressor) and Cro (**C**ontrol of **R**epressor and **O**thers). CI is responsible for lysogenic pathway and Cro is responsible for the lytic pathway. Another important gene *CII* is also transcribed along with Cro from the P_R promoter. High level of *cII* transcription helps the phage to stabilize *cI* and *int* gene expression. This moves the pathway towards lysogenic development.

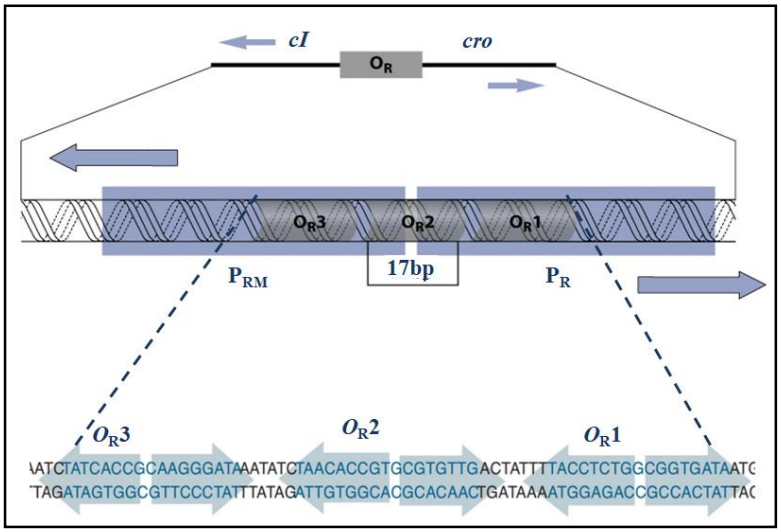

Figure 1.5. || The right operator region (O_R) of lambda phage. Figure courtesy: Ptashne 2004.

The inter play of CI and Cro with their cognate operators decide the developmental pathway of the bacteriophage, and is known as 'genetic switch' (Friedman 2001). Both these repressors regulate from O_R operator and take control over each other's expression. The O_R region consists of three 17bp partially palindromic sequences (Figure 1.5.). If CI takes control over O_R, it leads to lysogenic development and stops the transcription of *cro* gene from P_R. On the other hand, if Cro takes control over O_R, it leads to host lysis with the inhibition of CI expression from the P_{RM} promoter. The expressions of int and xis genes are also highly involved in this genetic switch. High levels of *int* expression help the phage genome to integrate into the host chromosome, whereas a balanced expression of the *int* and *xis* genes push the phage to lytic development (Echols & Guarneros 1983).

1.4.3. **Lytic development and regulation**

The Cro repressor is responsible for the lytic pathway. The Cro repressor containing sixty-six amino acids is a single domain protein with three alpha helices and three anti-parallel beta sheets (Owens

& Gussin 1983). As soon as the phage injects its genome into *E. coli*, the host transcription machinery starts transcription from the P_R and P_L promoters of the phage. The first two genes transcribed are *N* and *cro* by host RNA polymerase. Downstream of the *N* and *cro* genes, the host RNA polymerase encounters two terminators, tL and tR1 respectively. The N protein is responsible for antitermination of transcription at these terminators and facilitate the expression of a host of genes (Couturier & Dambly 1970; Roberts 1975). For the lytic development, Cro dimers bind strongly to O_R (specifically, O_R3). Affinity for O_R1 and O_R2 is roughly equal, and is less than that for O_R3 which allows the regulation in two directions (Figure 1.6.). First, Cro suppresses the expression of *cII* from P_{RE} (Reichardt 1975; Ptashne et al 1980) and secondly inhibits the P_{RM} for the synthesis of CI (Ptashne 1986).

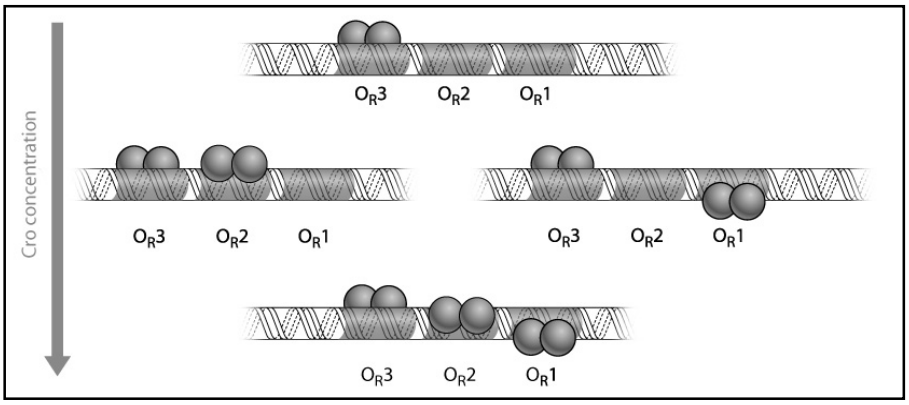

Figure 1.6. || Regulatory mechanism of Cro repressor on O_R. Figure courtesy: Ptashne 2004.

The N protein responsible for antitermination facilitates the expression of a second antiterminator, the *Q* gene (Couturier & Dambly 1970; Roberts 1975). Finally, the Q protein helps to express the late genes (*R* and *S*) from $P_{R'}$ to complete the lysis of *E. coli*. The gene products of *S* and *R* are crucial for host cell lysis and the release the progeny phages from the host cell. The R protein has murein transglycocylase activity and weakens the host peptidoglycan layer (Bienkowska-Szewczyk & Taylor 1980). The S protein generates pores on the cell membrane thereby enabling the release of the virions (Wilson 1982).

1.4.4. **Lysogenic development and regulation**

The dimers of λ CI repressor are chiefly responsible for the lysogenic development. After the expression of *cII* gene from P_{RE}, CII enhances the expression of *cI* from P_{RM} and *int* from P_i for the production of CI repressor and integrase respectively. CII is very unstable and is easily degraded by host Hfl protease. The CII stability is maintained by the expression of *cIII* gene. Soon after the expression of *cI*, it binds to O_R1 and consecutively at higher concentrations to O_R2 (Figure 1.7.). CI

binds to O_R3 with ten times less affinity as compared to O_R1. CI binding to O_R1 inhibits the expression of *cro* as well as the other lysis genes from the P_R promoter. Binding of CI to O_R2 enhances the RNA polymerase to transcribe the CI from P_{RM} promoter in a more efficient way -a mechanism known as positive feedback (Bushman et al 1989). Finally, the lysogenic development gets established with the help of the bacteriophage integrase (*int* gene product). As soon as the concentration of CI becomes very high (ten times higher than O_R2 binding concentration), the CI dimers will also bind to operator O_L. CI dimers binding to O_L and O_R result in the formation of a DNA loop structure, allowing CI dimers to form an octamer complex. Following the formation of the octamer complex, CI starts binding to O_R3 and inhibits its own transcription by blocking P_{RM}. Thus at high levels, λ repressor suppress its own rate of production (negative feedback). The consequence is a stable steady state with high repressor and low Cro concentration.

