The novel Antirepressor protein of *Staphylococcus aureus* temperate bacteriophage Phi11 and its role in the lysogenic-lytic switch

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **"The novel Antirepressor protein of *Staphylococcus aureus* temperate bacteriophage Phi11 and its role in the lysogenic-lytic switch"** submitted by Mr. Avijit Das, ID No. 2013PHXF0401G for award of Ph.D. degree of the Institute embodies original work done by him under my supervision.

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Abstract

Phi11, a temperate bacteriophage of *Staphylococcus aureus*, has been found to harbor a *cro* gene and a *cI* repressor gene, both of which play a very important role in the developmental pathway of Phi11. The Cro and CI repressor proteins have been found to bind specifically (with different affinity constant) to 15 bp partially palindromic repeat operator DNA (*O* DNA), located in the *cI-cro* intergenic region. *O* DNA consists of three 15 bp partially palindromic repeats (*O*1, *O*2 and *O*3). CI has binds to *O*1 and *O*2 with maximum affinity for *O*1; on the other hand, Cro binds only to *O*3. Surprisingly, the affinity of Cro repressor towards *O*3 is comparatively much lower than that of CI for *O*1 or *O*2.

To understand the mechanism of action of Cro, the effects exerted by various ions (cations and anions) upon the interaction between Cro and its cognate operator DNA have been studied by employing gel shift assays as well as circular dichroism spectral analysis. This study has revealed that NH_4^+ and $C_2H_3O_2^-$ ions better facilitated the binding of Cro to its cognate operator DNA as compared to Na^+, K^+ and Li^+. Interestingly, Mg^{2+}, CO_3^{2-} and $C_6H_5O_7^{3-}$ have an inhibitory effect upon this binding. The effect of the said ions upon the structure of Cro was also investigated by circular dichroism and it was found that other than $C_6H_5O_7^{3-}$ ions, none of the other ions destabilized the protein. On the other hand, Mg^{2+} and CO_3^{2-} ions maintained the structure of the protein but severely hampered its functional activity. $C_6H_5O_7^{3-}$ ions severely unfolded Cro and also inhibited its function. Considering all the data, NH_4^+ and $C_2H_3O_2^-$ ions appeared to be more suitable in maintaining the biological activity of Cro.

Interestingly, the genome of aureophage Phi11 reveals the presence of on early gene *gp07* (ORF7), which codes for the putative antirepressor protein (GenBank accession no. NC_004615.1). Antirepressor proteins are mainly involved in lytic cycle of various bacteriophages. The Phi11 Gp07 consists of two domains - an amino terminal Bro-N domain and a carboxy terminal KilA-C domain. Despite the important role of antirepressor proteins in the developmental pathway of phages, there are no reports on the purification and characterization of aureophage antirepressor proteins. In this work, study Gp07, its two domains and its deletion mutant (ΔGp07) have been cloned separately. The effects exerted by the overexpression of Gp07, ΔGp07 and its separate domains upon the growth rate as well as the morphology of the *Escherichia coli* cells have been studied. Taken together, these results indicate that Gp07, ΔGp07 as well as the carboxy-terminal domain of Gp07 upon overexpression, retards the growth rate of the *E. coli* cells and also induces filamentation in the cells. Moreover, the overexpressing cells also exhibit the presence of multiple nucleoids. The carboxy terminal KilA domain of Gp07

appears to be indispensible for its action upon the growth rate and morphology of the host cells. However, the growth inhibition and filamentation induced by the amino-terminal domain of Gp07 is temporal in nature.

The growth inhibitory effect of Gp07 upon the host cells makes it an interesting candidate for further characterization. However, the purification of Gp07, has proved to be very challenging. Being a lethal protein, upon overexpression it completely retards the growth of the host cells. In a bid to purify Gp07, a method was devised to overexpress and purify the full length Gp07 as carboxy terminal hexa histidine tagged variant. The recombinant protein was overexpressed in *E. coli* BL21(λDE3) cells. The time and temperature of induction by IPTG were optimized to obtain the overexpressed recombinant Gp07 in soluble form. Later, a gradient of imidazole and NaCl were used for successful purification of soluble Gp07 to homogeneity. It was found that Gp07 exists as a dimer in solution as is evident from gel filtration chromatography and glutaraldehyde cross-linking data. Further, it was observed that temperature has huge impact on the structural conformation of the protein.

Finally, the functional role of Gp07 in the developmental pathway of Phi11 was investigated. Antirepressor proteins of bacteriophages are chiefly involved in interfering with the function of the repressor protein and forcing the bacteriophage to adopt the lytic cycle. The results indicate that Gp07 functions as a novel antirepressor by regulating the developmental pathway of Phi11. It mediates its actions by enhancing the binding of the Cro repressor protein to its cognate operator. It was also observed that the CI repressor protein of Phi11 binds to the putative operator of Gp07 and regulates its expression. Moreover, it has been found that *S. aureus* transcriptional repressor *lexA* and co-protease *recA* genes play a crucial role in the lytic-lysogenic switching in Phi11. Finally, it has been identified that the Bro-N domain of Gp07 is actually responsible for enhancing the binding of Cro repressor to its cognate operator. Phi11 prophage induction is different from other bacteriophages. This work furnishes a first-hand report regarding the regulation involved in the developmental pathway of Phi11.

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List of abbreviations

ΔGp07	Eleven amino acids deletion mutant of Gp07
A_{595}	Absorbance at 595 nm
A_{600}	Absorbance at 600 nm
Amp	Ampicilin
Amp^R	Ampicilin resistance gene
APS	Ammonium per sulphate
bp	Base pair
BSA	Bovine serum albumin
CBB G250	Coomassie brilliant blue G-250
CBB R250	Coomassie brilliant blue R-250
CD	Circular dichroism
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsDNA	Double-stranded DNA
EDTA	Ethylenediamine tetra acetic acid
EMSA	Electrophoretic mobility shift assay
EtBr	Ethidium bromide
EtOH	Ethanol
Gp07	Gene product of ORF7
IPTG	Isopropyl thio-β-D-galactoside
Kan	Kanamycin
Kan^R	Kanamycin resistance gene
Kb	Kilo base pairs
kD	Kilodalton
LA	Luria bertani Agar
LB	Luria bertani broth
M	Molar
Mb	Mega base pairs
ml	Millilitre
mM	Millimolar
MOI	Multiplicity of infection

ng	Nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometer
O DNA	*cI-cro* intergenic region bearing the cognate operator DNA for Phi11 Cro
OD	Optical density
OD_{595}	Optical density at 595nm
OD_{600}	Optical density at 600nm
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pfu	Plaque-forming unit
pmol	Picomol
rCTD	Recombinant carboxy terminal domain (KilA-C) of Gp07
RNase	Ribonuclease
rNTD	Recombinant amino-terminal domain (Bro-N) of Gp07
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssDNA	Single-stranded DNA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) amino methane
Trp	Tryptophan
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultra violet
µg	Microgram
µl	Microlitre

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.

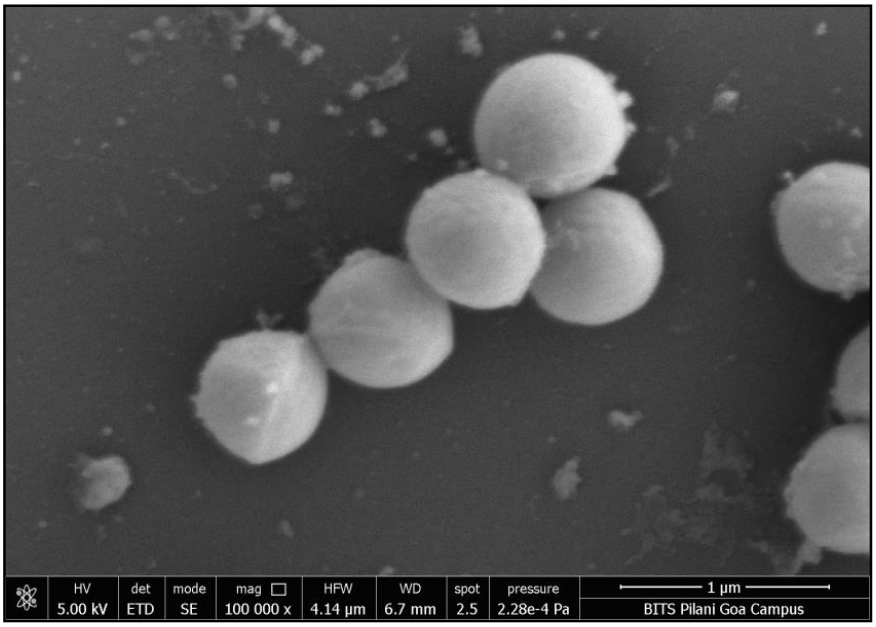

Figure 1.1. || Scanning electron micrograph of *S. aureus* RN4220, phage Phi11 host bacterium. Scale bars 1µm.

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).

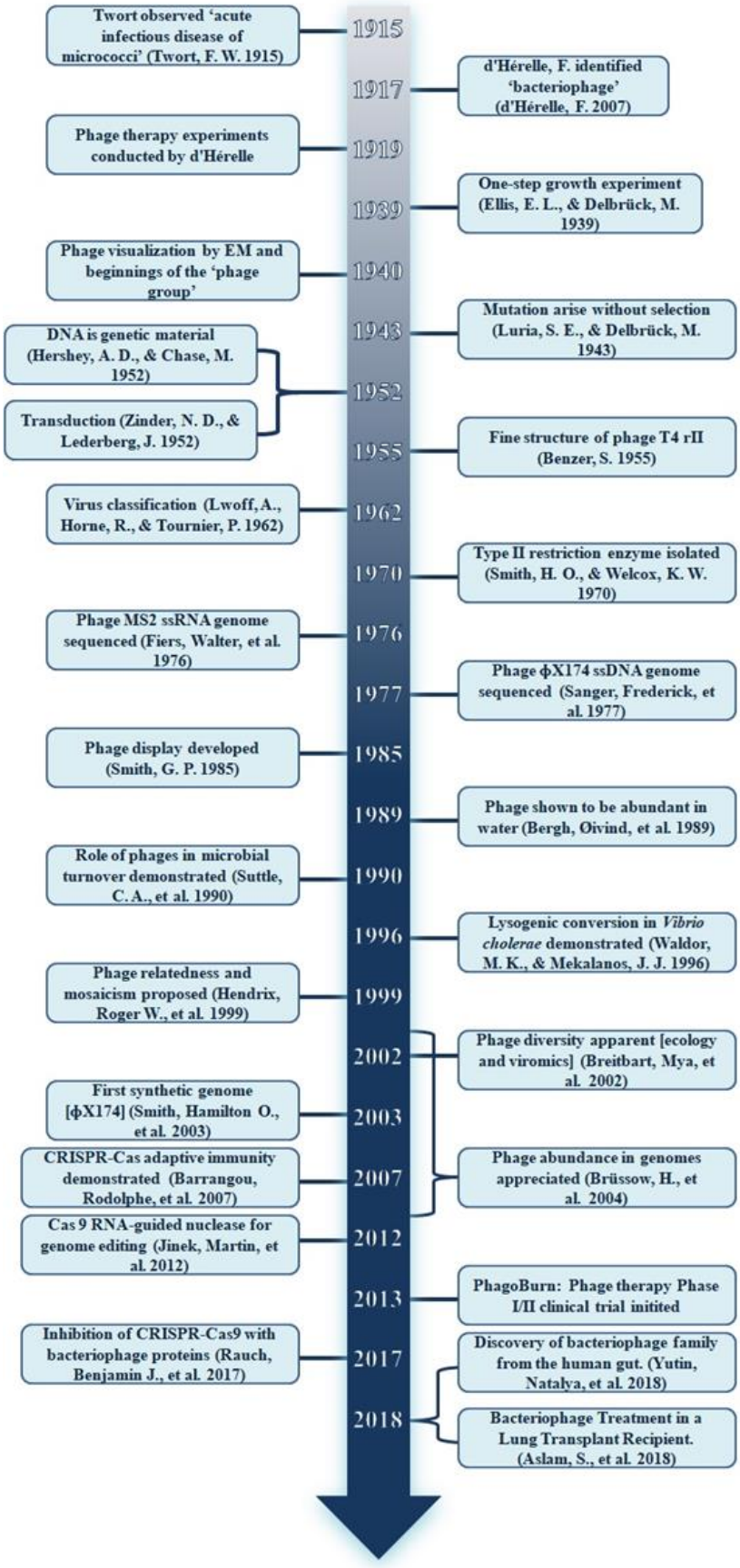

Figure 1.2. || Some major discoveries in the phage research.

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.).

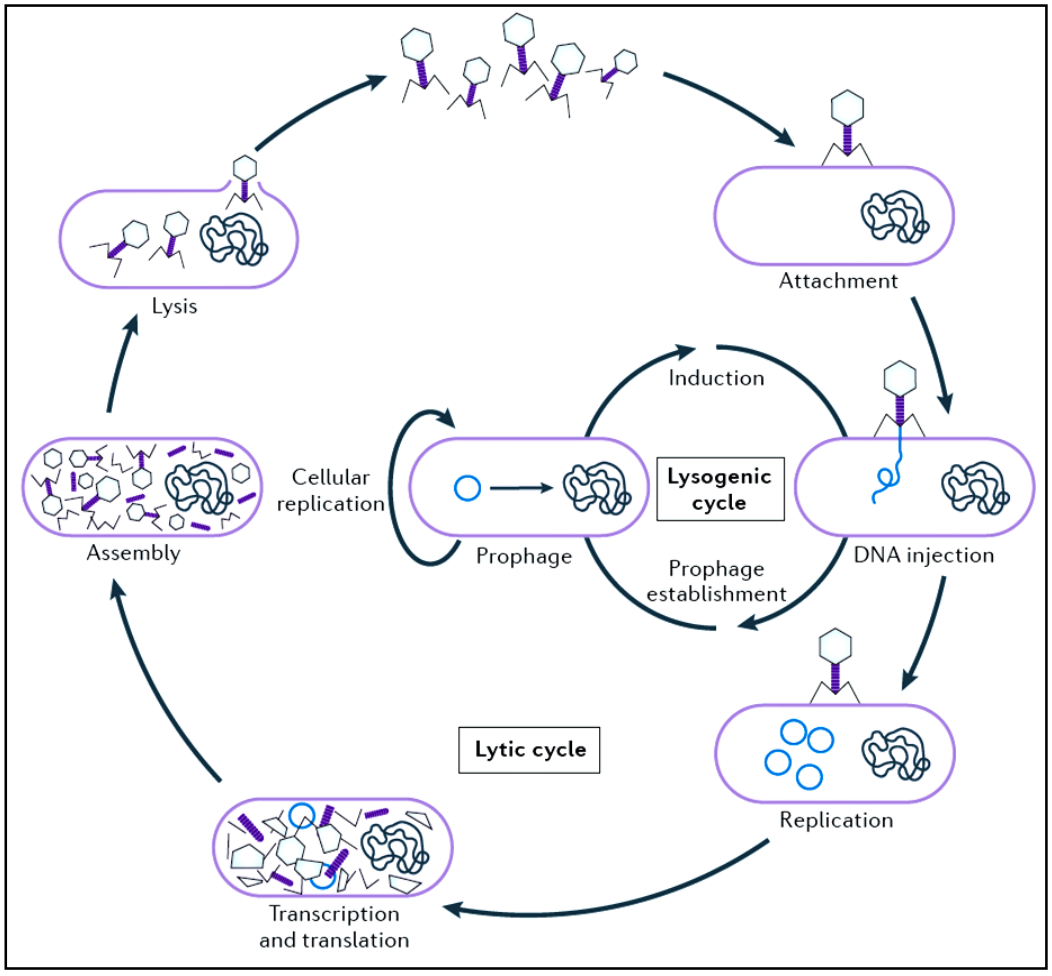

Figure 1.3. || Phage life cycles (lytic and lysogenic mode of development). Figure courtesy: Salmond & Fineran 2015.

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.

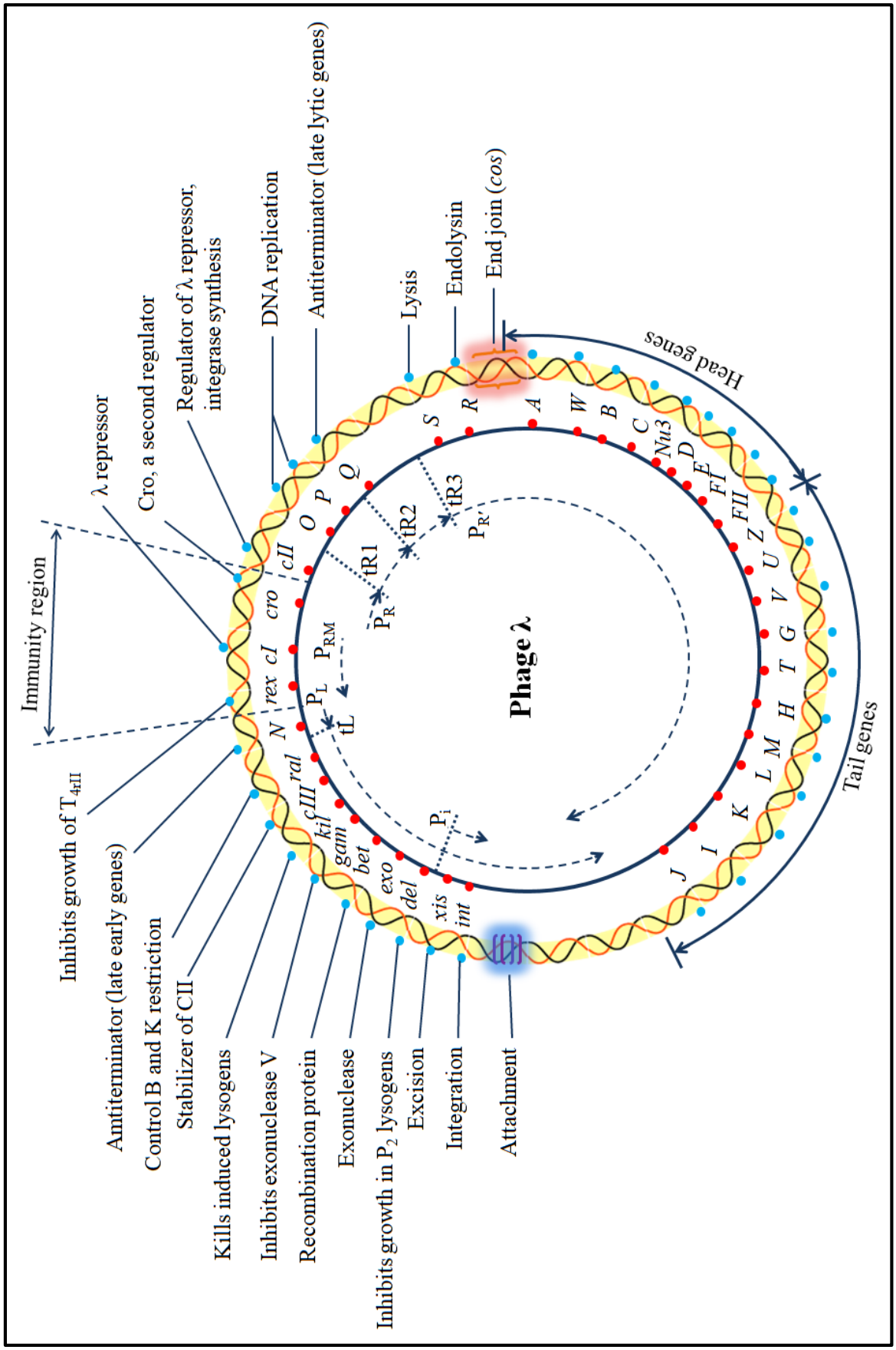

Figure 1.4. || The genetic map of model lambda (λ) phage.

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.

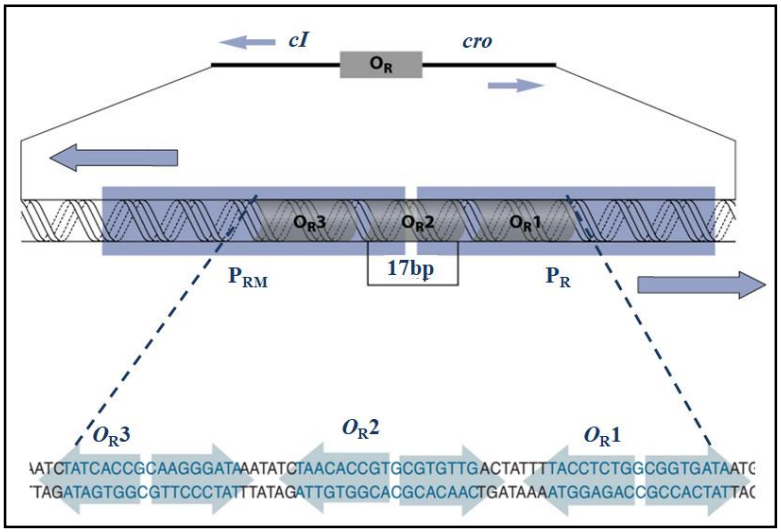

Figure 1.5. || The right operator region (O_R) of lambda phage. Figure courtesy: Ptashne 2004.

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).

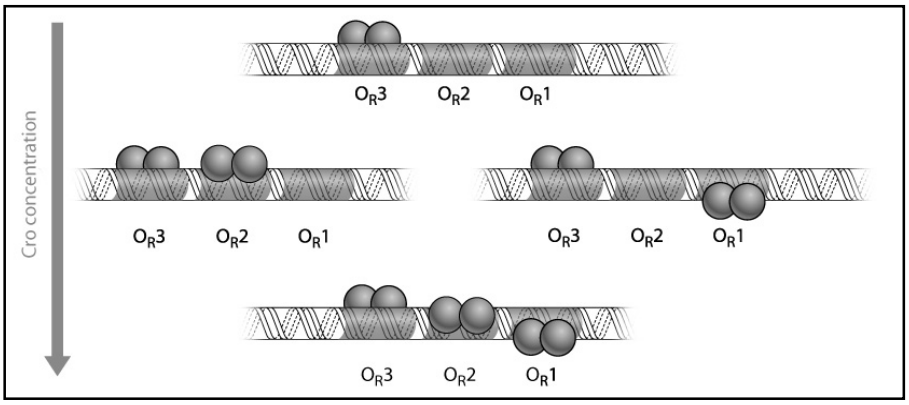

Figure 1.6. || Regulatory mechanism of Cro repressor on O_R. Figure courtesy: Ptashne 2004.

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.

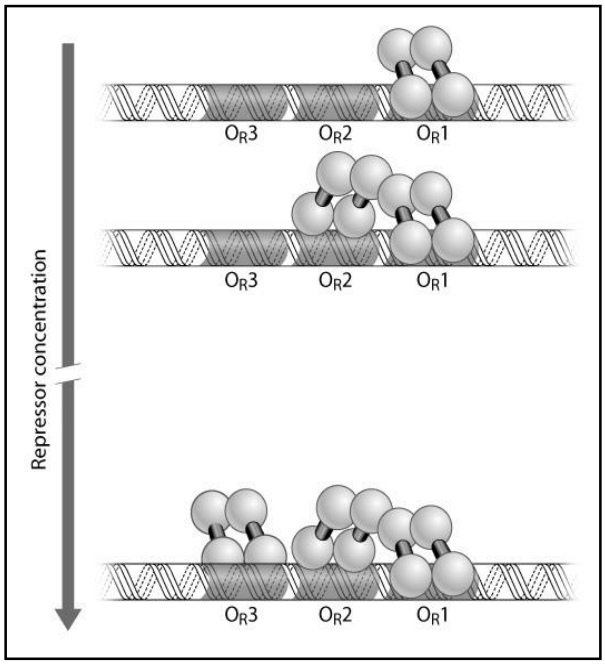

Figure 1.7. || Regulatory mechanism of CI repressor on O_R. Figure courtesy: Ptashne 2004.

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.

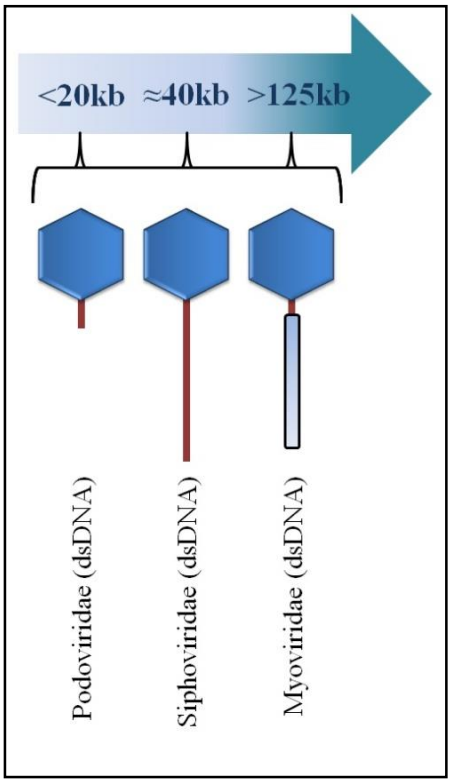

Figure 1.8. || Caudovirales phage families, morphologies (tail), genome types (dsDNA) and relative genome sizes. Note: Phage particles are not drawn to scale.

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.

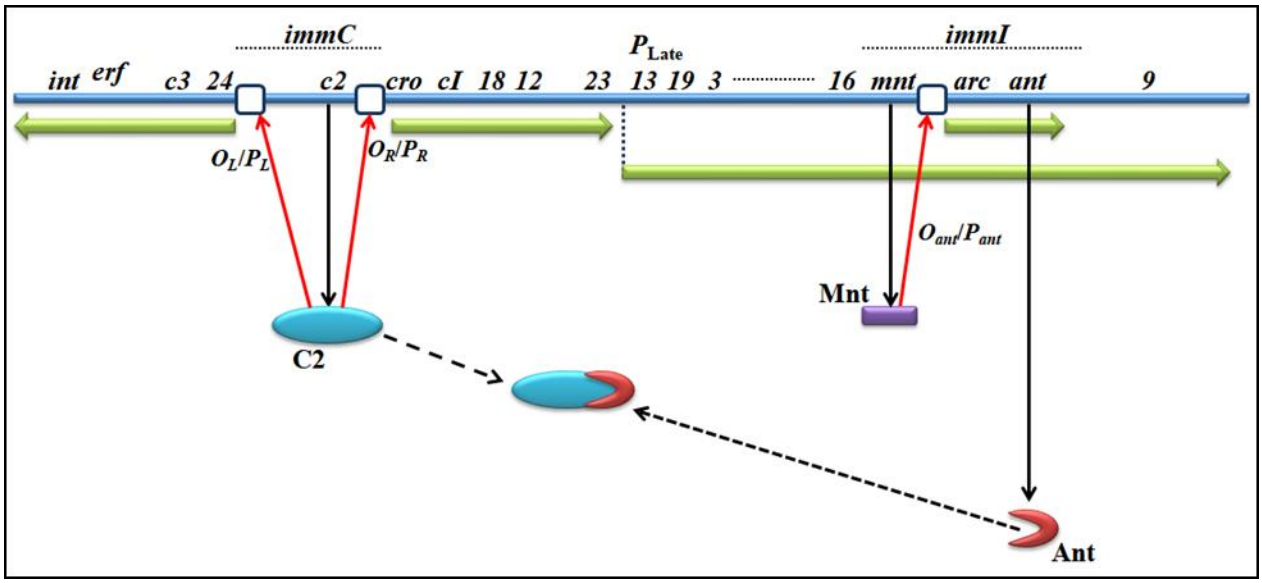

Figure 1.9. || The genetic map of *Salmonella typhimurium* phage P22. Red arrows point towards negative control during lysogeny by repressors acting at operator/promoter sites (O_L/P_L, O_R/P_R and O_{ant}/P_{ant}). C2 repressor inhibited by non-covalent binding of antirepressor (Ant).

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).

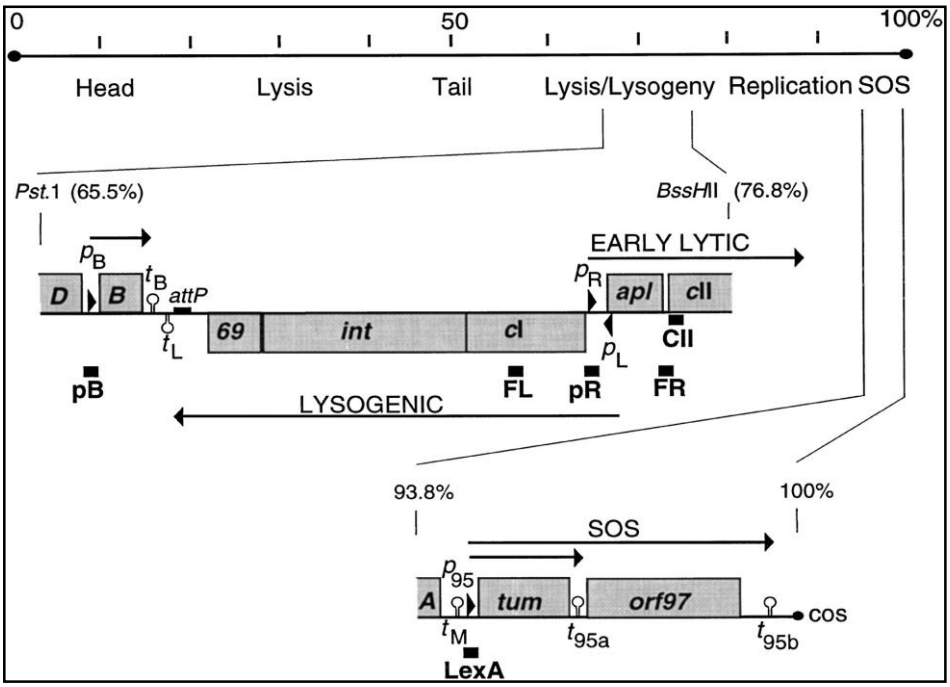

Figure 1.10. || Gene organization of the major control region of coliphage 186. Figure courtesy: Shearwin et al 1998.

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).

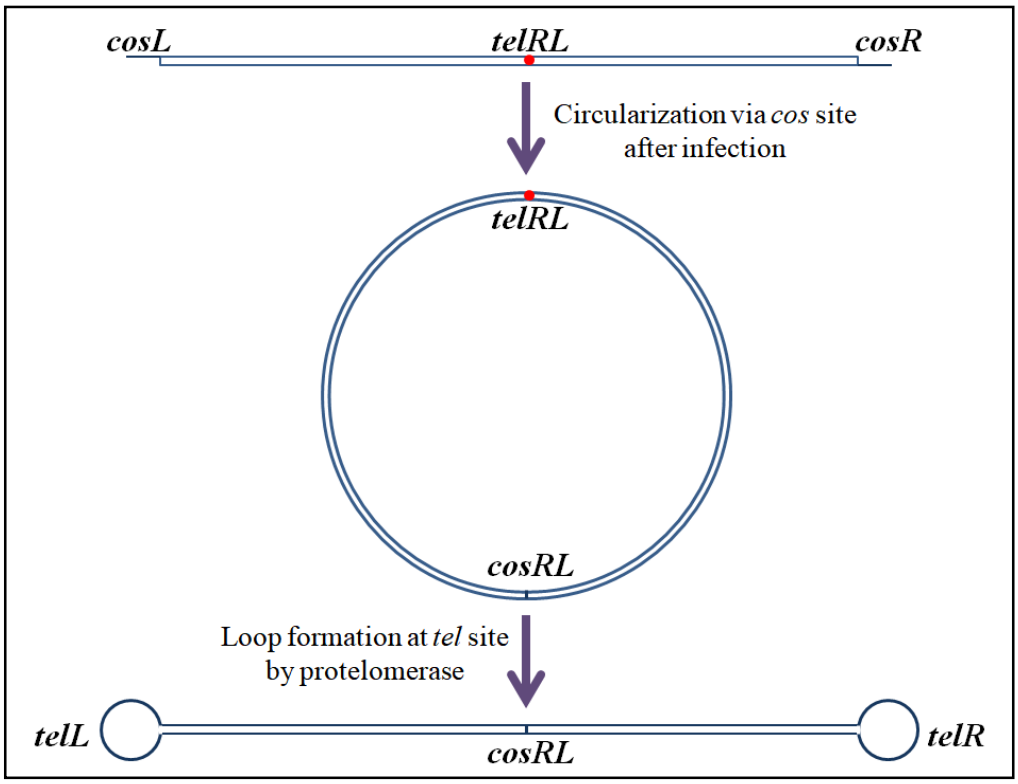

Figure 1.11. || Conversion of phage N15 dsDNA genome into linear plasmid.

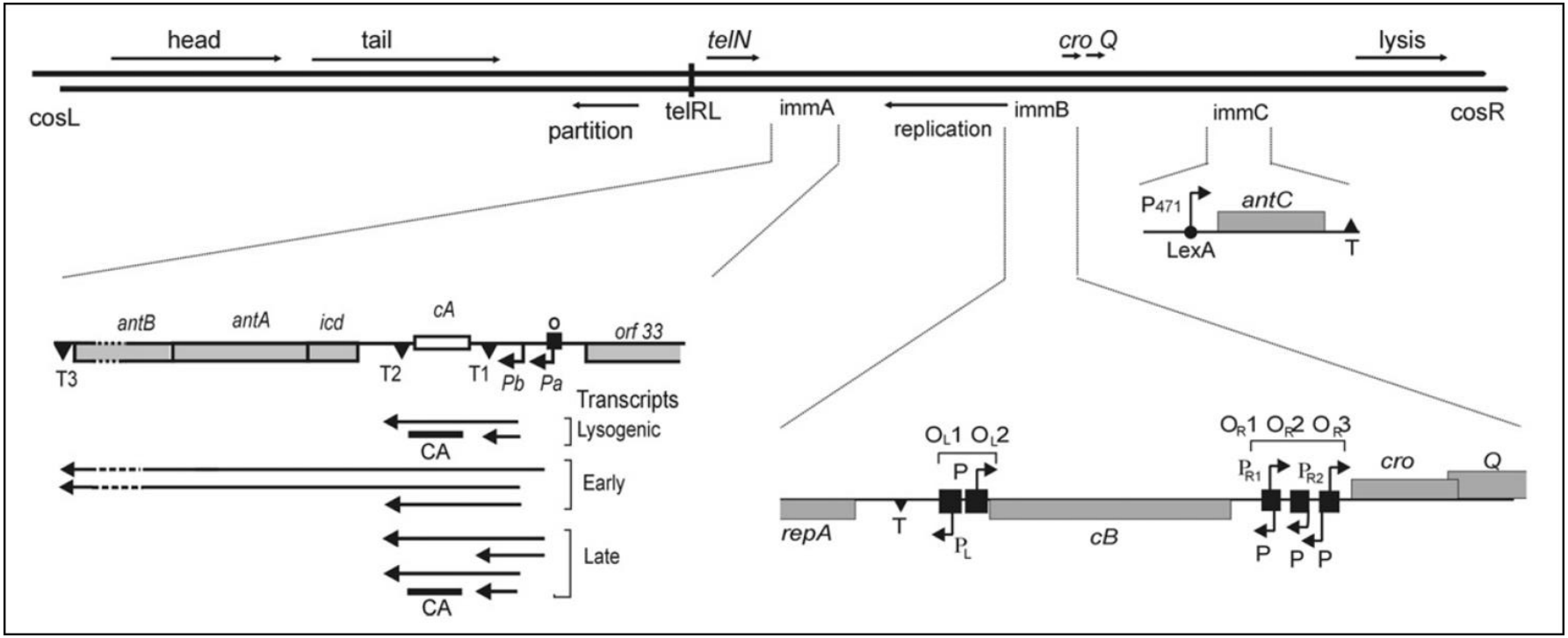

Figure 1.12. || Three lysogeny control regions (*immA*, *immB* and *immC*). Figure courtesy: Ravin 2011.

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).