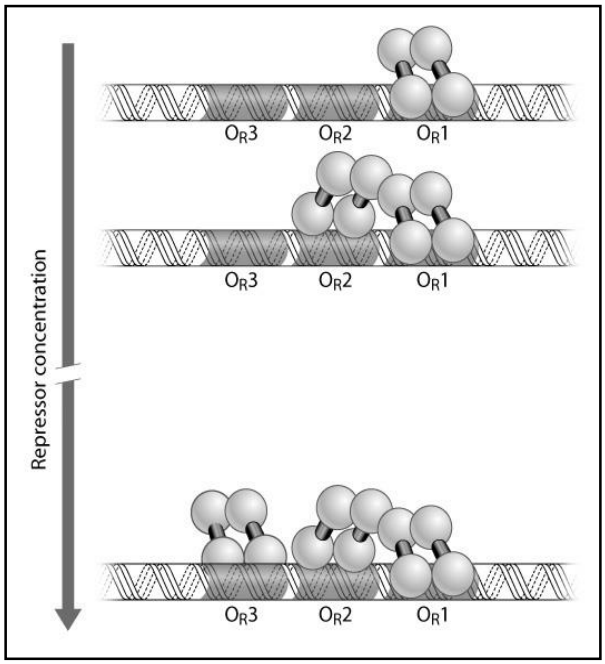

Figure 1.7. || Regulatory mechanism of CI repressor on O_R. Figure courtesy: Ptashne 2004.

1.4.5. **Induction**

Action of DNA damaging agents or stimulation of SOS response leads to the lytic-lysogenic shift, a process known as induction. The dormant state of prophage is triggered by elevated levels of environmental stress. The host protease RecA detects DNA damage and gets activated. Activated RecA is involved in the induction of SOS response by the activation of the auto-proteolytic activity of global repressor LexA. This auto- proteolysis of LexA leads to the production of damage repair proteins. The auto-cleavage site is similar with the CI auto-cleavage site. Upon induction, the activated RecA activates the auto-proteolytic activity of λ CI repressor. Finally, the functional

dimers of CI dissociate which renders CI non-functional in its operator binding ability. This stimulates the developmental pathway to switch from the lysogenic mode to the lytic mode.

1.4.6. **The genes of phage λ regulating host growth**

λ phage genome has a total of 73 ORFs (Open Reading Frames), some of whose products regulate host growth and show various effects on the infected *E. coli* cells. Noteworthy among these genes is the *kil* gene. The expression of this gene induces lower survival rate and extremely elongated and filamented *E. coli* cells without altering the DNA replication process (Greer 1975). The product of *kil* gene arrests the divisome formation in *E. coli* via ZipA-dependent inhibition of FtsZ assembly in *E. coli*. Apart from the *kil* gene, *rex* gene is also listed among the early genes of the phage and fall under the 'immunity region'. Under the control of P_L, *rex* gene produces two components RexA and RexB that abort lytic growth (Matz et al 1982). Exclusion of Rex causes termination of macromolecular synthesis, loss of active transport, the hydrolysis of ATP, and cell death. In response to lytic growth, formation of ion channels and depolarization of cytoplasmic membrane by RexB proteins takes place (Parma et al 1992).

1.5. ***S. aureus* and its phages (Aureophages)**

Bacteriophage and its host bacteria can be considered to have coevolved with respect to the evolutionary time scale (Golais et al 2013). Phage can coexist inside the bacterial cells in four different ways as a result of evolution (Golais et al 2013). One of the strategies is to exist as a replicating virus all through the lytic development; the second strategy employed by the phage is to exist as an unstable carrier form termed as pseudo-lysogeny and finally as a defective cryptic prophage.

Table 1.1. || ICTV classification of prokaryotic viruses (Order: Caudovirales).

Family	Morphology	Nucleic acid
Myoviridae	Non-enveloped, contractile tail	Linear dsDNA
Siphoviridae	Non-enveloped, noncontractile tail (long)	Linear dsDNA
Podoviridae	Non-enveloped, noncontractile tail (short)	Linear dsDNA

Initially, *S. aureus* phages were classified based on their reaction to polyclonal antiserum and neutralization of phage infections. A total of 11 serogroups (A-H and J-L) were identified based on the neutralization (Rippon 1956). Whereas serogroups phages A, B and F are temperate in nature. E, J and K group phages are non-infectious to *S. aureus*. International Committee on Taxonomy of

Viruses (ICTV) has classified phages according to morphology and genomic content. So far, phages that infect archaea and bacteria have been classified into twenty families (Adriaenssens et al 2018). Eighteen of these phage families contain DNA as their genetic material and only two families have RNA as genetic material. Caudovirales, a taxonomic order, contains phages with filamentous tails and double stranded DNA packed in an icosahedral capsid. All the identified *S. aureus* phages belong to this order and are further classified into three most important families based on their tail morphology (Table 1). Also, in these three families the genomes have different sizes (Class I: <20kb; Class II: ≈40kb and Class III: >125kb) (Figure 1.8.). *S. aureus* phages have been widely studied because of their medical importance. In 1985, Lee and Stewart studied virion proteins of 22 *Staphylococcus* phages using SDS-PAGE, including Phi11 (phi11 or φ11) and phi80α phages (Lee & Stewart 1985) and they categorized these two phages in Siphoviridae serogroup B. So far, these two temperate phages are among the best studied *S. aureus* phages, because of their high transducing efficiency, which can be used to transfer mutated genes between *S. aureus* strains. Also, it has been noted that these phages carry lysogenic (*cI*) and lytic (*cro*) genes in same orientation in their genome, similar to that of lambdoid phages (Ptashne 2004). Beside all these similarities, these phages are different from the model phage λ with respect to many regulatory genes. These phages have been described below.

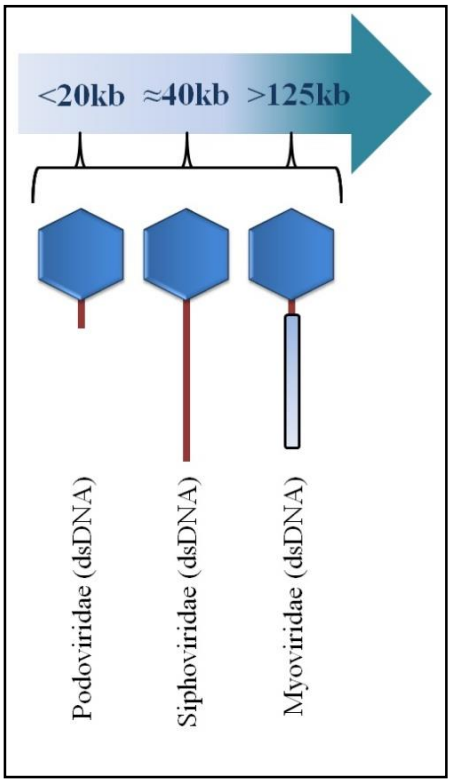

Figure 1.8. || Caudovirales phage families, morphologies (tail), genome types (dsDNA) and relative genome sizes. Note: Phage particles are not drawn to scale.

1.6. **The life cycles of different phages**

Every organism has its own genetic signature and regulates its life cycles differently. The λ phage is a well-studied model. Besides the *cro* repressor and *cI* repressor genes, other essential regulators like *cII, cIII, N, Q* and many other genes of λ are entirely absent in many phages. Hence these groups of phages have completely diverse regulators for their developmental pathway.