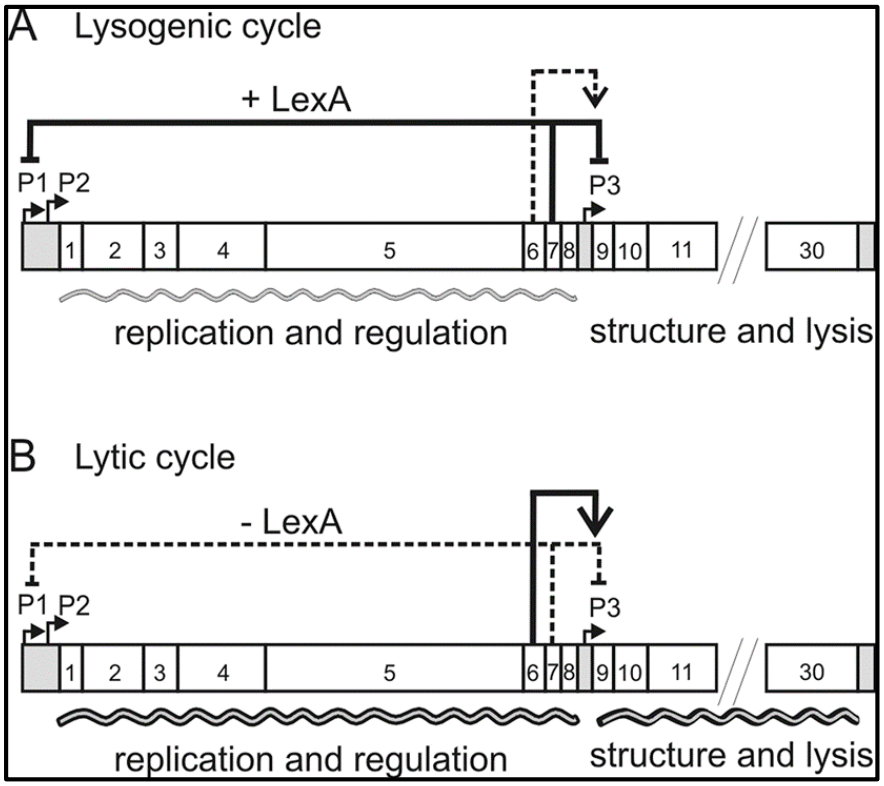

Figure 1.13. || Regulation of the lysogenic (A) and lytic (B) switch in the *B. thuringiensis* temperate phage GIL01. Figure courtesy: Fornelos et al 2018.

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.).

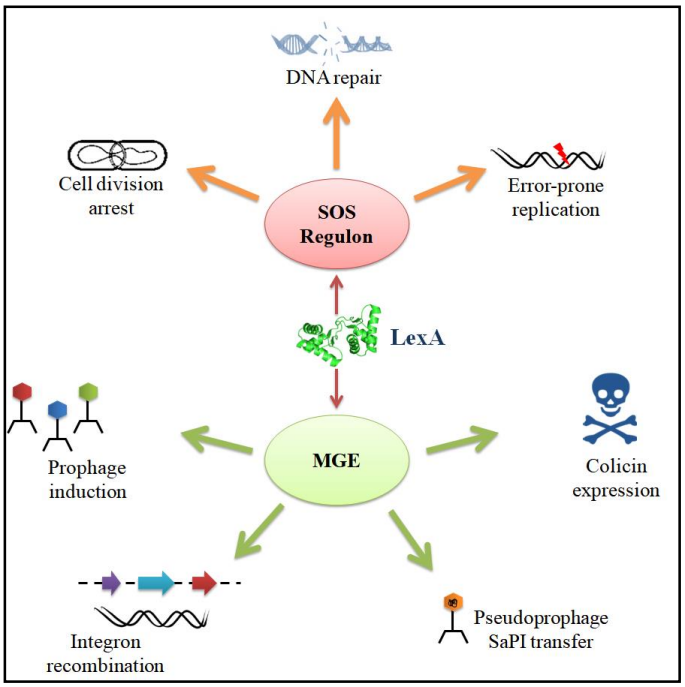

Figure 1.14. || LexA regulon overview. (Fornelos et al 2016)

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.

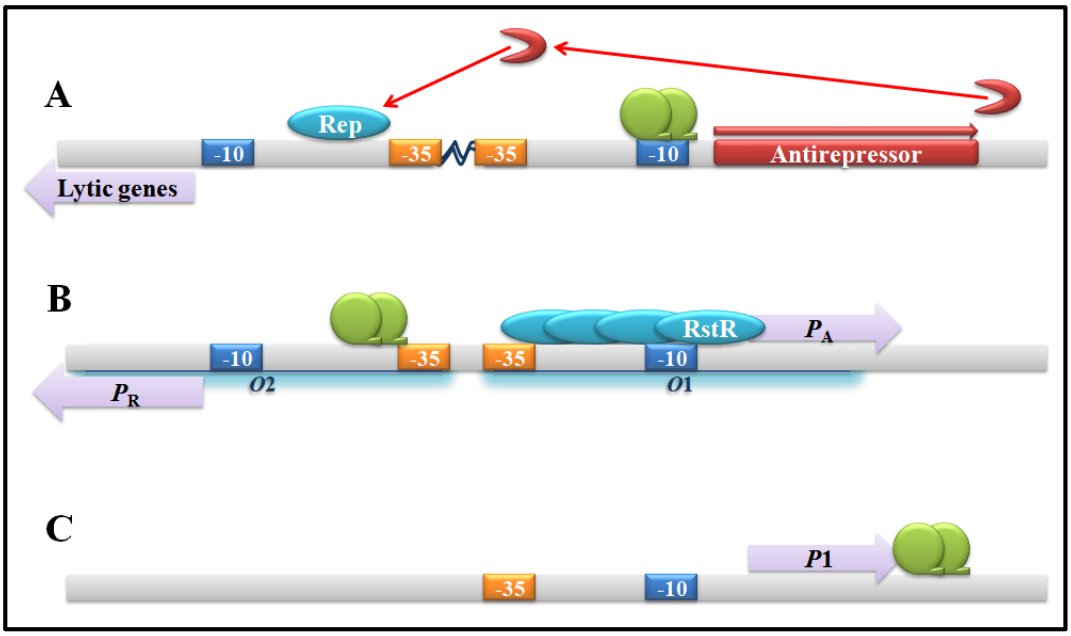

Figure 1.15. || LexA functions differently in different phages for lytic development. Green color indicates the LexA dimers. (A) Depicted the basic regulation pattern of phage 186, SPC32H, Gifsy and N15 through SOS response. (B) LexA regulation of *Vibrio cholerae* filamentous phage CTXφ. (C) Phage GIL01,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

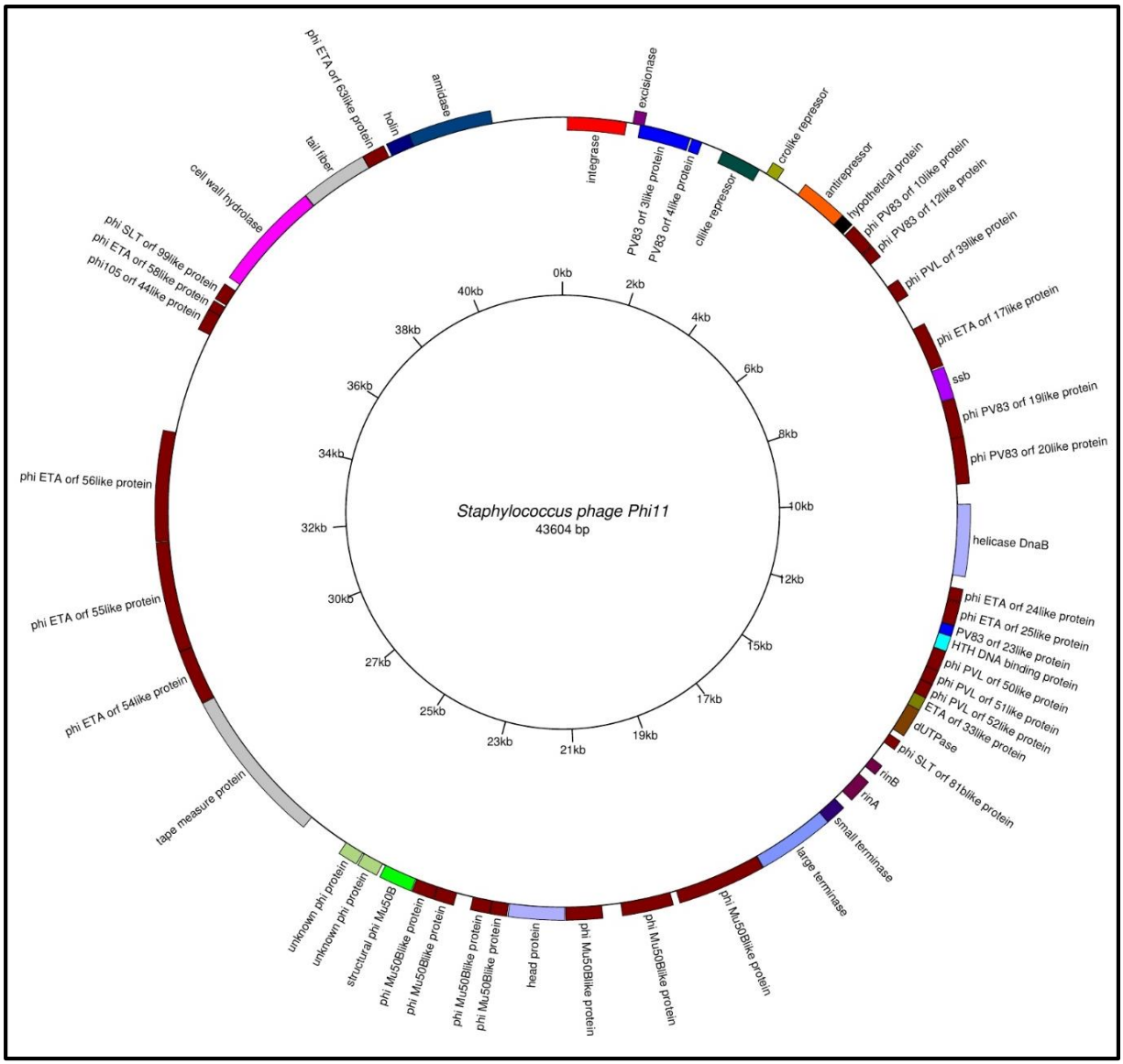

Figure 1.16. || The genetic map of model *S. aureus* phage Phi11.

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.

1.11. **References**

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Chapter 2

Changes in functional activity of Cro repressor mediated by various ions

2.1. **Introduction**

Temperate bacteriophages can adopt the lytic as well as the lysogenic mode of development based on the interaction of the phage encoded CI protein and the Cro protein with their cognate operator DNA sequences (Ptashne 1980; Susskind & Youderian 1983; Ptashne 1986; Ogawa et al 1988; Heinrich et al 1995; Kimsey & Waldor 2004; Koudelka et al 2004; Dodd et al 2005; Hatfull & Jacobs 2014). Repressor and Cro proteins are expressed by the temperate lambdoid phages during the early stages of infections. While repressor is involved in switching off the lytic pathway, Cro is involved in switching off the lysogenic pathway of temperate bacteriophages. Both Cro and CI repressor proteins of coliphage λ had been studied extensively at biochemical, biophysical, genetic and structural levels (Ptashne 1986). The *cI* and *cro* genes are particularly important in giving us a good insight about the gene regulation in the host bacteria as well as the infecting bacteriophages. Moreover, these regulatory proteins are also extensively utilized for construction of various expression vectors.

Bacteriophages of *S. aureus* include Phi11, phi 12, phi13, L45a, phi42 and phiSa3ms and it has been observed that most of these phages carry the *cI* and *cro* genes in divergent orientation in their genome, akin to that of lambdoid phages (Ptashne 1986). Phi11 is a member of serogroup B (Bachi 1980). The genome of Phi11 is a double stranded, linear DNA with a G+C content of 35-37% (Brown et al 1972). Additionally, the genome is circularly permuted (40% or more) (Kretschmer & Egan 1975; Lofdahl et al 1981) and is 5% (Lofdahl et al 1981) to 25% (Bachi 1980) terminally redundant.

Phi11 Cro is a 72 amino acid polypeptide and is 20-40% identical with Cro-like proteins of phages Tuc2009 (Kenny et al 2006), A2 (Ladero et al 2002) and so on. It is a moderately thermostable, single domain protein and binds to a single operator DNA (designated as *O*3) located in the *cI-cro* intergenic region (Das et al 2009).

The interaction of different regulatory proteins, such as lac repressor, bacteriophage repressor and Cro proteins, with their cognate operator DNA can be largely affected by different parameters such as ions, pH, temperature and so on (Mandal & Lieb 1976; Barkley et al 1981; Koblan & Ackers 1991; Relan et al 1997; Alberty & Bock 1953). Salts, in general, can play a very important role in influencing the biologically active conformation of proteins. The effects exerted by different salts upon different proteins can vary. Some salts might lead to aggregation, denaturation or unfolding of the protein and some might lead to minute changes in the domains of a multidomain protein. In

both cases the functional activity of the protein may or may not be affected (Bandhu et al 2009). However, there are a few ions such as magnesium cations which can stabilize double stranded DNA and help it to adopt its biologically active secondary and tertiary structures (Misra & Draper 1998). In fact, there are reports which state that stabilization of native DNA at room temperature require 100 times more concentration of NaCI as compared to $MgCl_2$ (Thomas 1954). It has also been reported that melting temperature of duplex DNA increases with increasing concentration of magnesium ions. A possible explanation for this observation could be that DNA unwinding is induced by the negatively charged phosphate groups which repel each other. The presence of counter ions like magnesium reduces this unwinding (Butzow & Eichhorn 1965). Whether such cations interfere with interaction of DNA binding proteins is yet to be established. The importance of ions upon repressor–operator interaction has already been reported for mycobacteriophage L1 (Bandhu et al 2009). Phi11 Cro repressor plays a very important role in the lytic regulation of Phi11; however, it has a very low affinity towards its cognate operator (*O* DNA, especially *O*3), as compared to lysogenic repressor CI (Das et al 2009). In spite of the importance of ions upon protein-DNA interaction, there are no reports regarding the effect exerted by different ions on the interaction of Phi11 Cro protein with its cognate operator DNA. In this regard, this chapter investigates the effect of different cations and anions on the structure and function of Phi11 Cro with special emphasis on its operator binding capacity. The results indicate that NH_4^+ and $C_2H_3O_2^-$ ions are required for optimal binding of Cro to its cognate operator DNA.

2.2. **Materials and Methods**

2.2.1. **Bacterial strains and growth conditions**

E. coli BL21 (λDE3) and *E. coli* XL1 Blue cells were grown at 37ºC in Luria broth (Sambrook & Russell 2001). The growth media were supplemented with kanamycin as per requirement. The *E. coli* strain BL21 (λDE3) harbouring recombinant *cro* was obtained from Prof. S. Sau (Bose Institute, India). The salts were purchased from Sigma, USA and SYBR Green II Stain-10,000X concentrate was purchased from ThermoFisher Scientific.

2.2.2. **Over-expression and purification of recombinant Cro**

Recombinant histidine tagged Cro was purified by Ni-NTA column using gravity flow technique as described previously (Das 2009). Briefly, an overnight primary culture of the *E. coli* strain BL21 (λDE3) harbouring recombinant *cro,* was grown in LB medium containing kanamycin (50µg/ml) by incubating in an incubator shaker at 37°C, 120 rpm. 10 ml of this primary culture was used to inoculate 500ml of LB medium containing kanamycin (50µg/ml) in two 1 litre conical flasks. Cells were allowed to grow at 37°C with shaking (at 120rpm) until an OD_{600} ~0.4-0.5 was reached. The culture was then transferred to a shaking incubator at 32°C and shaken at 120 rpm. After a 20 min cool-down period, IPTG was added to a final concentration of 0.5 mM. The induced cells were further allowed to grow for 3 h at 32°C and 140 rpm following which they were harvested by centrifugation (10 min, 6,500 rpm at 4°C). The cell pellet was washed with 0.9% NaCl and was stored at -80°C until use.

For purification of the recombinant Cro, the cell pellet was thawed and resuspended in 10 ml of ice-cold lysis buffer (Lysis buffer: 20 mM Tris-chloride buffer pH 8, 300 mM NaCl, 5% glycerol, 10 mM imidazole and 10 µg/ml of PMSF). The suspension was then lysed by sonication (Misonix Microson Sonifier Misonix XL2000 Sonicator System Ultrasonic Cell Disrupter) at 10 watts (RMS) for 12 min (Repeated cycles of 20 seconds with 2min intervals) at 4°C.Finally, the sonicated extract was centrifuged (45 min, 12,000rpm, 4°C) and the supernatant (cytoplasmic fraction) was separated from the pellet fraction. The recombinant Cro was purified from the supernantant fraction by Ni-NTA column chromatography according to manufacturer's protocol (ThermoFisher Scientific). The supernatant obtained after centrifugation was loaded onto Ni-NTA resin (pre-equilibrated with ice cold lysis buffer). The supernatant was allowed to pass through the column slowly to give adequate time for 6X histidine tagged Cro to bind to column material. This binding process was repeated thrice. After three rounds of binding, the flow-through was collected and stored at 4°C. The protein bound column was washed using 20 bead volumes of wash buffer (Wash buffer: 20mM Tris pH 8, 300mM NaCl, 25mM imidazole and 5% glycerol). Finally, the hexa His-tagged Cro was eluted

from the column using elution buffer (Elution buffer: 20mM Tris pH 8, 100mM NaCl, 300mM imidazole and 5% glycerol). The elution fractions were pulled together and kept for dialysis against dialysis buffer (Dialysis buffer: 20mM Tris pH 8, 200mM NaCl, 0mM imidazole, 5% glycerol) overnight at 4°C, to allow complete removal of imidazole. The dialyzed protein was stored at 4°C until further use.

2.2.3. **Qualitative estimation of the purified Cro using Tris-Glycine polyacrylamide gel electrophoresis (Tris-Glycine SDS-12% PAGE)**

The purified protein fractions were analyzed on Tris-Glycine SDS-12% PAGE (Sambrook & Russell 2001). The resolving gel (SDS-12% PAGE) was prepared by adding and mixing the different gel components (Table 2.1.) and the resultant solution was poured between the glass plates separated by spacers and allowed to polymerize. After allowing sufficient time for the resolving gel to polymerize, the 5% stacking gel mix (Table 2.1.) was prepared and added above the resolving gel with the insertion of a comb to form the lanes. Gel electrophoresis was carried out in presence of TGS buffer (5X TGS; tris-base 7.55gm, glycine 47 gm, 10% SDS 25ml and final volume 500ml by addition of water) initially at 90 Volts (V) and then at 120 V using 1X TGS running buffer till the optimal resolution was achieved.

Table 2.1. SDS poly-acrylamide gel percentage.

Components	**12% Resolving Gel[a]**	**15% Resolving Gel[a]**
H_2O	3.3ml	2.3ml
30% acrylamide	4ml	5ml
1.5M Tris HCl (pH 8.8)	2.5ml	2.5ml
10% SDS	0.1ml	0.1ml
10% APS	0.1ml	0.1ml
TEMED	0.012ml	0.012ml

Components	**5% Stacking Gel[b]**
H_2O	3.4ml
30% acrylamide	0.83ml
1M Tris HCl (pH 6.8)	0.63ml
10% SDS	0.05ml
10% APS	0.05ml
TEMED	0.005ml
[a] For the final 10ml. [b] For the final 5ml.	

2.2.4. **Staining of polyacrylamide gel using Coomassie Brilliant Blue-R250(CBB-R250)**

To stain the gel for visualising the protein bands, CBB-R250 has been used. The gel was taken out from the gel plates and washed with double distilled water. To visualize the protein bands in the gel, the gel was stained with CBB-R250 as described below (Ausubel et al 1998). Briefly, the washed polyacrylamide gel was soaked in freshly made CBB-R250 solution (Final volume 100ml: 40ml methanol, 10ml glacial acetic acid, 0.5gm CBB-R250, 50ml double distilled water) with rocking. After 20-30 min at room temperature, the staining solution was replaced with the destaining solution (given in appendix A). The gel was destained (at room temperature, on a rocker) until the gel background was clear and the protein bands were visible.

2.2.5. **Quantitative estimation of the purified Cro using Bradford method**

Bradford method was used for quantitative estimation of the purified protein (Bradford 1976). A 5X concentrated Bradford reagent (given in appendix A) was prepared and this preparation was kept at 4°C. To measure the concentration of a desired protein, the 5X Bradford reagent was diluted 1X with sterile double distilled water. Then a certain volume of the desired protein was added to the 1X Bradford reagent to make a final volume of 1 ml and the suspension was gently mixed. The mixture was allowed to stand at room temperature for 5 minutes till it turned blue in color. The intensity of the blue color was measured in a spectrophotometer at 595nm against an appropriate blank (1X Bradford reagent and sterile double distilled water). Initially a standard curve was constructed by using known concentrations of BSA (Bovine Serum Albumin). The unknown protein concentration was finally determined from the known standard curve. The molar concentration of Cro was calculated considering the theoretical molecular weight of Cro protein polymer from EMBOSS Pepstats (https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/).

2.2.6. **Gel shift assay and K_D (Apparent equilibrium dissociation constant) determination by image analysis**

Gel Shift Assay was carried out by standard procedure (Das et al 2007) in which various concentrations of the purified Cro protein were incubated with 90ng of *O* DNA which harbors *O*3 region (Figure 2.1.), in a binding buffer (10 mM Tris-Cl [pH 8.0], 200 mM NaCl, 5% glycerol) as described previously (Das et al 2009). The effect of the various cations and anions on Cro-*O* DNA interactions was investigated by replacing the 200mM NaCl in dialysis buffer with 200mM of KCl, LiCl, NH_4Cl, $MgCl_2$, Na_2CO_3, $C_2H_3NaO_2$ or $Na_3C_6H_5O_7$. The pH values of all the buffers were checked and adjusted to pH 8.0, before use. The purified Cro protein was thoroughly dialyzed against each of the above buffers and used for binding reactions and CD-spectra analysis.

To carry out gel shift assay, a 6% non-denaturing PAGE was used. Individual reaction mixtures were loaded onto different lanes of the polymerized 6% non-denaturing PAGE. Electrophoresis was carried out in cold for 5h at 80 V. Finally, the gel was stained with SYBR Green II Stain-10,000X concentrate in DMSO (ThermoFisher Scientific) and the stained native gel pictures were captured by E-Gel® Imager System with UV Light Base used SYBR® Filter (Invitrogen, Life Technologies).

The gel pictures were analyzed by ImageJ version 1.51d (Schneider, C.A., et al 2012). Plot profile function was used to determine the percentage of Cro-operator bound complex and graphs were drawn using sigmoidal fitting function of OriginPro 2016 Version b9.3.2.303 (Academic). The fitting curves had R-square value above 0.9. The K_D values were calculated from the graphs. Each experiment has been repeated thrice and the standard deviation values have been presented in the figures.

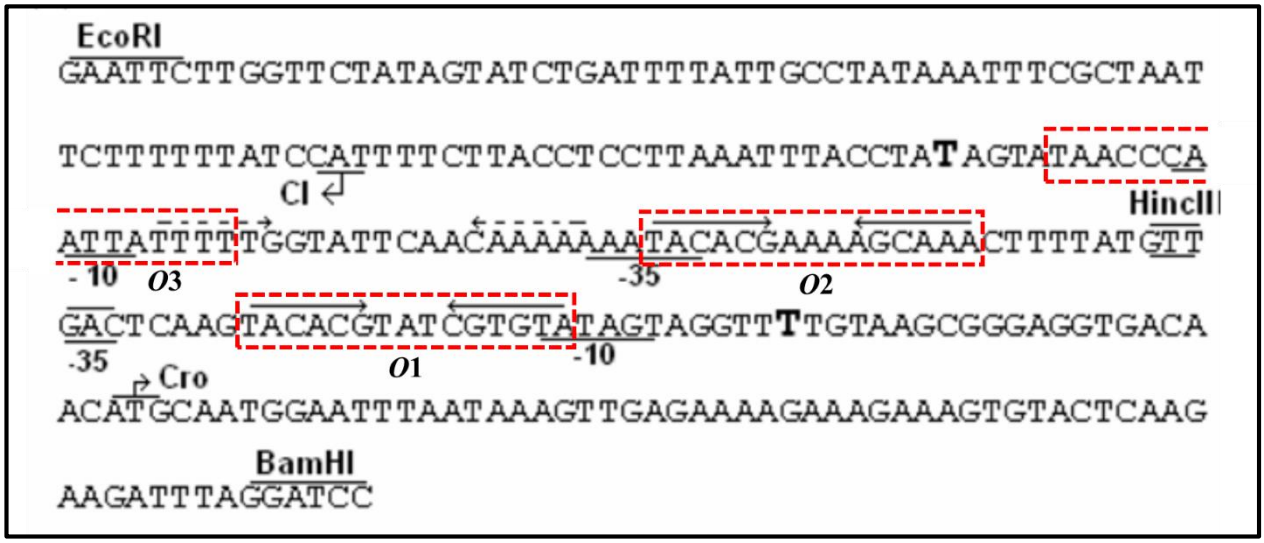

Figure 2.1. || Sequence of *cI-cro* intergenic region (designated as *O* DNA) of Phi11. The 15bp CI binding sites *O*1 and *O*2 are shown in red box. Cro binding site *O*3 (15bp) is shown in red box. Figure courtesy: (Das et al 2007).

2.2.7. **Secondary structure determination of Cro in presence of various ions by employing circular dichroism spectroscopy**

To investigate the effect of the various cations and anions on Cro-operator interactions, the 200mM NaCl in the binding buffer was replaced by 200mM of KCl, LiCl, NH_4Cl, $MgCl_2$, Na_2CO_3, $C_2H_3NaO_2$ or $Na_3C_6H_5O_7$. 30µM of Phi11 Cro was dialyzed in the desired buffers and the secondary structure of the protein in that particular buffer was analyzed by CD (200-240nm, an average of three scans were taken) by JASCO J-815 spectropolarimeter. Samples were transferred to a cuvette of 1mm path length prior to the recording of CD spectrum. The data were further analyzed by K2D2

web server (Perez-Iratxeta & Andrade-Navarro 2008) (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d2/).

2.2.8. **Oligomerization of Cro in presence of NaCl, $MgCl_2$ and Na_2CO_3**

To investigate the oligomeric status of Cro in presence of NaCl, $MgCl_2$ and Na_2CO_3, the aliquots of Cro (equilibrated with NaCl, $MgCl_2$ and Na_2CO_3) were run on a Tris-Glycine 12% PAGE without denaturation (Arndt et al 2012). To prevent denaturation, Cro (in the respective buffer) was mixed with 1X SDS sample buffer (without beta-mercaptoethanol) and loaded directly onto the gel without boiling. The gel was run at 50 V for approximately 8 hours in the cold room. The gel was then stained by CBB R-250, followed by destaining as described in section 2.2.4. To compare the oligomeric status of the native protein in comparison to its denatured state, the protein (in presence of $MgCl_2$ and Na_2CO_3) was run in native (Tris-Glycine 12% PAGE) as well as denaturing conditions (SDS-12% PAGE).

2.3 **Results**

2.3.1. **Cro retains its biological activity in presence of monovalent cations such as Na^+, K^+ and Li^+**

Gel shift assay was employed to measure the effect of monovalent cations Na^+, K^+ and Li^+ on the DNA binding activity of Phi11 Cro. Cro was equilibrated in NaCl, KCl or LiCl buffers and the equilibrium binding was studied. As is clear from the figures (Figure 2.2.A and 2.2.B, Figure 2.3.A and 2.3.B and Figure 2.4.A and 2.4.B), the apparent equilibrium dissociation constant, K_D for Cro-*O* DNA interaction in Na^+, K^+ and Li^+ buffers are 0.457, 0.437 and 0.427 micro molar (μM) respectively. This indicates that the three cations had similar effect upon the biological activity of Cro protein.

To further investigate the effect of Na^+, K^+ and Li^+ upon the structure of the Cro protein, CD spectral analysis (200-240nm) was carried out for Cro (equilibrated in NaCl, KCl and LiCl). The CD spectrum exhibited a plot of large negative ellipticity at 208nm for Cro equilibrated in all three buffers, namely, NaCl, KCl and LiCl (Figure 2.2.C, Figure 2.3.C and Figure 2.4.C respectively). K2D2 analysis of the CD spectrum for Cro in each of the three buffers indicated that the protein was approximately 68-75% α-helical, 1-2% β-stranded and 22-28% coiled in structure.

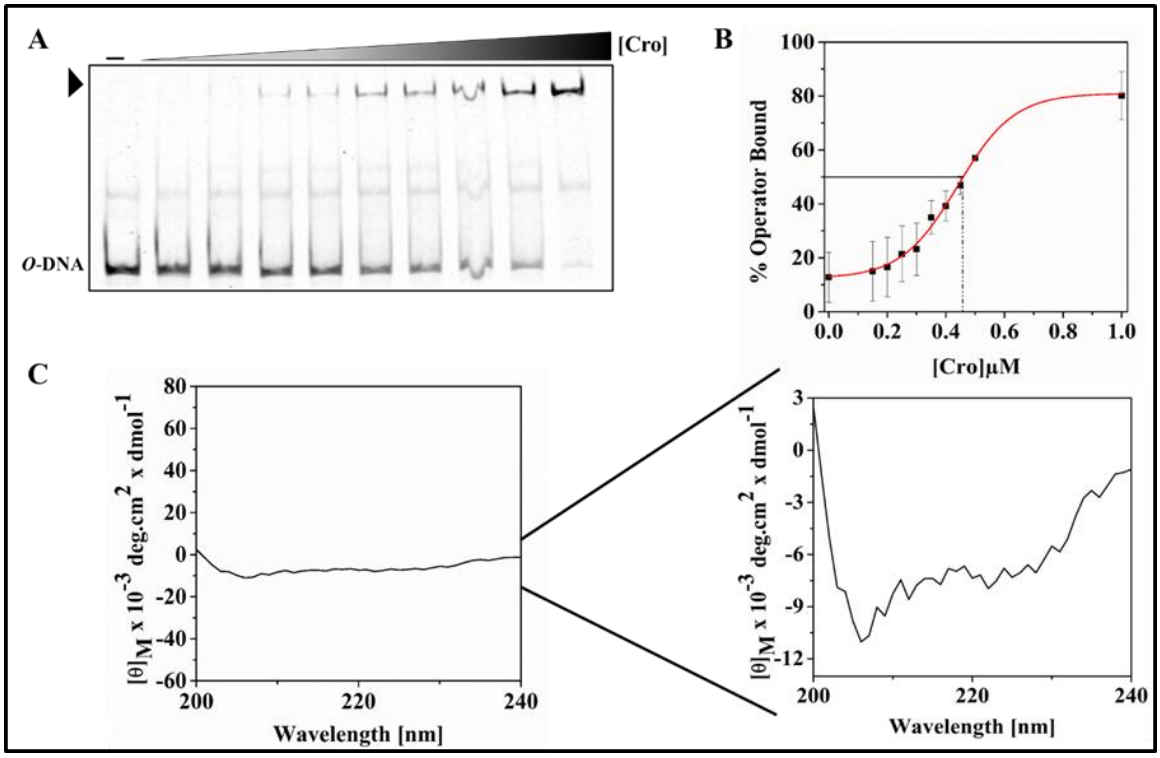

Figure 2.2. || Effect of NaCl on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(*O*) and Phi11 Cro (equilibrated with NaCl). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.15 μM-1 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in NaCl buffer.

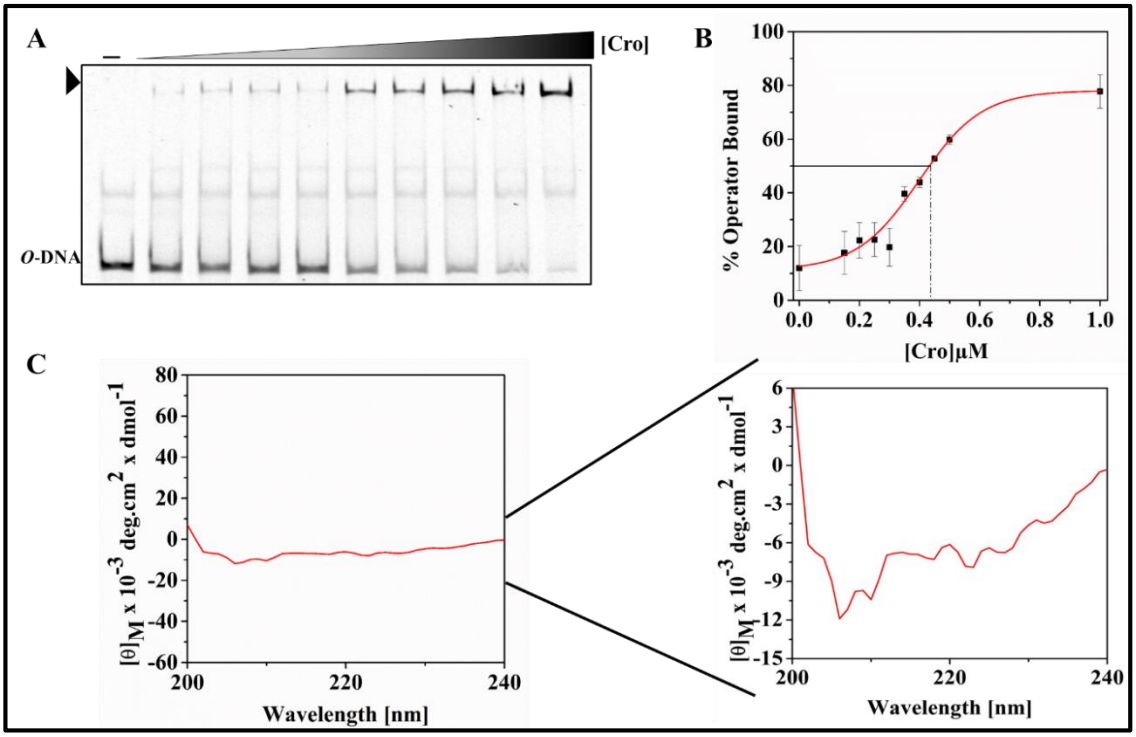

Figure 2.3. || Effect of KCl on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(*O*) and Phi11 Cro (equilibrated with KCl). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.15 μM-1 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in KCl buffer.

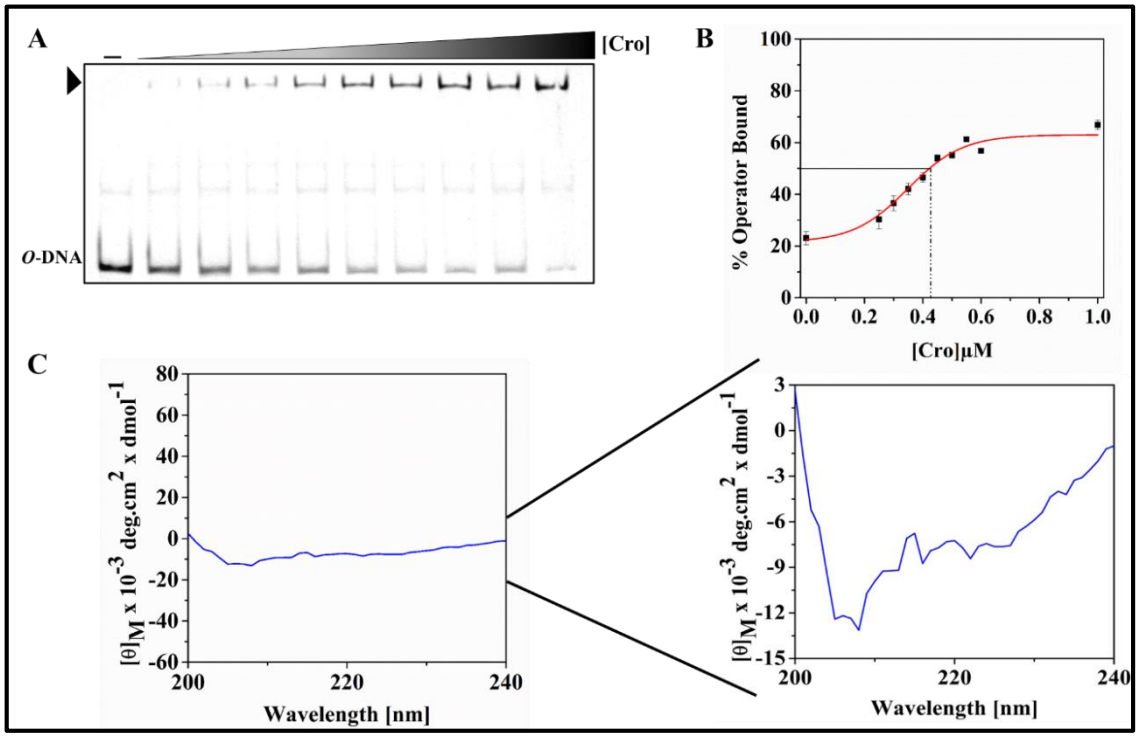

Figure 2.4. || Effect of LiCl on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(*O*) and Phi11 Cro (equilibrated with LiCl). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.25 μM-1 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in LiCl buffer.

2.3.2. **The monovalent cation NH_4^+ has a stimulatory effect upon the binding of Cro to its cognate operator DNA**

Cro protein, equilibrated in NH_4Cl buffer, was allowed to bind to *O* DNA. The complex thus formed was analysed by gel shift assay and the K_D in this case was found to be 0.376 μM (Figure 2.5.A and 2.5.B), which is lower than the K_D obtained for the previous cations.

To investigate the effect of NH_4^+ on the structure of the Cro protein, NH_4Cl buffer equilibrated Cro was subjected to CD spectral analysis (200-240nm). K2D2 analysis of CD spectrum obtained, however, indicated a spectrum similar to the other three cations. NH_4Cl buffer equilibrated Cro was found to be 63.05 % α-helical, 3.29% β-stranded and a 33.66 % coiled. In this case, the CD spectrum also exhibited a similar peak at 208nm as compared to that of the previous three cations (Figure 2.5.C).