1.6.1. ***Salmonella*** **phages**

Salmonella phages are well-studied phages. The development of *Salmonella* phages is quite different from the λ phage, involving different kinds of regulators. Antirepressor, a regulatory protein, is very much prominent in this group and is required for the switch from lysogenic to lytic development. Antirepressor mediated prophage induction is quite common in the evolution of phages. Studies on the regulatory mechanism of *Salmonella* phages revealed the presence of a different set of regulators and novel regulatory mechanisms. *Salmonella* phage Gifsy-1 and Gifsy-3 have different sets of repressor and antirepressor. Gifsy-1 repressor, GfoR interacts with antirepressor GfoA, whereas Gifsy-3 repressor GfhR interact with antirepressor GfhA and this hetero-complex neutralizes the action of repressor and pushes the development towards the lysis of host (Lemire et al 2011).

Temperate Podoviridae phage SPC32H is another well studied phage which exhibits unique regulatory mechanism. The repressor Rep acts against the antirepressor protein, Ant (Kim & Ryu 2013). Homo-tetramer repressor binds to operator to stop the transcription of Rep and a complex between Rep and ant form a stable hetero-octameric complex causing the disassembly of repressor from operator (Kim et al 2016). This mechanism guides the prophage towards the lytic cycle. Studies of Kim et al showed that Antirepressor does not compete for the DNA binding region of repressor. Crystal structure of the stable hetero-octameric complex proved that Ant binds to the amino and carboxy terminal domains of Rep dimmers and causes the dissociation of the Rep-DNA complex (Kim et al 2016).

Apart from the above phages, the complete regulatory mechanism was elucidated for P22, a *Salmonella typhimurium* phage (Figure 1.9.). Here, lysogeny is maintained by two repressors (C2 and Mnt) and the genome consists of two immunity regions- immC and immI (Susskind & Botstein 1978). Susskind et al aligned the λ genome with P22 and revealed that the organization and orientation of *cI* and *cro* genes are similar with functionally analogous genes of λ phage (Susskind & Youderian 1983). immC region codes for the *c2* gene and product of *c2* gene acts on O_L and O_R operators of P22 to repress the expression of lethal genes for the host cell. The amounts of C2 inside

the host maintain the prophage condition. The second immunity region codes for two more genes, important for regulation- Mnt repressor and Ant antirepressor. Expressions of *mnt* repress the expression of *ant* from the P_{ant} promoter. When Mnt is absent inside of the cell, Antirepressor binds non-covalently with C2 repressor and inhibit C2 function. Inactivation of C2 moves the developmental pathway towards lysis.

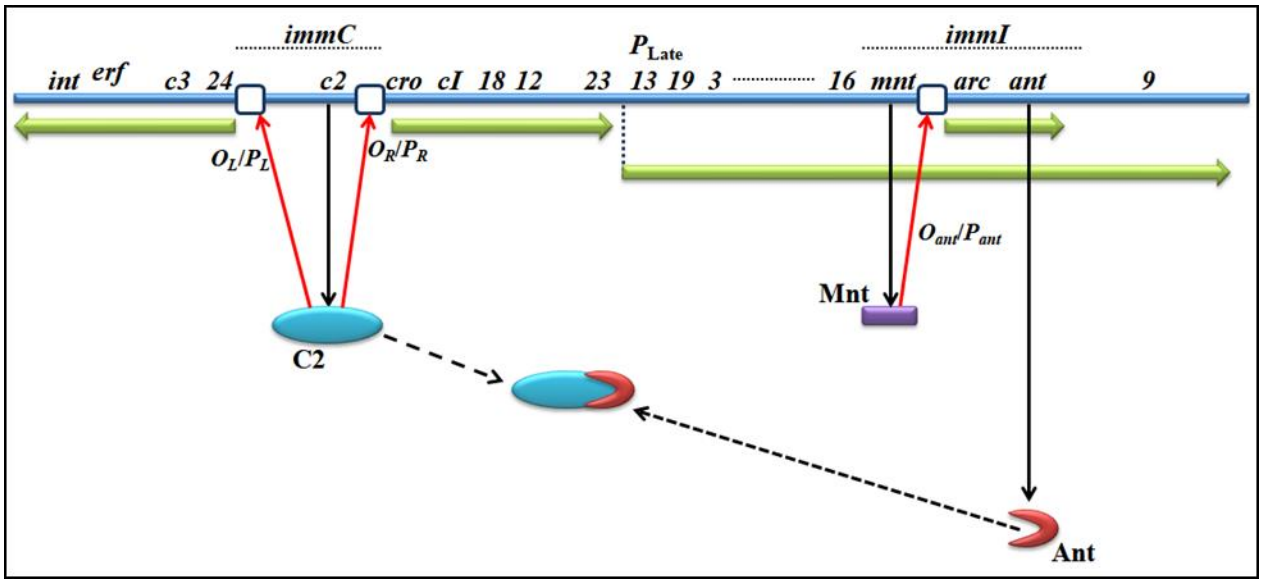

Figure 1.9. || The genetic map of *Salmonella typhimurium* phage P22. Red arrows point towards negative control during lysogeny by repressors acting at operator/promoter sites (O_L/P_L, O_R/P_R and O_{ant}/P_{ant}). C2 repressor inhibited by non-covalent binding of antirepressor (Ant).

1.6.2. **Temperate coliphage 186**

Coliphage 186 is a member of the non-inducible P2 phage, where repressor is not inactivated by RecA. The P2 prophage is non-inducible by ultraviolet irradiation, whereas induction of temperate coliphage 186 is mainly regulated by a small antirepressor Tum (Figure 1.10.). Repression of lytic promoters pR and pB is stimulating out by the Tum protein. Tum prevents CI repressor from binding to pR, pB, FL and FR operator sites (Figure 1.10.). Like λ phage, in coliphage 186, the lysogenic state is maintained by CI repressor and *cII* gene product (Lamont et al 1993). For the lytic development, excision and replication of the phage genome takes place from the pR promoter (Dodd et al 1990). Late genes are expressed by the activation of B protein, expressed form the pB promoter. Expression of Tum from the p95 promoter switches the pathway from lysogeny to lysis (Lamont et al 1989).