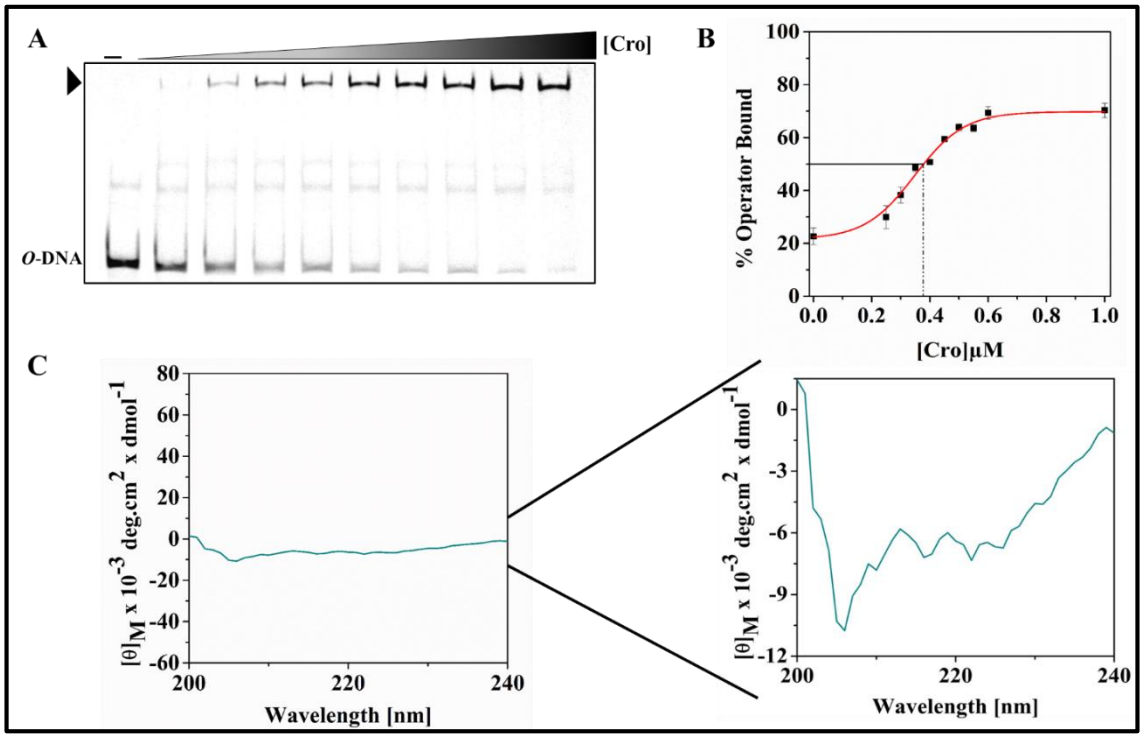

Figure 2.5. || Effect of NH_4Cl on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(*O*) and Phi11 Cro (equilibrated with NH_4Cl). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.25 μM-1 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in NH_4Cl buffer.

2.3.3. **Mg^{2+} does not unfold Cro but has an inhibitory effect upon the binding of Cro to its cognate operator DNA**

Cro protein, equilibrated in $MgCl_2$ buffer, was allowed to bind to *O* DNA. The complex thus formed was analyzed by gel shift assay and the K_D in this case was found to be 0.821 μM (Figure 2.6.A and 2.6.B).

However, the CD spectrum of Cro equilibrated with $MgCl_2$ buffer showed a plot of large negative ellipticity at 208nm and K2D2 analysis of the spectrum indicated that the protein was 75.57 % α-helical, 1.72 % β-stranded and 22.71% coiled in structure (Figure 2.6.C).

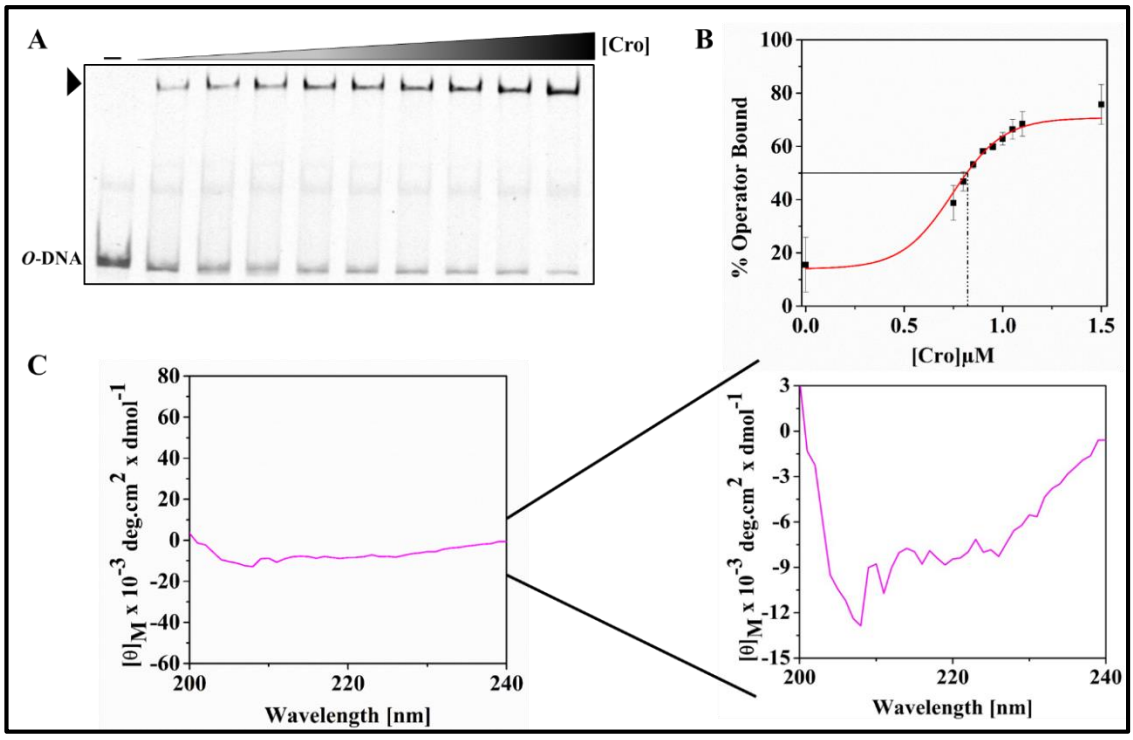

Figure 2.6. || Effect of $MgCl_2$ on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(O) and Phi11 Cro (equilibrated with $MgCl_2$). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.75 μM-1.5 μM. (B) The % operator bound to Cro in the O DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in $MgCl_2$ buffer.

2.3.4. **Effects of $C_2H_3O_2^-$ and CO_3^{2-} on Cro**

The K_D value of Cro-*O* DNA (Cro equilibrated with Na_2CO_3, $C_2H_3NaO_2$ buffers) interaction was studied using gel shift assay. It was found that the K_D value for Cro-operator interaction was 0.429μM in $C_2H_3NaO_2$ buffer (Figure 2.7.A and 2.7.B) and 0.753 μM in Na_2CO_3 buffer (Figure 2.8.A and 2.8.B). The CD spectrum of Cro equilibrated with Na_2CO_3 buffer showed a plot of large negative ellipticity at 208nm and K2D2 analysis of the spectrum indicated that the protein was 75.57 % α-helical, 1.72 % β-stranded and 22.71% coiled in structure (Figure 2.8.C).

On the other hand, the CD spectrum of Cro equilibrated with $C_2H_3NaO_2$ buffer showed a plot of comparatively smaller negative ellipticity at 208nm and K2D2 analysis of the spectrum indicated that the protein was 67.45 % α-helical, 3.24 % β-stranded and 29.26% coiled in structure (Figure 2.7.C).

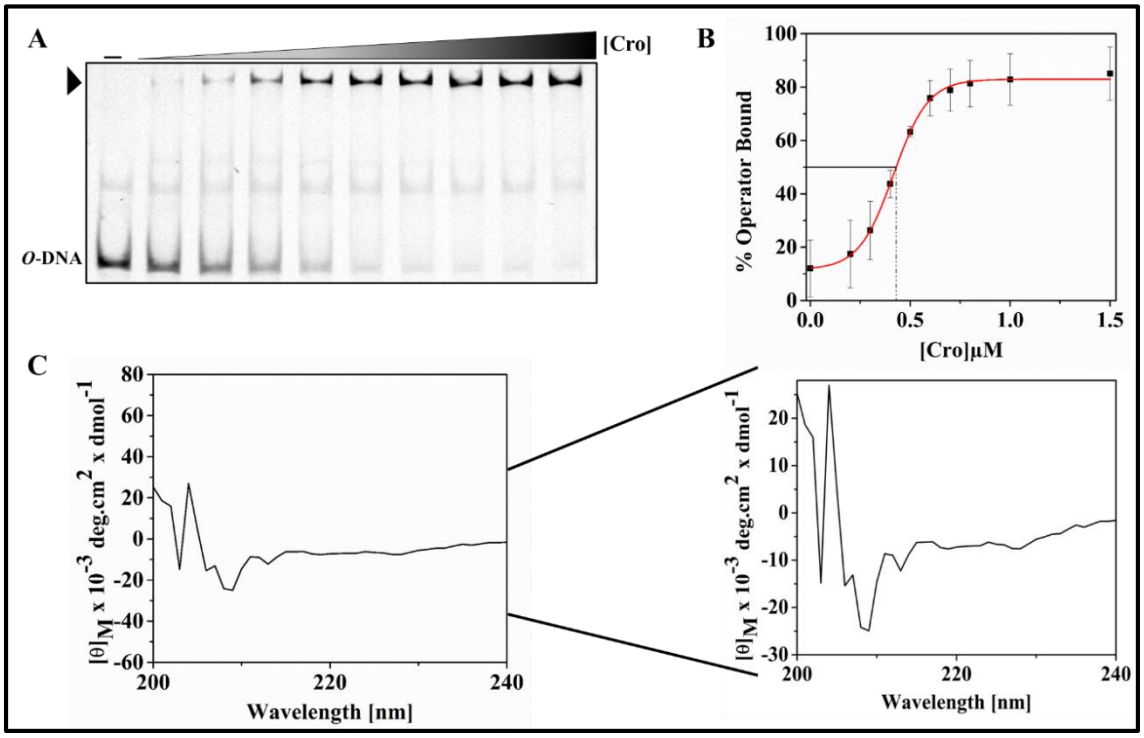

Figure 2.7. || Effect of $C_2H_3NaO_2$ on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(*O*) and Phi11 Cro (equilibrated with $C_2H_3NaO_2$). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.2 μM-1.5 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in $C_2H_3NaO_2$ buffer.

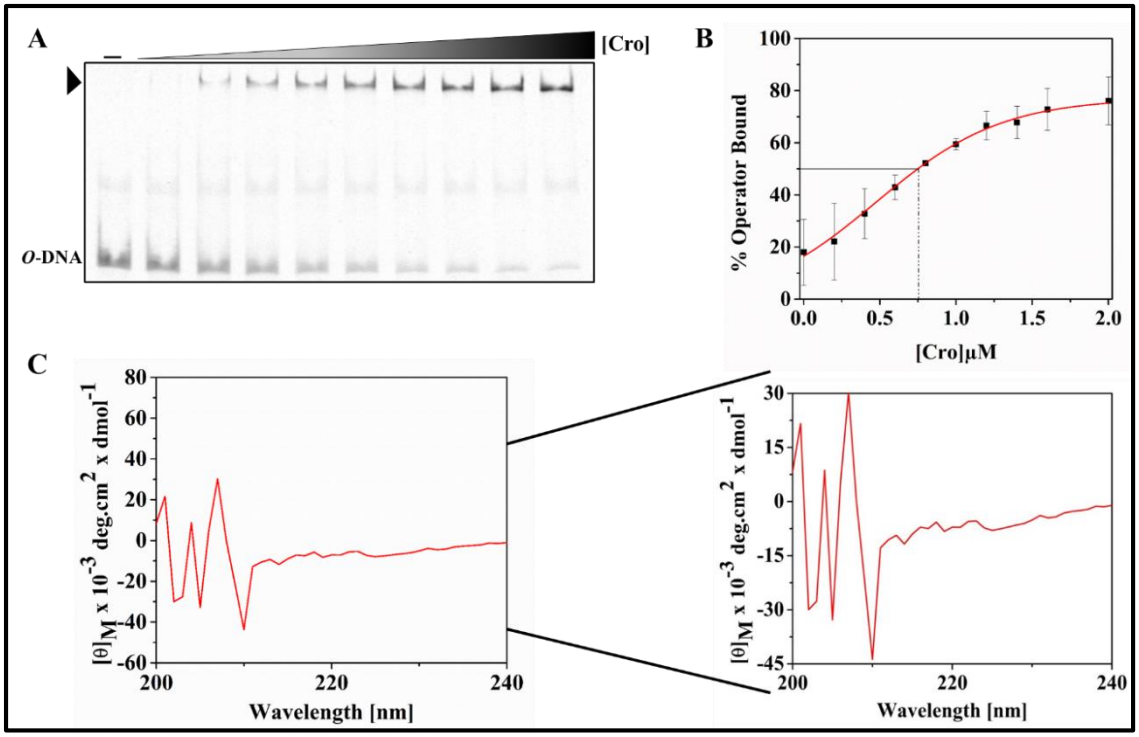

Figure 2.8. || Effect of Na_2CO_3 on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between operator DNA(*O*) and Phi11 Cro (equilibrated with Na_2CO_3). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.2 μM - 2 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in Na_2CO_3 buffer.

2.3.5. $C_6H_5O_7^{3-}$ has a profound effect on the structure and function of Phi11 Cro

Cro protein, after equilibration in $Na_3C_6H_5O_7$ buffer, was allowed to interact with *O* DNA and the K_D value of this interaction was found to be 1.846 μM (Figure 2.9.A and 2.9.B). To investigate the effect exerted by $C_6H_5O_7^{3-}$ upon the structure of Phi11 Cro, the CD spectra of $Na_3C_6H_5O_7$ equilibrated Cro was studied. It was found that Cro exhibited a much reduced peak at 208nm and was 7.96 % α-helical, 21.23 % β-stranded and 70.81% coiled in structure (Figure 2.9.C).

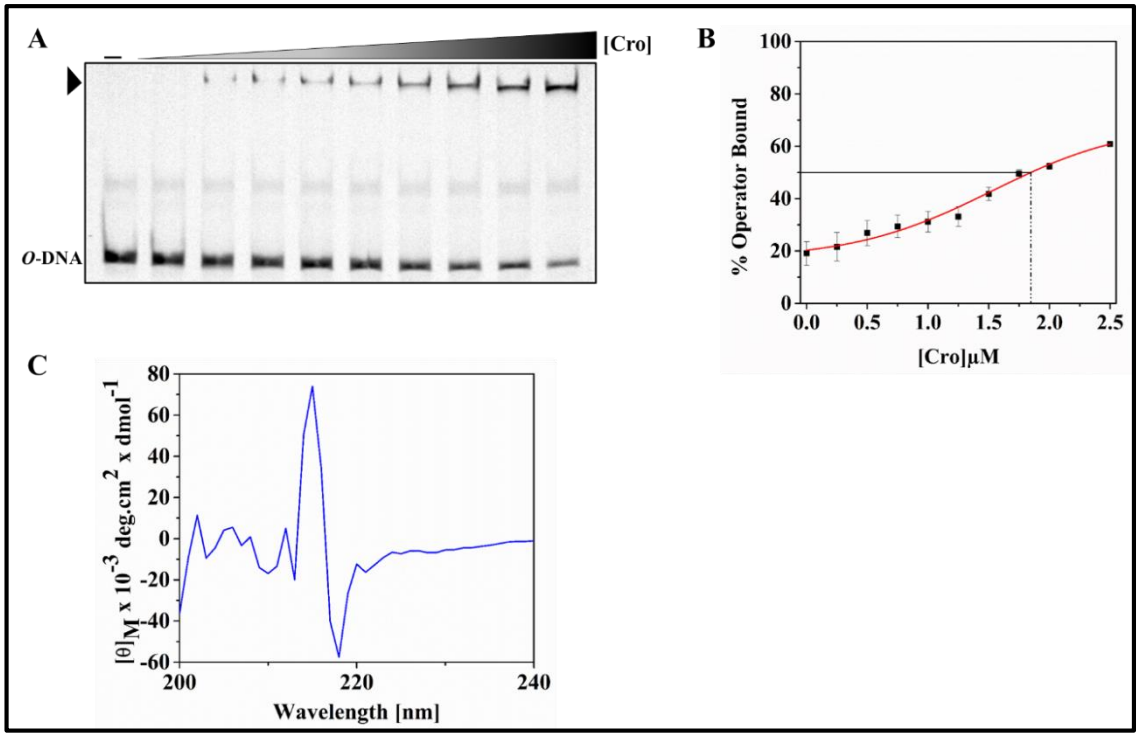

Figure 2.9. || Effect of $Na_3C_6H_5O_7$ on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between operator DNA(*O*) and Phi11 Cro (equilibrated with $Na_3C_6H_5O_7$). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.25 μM-2.5 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in $Na_3C_6H_5O_7$ buffer.

2.3.6. **Mg^{2+} and CO_3^{2-} induce oligomerization in Phi 11 Cro**

The oligomeric status of Phi11 Cro in presence of $MgCl_2$ buffer or Na_2CO_3 buffer was investigated. It was found that Cro, when equilibrated with $MgCl_2$ buffer or Na_2CO_3 buffer, actually existed as higher oligomeric species, unlike Cro equilibrated with NaCl buffer whereby it exists as a monomeric species (Figure 2.10.).

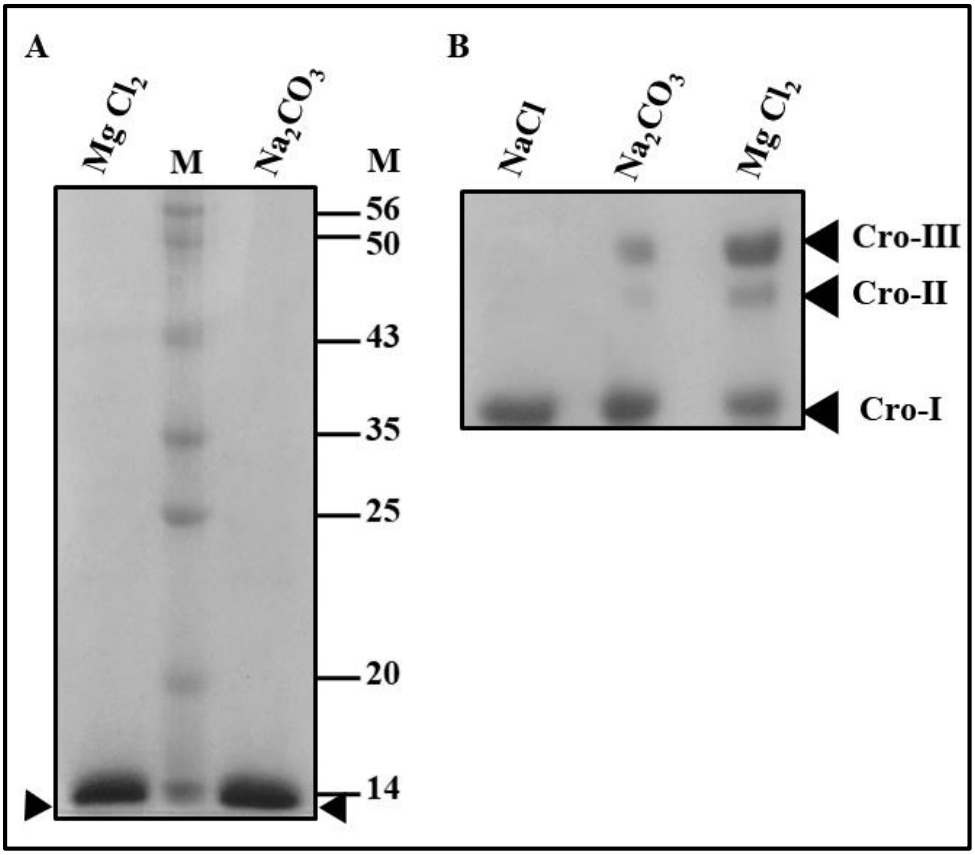

Figure 2.10. || Oligomeric status of Phi11 Cro in presence of buffers containing NaCl, $MgCl_2$ or Na_2CO_3.
(A) Denatured Cro (equilibrated with $MgCl_2$ and Na_2CO_3) run on SDS-12% PAGE. M stands for standard protein marker (size indicated on the right side of the gel). Arrowheads indicate the position of Cro. (B) Oligomerization of unboiled, Cro (in NaCl, $MgCl_2$ or Na_2CO_3) analysed by electrophoresis in 12% polyacrylamide gel (Materials and methods). Arrowhead Cro-I indicates the position of monomeric Cro. Arrowheads Cro- II and Cro-III represent the higher oligomers of Cro.

2.4. **Discussion**

Salts can play a very important role in influencing the biologically active conformation of proteins. The effects exerted by different salts upon different proteins can vary. Some salts might lead to aggregation, denaturation or unfolding of the protein and some might lead to minute changes in the domains of a multi-domain protein. In both cases the functional activity of the protein may or may not be affected (Bandhu et al 2009). In this study, we have employed gel shift assay to study the effect exerted by various anions and cations upon the functional activity of Phi11 Cro. We have also carried out CD spectral analysis (200-240nm) of Phi11 Cro (equilibrated with different salt buffers) to investigate the effect of the various salts upon the structure of the protein. We also attempted to investigate the structure-function relationship of Cro.

From the data obtained, it is clear that Phi11 Cro showed almost similar operator binding activity in presence of each of the three monovalent cations (Na^+, K^+ and Li^+) used. This indicates that the three cations had similar effect upon the biological activity of Cro protein. However, the K_D value of Cro-*O* DNA interaction in NH_4Cl buffer was found to be 0.376 μM, which is slightly lower than the K_D values obtained with NaCl, KCl and LiCl buffers. The data is indicative of the fact that in presence of NH_4^+, the interaction between Cro and its cognate operator is stronger as compared to Na^+, K^+ and Li^+. NH_4^+ possibly induces some modifications in the structure of either the Cro protein or *O* DNA or both, thereby strengthening the interaction.

The divalent ions, Mg^{2+} and CO_3^{2-}, were however found to severely hamper the Cro-*O* DNA interaction as indicated by their K_D values (approximately 2 times higher than the K_D obtained for the monovalent ions Na^+, K^+, Li^+ or $C_2H_3O_2^-$).

To investigate if the above salts had any effect upon the conformation of the protein, CD spectral analysis (200-240nm) was employed to check the conformation of Cro in presence of each of the ions. Surprisingly, it was found that when incubated in $MgCl_2$ buffer or Na_2CO_3 buffer, Cro exhibited the least randomness and maximum α-helical content. To explain this data, we assumed that Mg^{2+} and CO_3^{2-} might lead to multi-merisation of the protein which may partially block its operator binding site (Winter et al 2012). Previously, it has been had reported that micro molar concentrations Phi11 Cro exists as monomer in solution (Das et al 2009). In fact, our recent experiments showed that Cro equilibrated with $MgCl_2$ buffer or Na_2CO_3 buffer actually existed as higher oligomeric species, unlike Cro equilibrated with NaCl buffer whereby it exists as a monomeric species (Figure 2.10.).

CD spectra of Na^+, K^+, Li^+, NH_4^+ and $C_2H_3O_2^-$ equilibrated Cro showed similar peak at 208nm, indicating that these monovalent ions possibly maintain the biologically active conformation of Cro.

$C_6H_5O_7^{3-}$ ions led to a decrease in the affinity of Cro for its cognate operator, as indicated from the K_D value. Moreover, the CD spectral analysis indicates that $C_6H_5O_7^{3-}$ severely unfolded Cro. This unfolding of Cro in $Na_3C_6H_5O_7$ buffer strongly interfered with its functional activity.

Considering all the above data, it is clear that the structure of Phi11 Cro was maintained by NH_4^+ and $C_2H_3O_2^-$ allowed it to bind optimally to its cognate operator.

In spite of the changes of different cationic and anionic salt, the Cro repressor binding ability to *O* DNA has not changed significantly. Specially, cellular cations (Na^+ and K^+) does not shown effective changes in DNA-binding nature of lytic repressor Cro. Using the previous knowledge of low affinity of Cro repressor to its cognate operator DNA compare to CI repressor, we focused on a downstream gene (ORF7) of Cro (early express gene) for better understanding the involvement of ORF7 gene (designated as antirepressor in NCBI; NC_004615.1) product on Phi11 regulatory development. This chapter work has been published.

2.5. **References**

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Chapter 3

Expression of Gp07 causes filamentation in *Escherichia coli*

3.1. **Introduction**

The growing incidence of drug-resistant pathogenic bacteria is a major concern today. It has now become critical to devise novel therapeutic strategies and antibacterial alternatives to combat infections caused by multi-drug resistant pathogens. Several bacteriophages have been reported to utilize the host machinery for their survival. Such bacteriophages code for certain proteins which alter some of the essential host proteins. These phages encoded proteins can either be bacteriostatic or bactericidal in nature (Sau et al 2008). Information about such phage encoded proteins can be instrumental in designing drugs against pathogenic bacteria (Liu et al 2004).

Phi11 is a temperate bacteriophage (serogroup B and lytic group III) which infects *S. aureus* (Bachi 1980). It harbours a 43.6 kb (Iandolo et al 2002) double stranded linear DNA genome (G+C content of 35-37%) (Brown et al 1972). Being a temperate phage, Phi11 can undergo both the lytic as well as the lysogenic mode of development. The sequence of Phi11 *gp07* revealed the presence of two conserved domains - the amino terminal domain belonging to baculovirus repeated ORF[s] Bro family and the carboxy terminal domain belonging to ANT superfamily which bears homology to KilA domain (Zemskov et al 2000). The Bro proteins were proposed to be DNA-binding in nature and are involved in the regulation of viral and host transcription, replication and chromatin structure (Iyer et al 2002; Bideshi 2003). It has already been reported that bacteriophage lambda harbours a gene called the *kil* gene. Overexpression of the *kil* gene leads to inhibition of FtsZ ring formation, which in turn induces filamentation of *E. coli* (Haeusser 2014).

It has been observed that the affinity of Phi11 CI protein for its cognate operator is several times higher than that of Cro (Das et al 2009). This actually makes lysogeny the favoured mode of development. However, this is not observed in nature since the lytic pathway is essential for the phage to lyse out of the host cells when the latter is compromised (Fornelos et al 2016). Hence, there must exist some mechanism which will actually enhance the lytic mode of development to help the bacteriophage exit the compromised host. The genome of Phi11 shows the presence of ORF7. The early gene ORF7 (designated as Gp07) is annotated as putative antirepressor. However, no studies have been carried out on this gene, so far. It has been reported, that in several phages, the antirepressor protein functions to stimulate the lytic pathway (Fornelos et al 2016). To look into the function of *gp07*, this gene has cloned and overexpressed; additionally, its domains, as well as a truncated Gp07 (ΔGp07) have been cloned and overexpressed as histidine-tagged variants. ΔGp07 has been generated by deletion of 11 amino acid residues present at the amino terminal region of Gp07. Overexpression of the Gp07, ΔGp07 as well as the KilA-C domain exhibited an inhibitory

effect upon the cell division of *E. coli*. The Bro-N domain upon overexpression did exhibit some degree of toxicity; however, the inhibitory effect exerted by Gp07, ΔGp07 and the KilA-C domain expression far exceeded that of the Bro-N domain. This chapter offers a firsthand preliminary report about the cell growth inhibitory role of Phi11 *gp07*.

3.2. **Materials and methods**

3.2.1. **Bacterial strains, phage strains, and growth conditions**

S. aureus RN4220, *S. aureus* temperate phage Phi11 and pET28a (Novagen) were obtained from Prof. S Sau (Bose Institute, Kolkata, India). Phi11 has been grown in laboratory according to the method of Lee and Iandolo (Lee & Iandolo 1988). *S. aureus* RN4220 was grown at 37˚C in Trypticase soy broth (Lee & Iandolo 1986). *E. coli* BL21 (λDE3) and *E. coli* XL1 Blue cells were grown at 37˚C in Luria broth (Sambrook et al 2001). Appropriate antibiotics were added to the growth media as required.

3.2.2. **Phi11 phage Preparation**

High titer Phi11 phage was prepared by plate lysis method. Aliquot of diluted phage stock was mixed with 0.2 ml of *S. aureus* RN4220 (~0.8–1 OD_{600}) freshly grown in TSB containing 10 mg/ml $CaCl_2$. Adsorption of phage to *Staphylococcal* culture was allowed for 20 mins at room temperature (RT). Thereafter, this phage adsorbed *Staphylococcus* was mixed with 3ml of molten soft agar (maintained at 42ºC) and overlaid upon TSA plates. The plates were incubated overnight at 37ºC to obtain clear lysis zones. The incubated plates ware taken out from incubator and kept at RT to cool down. This was followed by addition of 5 ml TSB to each plate and the plates were kept at 4ºC, overnight, to enable the phage particles to diffuse into TSB. After incubation, the TSB containing the phage particles were collected in syringe and purified using a Millipore filter paper (0.2µm) to ensure that the lysate was free of any contaminating bacteria.

3.2.3. **Assay of viable phage particles in a lysate (PFU assay)**

The phage titer in a stock was determined by plaque assay method. The lysate stock was serially diluted with TSB. 0.1 ml of the phage lysate from the desired dilution was mixed with 0.3 ml of *S. aureus* RN4220 (~0.8–1 OD_{600}), freshly grown in TSB containing 10 mg/ml $CaCl_2$. Adsorption was allowed for 20 mins at RT. Thereafter, this phage adsorbed bacteria was mixed with 3 ml of molten soft agar (maintained at 42 ºC - 45 ºC) and overlaid upon TSA plates. The plates were incubated overnight at 37ºC. Next morning, the plaques were counted to estimate the plaque forming unit (PFU).

3.2.4. **Isolation of Phi11 genomic DNA**

Phi11 genomic DNA isolation was carried out as follows. Infection (of *S. aureus* RN4220 with Phi11) at high multiplicity was carried out for large scale phage propagation. 1000 ml of TSB was inoculated with 10ml overnight grown culture of *S. aureus* RN4220 and was incubated at 37ºC in a

shaker incubator, until the culture exhibited an OD 0.6. Cells were then infected with Phi11 at a MOI (multiplicity of infection) of 6 and incubated with shaking for another 8 h at 37ºC. The lysate was then incubated with 1µg/ml DNase I and 1µg/ml RNase A for another 30min. After that centrifuge was carried out at 12,000 rpm for 15min at 4ºC to separate out the supernatant, followed by addition of 10% polyethylene glycol (PEG-8000) and 0.5M NaCl. The mixture was further incubated for overnight at 4ºC. The suspension was further centrifuged at 22,000 rpm for 2 h and supernatants were taken out very carefully. The phage pellets were dissolved in Tris-EDTA (TE) buffer (pH 9.0) followed by addition of 20mM EDTA, 50µg/ml proteinase K and 0.5 % SDS, this mixture was incubated at 56ºC for 1 h. Then an equal volume of phenol and isoamyl-alcohol was added, followed by an equal volume of isoamyl-alcohol and chloroform were used to extract out the DNA from the mixture. Finally, the DNA was ethanol precipitated and dissolved in TE buffer (pH 8).

3.2.5. **Preparation of plasmid DNA**

3.2.5.1. **Boiling preparation of plasmid**

Plasmid DNA was isolated routinely as described (Sambrook et al 2001). This method is normally employed for small scale plasmid DNA preparation, whereby only 1.5 ml of overnight grown culture is used for plasmid isolation. The culture after harvesting is resuspended in STET buffer (given in appendix A) (Sambrook et al 2001), followed by addition of 2 µl of lysozyme (50 mg/ml). The entire mixture is boiled for 40 secs followed by centrifugation at 12000 rmp for 15 mins at 4º C. The supernatant was pipetted out to which 70 µl of 5.2 M ammonium acetate and 150 µl of isopropanol were added and incubate at room temperature for 5 min. This was followed by another round of centrifugation at 12000 rpm for 10 mins at 4º C. The pellet was then washed with 70% ethanol, dried and suspended in desired volume of TE buffer (pH 8).

3.2.5.2. **Kit based plasmid purification**

Plasmids DNA were routinely isolated from 5ml of bacterial cultures, using ThermoScientific plasmid extraction kit.

3.2.6. **DNA amplification using Polymerase Chain Reaction (PCR)**

DNA was amplified by PCR following standard method (Sambrook et al 2001). Briefly, the template DNA was prepared by the usual procedure of genomic DNA isolation or plasmid isolation described under section 3.2.4. and 3.2.5. respectively. About 100 ng to 150 ng of template DNA was mixed with the designed primers (1 µM of each of forward and reverse). This was followed by

addition of the following components: 0.25mM dNTP mix, 1 unit of Taq DNA Polymerase, 1X Taq Polymerase Buffer and finally the volume was made upto 50 µl with sterile double distilled water. Bio-Rad thermal-cycler was used to carried out the PCR reaction. The initial denaturation step was performed at 95°C for 5min followed by annealing of primers to the template DNA at the respective annealing temperature for 1 min .This was followed by extension reaction at 72ºC for desired time length (1000bp/min). The reaction was carried out for 35 cycles. The reaction was ended with a final extension step for 10 mins at 72°C. The PCR product was finally loaded into an agarose gel of desired percentage and eluted from agarose gel as described in section 3.2.8. and 3.2.9. respectively.

3.2.7. **Digestion of plasmid and genomic DNA using Restriction endonucleases**

Digestion of DNA with restriction endonucleases was carried out according to standard method (Sambrook et al 2001). A typical reaction mixture consisted of DNA and 1X digestion buffer (supplied along with the respective restriction endonuclease) in final reaction volume of 25µl. The desired restriction endonucleases were added to this mixture (ensuring that the final glycerol concentration in the reaction mixture does not exceed 2.5%). The mixture was then incubated at the recommended temperature for 4 h (in some cases incubation was also carried out overnight). Finally, the digested DNA was mixed with 1X loading dye (given in appendix A) and analysed by agarose gel electrophoresis.

3.2.8. **Agarose gel electrophoresis**

This was performed according to standard procedure (Sambrook et al 2001). Briefly, the desired amount of agarose (the percentage of agarose gel to be used depends on the size of the DNA fragment to be analyzed) was weighed and dissolved in 1X TAE by boiling with gentle swirling, till all the agarose dissolved completely. This molten agarose gel was cooled and a final concentration of 0.5 µg/ml ethidium bromide solution (given in appendix A) was added to it. The molten agarose was poured on a gel casting tray with a comb fitted to it (the teeth of the comb formed the sample wells). After the gel solidified the comb was removed very carefully. Finally, the gel was placed in the electrophoresis tank and submerged in 1X TAE buffer. Electrophoresis was carried out at 60-80 V after loading the samples (Mixed with 1X DNA loading dye). The setup was allowed to run till optimal resolution was achieved. DNA was visualized with long wavelength UV radiation (300 nm). Gels were documented by a Gel documentation system (E-Gel Imager System with UV Light Base, ThermoScientific).

3.2.9. **Purification of DNA bands from agarose gel**

Agarose gel extraction kit from ThermoScientific, was used for purifying DNA bands from agarose gel. Purification was carried out according to the manufacturer's protocol. Briefly, the agarose gel carrying the desired DNA bands are excised out using a sterile scalpel and collected in a 1.5ml autoclaved centrifuge tube. The weight of gel slice was recorded and to this was added the supplied binding buffer (binding buffer volume: gel slice weight in 1:1 ratio). This was incubated at 50-60^{o} C for 10 min, with repeated mixing every few minutes until the gel slice is completely dissolved. The mixture was incubated at room temperature to cool it down and then transferred to the supplied GeneJET purification column. This was then centrifuged for 1min at 12,000 rpm at room temperature. 700µl of the supplied wash buffer was added to the GeneJET purification column and the the flow through was discarded. To remove the residual wash buffer, the column was centrifuged for an additional 1 min at 12,000 rpm. The GeneJET purification column was transferred to a sterile 1.5mL micro-centrifuge tube. Finally, the supplied elution buffer (50µL) was added to the center of the purification column membrane and centrifuged for 1 min at 12,000 to collect the purified DNA fragment. The eluted DNA was stored at -20^{o} C until further use.

3.2.10. **Ligation of DNA**

Thermo Scientific DNA ligation kit was used to carry out the ligation of DNA. Basically, the DNA fragments to be ligated (vector and insert) were mixed in requisite molar ratio (vector DNA: insert DNA ratio was 1:3 in case of cohesive end ligation and 1:5 in case of blunt end ligation). The ligation reaction mixture further contained 10X ligation buffer (1X final concentration) and 1 µl of T4 DNA ligase. All the components of the ligation mixture were gently but thoroughly mixed to a final reaction volume of 10 µl. The ligation reaction mixture was incubated overnight at 4ºC (in case of cohesive end ligation) or at 22ºC (in case of blunt end ligation).

3.2.11. **Transformation of DNA**

DNA transformation is carried out in two steps. The first step involved the preparation of competent cells while the second step involved transformation of the desired DNA in the competent host cell. Both these steps are carried out by standard procedure (Sambrook et al 2001). The steps are as follows:

3.2.11.1. **Competent cell preparation**

A primary culture was prepared by inoculating a single healthy *E. coli* colony into 5 ml of sterile LB and allowing it to grow overnight at 37ºC with shaking. Next day, 0.1ml of the primary culture

was used to inoculate 10 ml of sterile LB. The cultrure was allowed to grow at 37ºC with shaking to reach the desired OD_{600} (0.4-0.5). The culture was then harvested by centrifugation at 5500 rpm for 10 mins at 4ºC. The supernatant was discarded and the cell pellet was resuspended in 10 ml of ice-cold 50 mM $CaCl_2$. This suspension was incubated in ice for 10 mins followed by centrifugation at 4000 rpm for 10 mins at 4ºC. Again, the cell pellet was resuspended in 5ml of chilled 50 mM $CaCl_2$ followed by centrifugation at 4000 rpm for 10 mins at 4ºC. Finally, the cell pellet was resuspended in 0.5 ml of chilled 50 mM $CaCl_2$ and incubated in ice for 16 hours.

3.2.11.2. **Transformation of plasmid DNA or ligation mixture**

100 µl of competent cell was taken in a microbiologically sterile eppendorf tube and mixed with DNA (10 µl of ligation mixture or ~50 ng of plasmid DNA). This mixture was incubated in ice for 45 mins following which it was subjected to heat shock treatment (42°C) for 90 seconds. Immediately after the heat shock treatment, the cells were chilled in ice and 500µl of sterile LB broth was added to it. This was incubated at 37°C with shaking for 45 mins. Finally, the transformation mixture was plated onto LBA plates containing proper antibiotic for selection of transformants and incubated overnight at 37°C for obtaining visible colonies.

3.2.12. **Bioinformatics analysis of Gp07**

Genome databases in NCBI (http://www.ncbi.nlm.nih.gov/) and Pfam (http://pfam.sanger.ac.uk/) were used for bioinformatics analysis of Gp07. EMBOSS programs (https://www.ebi.ac.uk/Tools/emboss/) were used for different bioinformatics analyses like molecular weight determination, charge and presence of different amino acid. Sequence similarity search was carried out by BLAST server (http://www.blast.ncbi.nlm.nih.gov/blast/Blast.cgi). The search for Phi11 *gp07* gene was carried out using BLASTP in different databases in Caudovirales order and 50% or more identity was taken as the threshold (Altschul et al 1990). Sequences similar to the *gp07* of Phi11 were detected from different non-redundant (NR) protein sequence databases by BLAST (blastp) program 2.2.32+. Alignment of full-length Gp07 protein was performed by Clustal Omega program 1.2.1 (http://www.ebi.ac.uk/Tools/msa/clustalo/). Using all the proteins which shared 50% identity to the Phi11 Gp07, a dendrogram was constructed using ClustalW2 Phylogeny program (https://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/) with neighbor-joining method.

3.2.13. **Cloning of *gp07*, its domains and truncated Gp07(ΔGp07)**

To clone Phi11 *gp07*, we employed a polymerase chain reaction with DreamTaq DNA Polymerase from ThermoFisher Scientific using primers C-gp07-F and C-gp07-R (Table 3.1.). The template used was Phi11 genomic DNA. The product obtained was 825bp in size and was cloned into pGEM-T Easy vector (Table 3.2.) according to the manufacturer's protocol (www.promega.com/protocols/). The recombinant plasmid (pGp07-T) thus obtained was transformed into competent *E. coli* XL1 Blue cells. A healthy recombinant harbouring pGp07-T carrying no mutation in the *gp07* (as confirmed by DNA sequencing) was chosen for further work. *gp07* was further subcloned into pET28a and designated as pGp07 (Table 3.2). This cloning has included ten extra amino acid residues (including six histidine residues) at the carboxy terminal end of the putative Gp07 protein.

Similarly, ΔGp07 (generated by deletion of 11 amino acid residues present at the amino terminal region of Gp07), Bro-N(rNTD) and KilA-C(rCTD) domains were PCR amplified from Phi11 genomic DNA using the primers C-Δgp07-F, C-gp07-R; C-gp07-F, C-Bro-R and N-KilAC-F, N-gp07-R respectively (Table 3.1.). The resulting PCR products of 792bp(ΔGp07), 420bp(rNTD) and 405bp(rCTD) were cloned into pGEM-T Easy vector (Table 3.2.), sequenced and further sub-cloned into pET28a (Table 3.2.). All the recombinant constructs were transformed into *E. coli* BL21(DE3) cells (Novagen, USA) and healthy transformants (carrying no mutation) were selected for further work.

Table 3.1. Primer used in this study to express the Gp07, rNTD, rCTD and ΔGp07.

Name	Sequence(5′-3′)[#]	Purpose
C-gp07-F	CCATGG**GAATGTGGGTGTTGAGGAAAAAGGAGG**	Forward primer for synthesis of Gp07 and rNTD
C-gp07-R	CTCGAG**CGCTCCCCCTAAATTAGCTTCATAAC**	Reverse primer for Synthesis of Gp07 and **Δ**Gp07
C-Bro-R	CTCGAG**GTCTGGATCTTTTAATGTTTGTTCAATTACATTG**	Reverse primer for the synthesis of rNTD
N-KilAC-F	CATATG**TACATCATTACAGTGTTGACTGAGTATAAGAAAG**	Forward primer for the synthesis of rCTD
N-gp07-R	CTCGAG**TTACGCTCCCCCTAAATTAGCTTCATAACC**	Reverse primer for the synthesis of rCTD
C-Δgp07-F	CCATGG**AAATGCAAGCATTACAAACATTTAATTTTAAAGAGC**	Forward primer for synthesis of **Δ**Gp07

#Used primer, restriction sites are underlined; for PCR, $\mathbf{T_m}$ is calculated for the bases in bold.

Table 3.2. Plasmid used in this study and their derivatives.

Plasmids	Source	Description
pGEM-T Easy	Promega	*amp*^{r}, *lacZ*, cloning vector
pET28a	Novagen	*kan*^{r}, T7 lac, His-tag, expression vector
pGp07-T	This study	Gp07 cloned in pGEM-T Easy vector
pGp07	This study	Gp07 cloned in pET28a
prNTD-T	This study	rNTD cloned in pGEM-T Easy vector
prNTD	This study	rNTD domain cloned in pET28a
prCTD-T	This study	rCTD domain cloned in pGEM-T Easy vector
prCTD	This study	rCTD domain cloned in pET28a
pΔGp07-T	This study	ΔGp07 cloned in pGEM-T Easy vector
pΔGp07	This study	ΔGp07 domain cloned in pET28a

3.2.14. **Overexpression assays of Gp07, its domains and ΔGp07 in *E. coli***

E. coli cells harbouring Gp07, rNTD, rCTD and ΔGp07 were separately grown in LB (with 50µg/ml kanamycin), overnight at 37˚C with shaking. At ~12-14 h, these cultures were subcultured to an OD_{600} of ~0.05 in LB (with 50µg/ml kanamycin) followed by induction with 0.5mM IPTG. Following induction, OD_{600} of the cultures were measured at 30 minutes. This was followed by OD_{600} measurements every 1 hour for 8 hours. The entire procedure was repeated three times and the mean values and the standard errors were calculated. The CI repressor protein of Phi11 (Das et al 2007) was used as a negative control.

3.2.15. **Examination of cell morphology of *E. coli* (harbouring Gp07, rNTD, rCTD or ΔGp07) using phase-contrast and fluorescence microscopy**

Following induction with IPTG, cells overexpressing either Gp07, rNTD, rCTD or ΔGp07 were harvested at two different time point (5 h and 8 h) and washed with 0.9% sterile NaCl solution. The washed cells were resuspended in saline, spread on a clean glass slide, air dried at room temperature and then fixed with methanol for 5 minutes at room temperature. The fixed cells were thoroughly washed with sterile distilled water, dried at room temperature, and then 10µl of poly-L-lysine (5 µg/ml of distilled water) was spread over the sample (Hiraga et al 1989).

To study the nucleoid structures formed in the *E. coli* cells (as a result of overexpressing either Gp07, rNTD, rCTD, ΔGp07 or none), the desired samples were stained with 10µl of DAPI solution [2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride, 4′, 6-Diamidino-2-phenylindole dihydrochloride] (Sigma-Aldrich Chemicals Pvt Limited) (Stock solution: 5µg/ml of DAPI in

saline), which binds specifically to DNA (Hiraga et al 1989). The cells were observed in a dark room, with the help of an Olympus IX51 inverted microscope oil immersion objective (100X), combining the phase-contrast system and the fluorescence system (a U-LH100HG apparatus). When the light of a halogen lamp was reduced to an appropriate level, the fluorescent nucleoid structures and cell shape were clearly visible at the same time. The microscope was also equipped with a ProgRes® CT3 camera (Jenoptik, USA). Photographs were captured and analyzed by ProgRes® CapturePro 2.8.8 software. The CI repressor protein of Phi11 was used as a negative control.

3.2.16. **Examination of *E. coli* cell morphology with scanning electron microscopy (SEM)**

To prepare samples for scanning electron microscopy, overnight cultures of *E. coli* cells carrying *gp07,* its domains and ΔGp07 (expressing either Gp07, rNTD, rCTD or ΔGp07) were separately allowed to grow to OD_{600} 0.05 in fresh LB at 37°C. Wild type *E. coli* as well as *E. coli* overexpressing Phi11 CI were used as negative control. The cells were induced with 0.5 mM IPTG and grown at 37°C with shaking (120 rpm) for another 8 h. The cells were then harvested by centrifugation for 10 min at 7500 g followed by washing with 1X PBS (pH 7) twice. Finally, the cell pellets were re-suspended in appropriate volume of 1X PBS and thinly smeared on clean cover-slips. The prepared cover-slips were kept for air drying. The dried cover slips were then washed three times with 1X PBS and the cells were pre-fixed with 3% glutaraldehyde (in 1X PBS) for 1 h at room temperature. The excess glutaraldehyde was removed by washing the cells, three times, with 1X PBS. The cells were then treated with 1% OsO_4 at 4^{o}C, and left overnight. Following this, the cells were again washed three times with 1X PBS at room temperature. Cells were then dehydrated using a series of ethanol solutions (10%, 30%, 50%, 70% and 90%, 5 min each) and finally transferred to 100% ethanol for 10min. To completely dehydrate the cells, the prepared slides were transferred to a CPD chamber (Leica EM CPD300). The specimens were placed onto foil which was glued onto a metal specimen holder after the CPD completion. The specimens were coated by gold-palladium mixture at 5nm thickness using Leica EM ACE200. The SEM images of cells were obtained using a FEI Quanta FEG 250 Scanning electron microscope.

3.3. **Results**

3.3.1. **Pfam analysis of Gp07**

Pfam (http://pfam.xfam.org) was used for full length protein sequence analyses. In Phi11 genome, amino acid sequence analysis by Pfam indicated that the locus tag "phi11_07" (ORF7, which was predicted as a Gp07) exists adjacent to the Cro-repressor (ORF6). The Gp07 protein consists of two conserved domains, an amino terminal domain (NTD) and the carboxy terminal domain (CTD). Out of the 274 amino acids of Gp07, the NTD ranges from 25th to 118th amino acids and belongs to baculovirus repeated ORF(s) Bro-N family (Zemskov et al 2000). The CTD ranges from 160th to 263rd amino acid residues of Gp07 and belongs to KilA-C domain under ANT superfamily (Figure 3.1.). KilA-C was initially reported as the carboxy terminal domain of phage PI KilA (Iyer et al 2002). According to Pfam analysis, the E-value for Phi11 Bro-N is 1.5e-27 and 5.4e-30 for KilA-C domain. Bro gene (5 members) has been reported in Bombyx mori nucleopolyhedrovirus (BmNPV), a double stranded virus infecting lepidopterans (Zemskov et al 2000). Further, there are reports of the existence of bro gene homologues in transposons, bacteriophages and probacteriophages (Riedel et al 1993; Ravin et al 1999 and Bideshi et al 2003). The Bro proteins are regulatory proteins involved in regulating transcription, replication in certain viruses and their host organisms.

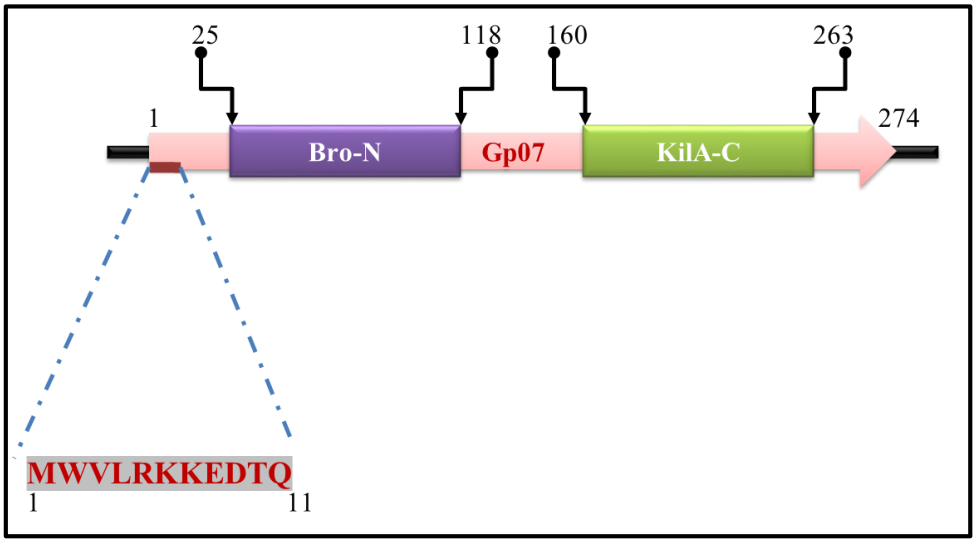


Figure 3.1. || Domain architecture of Gp07. Pfam analysis indicates that Gp07 harbours two domains - the amino terminal Bro domain and carboxy terminal KilA domain; the first eleven unique amino acids are coloured gray.

3.3.2. **Gp07 of Phi11 belongs to the Caudovirales**

According to International Committee on Taxonomy of Viruses (ICTV) classification, Caudovirales (the bacteriophages that have tails) is a taxonomic order within the kingdom Virus. There are at least 1442 phages in this order with complete genome sequence known to us. By BLASTP search

of non-redundant (NR) databases in Caudovirales order, it was observed that apart from the conserved residues, the stretch of the first eleven amino acids of Phi11 Gp07 protein are extremely unique (Figure 3.1.). These eleven amino acid residues are absent in all other proteins harbouring KilA-C and Bro-N domains, in Caudovirales order as well as outside the Caudovirales order (Figure 3.2. and 3.3.). All this twenty-six phages (vide Figure 3.3.) belong to Myoviridae and Siphoviridae family of viral systems. The neighbor-joining phylogenetic analyses revealed that the phage most closely related evolutionarily to phage Phi11 is *Staphylococcal* phage 42E (Figure 3.2.). It was reported that bro-like gene family is widespread among large double-stranded DNA viruses of invertebrates and bacteria (Bideshi et al 2003). Additionally, other reports revealed shuffling of domains which leads to different patterns of gene organizations (Iyer et al 2002). Phylogenetic analyses too display evolutionarily close neighbours belonging to different gene organizations (Figure 3.2.).

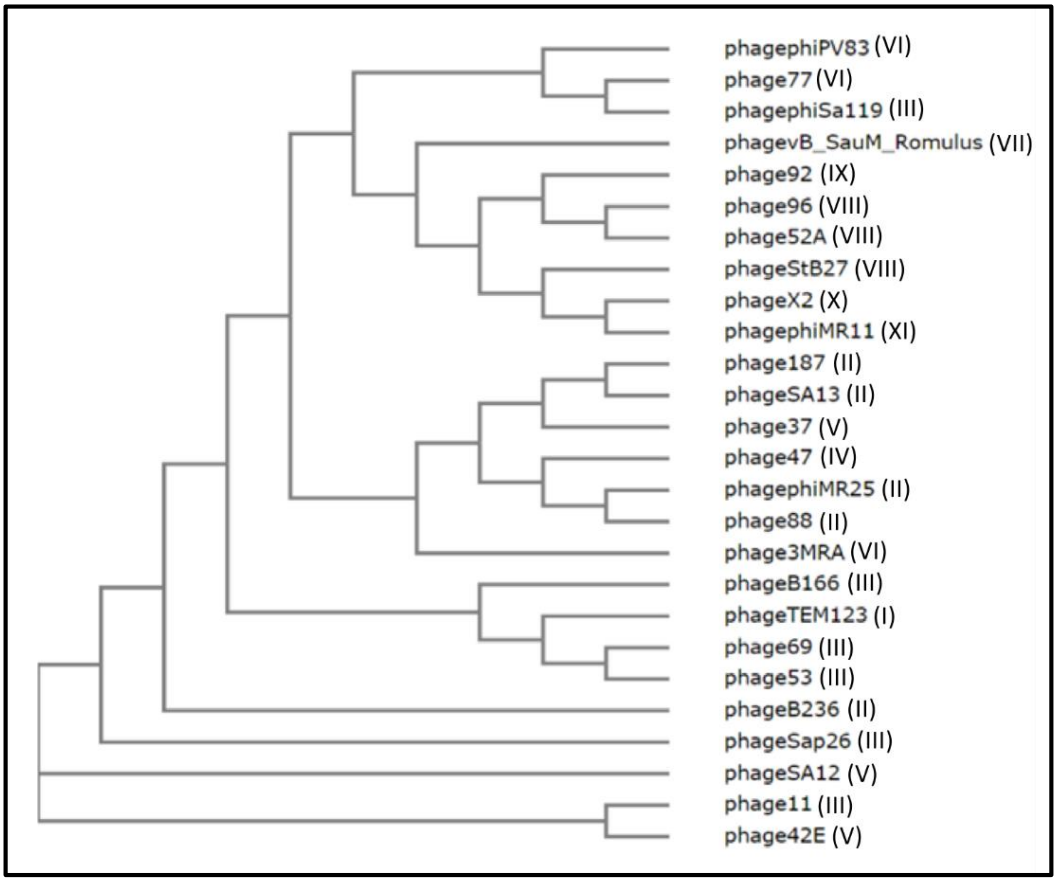


Figure 3.2. || Phylogenetic analysis. Phylogenetic analysis of Phi11 Gp07 has been aligned with ClustalO (See Materials and Methods for details) by neighbour-joining method. The Phi11 is evolutionary closely related with phage 42E. This analysis shows that distantly related phages with same gene organization.

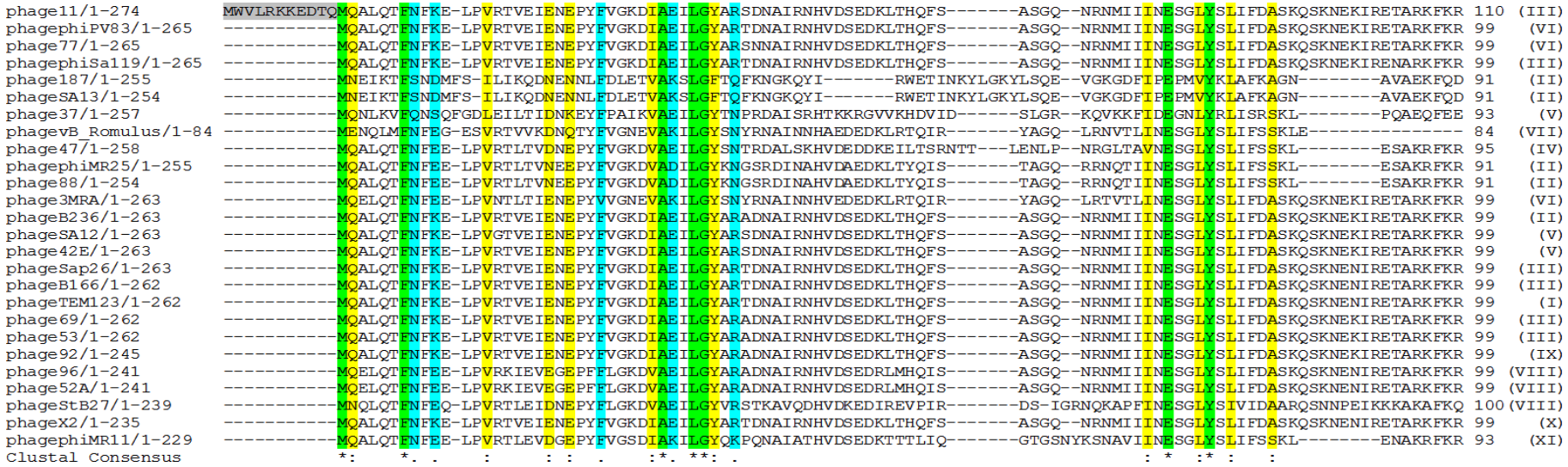


Figure 3.3. || Sequence analysis of the full-length protein of some phages in Caudovirales. Sequences are designated by their phage name, followed by protein length and finally gene organization type (Figure 3.8.). Phi11 Gp07 has been aligned with ClustalO (See Materials and Methods for details). The coloring reflects the conservation profile at clustal consensus of amino acids. '* 'identical residues (M,F,A,L,G,E,Y), all shaded green. ': 'highly conserved residues (Q,V,I,E,N,D,Y,F) shaded yellow. '. 'weakly conserved residues (S,D,E,F,V,R,Q,N,K) shaded turquoise. Phage Phi11 unique residues region (MWVLRKKEDTQ) all coloured gray.

3.3.3. **Inhibition of cell growth by expression of Gp07, its domains and ΔGp07**

E. coli cells harbouring either Gp07, rCTD or ΔGp07 (overexpressions were confirmed by SDS-12% PAGE, Figure 3.4.), upon induction by 0.5mM IPTG at 37ºC for 5 h, showed a rapid inhibition in growth rate (Figure 3.5.). This data is indicative of the growth inhibitory nature of Gp07. However, it was observed that there was a minor increase in OD_{600} of the cells after approximately 6 hours of induction of Gp07, rCTD or ΔGp07 (Figure 3.5.). On the other hand, *E. coli* cells harbouring rNTD (overexpression was confirmed by SDS-12% PAGE, Figure 3.4.) upon induction by 0.5mM IPTG at 37˚C for 3 h also showed inhibition in growth rate. However, in this case, the growth inhibition was much lower than that induced by Gp07, rCTD or ΔGp07 (Figure 3.5.). More interestingly, in this case, resumption of growth by the induced cells occurred earlier than that in case of Gp07, rCTD or ΔGp07 and there was a substantial increase in the optical density (OD_{600}) at the end of 5 h. In case of the negative control, CI, there was no significant change in the OD of the overexpressing cells as compared to the wild type cells.

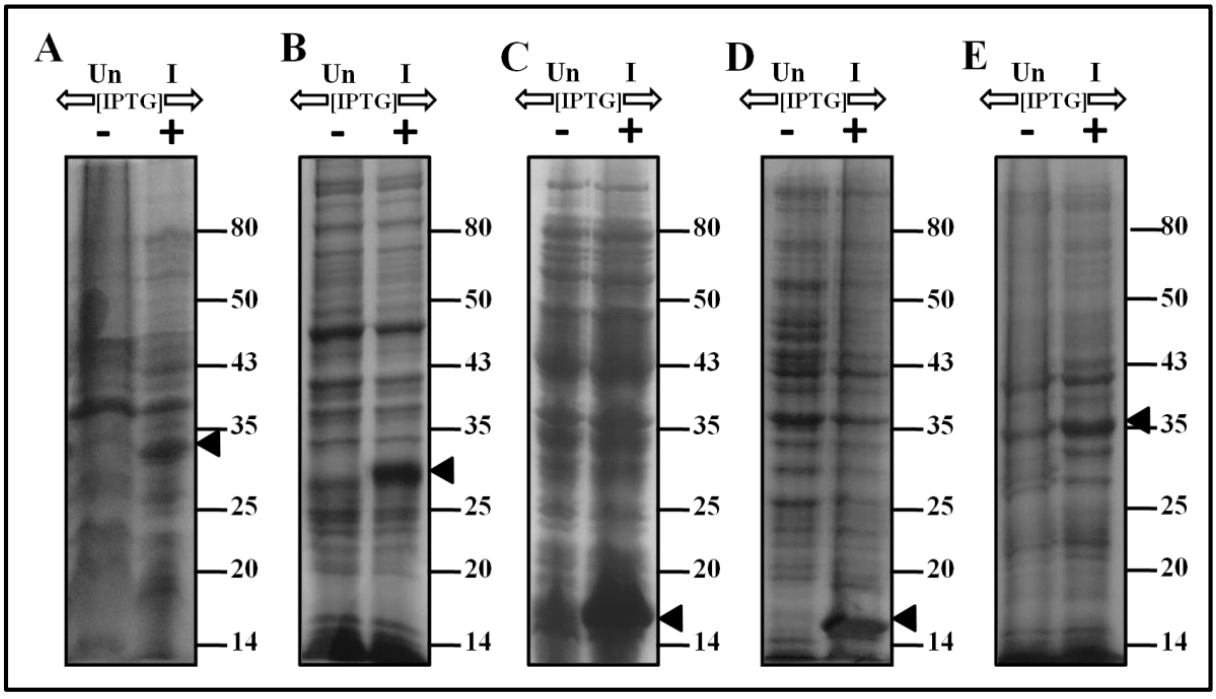


Figure 3.4. || Overexpression of Gp07, its domains (rNTD, rCTD), ΔGp07 and a control protein in *E. coli*. Overexpression of the recombinant proteins (induced with 0.5mM IPTG for 2 h at 37ºC) were analysed on SDS-12%-PAGE. (A) Overexpression of Gp07 (B) Overexpression of ΔGp07 (C) Overexpression of rNTD; (D) Overexpression of rCTD; (E) Overexpression of control protein. Arrowheads indicate the overexpressed protein. Un indicates uninduced cellular extract; I indicates induced cellular extract.

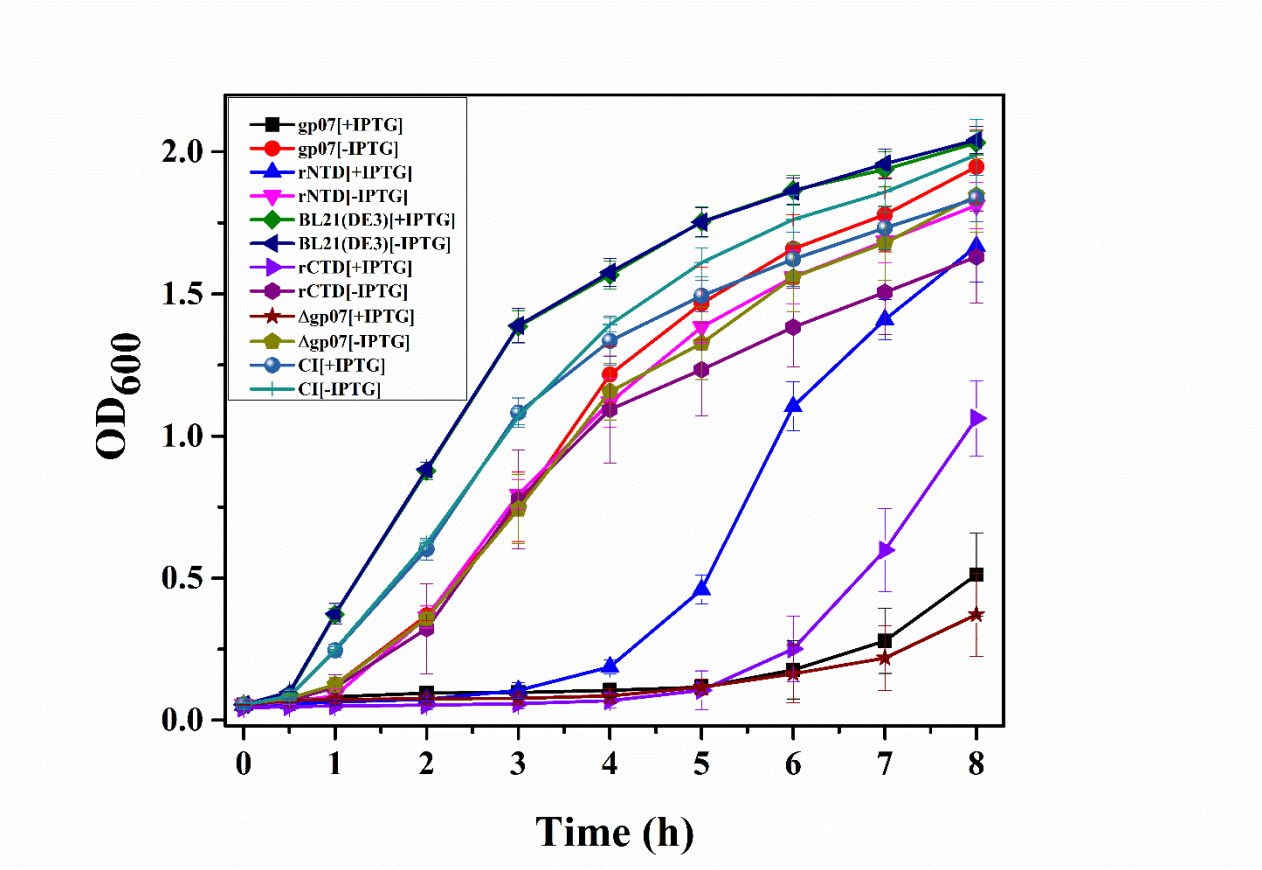
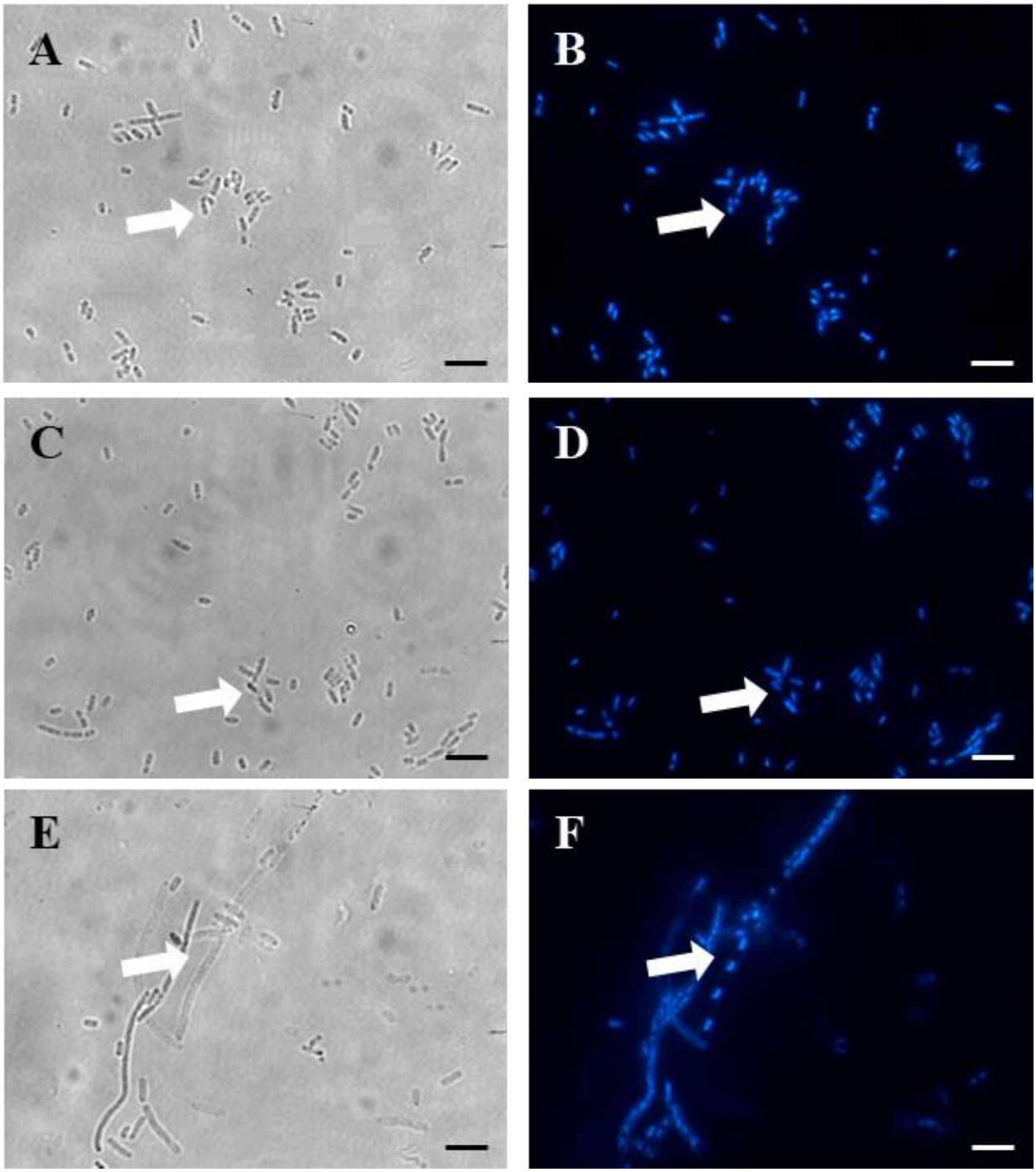


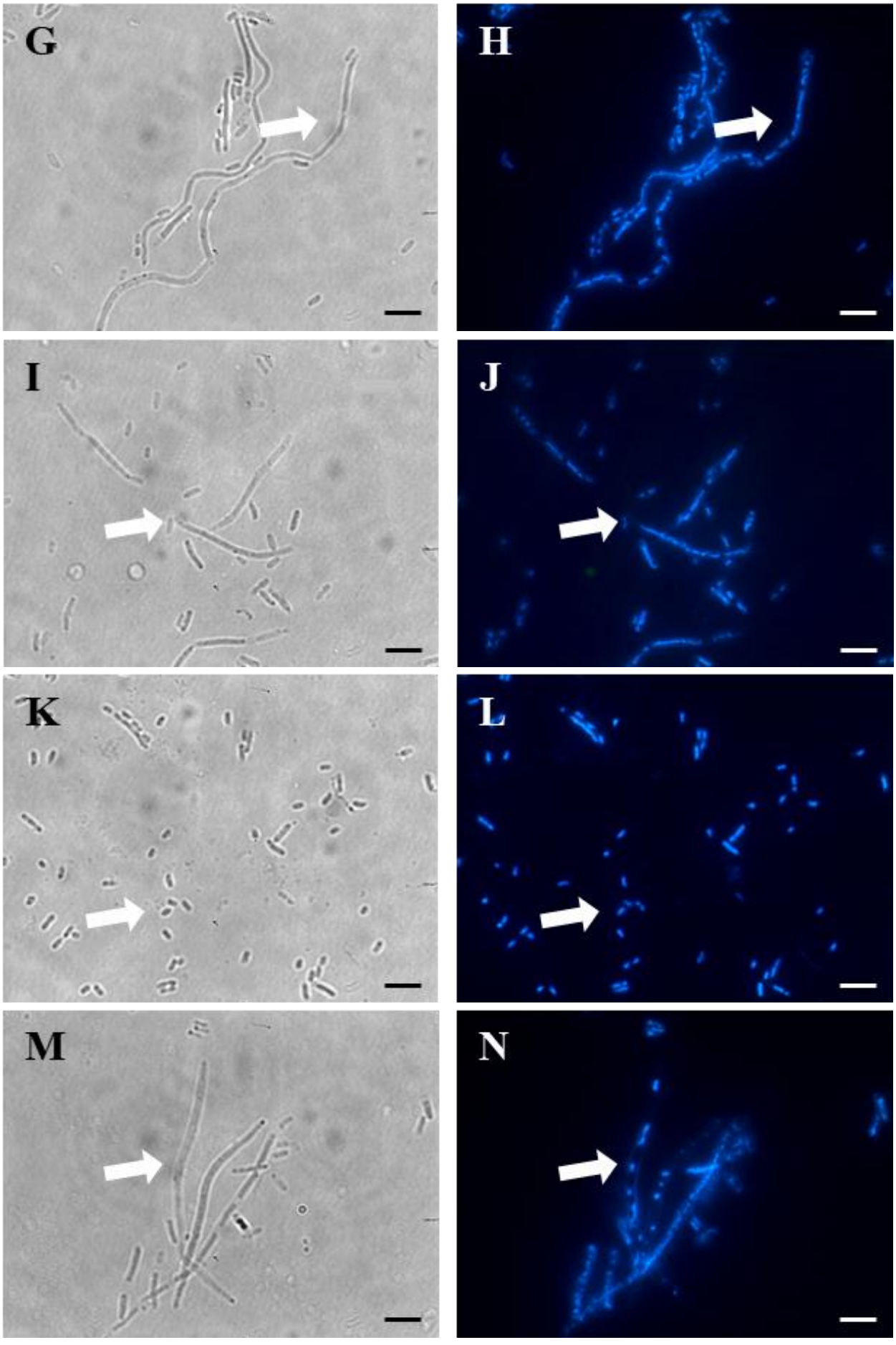
Figure 3.5. || Effect of Gp07, its domains and ΔGp07 upon the growth of *E. coli*.
Overexpression of Phi11 Gp07, rCTD as well as the ΔGp07 inhibited growth of the host cells. Overexpression of rNTD initially repressed host cell growth but resumption of cell growth was seen after a few hours. The overexpressed control protein (CI) had no effect upon the growth of *E. coli* cells. All experiments were performed in triplicate. The standard error of the mean was used to calculate the error bars. In case of those points where the experimental variations are too small, the error bars are not visible.