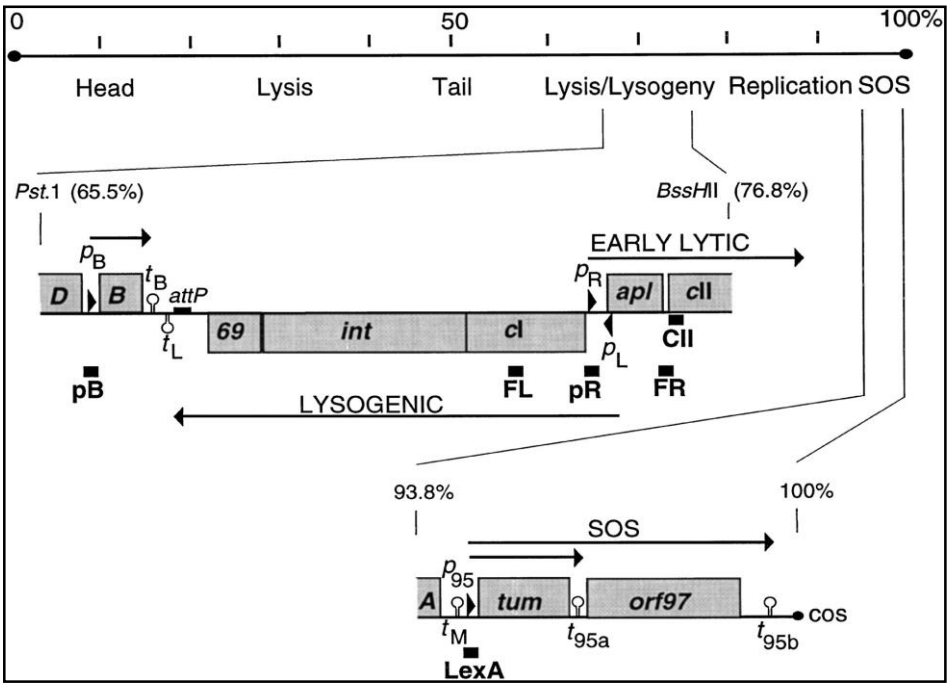

Figure 1.10. || Gene organization of the major control region of coliphage 186. Figure courtesy: Shearwin et al 1998.

1.6.3. **Lambdoid coli phage N15**

This temperate phage has a linear double stranded DNA genome of 46.36 kb. Inside the host cell, the linear plasmid gets circularized with the help of its 12bp *cos* site (Ravin & Ravin 1998). Finally, the genome convert to linear form and the lysogenic state is maintained (Figure 1.11.) (Ravin et al 2000). To maintain the lysogenic state in *E. coli*, the phage genome harbouring 59 ORFs, does not integrate into the host genome (Ravin et al 2000; Ravin 2011). The linear prophage retains three to five copies per bacterial genome. and maintains the lysogenic condition inside the host bacteria through three separate loci- *immA*, *immB* and *immC* (Figure 1.12.). The primary immunity region *immB* has important regulators and is involved with the regulation of *cro*, *Q* and late genes. The second immunity region *immA* is known as antirepressor operon (Ravin & Ravin 1999) and consist of three important genes- inhibitor of cell division (*icd*), antirepressor *antA* and *antB*. Soon after the infection, *immA* region uses the early promoters Pa and Pb and decides the developmental pathway to be followed (Figure 1.12.). Prophage induction is involved with *immC* region which encodes the antirepressor AntC (Mardanov & Ravin 2007). Expressions of antC gene directly inhibits the CB repressor (function as lytic repressor) from binding to the O site and direct the pathway towards lysis (Mardanov & Ravin 2007).

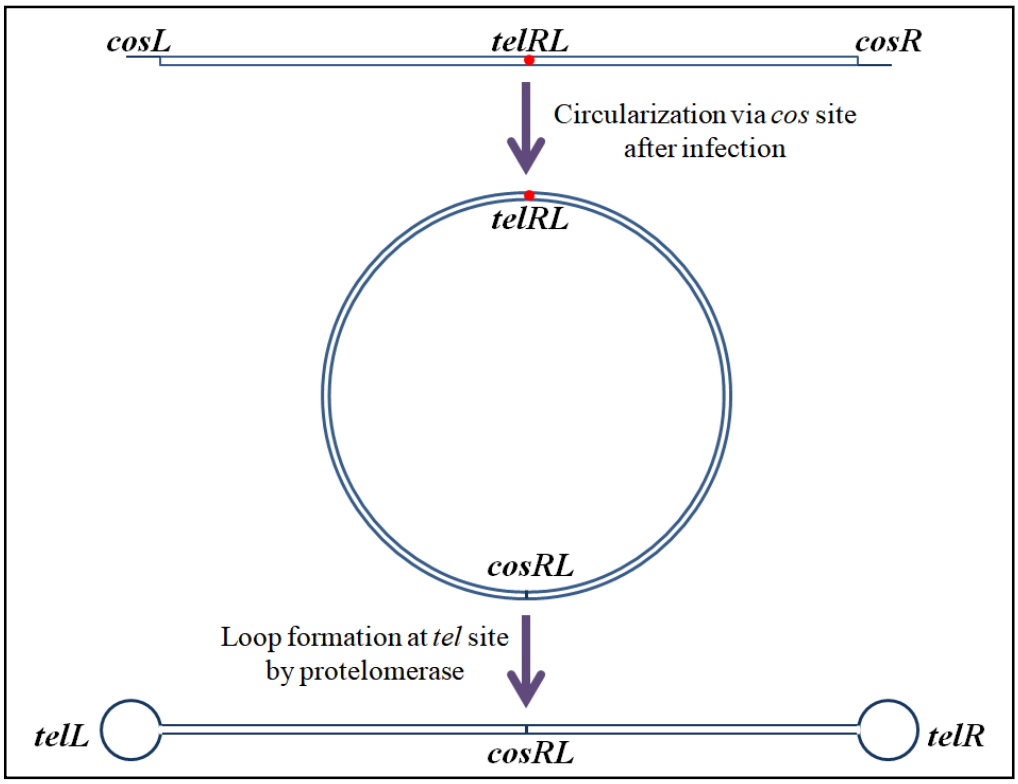

Figure 1.11. || Conversion of phage N15 dsDNA genome into linear plasmid.