3.3.4. **Microscopic observation of *E. coli* cells upon overexpression of Gp07, its domains and ΔGp07**

E. coli cells harbouring no plasmid and *E. coli* cells harbouring Gp07, induced with 0.5mM IPTG were examined under phase contrast microscope as well as SEM. It was observed that, the host cells carrying Gp07 upon induction became filamentous (Figures 3.6.E/3.6.G and 3.7.C). Moreover, irregular multiple nucleoid structures could be observed upon staining of the induced cells with DAPI (Figures 3.6.F/3.6.H). This suggests that normal DNA replication progressed in the cells.The filamentation was not reversible in case of *E. coli* cells overexpressing Gp07 (Figures 3.6.E/3.6.G and 3.7.C). Similar results were also observed in case of *E. coli* cells harbouring rCTD and ΔGp07 (Figure 3.5). In case of rNTD however, the filamentation was reversed at the end of 8 h as is evident from Figures 3.6.I/3.6.K and 3.7.D. Examination of the induced *E. coli* cells harbouring rNTD by phase contrast microscopy as well as SEM showed an increased number of *E. coli* cells with normal morphology at the end of 8 h (Figures 3.6.K and 3.7.D). This further strengthens the observation

that it is not the rNTD domain but the rCTD domain which is essential for the growth inhibitory effect of Gp07. In its absence, the rNTD alone cannot retain this activity of Gp07. The negative control, CI, had no effect on the morphology of the host cells (Figures 3.6.C/3.6.D and 3.7.B).

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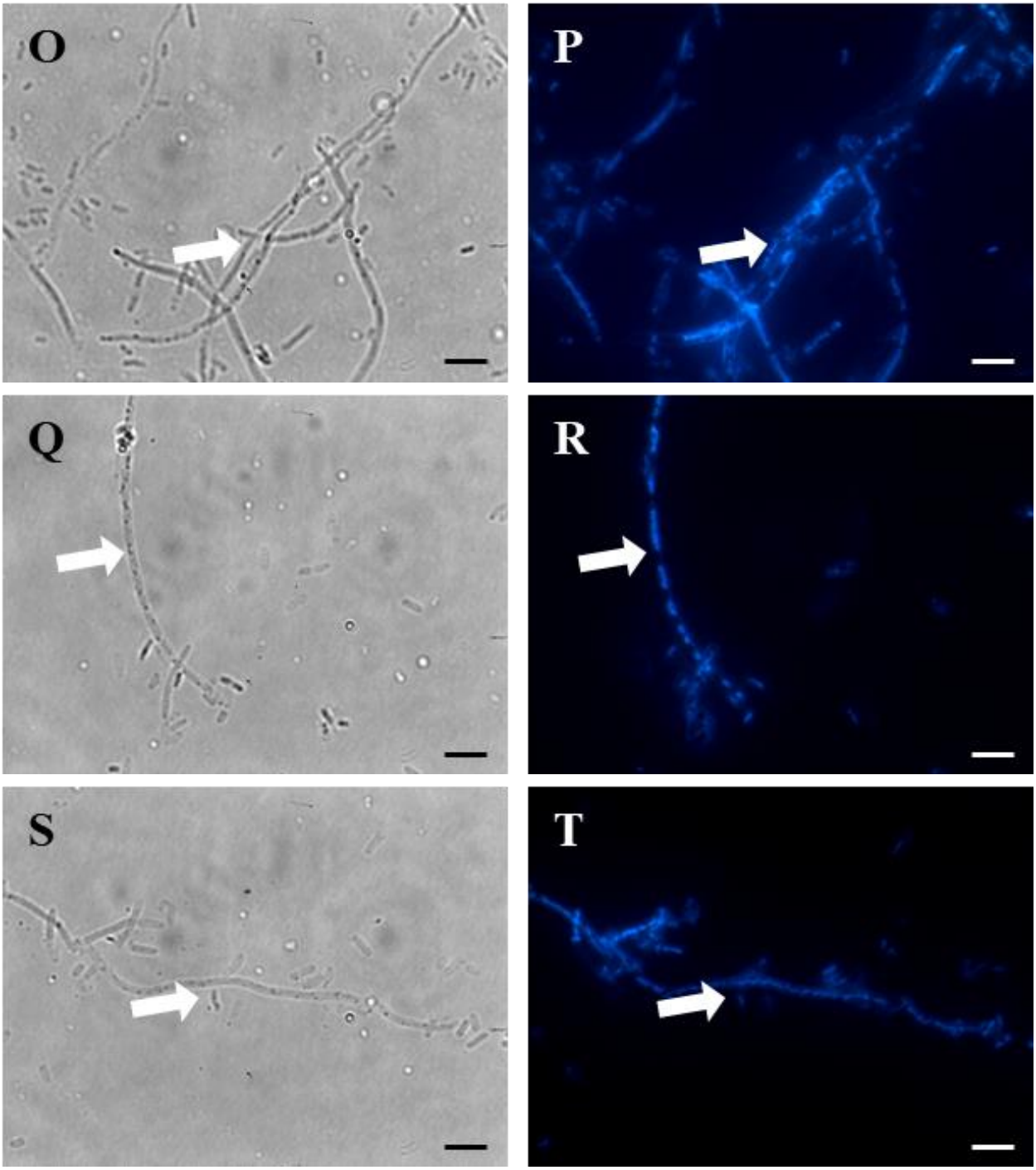
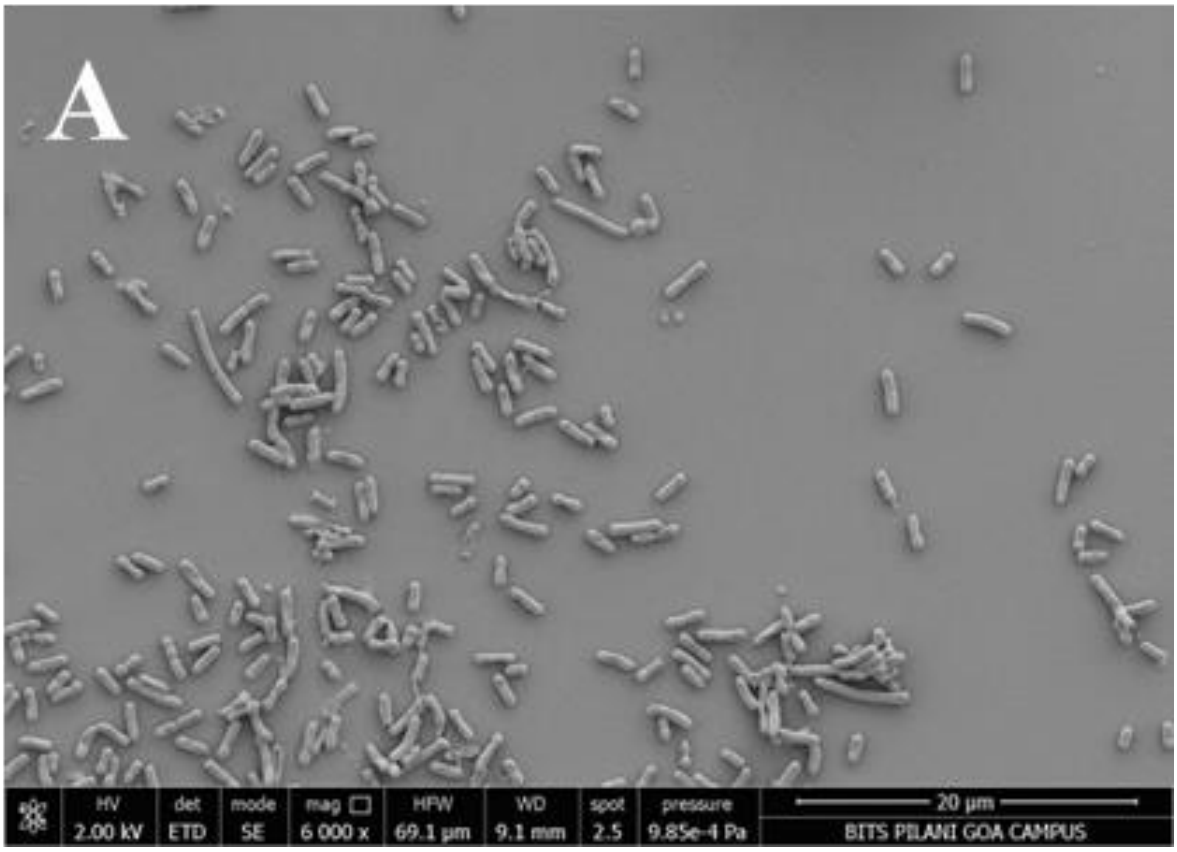


Figure 3.6. || Effect of overexpression of Gp07, its domains and ΔGp07 on the morphology of host cell.
(A) Morphology of wild type host cells (*E. coli*) under phase-contrast microscope at 100X magnification. (C) Morphology of *E. coli* cells overexpressing the control protein. (E,I,M,Q) Morphology of *E. coli* cells overexpressing Gp07, rNTD, rCTD and ΔGp07 respectively at 5h, as observed by phase contrast microscopy. (G,K,O,S) Morphology of *E. coli* cells overexpressing Gp07, rNTD, rCTD and ΔGp07 respectively at 8h, as observed by phase contrast microscopy. (B) DAPI stained normal host cells (*E. coli* BL21 DE3 not carrying any recombinant DNA) under fluorescence microscope. (D) DAPI stained *E. coli* cells overexpressing the control protein. (F,J,N,R) DAPI stained *E.coli* cells overexpressing Gp07, rNTD, rCTD and ΔGp07 respectively at 5h, under fluorescence microscope. (H,L,P,T) DAPI stained *E. coli* cells overexpressing Gp07, rNTD, rCTD and ΔGp07 respectively at 8h, under fluorescence microscope. All the scale bars are 5µm.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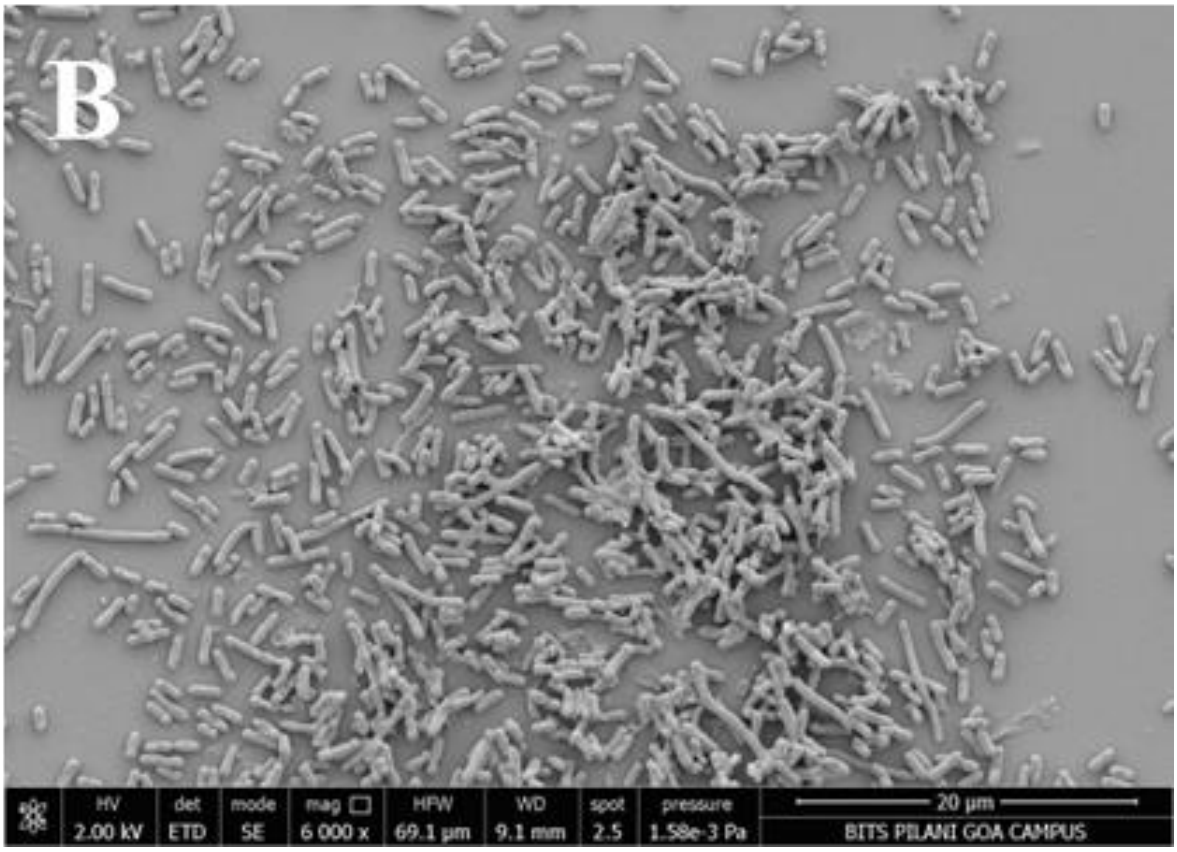
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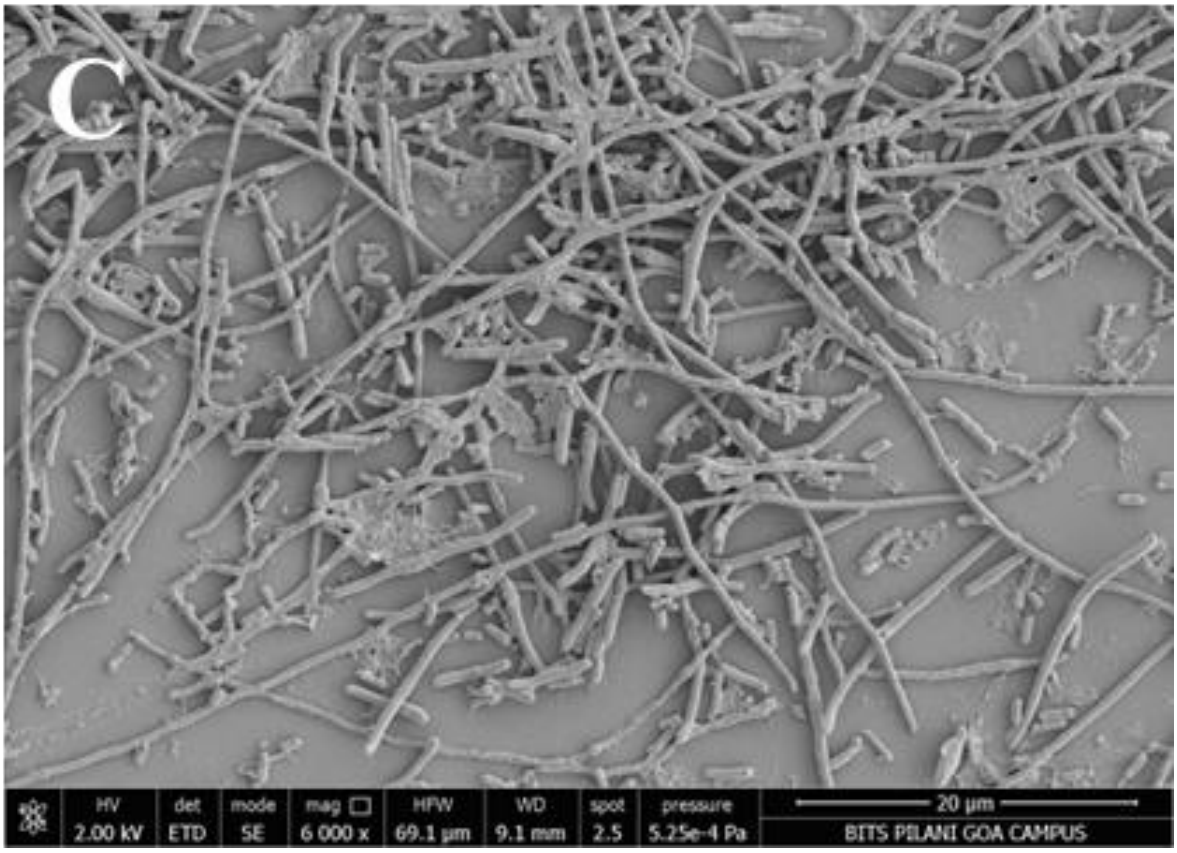
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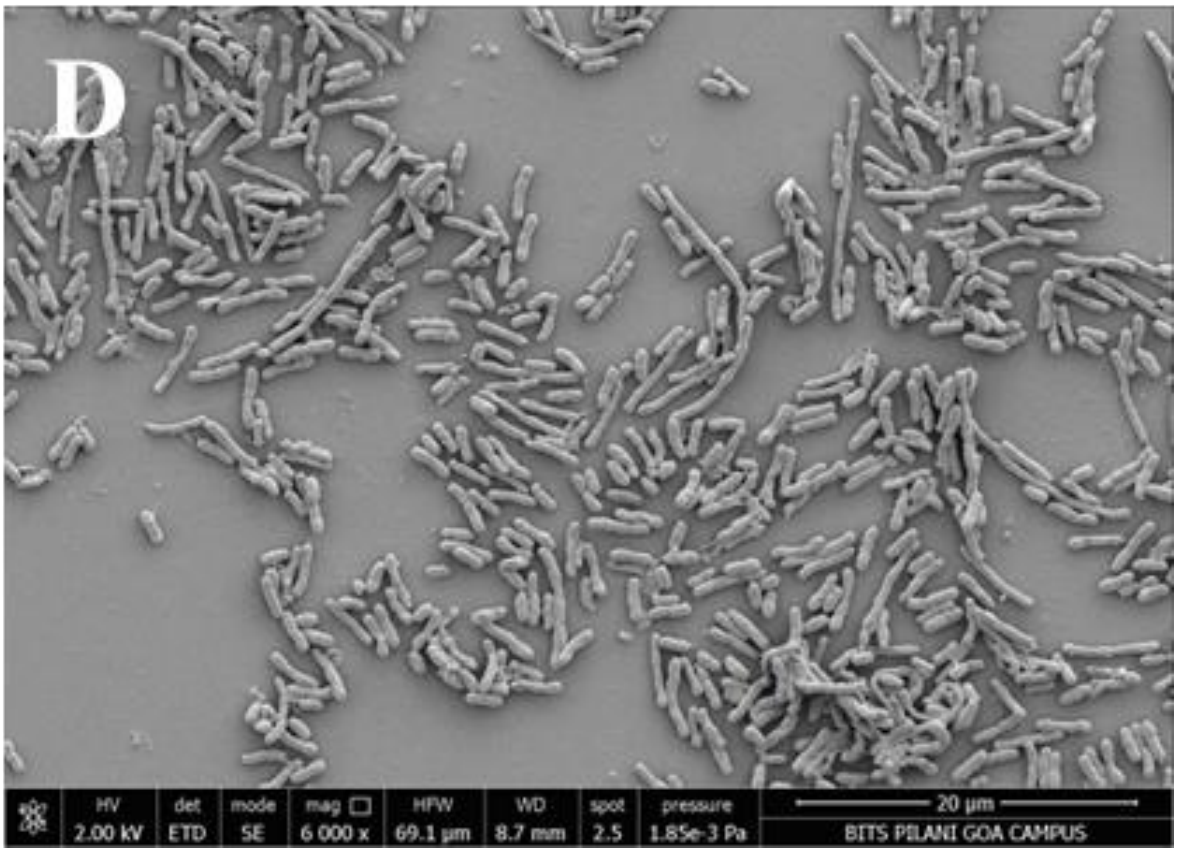
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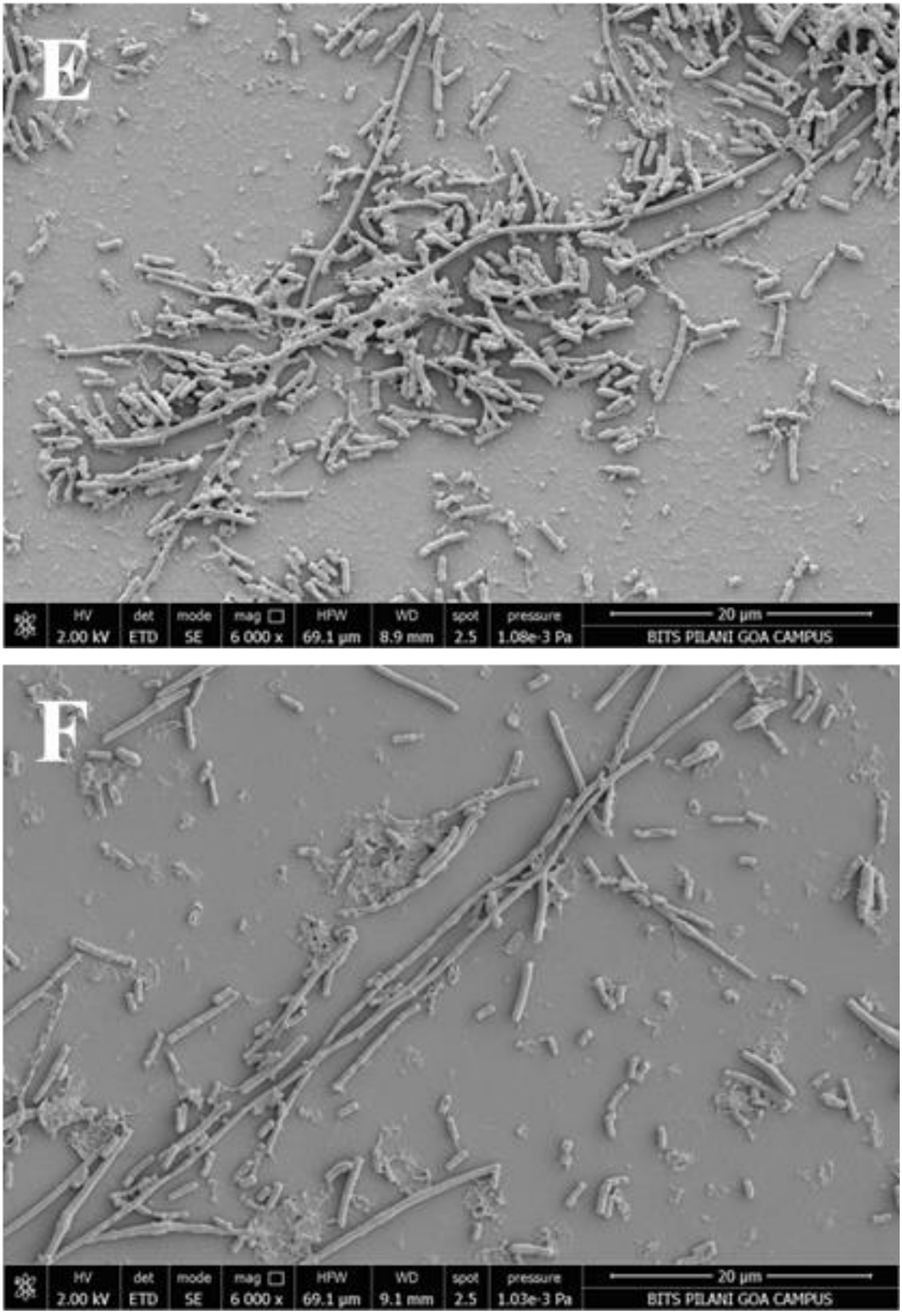

Figure 3.7. || Scanning electron micrographs of Wild type (WT), Control protein, Gp07, rNTD, rCTD and ΔGp07 expression in *E. coli*. (A) Wild type (WT), (B) Control protein (C) Gp07, (D) rNTD, (E) rCTD, (F) ΔGp07; all scale bars are 20 µm.

3.3.5. **Gene organization of Bro-N and KilA-C**

Domains are distinct functional and/or structural units in a protein. Usually they are responsible for a particular function or interaction, contributing to the overall role of a protein. The distribution of domains plays a very important role in gene regulations, such as, bacteriophage lytic-lysogenic control, toxin-antitoxin regulation (Chan et al 2014). Initial bioinformatics analysis shows Gp07 contain two essential domains (Bro-N and KilA-C), which are widely distributed in the genome of different organisms (Iyer et al 2002). We found eleven different gene organizations (Figure 3.8.), in the twenty-six bacteriophages (Table 3.3.) which we selected in caudovirales order with 50% or more identity as the threshold. In Types I-VI, with respect to the Bro-N domain, we found that the KilA-C domains lie downstream while HTH and HTH-S24 lie upstream; however, this arrangement is not observed in Types VII-XI. The intergenic region between HTH and HTH-S24 domains is very important for lytic-lysogenic regulation in Type III (Das et al 2007), and is present in most of twenty-six selected phages. The only exception is Type I *Staphylococcus* phage TEM123 (Figure 3.8.). Surprisingly, among the all analyzed *Staphylococcal* phages only Type VII belongs to Myoviridae family (Table 3.3.). In Types VIII-XI, an alternative gene organization is observed, whereby, the ORF06C domains lie downstream and HTH and HTH-S24 lie upstream of the Bro-N domain. In case of Type VII (phage vB SauM Romulus), the Bro-N domain is flanked by HPs (Hypothetical proteins) on both sides.

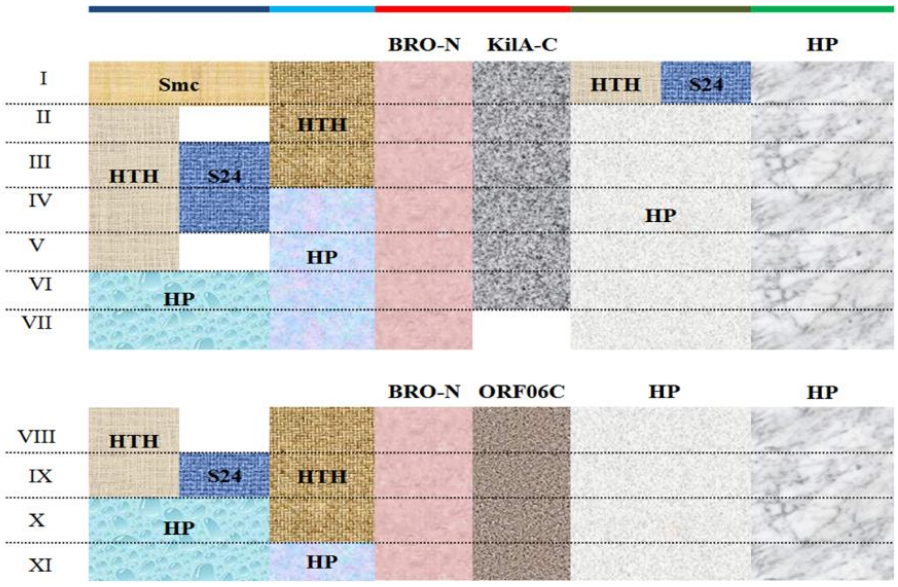

Figure 3.8. || Gene organization analyses of Caudovirales phage protein contain Bro-N domain. Polymorphisms associated with the gene organization of Gp07 and their neighboring genes found in 26 *Staphylococcus* phages. Eleven variations of gene organizations were found (Type I-XI). Top most color bar is indicating of five separate genes. In general, the Gp07 protein Bro-N domain was flanked upstream by KilA-C domain, for Type I–VI. For Type VIII–XI, the Bro-N cassette is flanked upstream by ORF06C. Other abbreviations used: Structural Maintenance of Chromosomes (Smc), Helix-Turn-Helix Xenobiotic Response Element family like proteins (HTH); Peptidase S24 LexA-like proteins (S24); hypothetical proteins (HP).

Table 3.3. 26 *Staphylococcus* phages are divided on eleven gene organizations type (Type I-XI).

Type	Family	Phage Name
I	Siphoviridae	*Staphylococcus* phage TEM123
II	Siphoviridae	*Staphylococcus* phage B236
	Siphoviridae	*Staphylococcus* virus 187
	Siphoviridae	*Staphylococcus* phage SA13
	Siphoviridae	*Staphylococcus* phage phiMR25
	Siphoviridae	*Staphylococcus* phage 88
III	Siphoviridae	*Staphylococcus* phage Phi11
	Siphoviridae	*Staphylococcus* virus SAP26
	Siphoviridae	*Staphylococcus* phage 69
	Siphoviridae	*Staphylococcus* phage 53
	Siphoviridae	*Staphylococcus* phage B166
	Siphoviridae	*Staphylococcus* phage phiSa119
IV	Siphoviridae	*Staphylococcus* phage 47
V	Siphoviridae	*Staphylococcus* phage 42E
	Siphoviridae	*Staphylococcus* phage SA12
	Siphoviridae	*Staphylococcus* phage 37
VI	Siphoviridae	*Staphylococcus* phage 3MRA
	Siphoviridae	*Staphylococcus* phage 77
	Siphoviridae	*Staphylococcus* prophage phiPV83
VII	Myoviridae	*Staphylococcus* phage vB_SauM_Romulus
VIII	Siphoviridae	*Staphylococcus* phage 96
	Siphoviridae	*Staphylococcus* phage 52A
	Siphoviridae	*Staphylococcus* phage StB27
IX	Siphoviridae	*Staphylococcus* phage 92
X	Siphoviridae	*Staphylococcus* phage X2
XI	Siphoviridae	*Staphylococcus* phage phiMR11

3.4. **Discussion**

This work shows that overexpression of Gp07, its carboxy terminal domain KilA-C (rCTD) as well as the deletion mutant of Gp07 (ΔGp07) significantly inhibited the growth of *E. coli* following induction with IPTG. Similar observations have already been reported in host cells expressing the *icd* gene of P1 (Riedel 1993), where, *E. coli* cells expressing the Icd protein of bacteriophage P1, become filamentous in nature. There have also been reports that the Rac prophage (Conter et al 1996) of *E. coli* harbours a gene called *kil* which codes for a 73 amino acid protein. The overexpression of this *kil* gene was shown to block cell division resulting in filamentation of the host cell. It has further been reported that this inhibition is relieved by excess FtsZ (Conter et al 1996). Studies on the Kim (Qin) prophage (Yang et al 2016) also indicate the inhibition of divisome formation in *E. coli*. The *kil* gene of bacteriophage lambda has also been reported to induce filamentation in host cells (Haeusser et al 2014). Haeusser et al have already reported that cell division in *E. coli* host cells are blocked by the Kil protein of bacteriophage λ. The Kil protein possibly ablates the FtsZ rings in *E. coli*, thereby blocking cell division. The block can however be inhibited by an abundance of FtsZ (Haeusser et al 2014).

Another interesting observation from this study indicated that *E. coli* cells overexpressing Gp07, its carboxy terminal domain KilA-C (rCTD) as well as the deletion mutant of Gp07 (ΔGp07), underwent a very slight increase in the optical density of the cells measured at 600 nm from 5-6 h following induction. Initially it was assumed that the growth inhibitory effect exerted by Gp07 might be temporal in nature and hence the slight increase in OD; however, cell morphology studies indicated that the cells upon Gp07, ΔGp07 and KilA-C expression became filamentous in nature, which is an indication of inhibition of cell division. This slight increase in the OD value can be attributed to the increase in length of the filamentous cells and not necessarily due to the resumption of cell growth.

Analysis of the amino acid sequence of Gp07 further indicates that the stretch of the first eleven amino acids of Phi11 Gp07 protein are extremely unique (Figures 3.1. and 3.3.). To check if the eleven amino acid residues had any role in the growth inhibitory effect of Gp07, these amino acids were deleted to generate the mutant, ΔGp07. It is interesting to note that ΔGp07 retained the growth inhibitory effect displayed by full length Gp07. Thus it can be assumed that the stretch of the first eleven amino acids of Phi11 Gp07 protein do not play any role in its growth inhibitory effect. Further bioinformatics analysis of Gp07 showed that the two identified domains, Bro-N and KilA-C are widely spread and present in different organisms. Considering cell growth kinetics

experiments, studies on cell morphology with phase-contrast and fluorescence microscopy as well as SEM data of Gp07 and its domains, it is clear that the carboxy terminal domain of Gp07 (KilA-C domain) is more important for its growth inhibitory function in *E. coli*. In case of the Bro-N domain, the data clearly suggests that the growth inhibition induced by the Bro-N domain alone, is temporal in nature.

3.5. **References**

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Chapter 4

Overexpression and purification of Gp07-a lethal protein

4.1. **Introduction**

Temperate bacteriophages are those which can adopt two modes of development-the lytic mode and the lysogenic mode. Some temperate bacteriophages can switch between these two developmental pathways. The switching is induced by certain conditions such as exposure of the lysogen to UV or other agents which can damage the DNA. Under such conditions, the prophage will switch to the lytic mode of development, thereby abandoning the damaged host cell - a process known as prophage induction. In temperate bacteriophages, particularly Phi11 developmental pathway is controlled by two essential repressors primarily, CI and Cro (Das et al 2007; Das et al 2009). However, 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.

Preliminary report showed that Gp07 upon overexpression led to considerable inhibition of the growth of the host *E. coli* cells; in fact, cell morphology studies confirmed the filamentous morphology of the Gp07 overexpressing cells (Chapter 3: Section 3.3.4.). Though Gp07 has a cell division inhibiting role, hence main objective was to assess its role in the developmental pathway of Phi11.

Despite the importance of antirepressor proteins in the developmental pathway of temperate phages, the aureophage Antirepessor proteins of aureophages have not yet been characterized. The novel architecture of Phi11 antirepressor (amino-terminal Bro and carboxy terminal KilA domain), as well as its inhibitory effect upon host cell division motivated us to carry out the cloning, overexpression and purification of the Phi11 Gp07. The preliminary role of Gp07 showed toxic effect on the expression host; the carboxy terminal domain alone also shows similar activity (Chapter 3). Here we describe a method to optimize the Gp07 expression conditions. This chapter work represents the first step towards the biochemical characterization of antirepressor from *Staphylococcal* phage Phi11 and indicates a possible protocol for efficient overexpression and purification of the protein in a functional, soluble form. This modified protocol might be useful in purification of other similar proteins carrying the KilA and Bro domains.

4.2. **Materials and methods**

4.2.1. **Strains and plasmids**

Phage Phi11, *S. aureus* RN4220, *E. coli* BL21 (λDE3) and *E. coli* XL1 Blue were grown in laboratory conditions as described in section 3.2.1. Appropriate antibiotics (ampicillin and kanamycin) were added to the growth media as required.

4.2.2. **Cloning of *gp07* into expression vector pET28a**

The genomic DNA of Phi11 was isolated as described in section 3.2.4. and used as template for amplification of *gp07* gene. Briefly, to clone Phi11 *gp07*, we employed a polymerase chain reaction with Taq polymerase (DreamTaq DNA polymerase from ThermoFisher Scientific) using primers C-*gp07*-F and C-*gp07*-R (Chapter 3: Table 3.1.). The product obtained was 825bp in size and was cloned into pGEM-T Easy vector according to the manufacturer's protocol (www.promega.com/protocols/) and denoted as pGp07-T. pGp07-T carrying *gp07* was double digested by the restriction enzymes, NcoI and XhoI and sub cloned into expression vector pET28a. pET28a and *gp07* gene cloning sites were generated by double digestion of pGp07-T and pET28a at two sites by the restriction enzyme NcoI and XhoI (restriction sites has been already added to the primer sequences at the time of primer design). PCR products and double digestion fragments were analyzed by electrophoresis on a 1.5% agarose gel and purified using the GeneJET Gel Extraction Kit (Thermo Scientific). pET28a carrying *gp07* has been designated as pGp07 and is derived from the pET28a vector. It has a Lac-regulated T7 promoter and kanamycin resistance. Gp07 cloned in this vector is fused to a carboxy-terminal tag of 8 residues (LEHHHHHH) with 2 amino-terminal residues (MG) to keep the *gp07* gene in-frame. Clones were screened by plasmid purification and followed by PCR and double digestion of recombinant pGp07-T and pGp07. The selected constructs were sequenced at the UDSC, Delhi to rule out any mutation.