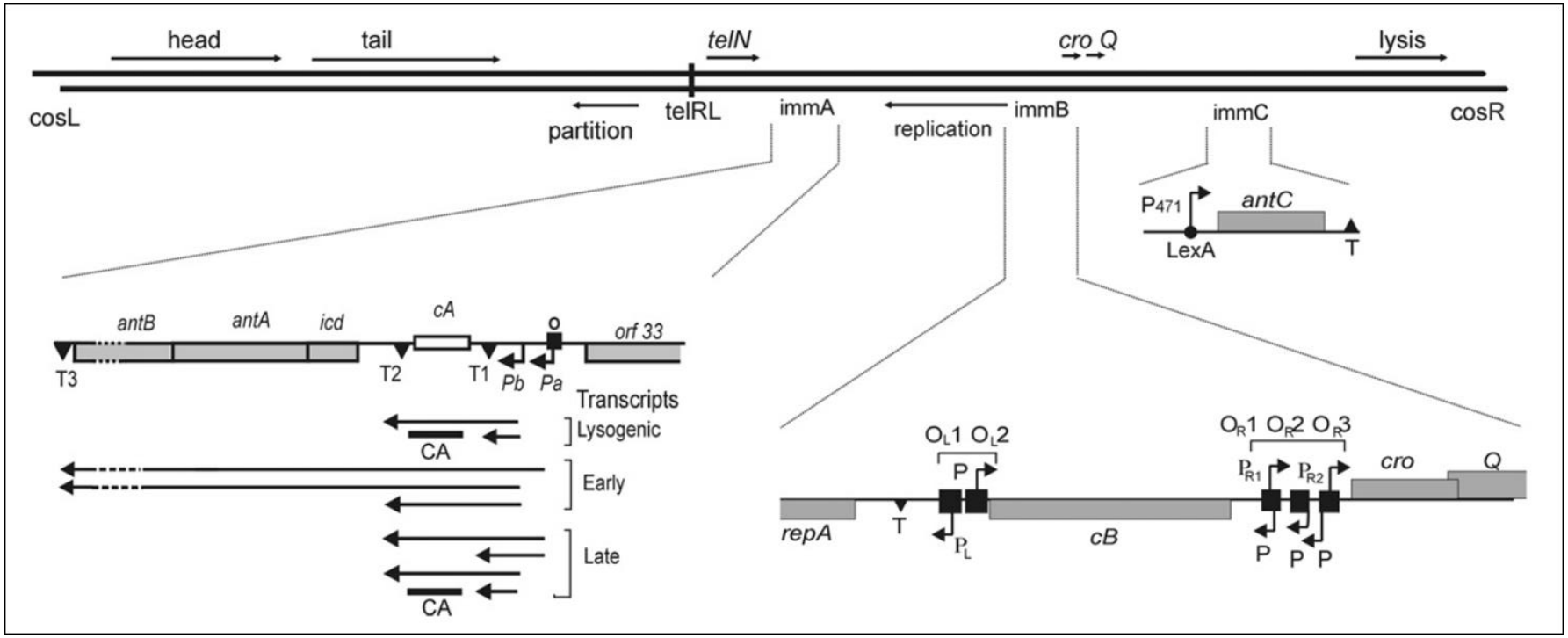

Figure 1.12. || Three lysogeny control regions (*immA*, *immB* and *immC*). Figure courtesy: Ravin 2011.

1.6.4. ***Bacillus thuringiensis*** **phage GIL01**

The family Tectiviridae consists of non-enveloped phages with double-stranded DNA as their genome. Gram-negative bacteria (*E. coli*) serve as normal host, where these phages do not form prophages. However, phages from the Tectiviridae use gram-positive bacteria as their host to form prophages. GIL01 is a temperate phage (15kb genome) with 30 potential ORFs of which six ORFs are homologous to the known proteins [excisionase (ORF1), DNA polymerase (ORF5), LexA-like repressor (ORF6), DNA packaging protein (ORF13), muramidase (ORF25) and muramidase (ORF30)]. The LexA repressor encoded by the host interacts with the gene product of *gp7* (ORF7) and represses the expression of early genes and structural components directly from the promoters P1, P2 and P3 to maintain lysogeny (Figure 1.13.). The induction of phage GIL01 takes place by the action of *gp7* gene product followed by inhibition of LexA binding to *dinBox1* and *dinBox1b* boxes (Fornelos et al 2016). Lytic response starts with DNA damage followed by RecA activation. The LexA repressor undergoes auto-cleavage, which results in derepression of promoters (P1 and P2). Derepression of P1 and P2 promoters facilitate the expression of the phage replication and regulatory genes to initiate the lytic cycle. Consequently, expression of *gp6* activates the expression from P3 to start the synthesis of structural and lysis genes, which facilitate host cell lysis and cell death (Figure 1.13.) (Fornelos et al 2018).

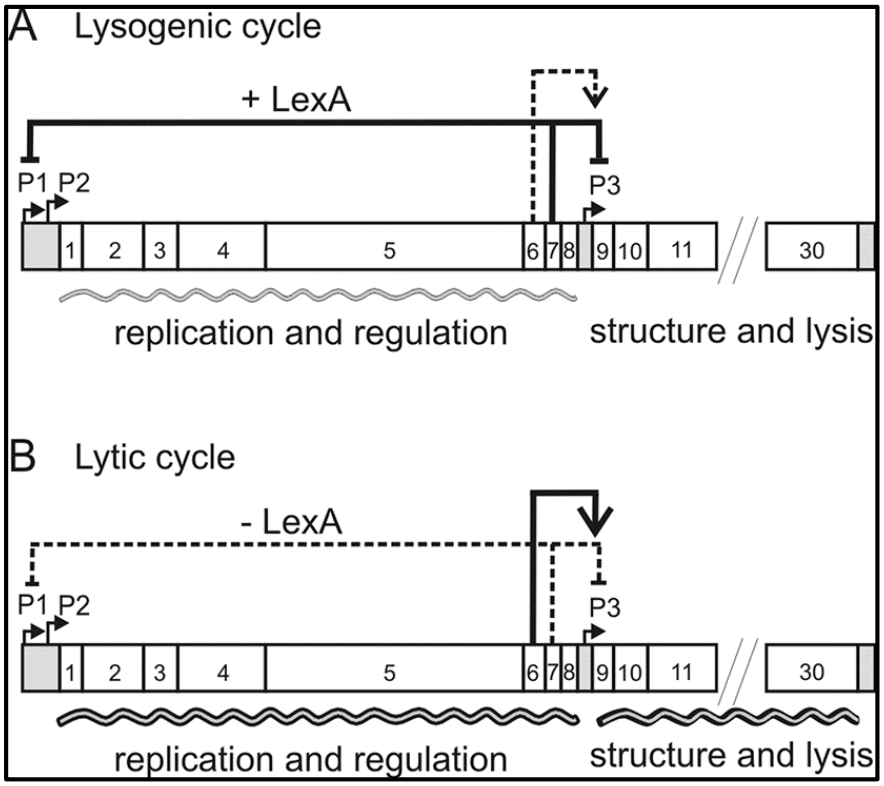

Figure 1.13. || Regulation of the lysogenic (A) and lytic (B) switch in the *B. thuringiensis* temperate phage GIL01. Figure courtesy: Fornelos et al 2018.

1.6.5. *Vibrio cholerae* phage CTXφ

The filamentous phage CTXφ uses *Vibrio cholerae* as its natural host. The phage has a 6.9 kb single-stranded DNA genome consisting of two regions (core region and RS2 region). This phage CTXφ also contains the genetic material considered essential by the bacterium for the production of cholera toxin (Boyd 2010). During most of its life span inside host, phage CTXφ remains as a prophage rather than a virion outside the host. For the injection of its genome, this phage use Toxin-Coregulated Pilus (TCP) and membrane receptor (TolQRA). As soon as the genome gets inserted into the host it gets circularized and is referred to as pCTXφ. The replication and development of CTXφ is control by the RS2 region (Boyd et al 2000). Repressor RstR is transcribed from the left promoter P_R, while the right promoter P_A transcribes the essential genes for the production of virions. RstR repressor has capability to bind O_R1, O_R2 and O_R3 and block the promoter P_A. Filamentous phage CTXφ does not kill its host. Lysogenic to lytic switch of CTXφ takes place after the completion of necessary proteins synthesis and packaging of single-stranded DNA inside of the protein capsid. Finally, the virions are released without killing the host (Davis & Waldor 2003).

1.7. **Phage development and role of SOS response**

The global response (SOS response) in bacteria, involves the repair of DNA damage followed by cell cycle arrest. This process is regulated by the global repressor, LexA, of SOS response. Single-stranded DNA stimulates the co-protease RecA and activates auto-proteolytic activity of global repressor LexA, thereby activating SOS response (Giese et al 2008; Rajendram 2015). In bacteria, the master repressor LexA not only arrests the cell cycle but is also involved in pathogenicity islands movement (SaPI transfer) (Ubeda et al 2007), virulence factor expression (Bisognano et al 2003), bacteriocins expression (Cascales et al 2007) and induction of prophages (Little 2005) (Figure 1.14.).