4.2.3. **Test expression with time scan for the Gp07 purification**

Vector pGp07 carrying *gp07* gene was transformed into *E. coli* BL21(λDE3) cells to generate expression clones. Small scale test expressions were carried out in 50ml cultures overexpressing the expression clones. Briefly, overnight cultures of the expression clone was prepared in 10ml of Luria bertani broth (LB) medium containing antibiotic (50 µg/ml kanamycin) in a 100ml conical flask, and the culture was grown overnight in a shaking incubator at 37°C, 120rpm. Three sets of 50ml of Luria Bertani broth containing kanamycin (50µg/ml) were inoculated with 1% overnight grown culture and incubated at 37°C with shaking at 120rpm, until an optical density of ~0.4-0.5 was reached at 600 nm (OD_{600}). Expression was then induced by adding 500µM IPTG and cultures

were incubated for three different time points (1hr, 2hr and 3hr) at 32°C with shaking at 140rpm. Cells were harvested separately by centrifugation (6,500rpm for 10 min at 4^{o}C) and resuspended in 10ml of lysis buffer [20 mM Tris-chloride buffer (pH 7.5), 500 mM NaCl, 5% glycerol, 10 mM imidazole] containing 10 µg/ml of PMSF (phenylmethane sulfonylfluoride) protease inhibitor EDTA-free. This suspension sonicated for 20 seconds on, 2min off cycle (incubated in ice) and five repetitive cycles were used to complete the sonication. Finally, the lysate was clarified by centrifugation (10,000rpm for 20 min, 4^{o}C) and the supernatant and pellet fraction were separated and the recombinant protein expression was evaluated at the three desired time points. The supernatant and pellet fractions were compared with the un-induced cells by analysis through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS 12%-PAGE). Gels were stained and destained as described in section 2.2.4 (Chapter 2).

4.2.4. **Large-scale protein expression and purification of His-tagged Gp07**

Overnight primary culture of *E. coli* BL21 (λDE3) carrying pGp07 was grown in LB medium containing kanamycin (50µg/ml) in an incubator shaker at 37° C, 120 rpm. 30 ml of the starter culture was used to inoculate 3 liters of LB medium containing kanamycin (50µg/ml) in ten 1 liter conical flasks. Cells were allowed to grow at 120rpm, and 37°C until an OD_{600} ~0.4-0.5 was reached. The culture was then transferred to a shaking incubator at 37°C and shaken at 120 rpm. After a 20 min cool-down period, IPTG was added to a final concentration of 0.5 mM. Cells were further cultured for 1 h at 32° C and 140 rpm. Cells were harvested by centrifugation (10 min, 6,500 rpm at 4°C). The cell pellet was washed with 0.9% NaCl and cell pellet was stored at -80° C until use.

Modified steps for two step IMAC purification has shown positive effect on purification of Gp07. Initially the cell pellet was resuspended in 10 ml of ice-cold lysis Buffer (Lysis buffer: 20 mM Tris-chloride buffer pH 7.5, 500 mM NaCl, 5% glycerol, 10 mM imidazole and 10 µg/ml of PMSF). Cells were lysed by sonication (Misonix Microson Sonifier Misonix XL2000 Sonicator System Ultrasonic Cell Disrupter) on ice at 10 watts (RMS) for 12 min (Repeated cycles of 20 seconds with 2min intervals). Finally, the sonicated extract was centrifuged (45 min, 12,000rpm, 4°C) and the supernatant (Cytoplasmic fraction) was separated from the pellet fraction. Gp07 was purified from the supernantant by Ni-NTA column chromatography according to manufacturer's protocol (ThermoFisher Scientific) using Wash buffer (Wash buffer: 20mM Tris pH 7.5, 500mM NaCl, 25mM Imidazole and 5% glycerol). Finally, the His-tagged Gp07 was eluted from the column using elution buffer (Elution buffer: 20mM Tris pH 7.5, 500mM NaCl, 300mM Imidazole and 5%

glycerol). The elution fractions were pooled together and kept for dialysis against dialysis buffer (Dialysis buffer: 20mM Tris pH 7.5, 500mM NaCl, 0mM Imidazole, 5% glycerol and 1mM EDTA) overnight to allow complete removal of imidazole and EDTA.

The dialysed protein thus obtained was further loaded onto the IMAC column for the next stage of purification. In this case, a different gradient of salt and imidazole concentrations (Wash buffer 1: 750mM NaCl and 25mM Imidazole, Wash buffer 2: 750mM NaCl and 30mM Imidazole, Wash buffer 3: 750mM NaCl and 40mM Imidazole, Wash buffer 4: 500mM NaCl and 60mM Imidazole) with the components of the wash buffer (Tris 20mM pH 7.5, Imidazole, NaCl, 5% glycerol) was incorporated. Finally, Gp07 was eluted using elution buffer (Tris 20mM pH 7.5, 300mM Imidazole, 500mM NaCl and 5% glycerol) and dialysed again for further characterization.

4.2.5. **Western blot analysis**

Western Blotting was performed according to manufacturer's protocol (GE Healthcare, Life Sciences), to confirm the identity of the purified protein. Briefly, the cells expressing the putative C-teminal His-tagged Gp07 were harvested. The putative Gp07 was purified by Ni-NTA affinity chromatography as discussed in section 4.2.4. The elution fraction carrying the purified putative Gp07 protein was run on a SDS-12% PAGE. The resolved proteins were transferred to Immobilon-PSQ Hydrophobic Polyvinylidene Fluoride (PVDF) (Millipore India Pvt. Ltd.) using a sandwich blotter (GE, miniVE System-Blot Module). Transfer was allowed to proceed for 1hr at 4°C at 50 Volts. Following transfer, the membrane was washed with Tris Buffered Saline (TBS) containing 50 mM Tris and 150 mM NaCl, pH 7.6 and blocked with 3% (w/v) BSA for an hour. The blocked membrane was again washed with TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20; pH 7.6) and twice with TBS, and finally incubated overnight, with TBS containing primary anti-His antibody (diluted 1:2000) at 4^{o}C. Next day, the blot was again washed twice with both TBST and TBS for 20 min each and incubated with alkaline phosphatase-tagged goat anti-mouse antibody (IgG1-AP) (diluted 1:2000) for 2h. This was again followed by washing twice with both TBST and TBS for 20 min each and the blot was stained with NBT/BCIP (given in appendix A) (Calbiochem®, Merck Millipore India Pvt. Ltd.) until the signal is clearly visible. The membrane was finally dried and photographed.

4.2.6. **Analytical gel filtration chromatography**

In order to determine the oligomeric status of the purified Gp07 in solution, analytical gel filtration chromatography using a SEC-s2000 column (Phenomenex, USA) was carried out. Briefly, 5 μM of

purified Gp07 was loaded onto a SEC-s2000 column that was equilibrated in dialysis buffer. The protein was eluted at a constant flow rate of 0.5ml/min. The absorbance of the eluted Gp07 was monitored at 280 nm. Marker proteins mix (Aqueous SEC 1, Phenomenex, USA) was separately eluted using same column and conditions. The retention factor of each protein was determined using its retention time, the total volume of the column, and the elution time. The plot of retention factors of marker proteins versus their log of molecular mass was used to calculate the molecular mass of Gp07.

4.2.6. **Glutaraldehyde cross-linking of Gp07**

To determine the protein-protein crosslinking reaction of Gp07 (5μM), the glutaraldehyde-mediated cross-linking experiment has been performed by standard procedure (Das et al 2009). Briefly, 5μM Gp07 (in 0.1M Na-phosphate (pH-7.4), 500mM NaCl and 5% glycerol) was incubated for 20 minutes at room temperature and then treated with 0.1% glutaraldehyde solution for 2 minutes in 20 μl total reaction volume. The reaction was stopped by addition of a SDS sample buffer. The reaction mixtures were boiled for 2 minutes and analysed by an SDS-12% PAGE.

4.2.7. **Spectroscopic studies on Gp07**

The effect of temperature on the secondary structural elements of Gp07 was investigated from the far-UV CD (200-250 nm) spectra of the protein recorded on a Jasco-715 spectropolarimeter. A cell with a path length of 1 mm was used and 5μM of Gp07 was incubated for 5min at different temperatures (15^{o}C, 25^{o}C, 35^{o}C, 45^{o}C, 55^{o}C, 65^{o}C, 75^{o}C and 85^{o}C) (Creighton 1997). The spectra of the protein so obtained were analyzed by BeStSel tool (Micsonai et al 2018). The buffer values were subtracted from the corresponding spectra of protein samples. The scans were repeated thrice for each temperature. PSI-PRED was used to analyse the putative secondary conformation of Gp07 (Buchan et al 2013).

To investigate the effects of GdnCl on the tryptophan (Trp) residues of Gp07, the fluorescence of the samples was measured at room temperature in a disposable 10 mm path length polystyrene cuvette. The intrinsic tryptophan fluorescence spectra (λ_{ex}= 295 nm and λ_{em}= 300-400 nm) of Gp07 (2.5 μM each) were recorded in the presence of GdnCl (using 0M, 1M, 2M, 3M, 4M, 5M and 6M) using a Jasco-FP8500 spectrofluorimeter by the method of Albrecht et al (Albrecht 2008). All resultant spectra were obtained by the subtraction of the control. Three repeat scans per sample were made and the λ_{max} were analyzed.

4.3. **Results**

4.3.1. **Cloning of *gp07* into pET28a expression vector**

gp07 gene was PCR amplified as described above (Materials and methods) and a DNA band of 825bp was observed on a 1.5% agarose gel. This band was gel purified and the product was ligated into a pGEM-T easy vector and transformed into XL1 Blue competent *E. coli* cells. The transformants that had grown on the LB agar plates supplemented with ampicillin (100µg/ml) were subjected to blue white screening and 10 white colonies were selected for further analysis. The 10 colonies were further screened and selected for the presence of *gp07* gene by PCR using forward primer and reverse primer of *gp07*; the DNA isolated from the 10 selected colonies were further subjected to double digestion with NdeI and XhoI. All the ten transformants were found to be positive for the presence of *gp07* and gave a band at 825bp (Figure 4.1.A and 4.1.B) when run on a 1.5% agarose gel.

The sequences of the oligonucleotide primers utilized to generate the pGp07 have already been described (Chapter 3: Table 3.1. and Table 3.2.). The recombinant DNA from a healthy transformant was sequenced to rule out any mutation. Further, a healthy E. coli XL1 Blue single colony harbouring pGp07-T (containing no mutation) was digested with NcoI and XhoI and the *gp07* gene was subcloned into pET28a vector also digested with NcoI and XhoI. Eight transformants were obtained out of which five harboured the recombinant plasmid pGp07 (Figure 4.1.C and 4.1.D). Plasmid DNA was isolated from all the five positive transformants and were individually transformed into *E. coli* BL21 (λDE3) for optimizing the conditions for the overexpression of Gp07.

4.3.2. **Test expression with time scan for the Gp07 purification**

All 5 positive constructs pGp07 were employed for evaluating the optimal conditions for over expression of Gp07 protein production by small-scale test expression (from 25 ml cultures) (Figure 4.2.A). Of the 5 selected positive clones, only construct-I (Cn-I) and to some extent, Cn-II showed optimal recombinant protein expression. However, the growth of *E. coli* BL21(λDE3) transformed with Cn-I was severely impaired, especially after the addition of the inducing agent (IPTG). For the remaining constructs (Cn-IV and Cn-VIII), protein expression levels were found to be very low, while Cn-V, failed to produce any amounts of recombinant Gp07 (Figure 4.2.A).

Test expression results indicated that Gp07 could be produced in a soluble form in optimal quantities in the host cells (Figure 4.2.B). It was further found that high levels of soluble Gp07

protein is produced during 1h of induction with IPTG at 32ºC. When the time of induction was increased to 2h and 3h, soluble protein expression was highly impaired (Figure 4.2.B). Fortunately, a large percentage of Gp07 appeared in the supernatant fraction obtained after sonication, when induced for 1h. Taken together, these results from cloning and small-scale test expression identified the best combination of time and temperature of induction for the production of the Gp07 in a soluble form.

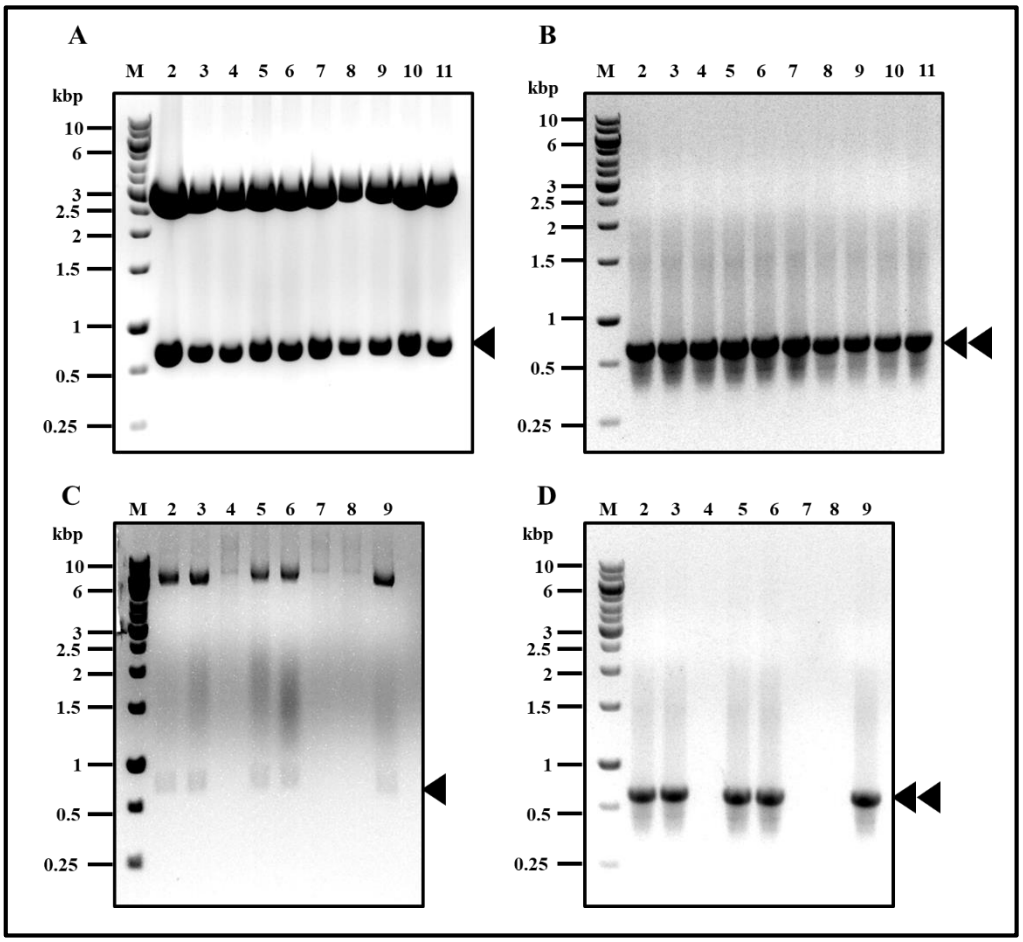

Figure 4.1. || Confirmation of recombinant Gp07 clones.
(A) Confirmation of pGp07-T clones by DNA double-digestion using the restriction enzymes NdeI and XhoI. The double-digested products show two individual bands approximately 3 kbp and 825 bp representing the vector and the insert, respectively (Lanes 2–11). (B) Confirmation of selected recombinants (pGp07-T) by PCR amplification. (C) Confirmation of pGp07 clones by DNA double-digestion using the restriction enzymes NdeI and XhoI. The double-digested products show bands approximately 825 bp representing the insert (Lanes 2–9). (D) Confirmation of selected recombinants (pGp07) by PCR amplification. All analysis were carried out on 1.5% agarose gel. M is the 1 kbp DNA ladder. Single arrowhead indicates the digestion product whereas PCR products were indicated by double arrow head.

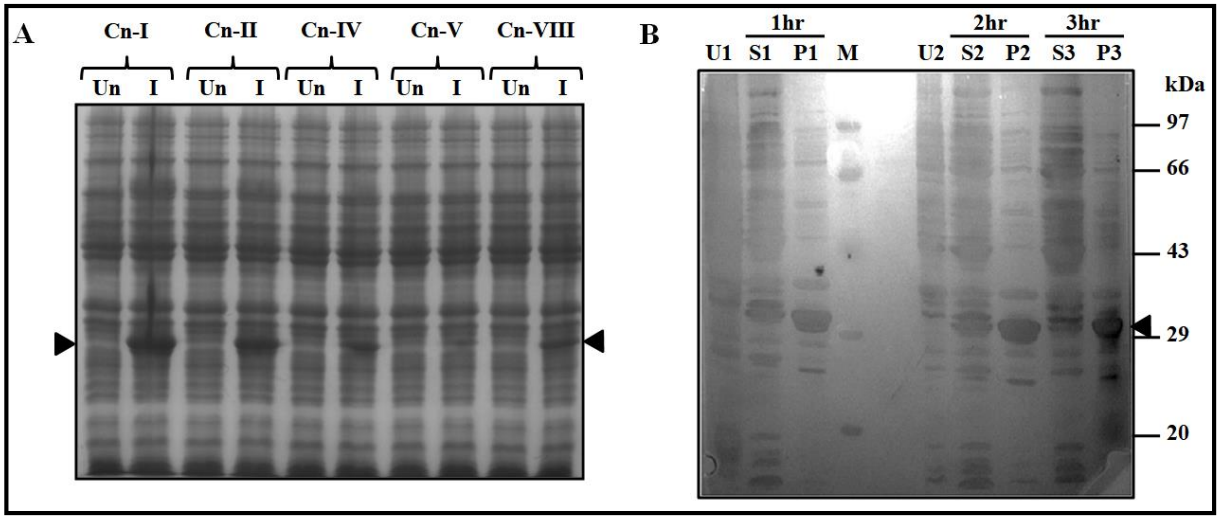

Figure 4.2. || Gp07 overexpression.
(A) Protein gel demonstrating heterologous expression of recombinant protein Gp07. The selected recombinants were over-expressed and analyzed in SDS-12% PAGE. Cn represented as construct number. (B**)** Optimization of time for overexpression and purification of Gp07. U indicates Un-induced, S indicates induced supernatant, P indicates induced pellet; Molecular masses of the marker (M) proteins (in kDa) were shown at the right side of the gel, and arrow head indicates Gp07. U1, U2 indicate uninduced control at 1hr and 2 hr respectively. S1, S2 and S3 indicate induced supernatant at 1 hr, 2hr and 3hr respectively. P1, P2 and P3 indicate induced pellet fraction at 1hr, 2hr and 3hr respectively.

4.3.3. **Large-scale protein expression and purification of His-tagged Gp07**

Cn-I was selected for large scale production of His-tagged Gp07. Large-scale protein production was performed in *E. coli* BL21(λDE3) cultured in 3 liters of LB media containing 50µg/ml kanamycin. The first stage of IMAC (Ni^{2+}-NTA) successfully enriched Gp07 from clarified cell lysates and the different fractions of washes and elutes were analyzed by SDS-12%PAGE (Figure 4.3.A). As is clear from the Figure 4.3.A, Gp07 could not be purified to homogeneity after the first IMAC and the presence of contaminating proteins are largely evident. Recombinant Gp07 obtained from the first IMAC stage was dialysed overnight at 4^oC with the modified dialysis buffer (20mM Tris pH 7.5, 500mM NaCl, 0mM Imidazole, 5% glycerol and 1mM EDTA). To remove proteins that bound non-specifically during the first IMAC stage, the dialyzed Gp07 was subjected to a second Ni^{2+} affinity chromatography step. In this step, most of non-specific proteins were found in the wash 1 fraction (W1 lane in Figure 4.3.B), and purified recombinant Gp07 was eluted from the resin with buffer containing 300 mM imidazole (E1-E9 lanes in Figure 4.3.C). A substantial amount of Gp07 was also found in the flow through fraction (F lane in Figure 4.3.B). Thus, a single band purification of the Gp07 was achieved as can be seen in the elution fraction (Figure 4.3.C). The different elution fractions were pooled together and dialyzed against dialysis buffer as per the

requirements of further experiments. The final concentration of the dialyzed recombinant protein was found to be ~3.2mg/3liters of culture estimated by Bradford protein estimation assay.

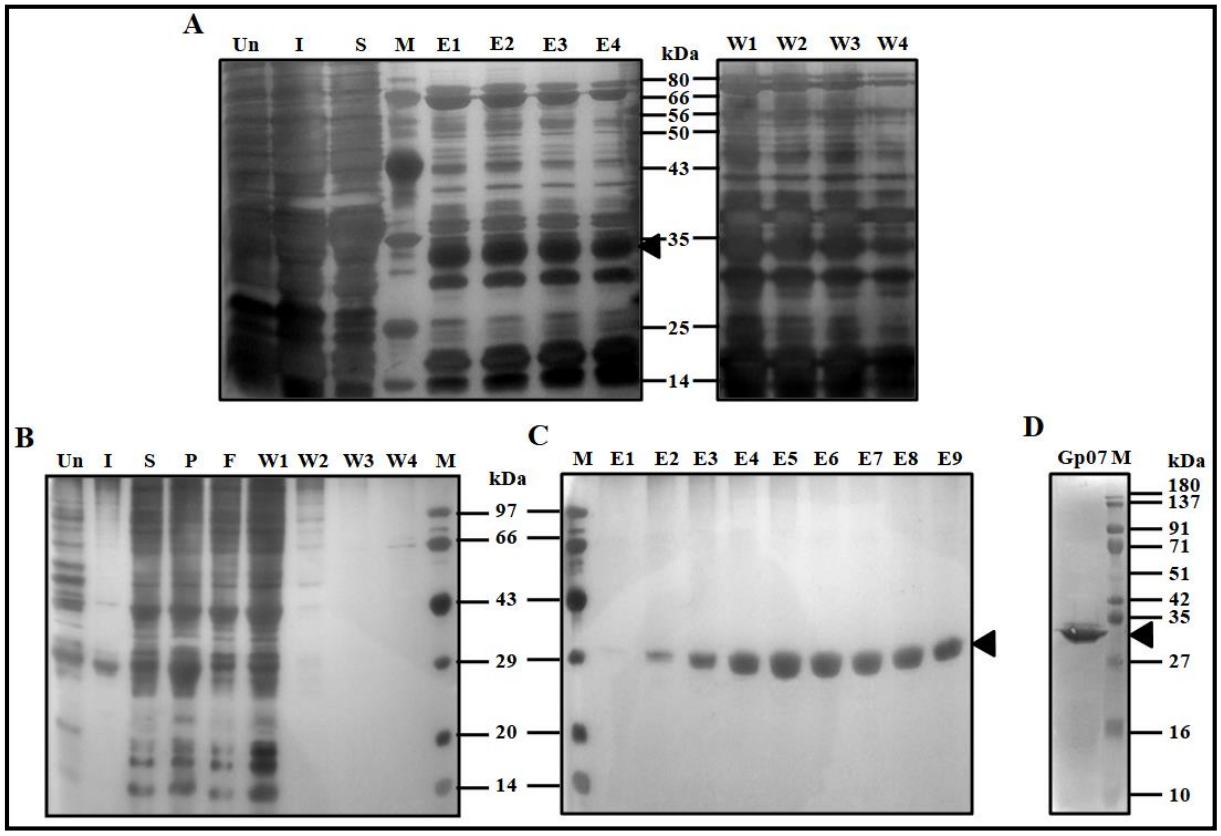

Figure 4.3. || Purification of Gp07, fractions analysed by SDS-12% PAGE.
(A) E indicate elution fractions (E1-E4), Molecular masses of the marker (M) proteins (in kDa) were shown on the right side of the gel, and arrow head indicate Gp07. (B) Purification of Gp07. All fractions were prepared from Gp07 over-expressed cell extract. Un-induced (Un), induced (I), supernatant (S), pellet (P), flow-thorough (F), washes (W1, W2, W3 and W4). (C) Elution fractions (E1-E9 after 2nd stage of IMAC purification) are loaded in different lanes. Arrowhead indicates Gp07. Molecular masses of the marker (M) proteins (in kDa) were shown. (D) Western blotting analysis of purified Gp07 protein expressed in *E. coli* BL21(λDE3) cells. Arrowhead denoted Gp07 that interacted with anti-his antibody. Molecular masses of the marker (M) proteins (in kDa) are shown at the right side.

4.3.4. **Oligomeric state of Gp07**

To gain information about the oligomeric status of Gp07 in solution, the protein (~5 µM) was subjected to analytical gel filtration chromatography. The Gp07 chromatogram primarily shows a single peak with the retention time of 24.08 min (Figure 4.4.A). In comparison with the retention time of some standard proteins, the elution volume of Gp07 indicates that its apparent molecular mass is 62.7 kDa (Figure 4.4.B). As the theoretical mass of Gp07 is 32.8 kDa, this protein mostly exists as a dimer in the aqueous solution. The data together indicates that repressor forms significant amount of dimers in solution at ~5 µM concentration. A glutaraldehyde-mediated cross-linking of Gp07 was performed as stated in the methodology. There is a formation of ~65 kDa protein species

in the presence of glutaraldehyde, suggesting the dimerization of Gp07 in the aqueous solution (Figure 4.4.C).

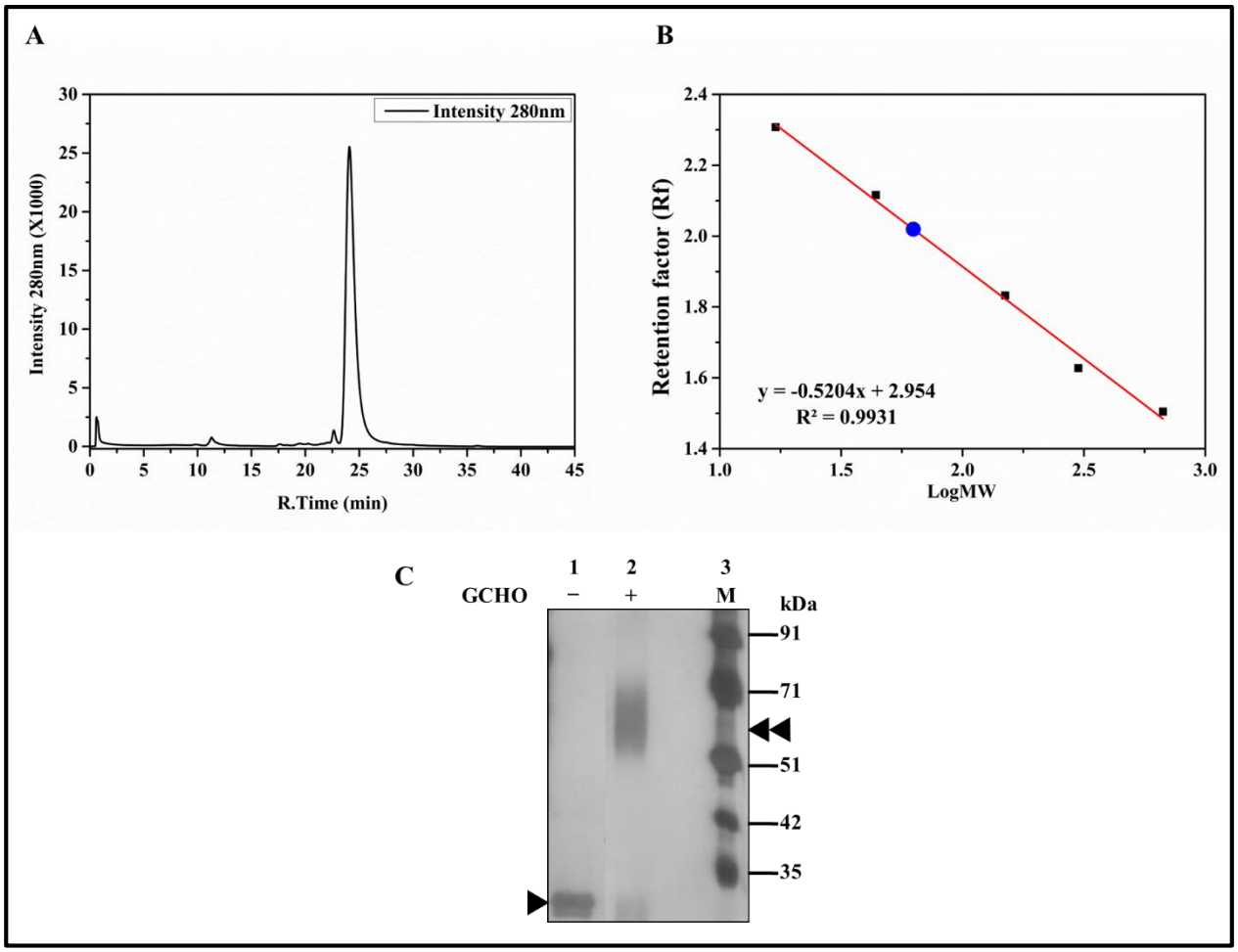

Figure 4.4. || Oligomerization analysis of Gp07 by gel filtration chromatography.
(A) Analytical size-exclusion chromatography of purified full-length Gp07 with a SEC-s2000 column (Phenomenex). (B) The Rf values of different marker proteins, determined from their elution profiles, were plotted against their respective molecular masses. The calibration curve is shown, where Log MW is the logarithm of the molecular weight and Rf is the retention factor. The blue dot shows the Log MW of Gp07. The equation resulting from this graph was used to determine the mass of Gp07. (C) Characterization of the oligomeric state of Gp07 by glutaraldehyde (GCHO) cross-linking. Gp07 dimer was determined in relation to molecular mass standards (M). Lane 1: Gp07 in absence of GCHO, lane 2: Gp07 in presence of GCHO and lane 3: molecular standard marker. Reaction mixtures were analyzed by 12% Tris-glycine SDS-PAGE. Dimer and monomer form of Gp07, indicate by single and double arrowhead respectively.

4.3.5. **Thermal denaturation of Gp07 monitored by CD spectroscopy**

The far UV CD results clearly indicates that the negative chirality of the CD signal decreases with rise in temperature (Figure 4.5.A). This occurrence is most noticeable between 35˚C and 45˚C. From 15°C to 35°C, the CD spectrum is dominated by α-helical structure while at higher temperatures (45°C-85°C) the CD spectra is dominated by β-sheet (anti-parallel) and random coil. It is well documented that rise in temperature normally disturbs the native conformation of proteins

and sponsor aggregation (Carpenter et al 1999). Thus, the aggregation of Gp07 is probably induced by the rise in temperature which is reflected in the loss in CD signal. Additionally, it is observed that at temperatures ranging from 65˚C-85˚C, the loss in CD signal reaches a plateau (Figure 4.5.B). Probably at temperatures at and above 65˚C there is a saturation in the aggregation process, leading to superimposition of the curves. The change in the CD data was best-fitted by a two-state process, and the midpoint temperature of transition (T) was 50.38 ± 1.9°C.

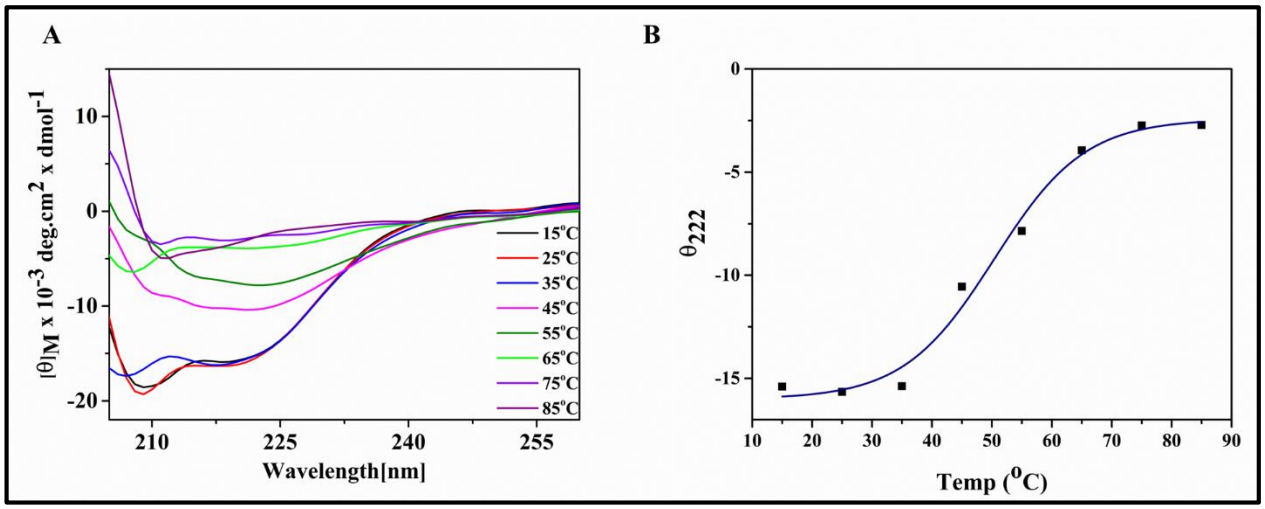

Figure 4.5. || Temperature-induced unfolding of Gp07.
(A) Far-UV CD spectra of Gp07 in the presence of indicated temperature. (B) Rising temperature leads to changes in the ellipticity at 222nm.

4.3.6. **GdnHCl mediated changes in structure of Gp07**

Intrinsic tryptophan fluorescence studies (λ_{ex} at 295 nm) were performed to study any changes in the architecture of Gp07 due to the effect of the GdnHCl. The fluorescence spectra after unfolding of Gp07 showed a shift in the emission maxima (λ_{max}) (Figure 4.6.A). This shift was more prominent with higher GdnHCl concentration. Out of four tryptophan residues in Gp07, one of the residues is present as the second amino acid from the amino terminal of the protein, and is not a part of the alpha helix or beta sheet (Figure 4.6.B). The other two tryptophans are approximately at the middle, one being at the 111th position (and is a part of the alpha helix) while the other is at the 216th position (is a part of the beta sheet). The last tryptophan residue is present at the carboxy terminal (250th position) of the protein (is a part of the beta sheet). These three tryptophan residues (at 111th, 216th and 250th position) possibly contribute to the red shift. A larger shift in λ_{max} for an unfolded protein indicates a complete exposure of a buried tryptophan upon unfolding. In case of Gp07, a large shift (from λ_{max}338 to λ_{max}354) with rising GdnHCl concentration is indicative of a complete exposure of the tryptophan residues upon unfolding of the protein.

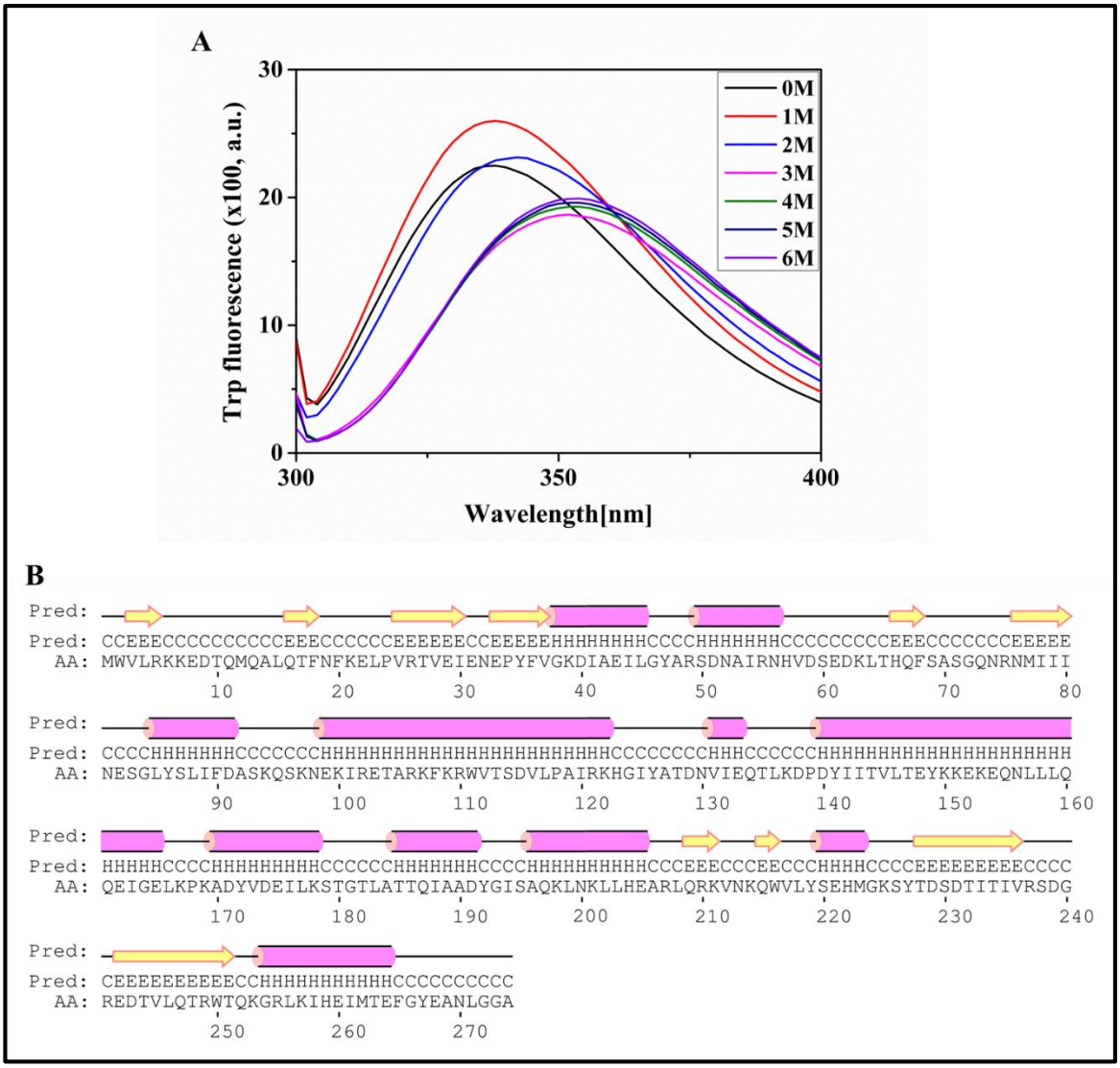

Figure 4.6. || GdnHCl-induced unfolding of Gp07.