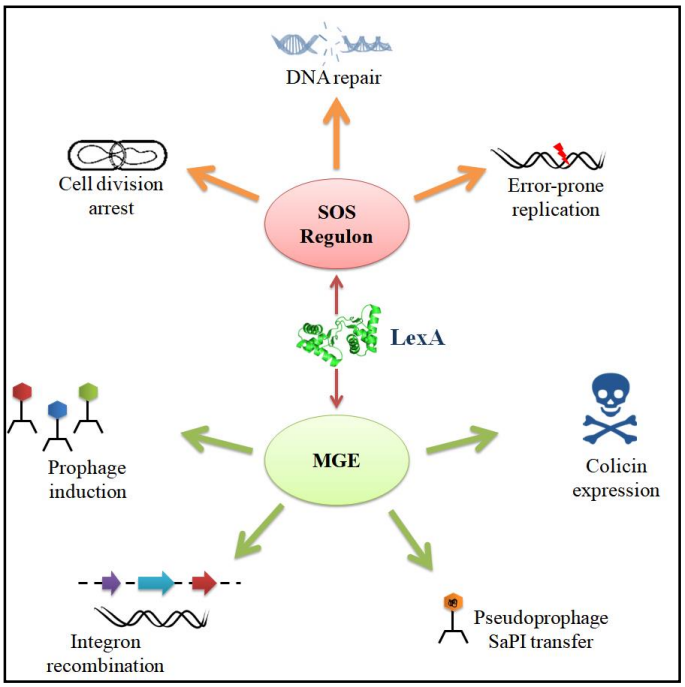

Figure 1.14. || LexA regulon overview. (Fornelos et al 2016)

1.7.1. **LexA and life cycles of temperate phages**

Since the discovery of temperate phages, the scientific research community have shown that lysogenic development is entwined with SOS response (Little 2005). In λ phage, the prophage condition is maintained by CI and the phage genome dormantly stays within bacterial chromosome, without expressing its own genes (Hochschild & Lewis 2009). However, DNA damage leads to the activation of SOS response and induces the autolysis of CI. In many phages, the encoded repressor is non-cleavable and prophage induction takes place under the control of the global repressor LexA. Many phages also encode a second regulatory protein antirepressor, which counteracts the repressor function. Several temperate phages show similar regulation using antirpressor, for eg, coliphage 186 has Tum protein as antirepressor and phage SPC32H has antirepressor Ant protein. LexA binds to the SOS box, upstream of the antirepressor genes and directly controls the working of the antirepressor protein (Shearwin et al 1998; Mardanov & Ravin 2007; Lemire et al 2011; Kim & Ryu 2013) (Figure 1.15.A). DNA damage activates the auto-cleavage activity of LexA and reduces the concentration of LexA, this is followed by the transcription of antirepressor and finally the pathway shifts towards lysis of the infected host. In filamentous phage CTXφ, the LexA and RstR bind simultaneously to *O*1 operator and repress the transcription of RstA repressor from P_A promoter, whereas this binding induces the expression of RstR repressors from P_R promoter (Figure 1.15.B) (Kamruzzaman et al 2014). SOS induction leads to the autocleavage of LexA, which finally encourages the phage to increase copies of its genome (Kimsey & Waldor 2009; Quinones et al

2005). Like the CTXφ phage, phage GIL01also does not integrate its genome with the host genome. Moreover, the genome exists as a linear plasmid inside the host cell to maintain lysogeny. However, the GIL01 lytic-lysogenic switch regulation is different from that of other phages (Figure 1.15.C). In this case, LexA directly binds with P1 promoter of the phage and negatively controls the expression of the phage genes. GIL01 harbours a 50-amino acid *gp7* gene product of ORF7; this small protein plays a crucial role in protecting LexA and maintaining the prophage (Fornelos et al 2016). Non-covalent binding of LexA with *gp7* gene product enhances LexA binding to poor affinity promoters. Furthermore, some phages have developed their own regulators to manipulate or hijack the host system using the host LexA.

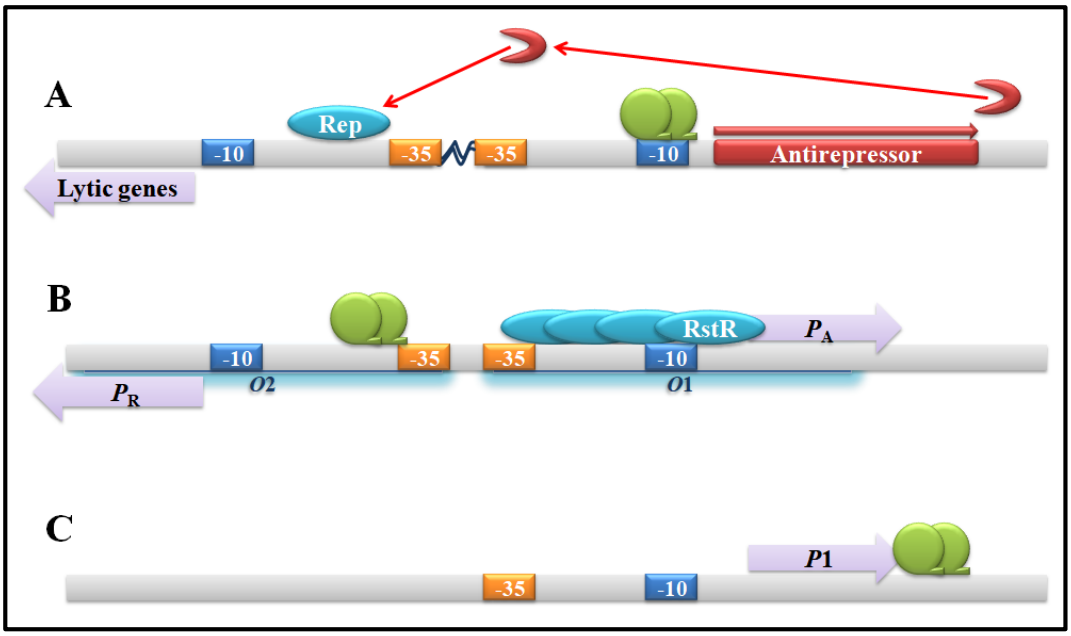

Figure 1.15. || LexA functions differently in different phages for lytic development. Green color indicates the LexA dimers. (A) Depicted the basic regulation pattern of phage 186, SPC32H, Gifsy and N15 through SOS response. (B) LexA regulation of *Vibrio cholerae* filamentous phage CTXφ. (C) Phage GIL01, LexA binds to P1 promoter and maintains prophage.