(A) Intrinsic Trp fluorescence spectra of Gp07 at 0-6 M GdnHCl. (B) Gp07 secondary structure predicted with the PSI-PRED server. The Gp07 secondary structure showed to be organized in coil regions interrupted with short beta sheets and alpha helices. The PSI-PRED model showed eleven alpha helices (pink barrels) and ten beta sheets (yellow arrows).

4.4. **Discussion**

This work reports the cloning, overexpression and purification the Phi11 Gp07 protein for the first time, in a heterologous *E. coli* system, as a histidine tagged variant. Due to the lethal effect of Gp07 overexpression upon the host cells (Chapter 3) (Das et al 2018), it is extremely difficult to overexpress and purify the protein.

This chapter describes, the optimization of the time of induction at which the protein appears in the soluble extract. Further, a two stage IMAC purification has been employed which leads to purification of the protein to homogeneity. The oligomeric status of the protein as well as the effect of different temperatures upon the secondary structure of Gp07 has also been investigated in this chapter. The status of the tryptophan residues (buried or exposed) has also been reported. Despite the comparatively low protein yields obtained here, the present work paves the way for the further biochemical and structural characterization of Gp07.

4.5. **References**

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Chapter 5

The role of Gp07 in the developmental pathway of Phi11

5.1. **Introduction**

Temperate bacteriophages are those which can adopt two modes of development-the lytic mode and the lysogenic mode. Temperate bacteriophages can also switch between these two developmental pathways. The switching is induced by certain conditions such as exposure of the lysogen to UV or other agents, which can damage the DNA. Under such conditions, the prophage will switch to the lytic mode of development, thereby abandoning the damaged host cell - a process known as prophage induction. In λ phage, it has been reported that the switch from lysogenic mode to the lytic mode is mediated by a protein RecA. RecA is the transcriptional activator of the SOS genes and induces the cleavage of lambda CI repressor protein, thereby favouring the lytic mode of development (Little & Mount 1982; Nohmi et al 1988; Dutreix et al 1989; Kim & Little 1993).

However, 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). In most of the temperate bacteriophages harbouring antirepressor gene, prophage induction is effected by binding of the antirepressor protein to the corresponding repressor protein. Formation of the antirepressor-repressor complex leads to dissociation of the repressor protein from the cognate operator, thereby paving the way for prophage induction (Riedel et al 1993; Lemire et al 2011).

Gp07 and its deletion mutants have cloned, overexpressed and purified (Chapter 3 and 4), as histidine tagged variants. This chapter analyzes its role in the developmental pathway of Phi11. Gp07 has been annotated as antirepressor (NC_004615.1). It is also referred to as transcription control protein (TCP) (Xia & Wolz 2014). The work presented in this chapter shows that LexA and RecA play a very important role in the lytic-lysogenic switch in Phi11, as has already been reported in λ phage (Janion 2008). However, the results obtained indicate that the mechanism of action of Gp07 is different from that of the other antirepressor proteins. In case of Gp07, it has been found that the protein does not interfere with the binding of CI to its cognate operator; instead, it enhances the binding of Phi11 Cro protein to its cognate operator, thereby promoting the lytic cycle. So, far no work has been done either on the structure or function of Gp07. By using extremely purified Gp07 and Cro proteins, this chapter shows that Gp07 specifically stimulates effective binding of Cro repressor to its operator sites. It is also hypothesized that Gp07, Cro and *cI-cro* intergenic region

complex is very important for lysis of the host cells and possibly constitute a part of the genetic switch involved in the developmental pathway of Phi11.

5.2. **Materials and methods**

ATP-γ-S and PMSF were procured from Sigma-Aldrich, and oligonucleotides were purchased from Integrated DNA Technologies (Bioresource Biotech Pvt. Ltd.). IPTG, kanamycin and ampicillin were purchased from Hi-media pvt. ltd. All other chemical and molecular biological reagents were from Thermo Scientific ACS grade. All the primers were synthesised by Integrated DNA Technologies (Bioresource Biotech Pvt. Ltd.), and all the constructs were confirmed by sequencing analysis at Central Instrumentation Facilities (CIF), UDSC (New Delhi, India). The native DNA gel staining solution, SYBR green was purchased from ThermoFisher scientific.

5.2.1. **Basic molecular biological methods**

All molecular biological methods such as plasmid DNA isolation, polymerase chain reaction (PCR), restriction enzyme digestion, agarose gel electrophoresis, labelling of DNA fragment by [γ-^{32}P] ATP, DNA ligation, competent *E. coli* cell preparation, DNA transformation, estimation of protein and DNA, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), staining of polyacrylamide gel, native polyacrylamide gel electrophoresis, isolation of genomic DNA from *S. aureus* Newman, and sequencing of DNA fragments were performed as reported earlier (Sambrook & Russell 2001; Ausubel 2002). The molar concentration of recombinant proteins was estimated using the molecular mass of their relevant monomers. The equilibrium dissociation constant (K_D) was determined essentially by a procedure as reported earlier (Das & Biswas 2016).

5.2.2. **Phage, bacterial strains and growth conditions**

Phi11 has been grown in our laboratory according to the method of Lee and Iandolo (Lee & Iandolo 1988). *S. aureus* RN4220 was grown at 37 °C in trypticase soy broth (Lee & Iandolo 1986). *E. coli* BL21 (λDE3) Star and *E. coli* XL1 Blue cells were grown at 37 °C in luria broth (Sambrook et al 2001). Appropriate antibiotics (ampicillin and kanamycin) were added to the growth media as required.

5.2.3. **Plasmid construction**

Staphylococcus aureus Newman genomic DNA was extracted by standard method (Chapaval et al 2008). The *lexA* and *recA* genes were amplified from the isolated genomic DNA (GenBank: AP009351.1) by PCR using specific sets of primers (Table 5.1.). The purified PCR fragments were digested with NdeI and XhoI and cloned into pET28a to yield the recombinant plasmids pLexA and pRecA respectively (Table 5.2.).

Cloning of Phi11 *gp07* and its deletion mutants have been described (Chapter 3) (Table 3.1. and Table 3.2.). Cloning of *cI* repressor and *cro* repressor have been described previously (Das et al 2007; Das et al 2009). The *cro* gene was also cloned into the expression vector p^{TEV}Ovi/MB, using the N-*cro*-F and N-*cro*-R primers for further studies (Table 5.1. and Table 5.2.).

5.2.4. **Cloning of putative operator DNA**

The putative operator sequences were amplified using specific primers and DreamTaq DNA polymerase, in a polymerase chain reaction. The primers p-*gp07*-F and p-*gp07*-R were used for amplifying the putative operator region of *gp07*, while p-*lexA*-F and p-*lexA*-R were used for amplification of the putative operator region of *lexA*. p-*recA*-F and p-*recA*-R were used for amplification of the putative operator region of *recA* (Table 5.1.). The PCR products were finally cloned into the pGEM-T vector according to the manufacturer's instructions (Promega, USA). The resulting recombinant plasmids were named P*gp07*-T, P*lexA*-T and P*recA*-T respectively. Cloning of the *cI-cro* intergenic region (*O* DNA) has been described previously (Table 5.1.) (Das et al 2007).

5.2.5. **Over-expression and purification of the recombinant proteins**

To over-express the recombinant histidine tagged proteins, *E. coli* BL21(λDE3) Star cells were used. Recombinant CI (Das et al 2007), Cro (Das et al 2009) and Gp07 (Das & Biswas 2019) were overexpressed in *E. coli* BL21(λDE3) from plasmids pSAU1220, pSAU1259 and pGp07 and purified as previously described (Chapter 4, Section 4.2.4.) (Das et al 2007; Das et al 2009). To induce the overexpression of the histidine tagged LexA, RecA, rNTD, CI, rCro and Cro proteins (Table 5.3.), *E. coli* BL21(λDE3) cells harboring pLexA, pRecA, prNTD, pCI, pCro and pNCro were separately grown in luria bertani broth (supplemented with 50µg/ml kanamycin) at 37° C to an OD_{600} of ~0.6 (Table 5.3.). The cultures were then induced with 0.5mM IPTG and growth was continued for another 3h at 32^{o} C. *E. coli* BL21(λDE3) harbouring the recombinant plasmids were harvested (CI overexpression was carried out at 14^{o} C, for 12-14hr with 0.1mM IPTG), while the *E. coli* BL21(λDE3) cells harbouring pGp07, pΔGp07 and prCTD plasmids (Table 5.2.) were induced for 1hr at 32^{o} C for the over-expression of Gp07, ΔGp07 and rCTD. The resulting cell pellets were washed with 0.9% NaCl and stored at -80^{o} C.

Finally, the harvested cells were taken out as per the requirement of the experiments and the over-expressed recombinant proteins were purified using the Ni-NTA affinity chromatography according to manufacturer's protocol (ThermoFisher Scientific) with some modifications in lysis, wash and elution buffers as listed (Table 5.4). Finally, the eluted recombinant proteins fractions are checked

in SDS-12% PAGE (Figure 5.1) and dialysed against their specific dialysis buffers to completely remove imidazole. A gradient dialysis was employed such that the protein was stored in a buffer as per the downstream requirements (Table 5.4). Briefly, NaCl was removed in a step by step manner using dialysis buffer Buf-Di1 to reduce the NaCl concentration to 400mM and buffer Buf-Di2 was used to reduce the NaCl concentration to 200mM, whereas EDTA was removed by using the buffer Buf-Di3 (Table 5.4).

5.2.6. **Bioinformatic analysis**

S. aureus Newman (AP009351.1) and Phi11 (NC_004615.1) genome were retrieved from genomic databases of NCBI (https://www.ncbi.nlm.nih.gov/genome/) for bioinformatics analysis. EMBOSS programs (https://www.ebi.ac.uk/Tools/emboss/) were used for different bioinformatic analyses like molecular weight determination, charge, presence of different amino acid, solubility. LASAGNA aligned experimentally validated *S. aureus* LexA repressor binding boxes were collected from the CollecTF database (Cirz et al 2007; Lee & Huang 2013; Kılıç et al 2014). Alignment of promoter regions of *gp07*, *lexA* and *recA* with known regions of gram positive *lexA* binding regions were performed using Clustal Omega program 1.2.1 (Sievers et al 2011).

5.2.7. **Gel retardation assays**

To analyze the effects exerted by Gp07 on the binding of CI and Cro to *O* DNA, gel retardation assay was employed. The assay reaction consisted of various concentrations of CI, Cro or Gp07 protein which were incubated with 90ng of operator DNA, in a binding Buf-Di2 (Table 5.4.) for 20 min in ice as described previously (Das et al 2007). Further, the CI-*O* DNA or Cro-*O* DNA complex formed was incubated with Gp07 or ΔGp07 or Bro-N or KilA-C separately for 30 min to check the effect of the proteins on the stability of the complex formed. Gel shift assay were employed to check these DNA- protein interactions. The interaction of Gp07 with the 269 bp *O* DNA was also analysed by gel shift assay. The binding of LexA to a 145bp fragment (bioinformatically identified LexA box situated in this sequence, Section 5.2.6.) of p-*gp07* (446bp) was analysed (Table 5.2.). Finally, the 30 bp (this carries a 14 bp LexA binding operator is situated in the centre please refer) duplex oligonucleotide was used to check the LexA binding to *gp07* promoter. Furthermore, the binding of LexA to the operator of *lexA* (143 bp) and *recA* (186 bp) were analysed similarly (Cirz et al 2007; Das et al 2007). CI and Cro binding to the promoter of *gp07* was also analysed by using 446 bp *cro-gp07* intergenic region (which harbours the said promoter). To carry out gel shift assay, 6% and 8%

non-denaturing PAGE were used. Finally, the gel was stained by SYBR Green II (ThermoFisher Scientific) and analysed as described previously (Das & Biswas 2016).

Table 5.1. Primers used in this study to clone our desired genes and promoter regions.

Name	Purpose	Sequence(5′-3′)[#]	Reference
PCR5		GAATTC**AACATGCAATGGAATTTAATAAAG**	Das et al 2009
	Forward primer for synthesis of *cro*		
PCR4		AAGCTT**GCATTTGTTATGTCTCC**	
	Reverse primer for synthesis of *cro*		
PCI5		GAATTC**AAAATGGATAAAAAAGAATTAG**	Das et al 2007
	Forward primer for synthesis of *cI*		
PCI4		AAGCTT**AGGCGCTATTAATCAC**	
	Reverse primer for synthesis of *cI*		
C-*gp07*-F		CCATGG**GAATGTGGGTGTTGAGGAAAAAGGAGG**	Chapter 3
	Forward primer for synthesis of Gp07 and rNTD		
C-*gp07*-R		CTCGAG**CGCTCCCCCTAAATTAGCTTCATAAC**	
	Reverse primer for synthesis of *gp07* and Δ*gp07*		
C-*Bro*-R		CTCGAG**GTCTGGATCTTTTAATGTTTGTTCAATTACATTG**	
	Reverse primer for the synthesis of rNTD		
N-*KilAC*-F		CATATG**TACATCATTACAGTGTTGACTGAGTATAAGAAAG**	
	Forward primer for the synthesis of rCTD		
N-*gp07*-R		CTCGAG**TTACGCTCCCCCTAAATTAGCTTCATAACC**	
	Reverse primer for the synthesis of rCTD		
C-Δ*gp07*-F		CCATGG**AAATGCAAGCATTACAAACATTTAATTTTAAAGAGC**	
	Forward primer for synthesis of Δ*gp07*		
N-*lexA*-F		CAT**ATG**AGAGAATTAACAAAACGACAAAGC	This study
	Forward primer for synthesis of *lexA*		
N-*lexA*-R		CTCGAG**TTACATTTCGCGGTACAAACCAATTAC**	
	Reverse primer for synthesis of *lexA*		
N-*recA*-F		***CAT^{A}TG**GATAACGATCGTCAAAAAGC	
	Forward primer for synthesis of *recA*		
N-*recA*-R		CTCGAG**CTATTCTTCGTCAAATAATGACTTTGG**	
	Reverse primer for synthesis of *recA*		
N-*cro*-F		CAT**ATG**CAATGGAATTTAATAAAGTTGAG	
	Forward primer for synthesis of *cro*		
N-*cro*-R		CTCGAG**TTAAGATGTTTGTTTGCGTTTCG**	
	Reverse primer for synthesis of *cro*		
pHC1		GGATCCTAAATCTTCTTGAGTAC	Das et al 2007
	Synthesis of *O* DNA		
pHC2		GAATTCTTGGTTCTATAGTATCTG	
	Synthesis of *O* DNA		
p-*gp07*-F		**AGGAGACATAACAAATGCAAGACC**	This study
	Synthesis of *gp07* promoter		
p-*gp07*-R		**TCAGCAGACGGTTATCGCAATG**	
	Synthesis of *gp07* promoter and 192 bp region of *gp07* promoter		
p-192*gp07*		**AACGGTGTTGAAAGCGATAACAAAG**	
	Synthesis of 192 bp region of *gp07* promoter		
p-*gp07*-F′		**AAATTGAAGCTTATGATAAATCACTTAAAG**	
	Synthesis of 145bp region of *gp07* promoter		
p-*gp07*-R′		**ATAGCACTTCGTAGCTTTTTAGTAATTTC**	
	Synthesis of 145bp region of *gp07* promoter		
p-*lexA*-F		**CATTTTCACTCCTAGAACATTTGTTTGTATTAG**	
	Synthesis of *lexA* promoter		
p-*lexA*-R		**TATATAGGCACTCCCTAAATTAATTTAAATACAG**	
	Synthesis of *lexA* promoter		
p-*recA*-F		**ATTGTATTATCGATAAAAATATAAGCACG**	
	Synthesis of *recA* promoter		
p-*recA*-R		**TTTTTGACGATCGTTATCCAAAGC**	
	Synthesis of *recA* promoter		

#Restriction sites are underlined; for PCR, $\mathbf{T_m}$ is calculated for the bases in bold.
*T(TTG)→A(ATG) Transversion of the star codon for *recA* expression. Changed nucleotide written in superscript.

Table 5.2. Plasmids used in this study.

Name	Description	Purpose	Reference
p^{TEV}Ovi/MB	*kan*^{r}, T7 lac, His-tag, TEV, expression vector	Expression of His tag variant proteins with TEV cleavage site	This study
pET28a(+)	*kan*^{r}, T7 lac, His-tag, thrombin, expression vector	Expression of His tag variant proteins with thrombin cleavage site	Chapter 3
pGEM-T	*amp*^{r}, *lacZ*, cloning vector	Used for sub-cloning and promoter regions cloning	
pGp07-T	*gp07* gene cloned in pGEM-T Easy vector	Cloning of PCR amplified *gp07* gene	
pGp07	*gp07* gene cloned in pET28a(+)	Over-expression of Gp07	
prNTD-T	rNTD cloned in pGEM-T Easy vector	Cloning of PCR amplified *bro*-N gene	
prNTD	rNTD domain cloned in pET28a(+)	Over-expression of Bro-N	
prCTD-T	rCTD domain cloned in pGEM-T Easy vector	Cloning of PCR amplified *kilA*-C gene	
prCTD	rCTD domain cloned in pET28a(+)	Over-expression of KilA-C	
pΔGp07-T	Δ*gp07* cloned in pGEM-T Easy vector	Cloning of PCR amplified *gp07* gene	
pΔGp07	Δ*gp07* domain cloned in pET28a(+)	Over-expression of ΔGp07	
pLexA	*lexA* gene cloned in pET28a(+)	Over-expression of LexA	This study
pRecA	*recA* gene cloned in pET28a(+)	Over-expression of RecA	This study
pCro	*cro* gene cloned in p^{TEV}Ovi/MB	Over-expression of TEV cleavage site containing Cro	This study
pSAU1259	*cro* gene cloned in pET28a(+)	Over-expression of Cro	Das, M., et al. 2009
pSAU1220	*cI* gene cloned in pET28a(+)	Over-expression of CI	Das, M., et al. 2007
pSAU1201	*O* DNA promoter cloned	To check the binding of CI, Cro, Gp07 protein to *O* DNA promoter	
P*gp07*-T	*gp07* promoter cloned in pGEM-T Easy vector	To check the binding of CI, Cro, Gp07, LexA protein to *gp07* promoter	This study
P*lexA*-T	*lexA* promoter cloned in pGEM-T Easy vector	To check the binding of LexA protein to *lexA* promoter	This study
P*recA*-T	*recA* promoter cloned in pGEM-T Easy vector	To check the binding of LexA protein to *recA* promoter	This study

Table 5.3. Nomenclature of the *S. aureus* and Phi11 recombinant proteins in this study.

Host/Source	Protein[a]	Nomenclature[#]	Description
Phage Phi11	Cro	Cro	Cro repressor with His-tag
	Cro	rCro	Cro repressor without His-tag
	CI	CI	CI repressor
	Gp07	Gp07	Anti-repressor, as annotated in NCBI
	ΔGp07	ΔGp07	Eleven amino acids deletion mutant of Gp07
	Bro-N	rNTD	NTD of Gp07
	KilA-C	rCTD	CTD of Gp07
S. aureus	LexA	LexA	LexA repressor
	RecA	RecA	RecA Co-protease

[a]Recombinant proteins. [#]As annotated in this Chapter.

Table 5.4. Buffers used in this study.

Name[#]	Composition	Used for proteins
Buf-Ly1	20mM Tris(pH 7.5), 750mM NaCl, 10mM Imidazole, 5% Glycerol	Gp07, ΔGp07, rCTD
Buf-Ly2	20mM Tris(pH 7.5), 500mM NaCl, 10mM Imidazole, 5% Glycerol	CI, rNTD, LexA
Buf-Ly3	20mM Tris(pH 7.5), 300mM NaCl, 10mM Imidazole, 5% Glycerol	Cro, rCro, RecA
Buf-Wa1	20mM Tris(pH 7.5), 750mM NaCl, 25mM Imidazole, 5% Glycerol	Gp07, ΔGp07, rCTD
Buf-Wa2	20mM Tris(pH 7.5), 750mM NaCl, 30mM Imidazole, 5% Glycerol	Gp07, ΔGp07, rCTD
Buf-Wa3	20mM Tris(pH 7.5), 500mM NaCl, 40mM Imidazole, 5% Glycerol	Gp07, ΔGp07, rCTD
Buf-Wa4	20mM Tris(pH 7.5), 500mM NaCl, 60mM Imidazole, 5% Glycerol	CI, rLexA
Buf-Wa5	20mM Tris(pH 7.5), 300mM NaCl, 25mM Imidazole, 5% Glycerol	Cro, rCro, rNTD, RecA
Buf-El1	20mM Tris(pH 7.5), 400mM NaCl, 300mM Imidazole, 5% Glycerol	Gp07, ΔGp07, rCTD
Buf-El2	20mM Tris(pH 7.5), 200mM NaCl, 300mM Imidazole, 5% Glycerol	CI, rNTD, LexA
Buf-El3	20mM Tris(pH 7.5), 100mM NaCl, 300mM Imidazole, 5% Glycerol	Cro, rCro, RecA
Buf-Di1	20mM Tris(pH 7.5), 400mM NaCl, 5% Glycerol, 1mM EDTA	Gp07, ΔGp07, rCTD, rNTD
Buf-Di2	20mM Tris(pH 7.5), 200mM NaCl, 5% Glycerol, 1mM EDTA	Gp07, ΔGp07, rCTD, rNTD, CI, LexA, Cro, rCro, RecA
Buf-Di3	20mM Tris(pH 7.5), 200mM NaCl, 5% Glycerol	Gp07, ΔGp07, rNTD, CI, LexA
Buf-Di4	20mM Tris(pH 7.5), 50mM NaCl, 5% Glycerol	RecA
Buf-B1	20mM Tris(pH 7.5), 400mM NaCl, 5% Glycerol	Gp07, ΔGp07, rNTD
Buf-B2	20mM Tris(pH 7.5), 500mM NaCl, 5% Glycerol	Gp07, ΔGp07, rNTD

#Buf: Buffer, Ly: Lysis, Wa: Wash, El: Elution, Di: Dialysis, Bi: Binding

5.2.8. **DNase I footprinting assay**

Footprinting assay was performed by a standard method with minor modifications (Ganguly et al 2009). p-*gp07*-R and p-192*gp07* primers were used to amplify the 192 bp region of *gp07* promoter to perform the DNase I footprinting. Briefly, the 192 bp region was amplified using the ^{32}P labelled primer (p-*gp07*-R primer) (~5000 cpm) and incubated in the presence or absence of CI on ice for 20min. The reaction mixtures were made 1 mM with MgCl2 and treated with 0.15 units of DNase I for 5 min at room temperature. After terminating the reaction with a stop solution (10 mM EDTA, pH 8.0), the digested DNA fragments were purified by phenol:chloroform extraction (1:1) followed by ethanol precipitation. Finally, the purified reaction mixtures were resuspended in sequencing gel loading buffer (98% deionized formamide, 10 mM EDTA (pH 8.0) and 0.025% bromophenol blue). The adenine plus guanine (A + G) and guanine (G) sequencing ladders were produced from the same labelled DNA fragments as described (Belikov & Wieslander 1995; Ganguly et al 2009). Briefly, A + G sequencing ladder was prepared by mixing 10µl of formic acid with 5µl of labeled DNA (p-*gp07*) and incubated at 37^{o} C for 30 minutes. After incubation, a speed-vac was employed to dry the sample. 100µl of autoclaved sterile water was added twice and the sample was dried in speed-vac. 90µl of sterile autoclaved water was then added to the dried sample followed by addition of 10µl of piperidine. The sample so prepared was boiled for 15 minutes. Again, 100µl of autoclaved sterile water was added twice to the boiled sample followed by drying in speed-vac. Similarly, G sequencing ladder was prepared by mixing 5µl of labelled DNA(p-*gp07*) with 75µl of dialysis buffer (Buf-Di3, Table 5.4.) and 20µl of 1% DMS; this mixture was incubated for 2 minutes in room temperature following which 25µl of DMS stop solution was added. Immediately, 100% chilled ethanol was added to the mixture and centrifuged for 30 minutes at 4^{o} C at 12,000 rpm. Centrifugation was repeated with 75% chilled ethanol. Finally, ethanol was discarded and speed-vac was applied to dry the sample. 90µl of sterile autoclaved water was added to the dried sample followed by addition of 10µl of piperidine. This mixture was boiled for 15 minutes. 100µl of autoclaved, sterile water was added twice to the boiled sample. The sample was dried in speed-vac. Finally, the DNA fragments, separated by the urea-8% PAGE, were analyzed.

5.2.9. **Pull-down assay**

To check the interaction of Gp07 or ΔGp07 or rNTD with untagged Cro, pull-down assays were performed with minor modification (Nguyen & Goodrich 2006). Briefly, the purified Gp07, ΔGp07 and Bro-N recombinant proteins were treated using SAN (Salt Active Nuclease, Sigma) at a final concentration of 0.1 U/µl at 10° C, to remove any DNA or RNA contamination. Further, the treated Gp07, ΔGp07 and Bro-N bait proteins were incubated separately with HisPur™ Ni-NTA resin

beads (Thermo Scientific) in Buf-B1 (Table 5.4.) in a total volume of 50 µl in ice for 2 h. The bound proteins slurry was washed twice with Buf-B2 (Table 5.4.) containing 500 mM NaCl and equilibrated with the binding Buf-Di3. The tag less purified Cro protein was added to the slurry and incubated in ice for another 30 min. The agarose beads were vigorously washed four times (each time with ten beads volume) with the binding Buf-Di3 (Table 5.4.), and the bound proteins were analyzed by SDS-15% PAGE as described (Nguyen & Goodrich 2006).

5.2.10. **RecA-mediated cleavage of LexA and CI**

RecA-mediated cleavage reactions (100 µl) were performed at 37° C. The premix preparation was prepared at 37° C, which included 2 µM recombinant RecA coprotease, 6 µM of the indicated single-stranded oligonucleotide (N-*gp07*-R) (Table 5.1.), 1 mM ATP-γ-S, 50 mM Tris pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$ and pre-incubated for 30 minutes at 37° C. The cleavage reaction was initiated by addition of 10 µM of purified CI or LexA repressor. 14µl of sample was removed from the reaction mixture at desired time points, immediately mixed with SDS-PAGE sample buffer and heated for 5 min to stop the reaction. Reactions were analyzed on SDS-15% PAGE and stained with coomassie brilliant blue. Fraction of uncleaved protein (CI or LexA) at each time point was calculated by subtracting the protein band intensity at each point from the band intensity of the protein band at zero-time point and finally the percentage of uncleaved protein was calculated. The net intensities of the undigested products were measured by ImageJ (Abràmoff et al 2004).

5.3. **Results**

5.3.1. **Purification of the constructed recombinant proteins (from Phi11 and *S. aureus)***

The His-tagged recombinant proteins were purified as described in materials and methods (Section: 5.2.5.). All the Ni-NTA purified recombinant proteins were analyzed in SDS-12% PAGE to check the purity (Figure 5.1.).

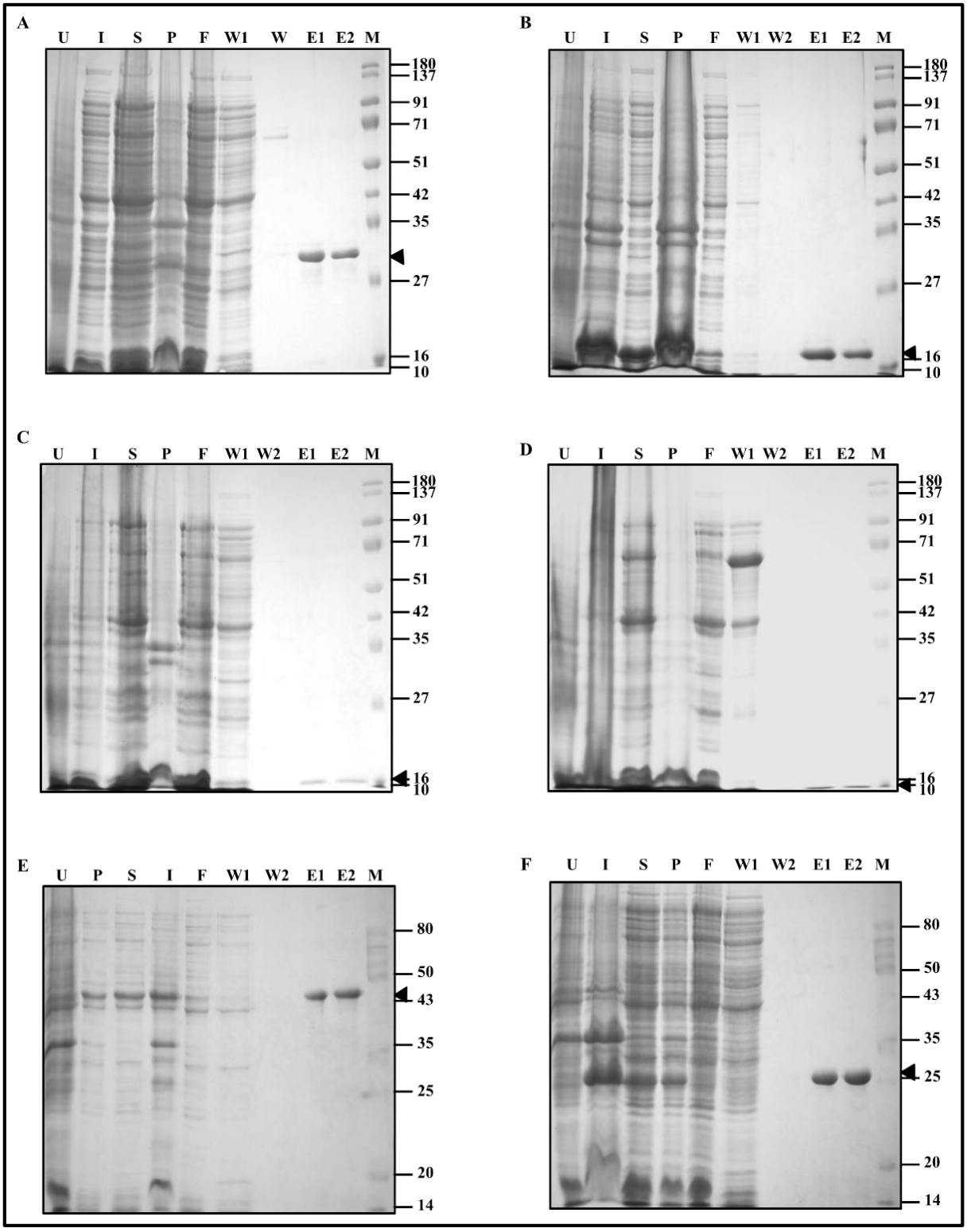

Figure 5.1. || Purification of the constructed recombinant proteins (from Phi11 and *S. aureus*). The purification profile of different histidine-tagged proteins prepared from different cell extracts (See materials and methods for details, Section 5.2.5.), were analyzed by SDS-12% PAGE. Panel A: purification profile of ΔGp07; Panel B: purification profile of rNTD; Panel C: purification profile of rCTD; Panel D: purification profile of rCro; Panel E: purification profile of RecA and Panel F: purification profile of LexA. Lanes U, I, S, P, F, W1, W2 and E1/E2, indicates un-induced, induced, supernatant, pellet, flow-thorough, wash-1, wash-2 and elution fractions, respectively. Arrowheads indicate the eluted protein. Molecular masses of the marker proteins (in kDa) are shown at the right side of each gel.

5.3.2. **Gp07 has no effect upon the binding of CI to its cognate operator**

The *cI* gene (encoding lysogenic repressor CI) and *cro* gene (encoding lytic repressor Cro) of Phi11 are adjacent genes but oriented divergently (Iandolo et al 2002). The *cI-cro* intergenic region harbors the divergent promoters for both *cI* and *cro* genes. A 269 bp *cI-cro* intergenic region is referred to as *O* DNA (Das et al 2007). *O* DNA when digested with HincII, resulted in the production of a large fragment designated as O_R and a smaller fragment O_L (Das et al 2007). This *O* DNA has three 15bp inverted repeats with partial two-fold symmetry which were designated as *O*1, *O*2 and *O*3 (Das et al 2009). *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* (Ganguly et al 2009).

CI repressor has two binding sites, *O*1 and *O*2 in *cI-cro* intergenic region (*O* DNA), hence repression by CI can move the developmental pathway of Phi11 towards the lysogenic pathway (Ganguly et al 2009). Gp07 (designated as antirepressor in NCBI; NC_004615.1) is situated downstream of *cro* gene. Upon incubation of CI-*O* DNA complex with various concentration of Gp07, there was no change in the affinity of CI for *O* DNA as is evident from our results (Figure 5.2.).

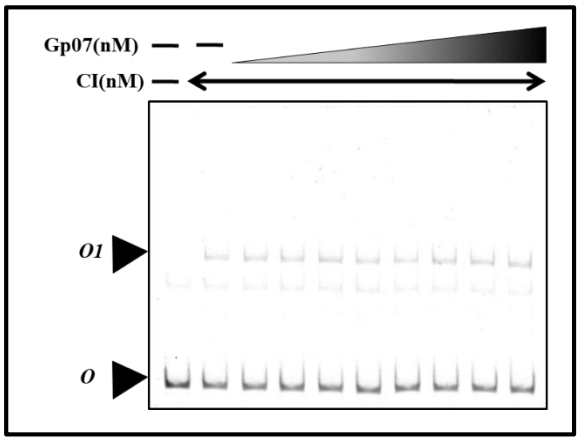

Figure 5.2. || Analysis of the effect of Gp07 upon the binding of CI to *O* DNA. 6% non-denaturing PAGE showing the interaction between operator DNA(*O*), Gp07 and CI. Here a fixed concentration of CI is allowed to bind to *cI-cro* intergenic region (*O* DNA), in presence of increasing concentrations of Gp07. Arrowheads represents the *O* DNA-CI complex(*O*1) and free *O* DNA(*O*). The concentration of Gp07 ranges from 0.5 μM - 1.5 μM. Negative symbol indicates the absence of Gp07 and CI.

5.3.3. **Gp07 has no binding site in *O* DNA**

Gp07 is a novel two domain protein (Das, A. & Biswas, M., 2019). It consists of an amino-terminal Bro-N domain (NTD) and a carboxy-terminal domain designated as KilA-C (CTD). These two domains are shuffled randomly in prokaryotes, prokaryotic viruses and eukaryotes (Iyer et al 2002). The Bro-N domain has been predicted to have non-specific DNA binding ability (Zemskov et al 2000; Iyer et al 2002). Also, it has been noticed, that disruption of Bro-N domain reduces the replication efficiency in *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) (Bideshi et al 2003). The Bro-N enhances replication during the occlusion phase of replication to maintain the virulence of viruses and bacteriophages (Bideshi et al 2003). KilA-C domain is also known to possesses DNA-binding activity of the viral regulatory proteins (Iyer et al 2002). In case of Phi11, Gp07 harbours both the Bro-N as well as KilA-C domains. The DNA binding abilities of Gp07 with respect to *O* DNA has been analysed. However, surprisingly Gp07 does not possess any DNA binding property as is evident from the Gel shift assay results (Figure 5.3.).

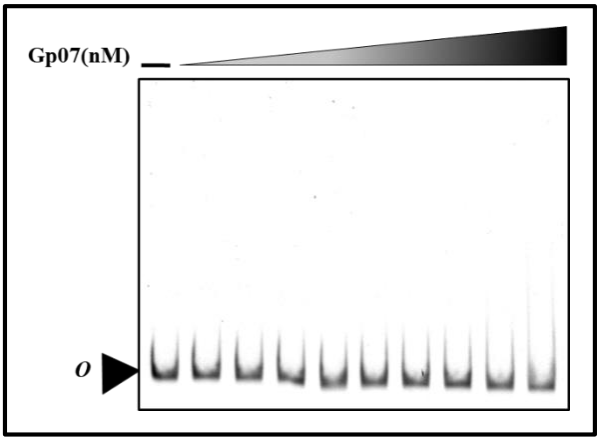

Figure 5.3. || Analysis of interaction of Gp07 with 269 bp *cI-cro* intergenic region (*O* DNA). Gp07 was incubated with 90ng operator DNA(*O*) and analysed on a 6% non-denaturing PAGE. Arrowhead represents the free *O* DNA. The concentration of Gp07 ranges from 0.1 µM - 2.5 µM. Negative symbol indicates the absence of Gp07.