1.8. **Bacteriophage Phi11**

Bacteriophage Phi11 was first reported in 1967 as P11 phage (Novick 1967) and its nucleotide sequence was first reported in 2002 (Iandolo et al 2002). Phi11 is a temperate bacteriophage of *S. aureus* and can hence choose either the lytic or the lysogenic mode of development. Of the temperate *S. aureus* phages, Phi11 was studied most extensively at molecular level and exploited in construction of vectors and in gene transfer among *S. aureus* strains. Several temperature sensitive, clear plaque and virulent mutants of Phi11 were isolated, mapped and characterized to some extent (Novick 1967; Kretschmer & Egan 1975). Its lysogenization frequency

varies from 1-10% and it also inhibits host protein synthesis after 30-40 min of induction (Chapple & Stewart 1987). It harbors 53 genes (NCBI Ref. Seq.: NC_004615) including two repressor genes, *cI* and *cro*, at one end of its genome (Iandolo et al 2002) (Figure 1.16.). The *cI* and *cro* genes are adjacent genes located in divergent orientation like that of lambdoid phages.

1.8.1. **Life cycle of aureophage Phi11**

Lysogenization in Phi11 is effected by integration of the *attP* site (phage DNA attachment site) of phage DNA with the *attB* (bacterial DNA attachment) site in the bacterial DNA thereby generating two new sites, *attL* and *attR*, at the right and left junctions between the prophage and the host chromosome, respectively (Pattee et al 1977). The *int* and *xis* genes of Phi11 have been cloned;

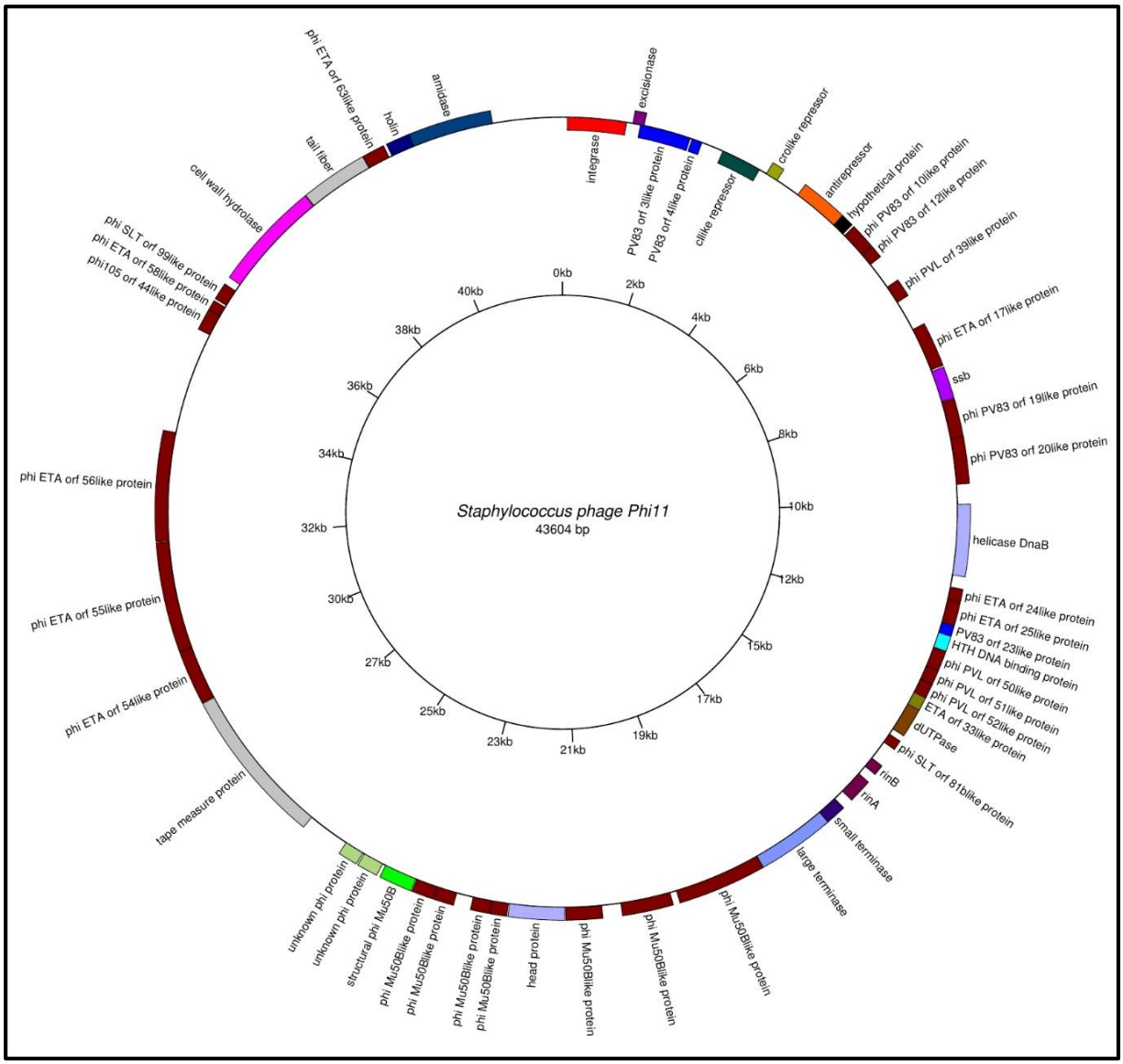

Figure 1.16. || The genetic map of model *S. aureus* phage Phi11.

however, when compared to the *int* and *xis* genes of phage L54a (another *S. aureus* bacteriophage), no global homology could be found between them either at DNA or protein level, though there is significant homology at the potential regulatory regions, at the DNA level (Lee & Iandolo 1988).

1.8.2. **The lysogeny module**

The key components of the lysogeny module of Phi11 are integrase gene, excisionase gene, *cI*-like repressor gene and *cro*-like repressor gene (Iandolo et al 2002). While the integrase gene and the *cro*-like repressor gene are transcribed from one strand in the same direction, *cI*-like repressor gene and the excisionase gene use the opposite strand for their transcription (Iandolo et al 2002). Lucchini et al in 1999, suggested that proteins involved in the lysogenic development of temperate phages usually interact with a target DNA having a conserved sequence (Lucchini et al 1999). The presence of such a site was also detected in phages Phi11, phi12, phi13, Mu50B, phiETA, phiPVL, phiSLT and phiPV83, and was found to occur between the *int* and Orf-C (and *int* and *xis*) as a stem-loop consisting of a 9 base inverted repeat with a 3 base loop (ATTTAGTACtagGTACTAAAT) (Iandolo et al 2002).

1.8.3. **The putative *cI* repressor gene**

Blast analysis of ORF5 suggests that the 748 bp ORF5 of Phi11 functions as *cI*-like repressor (Iandolo et al 2002). The CI repressor protein of Phi11 is responsible for lysogenic development of the phage. Akin to the CI protein of phage lambda, Phi11 CI also harbors an amino terminal domain (NTD) and a carboxy terminal domain. Reports indicate that Phi11 CI shares 19% identity and 38% positivity to λ CI at the protein level (Das et al 2007). Phi11 CI monomer cosists of 239 amino acids. The amino terminal domain harbors a putative helix-turn-helix (HTH) motif and is involved in Phi11 operator DNA binding (Das et al 2007; Biswas et al 2014). It has also been reported that the tentative HTH motif of the amino terminal domain belongs to the HTH-XRE family of transcriptional regulators (Das et al 2007). The carboxy terminal domain shares significant similarity to the carboxy terminal domains of the *E. coli* LexA and lambdoid phage repressors indicating its participation in the dimerization of Phi11 CI (Das et al 2007; Ganguly et al 2009). Biswas et al in 2014, have reported that the DNA binding activity of Phi11 CI amino terminal domain appeared to be similar to that of full-length CI, though they are structurally different (Biswas et al 2014). Furthermore, it has been found that Phi11 CI NTD exists as a dimer in solution; this is surprising as the oligomerization activity of CI was thought to reside in its CTD. Thus the

uniqueness of Phi11 lies in the fact that its DNA binding domain also possesses dimerization property (Biswas et al 2014).

1.8.4. **Autocleavage of Phi11 CI repressor**

RecA or alakaline pH mediated autocleavage is a well-known phenomenon in proteins containing Ala-Gly (Little 1984), Cys-Gly (Nohmi et al 1988), and Leu-Gly (Koudelka et al 2004) dipeptide. The CI monomer of Phi11 harbors a very small protease-sensitive region between its NTD and CTD (Ganguly et al 2009). The primary sequence of Phi11 CI indicates the presence of an Ala-Gly dipeptide at its 130th and 131st positions. Thus RecA or alakaline pH mediated autocleavage at this dipeptide will result in two fragments- a ~19 kDa histidine tagged N-terminal fragment and a ~13 kDa C-terminal fragment. This is further supported by the fact that the 19 kDa protein lighted up when subjected to western blot analysis with anti-his antibody (Das et al 2007).

1.8.5. **The lytic module**

Depending on the environmental conditions, the phage genome starts synthesing the *cro* gene, thereby triggering the induction of the lytic cycle and phage. The lytic mode of development involves the synthesis of the late genes and the head genes.

1.8.6. **The putative *cro* gene**

The single domain Cro of Phi11 shows some unusual dissimilarities when compared to bacteriophage λ. Blast analysis shows that the Phi11 ORF6 encodes a *cro*-like repressor and is composed of only 72 amino acids and HTH-XRE family of transcriptional regulators. Interestingly, Phi11 Cro is thermostable and aggregation of Phi11 Cro began at ~63°C (Das et al 2007). Besides forming a complex with its cognate operator DNA, Phi11 Cro shows smaller amounts of dimerization capabilities at higher concentration (μM) (Das et al 2007).

1.8.7. **The *cI-cro* intergenic region contains binding sites for repressors**

The *cI* (encoding CI) and *cro* (encoding Cro repressor) genes of Phi11 are adjacent genes but oriented divergently (Iandolo et al 2002). The *cI-cro* intergenic region harbors the divergent promoters for both the above genes. A 269 bp *cI-cro* intergenic region (referred to as *O* DNA or O_LO_R DNA) was amplified from the Phi11 genome, cloned and used for studying its interactions with the CI and Cro proteins. Furthermore, O_LO_R DNA digested with HincII resulted in the production of a large fragment designated as O_R and a smaller fragment O_L. Sequence analysis of

O_LO_R region by software program indicated the presence of at least three 15bp inverted repeats with partial two-fold symmetry and were designated as *O*1, *O*2 and *O*3 (Das et al 2007). *O*1 was located in O_R region, while *O*2 and *O*3 localized in the O_L region. While *O*1 was found to overlap with the promoter of *cro*, *O*2 and *O*3 overlapped with the promoter for *cI*.

1.8.8. **Interaction of Phi11 CI and Cro proteins with the *cI-cro* intergenic region**

CI was allowed to bind to O_LO_R DNA as well as to O_L and O_R DNA and the results were analysed by standard gel shift assay (Ganguly et al 2009). Results indicated that, CI binds to both O_L as well as O_R DNA and the binding is specific. Gel Shift Assay results also showed that CI, incubated separately either with O_L or O_R DNA, gave rise to a single shifted complex. Moreover, it was found that CI has a slightly higher affinity for O_R as compared to O_L, as was evident from the equilibrium dissociation constant (~39 nM for O_R and ~30 nM for O_L). It was also found that CI, upon binding to O_LO_R DNA results in two shifted complexes-I and II, with I being the major complex. However, increasing concentrations of CI (55nM) results in nearly equal amounts of both I and II. This data suggests that O_LO_R DNA contains at least 2 binding sites for CI. Probably CI first binds to O_R and with increasing concentrations starts binding to O_L, thereby indicating that the binding is cooperative in nature (Ganguly et al 2009).

Cro was allowed to bind to O_L DNA and the results were analysed by standard Gel Shift Assay. The distinct binding pattern of Phi11 Cro is interesting. Phi11 Cro shows affinity only to the 15bp O3 operator and stops the synthesis of *cI* gene, whereas phage λ Cro protein has affinity for all its cognate operators. Moreover, bacteriophage λ Cro can auto regulate its own synthesis; such an autoregulation appears to be absent in Phi11 (Das et al 2007).

1.9. **Gaps in existing research**

Bacteriophage antirepressor protein plays a critical role in converting the lysogenic pathway of the bacteriophage to lytic pathway under certain conditions such as heat shock or UV light (Donch et al 1970; Heinemann 1971; Kanter & Harriman 1972; Jordan et al 1973; Little & Mount 1982). Most importantly, it has already been shown that in P1201(a corynephage), the antirepressor proteins are actually toxic to the host bacterial cell (Kuana et al). As described in section 1.8, the role played by Phi11 ORF7 (annotated as antirepressor in NCBI) in Phi11 developmental pathway is not yet explored to a large extent. Hence the gaps in existing research are as follows:

- The ORF7 of *S. aureus* phage Phi11 has not been studied at the structural or functional level; hence there is no information regarding its structure and function. This present work attempts to make an in depth study into the structure and function of Phi11 ORF7 gene product.
- There has been not reports regarding the toxic effect exerted by Phi11 antirepressor protein upon the host cell. The current study focuses on this aspect as well.
- Despite the importance of antirepressor proteins in the phages development there is no information about the antirepressor proteins of *S. aureus* temperate bacteriophages. Hence, a detailed characterization of the putative antirepressor protein of Phi11 will greatly enrich our knowledge regarding the developmental pathway of Phi11.
- The involvement of *S. aureus* genes (mainly the SOS response genes) upon the developmental pathway of Phi11 is yet to be explored.

1.10. **Objectives of the proposed research**

A handful of temperate bacteriophages such as Coliphage 186 (Shearwin et al 1998), P4 (Liu et al 1998), P1 (Riedel et al 1993), Gifsy1 and Gifsy3 (Lemire et al 2011) have been reported to harbor an antirepressor gene whose product is involved in the lysogenic to lytic switch. In Coliphage 186, it was found that prophage induction was effected by the Tum protein whose expression is controlled by the host LexA (Lamont et al 1989; Brumby et al 1996).

Hence the main objectives of the research proposal are:

A. Cloning, overexpression and purification of the putative antirepressor gene (ant), the Bro-N domain and the KilA-C domain.
B. Characterization of the putative antirepressor gene of Phi11.
C. Generation of deletion mutants of the Antirepressor protein.
D. Identification of the host genes involved in regulation of the putative antirepressor.
E. Studies on the toxic effects exerted by the overexpression of Phi11 ORF7 upon the host cells.
F. Deciphering the interplay between the different Phi11 proteins and host genes/proteins involved in determining the developmental pathway of Phi11.

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