5.3.4. **Gp07 greatly enhances the binding of Cro to its cognate operator**

The effect exerted by Gp07 on the binding of Cro to *O* DNA was also analysed. In this case, it was found that the presence of Gp07 greatly enhances the binding of Cro to its cognate operator. While in absence of Gp07, there is only ~49% binding of 400nM Cro to 90ng *O* DNA, in presence of increasing concentration of Gp07 (0.5µM to 1.5µM) there is almost ~97% binding of 400nM Cro to 90ng *O* DNA (Figure 5.4. and Figure 5.6.D).

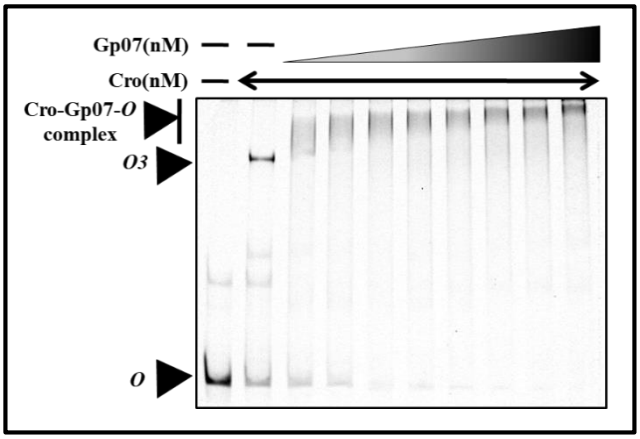

Figure 5.4. || Analysis of Cro and *O* DNA binding enhancement by Gp07. 6% non-denaturing PAGE showing the interaction between 90ng operator DNA(*O*) and Phi11 Cro (400nM) in presence of increasing concentrations of Gp07. The concentration of Gp07 ranges from 0.5 µM - 1.5 µM. Arrowheads with bar represent the super shifted complexes, Cro-Gp07-*O* DNA. Arrowheads *O3* represents the Cro-*O* DNA complex. Arrowheads *O* represents the free *O* DNA. Negative symbol indicates the absence of Gp07. The gel was scanned using ImageJ and % of *O* DNA bound were calculated (Figure 5.6.D).

5.3.5. **Interaction of CI and Cro with the putative operator region of Gp07**

The intergenic region between *cro* and *gp07* (446bp; designated as p-*gp07*) possibly harbors the promoter of *gp07*. To check, if *gp07* is under any transcriptional regulation by CI and Cro, the binding of CI and Cro (separately) to p-*gp07* was analysed. The enzyme mobility shift assay (EMSA) results indicate that, CI has a binding site in p-*gp07* (Figure 5.5.B.). CI binds to p-*gp07* with K_D 0.28 µM (Figure 5.5.C.). Further to identify the exact CI binding region in p-*gp07*, have a DNase I footprinting assay was also performed (Figure 5.5.D). The footprinting results clearly indicate that a 15 bp region in p-*gp07* is protected by CI from DNase I digestion (Figure 5.5.E). This 15 bp CI binding region has been designated as *P*2. It was further confirmed that CI binds to *P*2 by gel shift assay with a 30 bp oligonucleotide, harbouring *P*2 of *gp07* promoter (Figure 5.5.F). Cro on the other hand, has no binding site on p-*gp07*, and hence possibly does not regulate Gp07 (Figure 5.5.A).

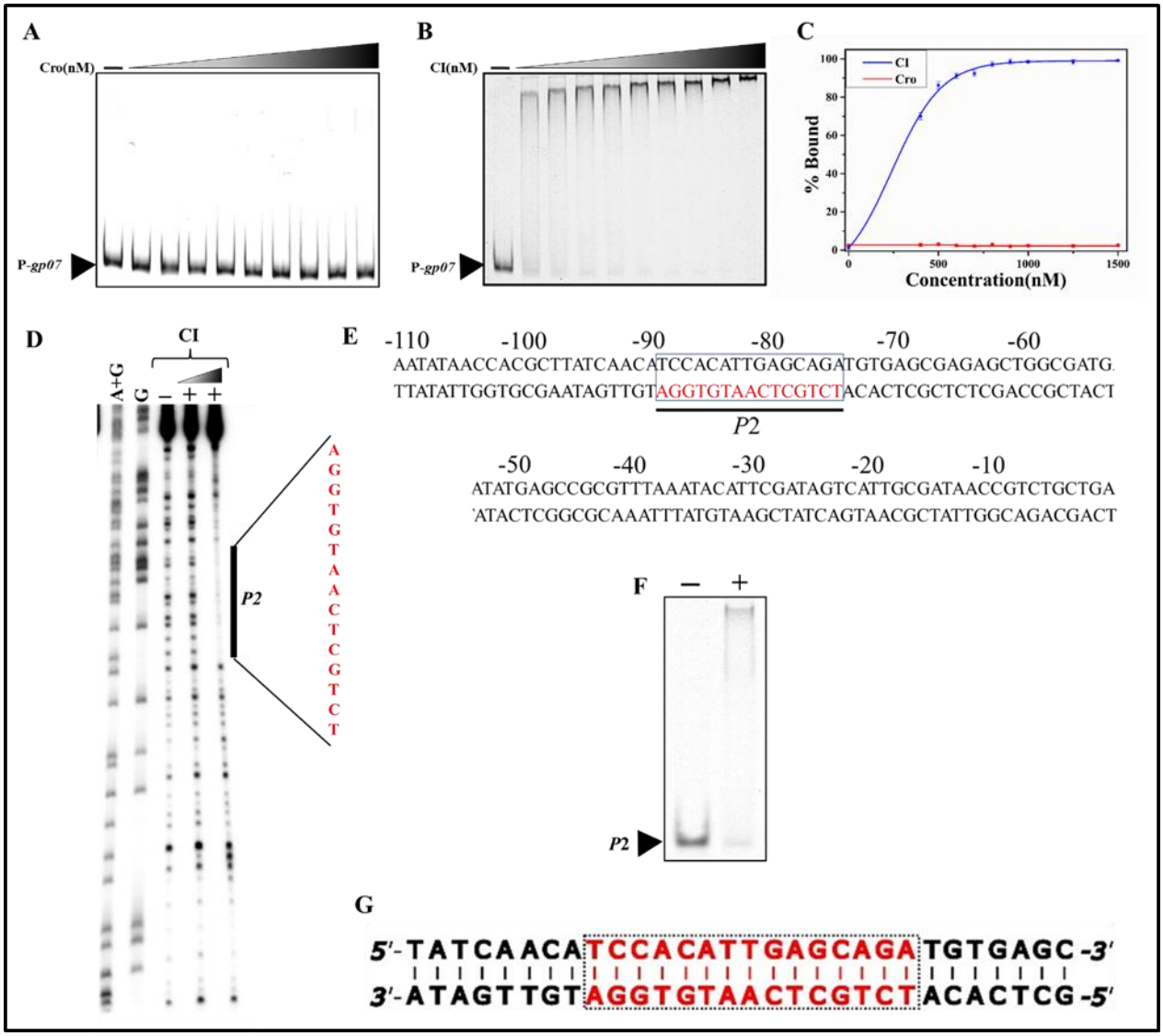

Figure 5.5. || CI and Cro repressors binding to p-*gp07*. A 6% non-denaturing PAGE showing the interaction between 90ng of p-*gp07* and increasing concentrations of Cro (A) or CI (B). Arrowheads represent the free p-*gp07*. The concentration of Cro and CI ranges from 0.4 µM - 1.5 µM. Negative symbol indicates the absence of Cro and CI. (C) The gels were analyzed using ImageJ and graphically represented to calculate K_D. (D) Footprinting assay. Autoradiogram of DNase I footprinting assays produced using the labelled lower strand of *P*2 in presence of CI (E). The labelled DNAs were incubated with 250 and 1000 nM CI (+) or without (−) CI prior to DNase I digestion as described (See materials and methods for details, Section 5.2.8.). The resulting DNA fragments were identified using the A+G and G markers. The location of the 15 bp *P*2 region is represented by a solid bar (E). (F) Repression of *gp07* by binding of CI repressor at *P*2 box. 8% non-denaturing PAGE showing the interaction between 60ng of 30 bp duplex oligonucleotides (harbouring *P*2) with 500nM of CI. Arrowhead *P*2 represent the unbound oligonucleotides. Negative symbol indicates the absence and positive symbol indicates presence of CI. (G) Sequence of the 30 bp duplex oligonucleotide harbouring the CI binding region (*P*2) which has been used for gel shift assay (Figure 5.5.F) to verify the predicted 15bp (*P*2) consensus box (red) in the centre of 30bp region (predicted by DNase I footprinting).

5.3.6. **Effect of NTD, CTD and ΔGp07 on the binding of Cro to *O* DNA**

Based on our previous bioinformatic analysis, ΔGp07 has been constructed where 11 amino acids were deleted from amino terminal of Gp07. Gel shift assay using Cro and *O* DNA in presence of ΔGp07, also showed enhancement in the affinity of Cro for *O* DNA (similar to that exhibited in presence of Gp07 (Figure 5.4.); thus ΔGp07 also enhances the DNA-binding activity of Cro repressor (Figure 5.6.B). This indicates that the extra eleven amino acids at the amino terminal of Gp07 has possibly no role in the binding of Cro to *O* DNA.

The enhancement in the affinity of Cro for *O* DNA was further analysed in the presence of the individual domains of Gp07, that is either NTD (Bro-N) or CTD (KilA-C) (Figure 5.6.A and Figure 5.6.C). Interestingly, it has been observed that the binding of Cro to *O* DNA is enhanced only in presence of rNTD, rCTD has not effect on the binding of Cro to *O* DNA (Figure 5.6.C and Figure 5.6.D).

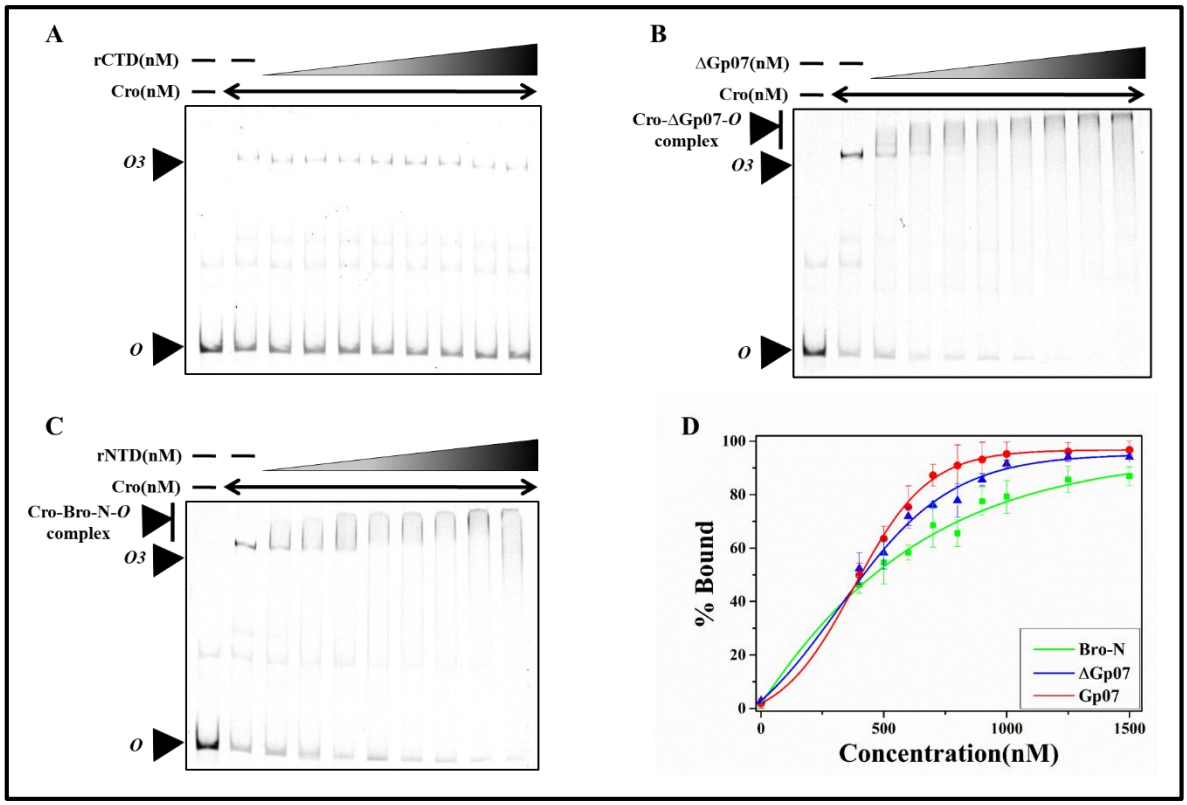

Figure 5.6. || Analysis of the interaction between Cro and *O* DNA in presence of rCTD (KilA-C), ΔGp07 and rNTD (Bro-N). 6% non-denaturing PAGE showing the interaction between 90ng operator DNA(*O*) and Phi11 Cro (400nM) in presence of increasing concentrations of rCTD (A) or ΔGp07 (B) or rNTD (C). The concentration of rCTD, ΔGp07 and rNTD ranges from 0.5 μM - 1.5 μM. Arrowheads with bar represent the super shifted complexes, Cro-ΔGp07-*O* DNA and Cro-Bro-N-*O* DNA respectively. Arrowheads *O* represents the free *O* DNA. Negative symbol indicates the absence of rCTD, ΔGp07, rNTD and Cro. (D) Graphical representation of the percentage of *O* DNA bound.

5.3.7. **Interaction of LexA with the putative operator of Gp07**

LexA, the master repressor, regulates many genes which are related or unrelated to cell growth (Fornelos et al 2016). Besides the preliminary function of this repressor, it has been reported to play a crucial role in lytic-lysogenic development in many phages (Fornelos et al 2016). For studying the role of LexA in the developmental pathway of Phi11, *S. aureus*, LexA has been purified. Initially, bioinformatic analysis was carried out to identify the possible binding region of LexA, in *gp07* promoter region (Figure 5.7. and Table 5.5.). LexA was incubated with the predicted binding region of p-*gp07* with (a 30 bp duplex DNA carrying a 14 bp LexA box situated in its center, as has been bioinformatically identified and described in materials and methods). The results indicate that the LexA binding region is indeed situated in p-*gp07* (Figure 5.8.A and Figure 5.8.B) and is designated as *L*1. Also, LexA was incubated with the promoters of *recA* and *lexA*, which are already predicted to harbour LexA binding regions (Figure 5.7.) (Table 5.5.) (Cirz et al 2007). LexA showed different binding affinities towards the promoter of *recA* (186 bp), *lexA* (143 bp) and *gp07* (145 bp) with K_D values of 340.8 nM, 387.4 nM and 267.3 nM respectively (Figure 5.9.). This clearly indicate that LexA inhibits the promoter of Phi11 *gp07* at a very low concentration, whereas it binds to the *recA* promoter at a comparatively higher concentration. The binding of LexA to its own promoter occurs at a very high concentration. From this data it can be concludde that LexA functions to inhibit the synthesis of *gp07* at a very low concentration; once *gp07* synthesis is repressed, LexA inhibits the synthesis of *recA*, thereby paving the way for lysogenic mode of development. Finally, it auto-regulates its own production.

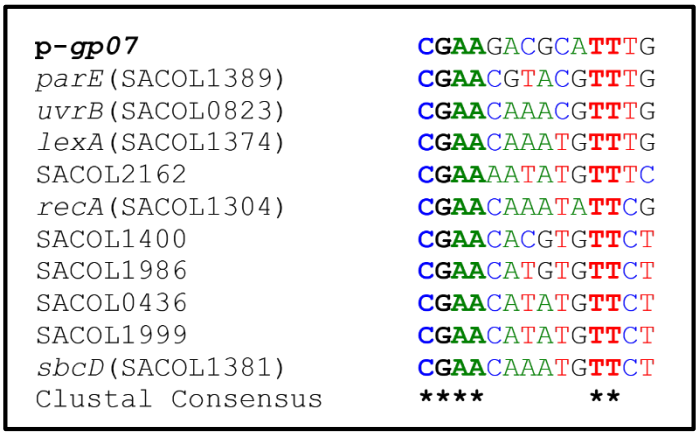

Figure 5.7. || Sequence analysis of 14bp LexA binding box. *gp07* promoter aligned with known LexA binding boxes, collected from CollecTF using ClustalO (See Materials and Methods for details). The colouring reflects the conservation profile at clustal consensus of nucleotides. '*' identical residues. Sequence of phage Phi11 LexA binding consensus (CGAAGACGCATTTG), sequence name is in bold front.

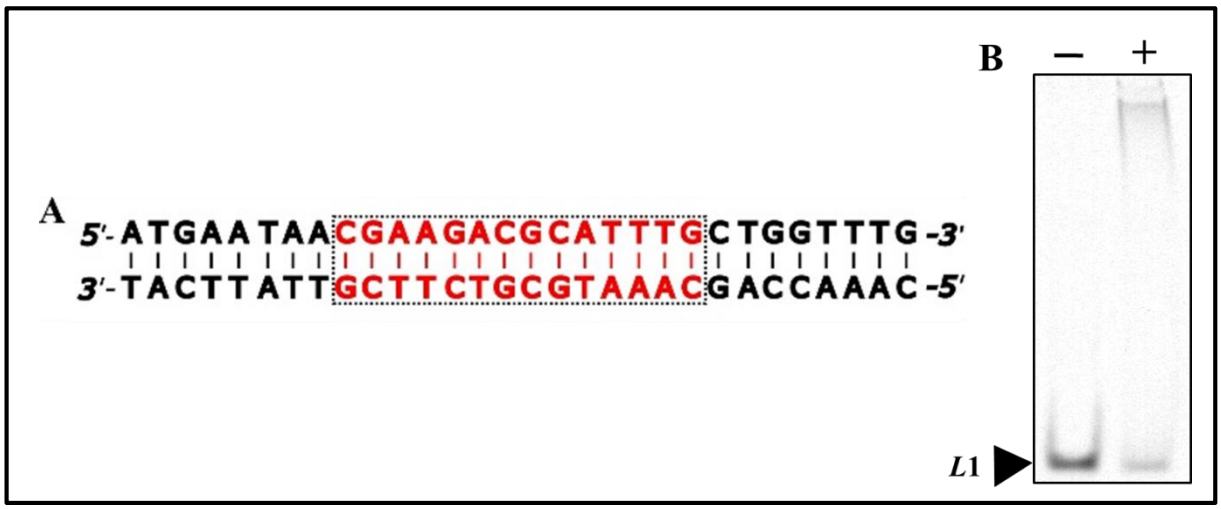

Figure 5.8. || Repression of *gp07* by LexA repressor. A 30 bp duplex oligonucleotides harbouring the LexA binding box (A), has been used for gel shift assay (B) to validate the *in-silico* predicted 14bp (*L*1) consensus box (red) in the center of 30bp region. (B) 8% non-denaturing PAGE showing the interaction between 60ng of 30 bp duplex oligonucleotides (*L*1) with 500nM of LexA. Arrowhead *L*1 represent the free oligonucleotides. Negative symbol indicates the absence and positive symbol indicates presence of LexA.

Table 5.5. *In-silico* search identified putative LexA-binding sites from the *S. aureus* and phage Phi11 genome.

Gene	Operator site(LexA box)	Strand[a]	d^{TSS}	Position
lexA (*S. aureus*)				
	CGAACAAATGTTTG	-	-71	1375709
recA (*S. aureus*)				
	CGAACAAATATTCG	+	-100	1316080
gp07 (Phage Phi11)				
	CGAAGACGCATTTG	+	-293	4135

"a" Positive and negative symbols denote the strand on which sites were detected with respect to the leading strand in the annotated genome. The d^{TSS} columns indicate the distance from the predicted translation start site (TLS). The absolute genome position of the site is also given.

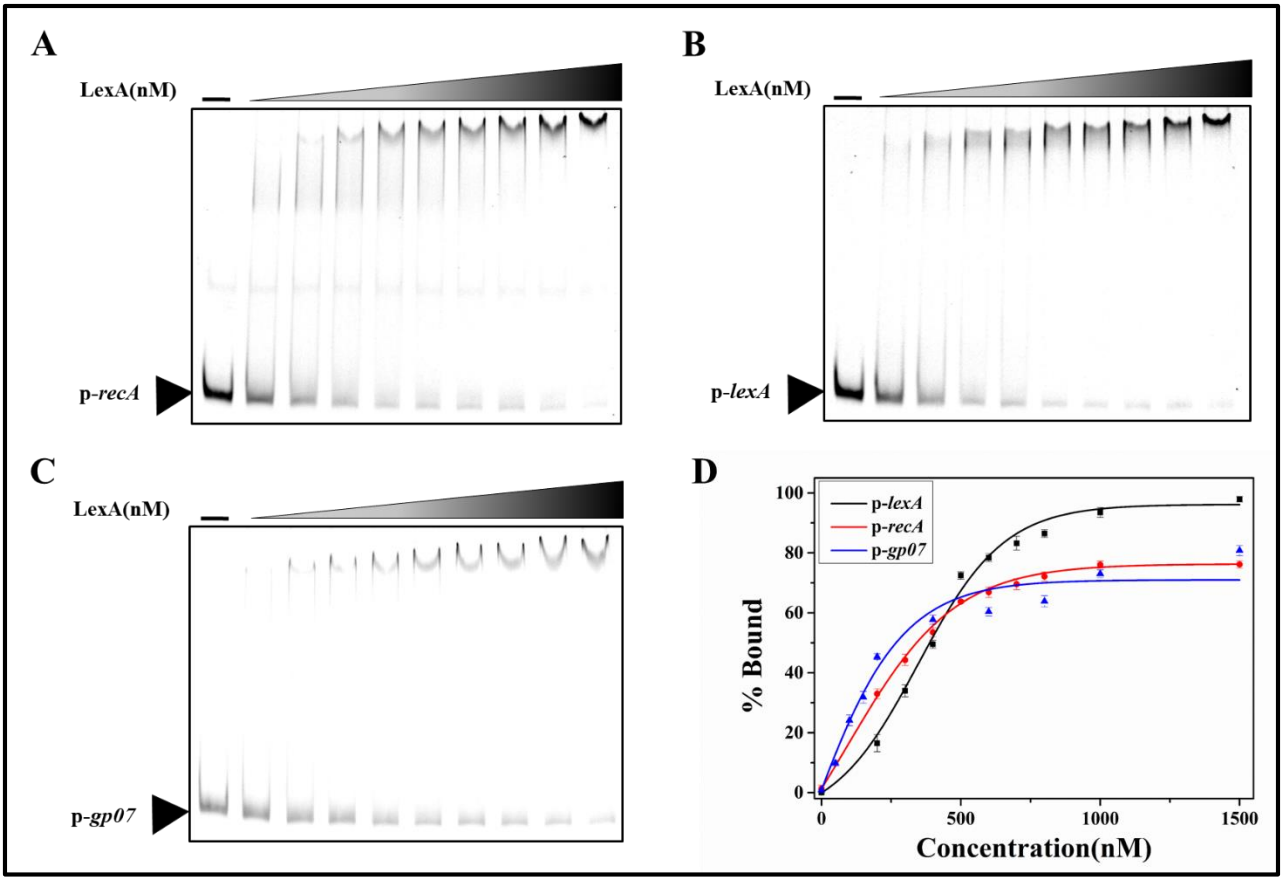

Figure 5.9. || LexA mediated repression of *recA*, *lexA* and *gp07* expression. 6% non-denaturing PAGE showing the interaction between *S. aureus* LexA and 90ng putative promoter DNA of (A) *recA*(p-*recA*), (B) *lexA*(p-*lexA*) and (C) *gp07*(p-*gp07*). Arrowheads represents the free promoter DNA. Negative symbol indicates the absence of LexA. (D) The gels were scanned using ImageJ and % of DNA bound were calculated.

5.3.8. **Cleavage of Phi11 CI and host LexA with host RecA**

The SOS response is very intimately involved in a bacteriophage's lytic lysogenic switch (Fornelos et al 2016). Repressor LexA and co-protease RecA play a crucial role in this respect (Fornelos et al 2016). Many bateriophages produce non-cleavable CI repressor unlike λ phage. In such cases, the lytic-lysogenic switch is regulated by antirepressor (Shearwin et al 1998; Mardanov & Ravin 2007; Lemire et al 2011; Kim & Ryu 2013). However, in several different bacterial group such as λ, LexA was found to be cleaved by RecA at Ala-Gly bond (Mo et al 2014). As an exception, in case of *Enterobacteria* phage phi80, the CI repressor was cleaved by co-protease RecA not at the usual Ala-Gly bond, but in at the Cys-Gly bond (Eguchi et al 1988). In case of Phi11, the CI repressor is found to possess both Ala-Gly and Cys-Gly bond (Figure 5.10.). Phi11 CI undergoes cleavage by RecA as is clear from our data (Figure 5.11.A). However, the rate of cleavage is much more higher with LexA as a substrate as compared to CI (Figure 5.11.C). The N-terminal sequencing of fragments generated after cleavage of CI by RecA has been performed (The Protein Facility, Iowa

State University, USA). Interestingly, despite the presence of Cys-Gly bond, Phi11 repressor was getting cleaved at Ala-Gly bond (Figure 5.10.).

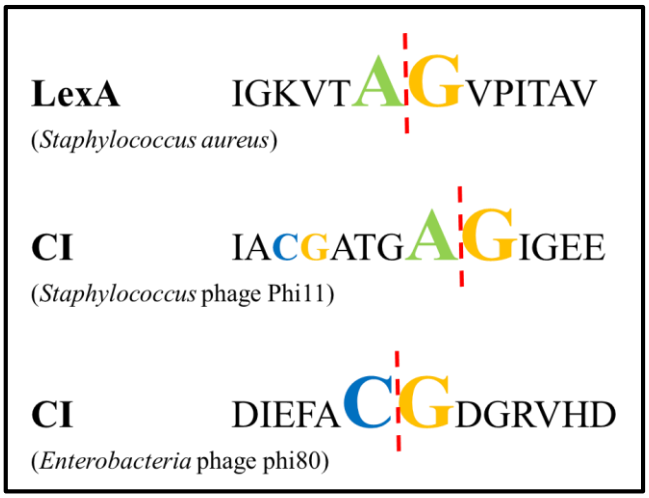

Figure 5.10. || RecA mediated cleavage sites of CI and LexA repressor. *S. aureus* LexA has Ala-Gly (AG) site, whereas CI repressor of phage Phi11 has both Cys-Gly (CG) and AG sites. Our amino terminal sequencing of CI fragments (Figure 5.11.) indicate that CI is cleaved at AG site and not at CG site like *Enterobacteria* phage phi80 (Eguchi et al 1988).

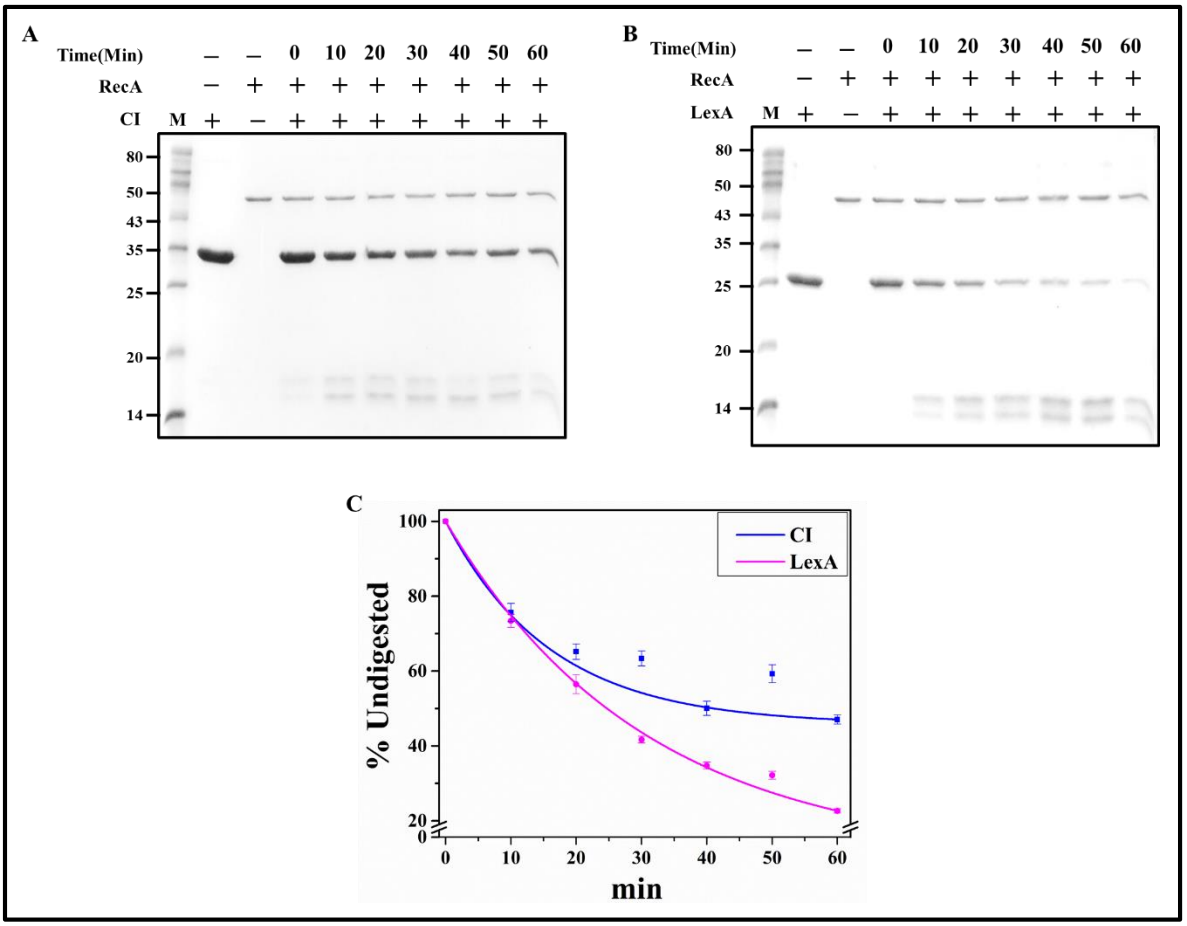

Figure 5.11. || RecA mediated cleavage of CI and LexA. RecA in presence of ssDNA (N-*gp07*-R, Table 5.1.) induce self-cleavage of LexA and CI repressor. RecA induced CI (A) and LexA (B) cleavage at different time (0 to 60 min) points at 37 ^{o}C. Left side of the gel indicates the protein marker (M) in kDa. The gels were scanned using ImageJ and percentage of Undigested protein was calculated (C).

5.3.9. **Pull-down assay to identify protein-protein interaction**

Although both the domains of Gp07 were reported as DNA-binding domains, neither of them exhibited this property. To perform the pull-down assay, His-tagged Cro has been purified by Ni-NTA chromatography and the tag was removed from using TEV enzyme to make tagless Cro protein (Section: 5.2.9.) (Figure 5.12.). Further, the protein-protein interaction was studied to understand DNA-binding enhancement effect of Cro repressor using Gp07, ΔGp07 and Bro-N. Surprisingly, our pull-down analysis did not show, any possible interaction between Cro with Gp07 or ΔGp07 or rNTD (Figure 5.13.). Hence further characterization of the enhanced Cro binding has to be explored.

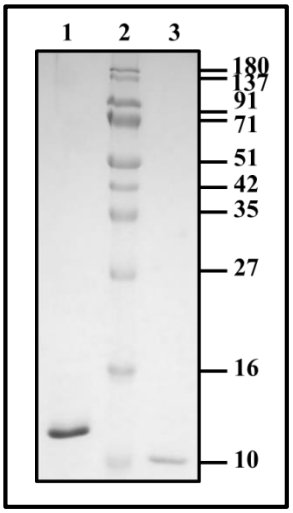

Figure 5.12. || Purification of tag-less Cro. Purification of the histidine tagged Cro (Lane 1) by Ni-NTA affinity chromatography. After TEV protease digestion, the tag-less Cro (Lane 3) was purified and the highly purified protein fractions were analysed by Tris-glycine SDS-15% PAGE. Molecular masses of the marker (M; Lane 2) proteins (in kDa) are shown on the right side of the gel.

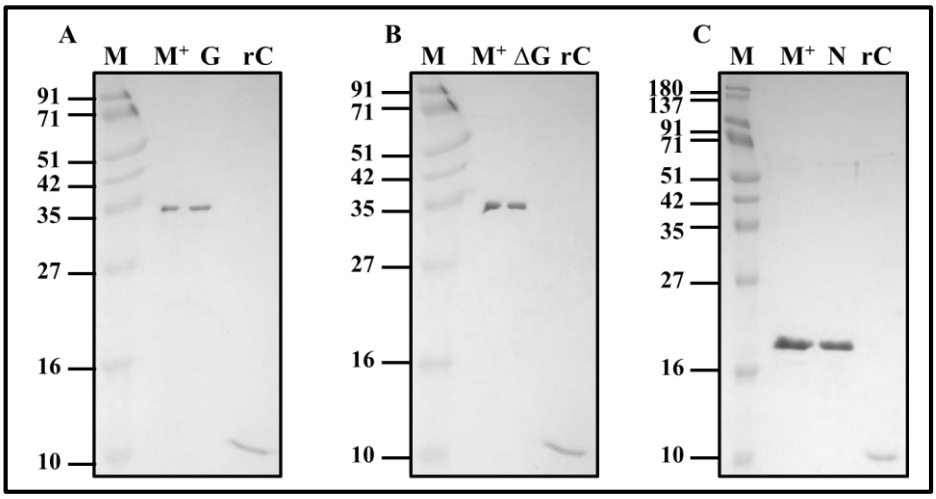

Figure 5.13. || Pull down assay. Cro with Gp07(A), ΔGp07(B) or rNTD(C). M represent the marker lane (kDa are indicated left side of the gels), M^+ represent the reaction mix (Gp07 with Cro, ΔGp07 with Cro or rNTD with Cro), G represent the Gp07, ΔG represent the ΔGp07, N represent the rNTD and rC represent the tag less Cro. Assays were analyzed in SDS 15%-PAGE.

5.4. **Discussion**

Phi11, an aureophage, is a temperate phage. However, unlike λ, Phi11 does not possess the additional regulatory genes, like *cII* and *cIII*. Instead it harbours a gene, *gp07*, which has been annotated as antirepressor. In Phi11, the *cI* and *cro* repressors are the key players in the developmental switch with additional backup from antirepressor. The regulatory lytic repressor, Cro, binds to its cognate operator at high concentrations and also forms dimer at high concentration (μM in concentration) (Das et al 2009). CI has two binding sites in *cI-cro* inter genic region, namely, *O*1 and *O*2. CI shows binds to *O*1 operator at a low concentration, followed by a higher concentration binding to *O*2 (Ganguly et al 2009). These binding interactions repress the transcription of *cro* gene. In case of phage λ, low level of Cro repressor stimulate repression of pRM and is essential for lytic growth (Lee et al 2018). In contrast, Phi11 needs higher amount of Cro to support its lytic development (Das et al 2009). Interestingly, Gp07 helps Cro to bind to its cognate operator (*O*3) at a lower concentration and thus repress the lysogenic development. Hence, Gp07 possibly functions as a co-repressor or antirepressor. Furthermore, the enhancement of the binding interaction between Cro and its cognate operator is solely the role of Bro-N domain of Gp07.

The promoters of antirepressor are repressed by CI repressor, as is the case with most phages carrying antirepressor as potent regulator for the lytic development, (Fornelos et al 2016). Also the host SOS genes are highly involved in the lytic development of bacteriophages (Fornelos et al 2016). *Enterobacteria* phage 186, *Salmonella* phage SPC32H, *Salmonella* phage Gifsy and lambdoid coliphage N15 regulate their developmental pathway through SOS response (Shearwin et al 1998; Lemire et al 2011; Ravin 2011). Moreover, In *Vibrio cholerae* filamentous phage CTXφ, the LexA and CI repressor binds simultaneously to *O*1 operator and repress the transcription from P_A promoter, where this binding enhances the expression of RstR repressors from PR promoter (Kimsey & Waldor 2009), which helps the host cell to maintain prophage condition. Similarly, the Phi11 CI and LexA repressor repress the Gp07 expression. Further, in Phi11, the SOS induction leads to the inactivation of global repressor LexA followed by auto-cleavage by RecA and lowers the LexA concentration. This causes the phage to increase its genome number and move towards the lytic development. The observations from this study indicates that the regulatory units and regulatory pattern in Phi11 is completely distinct from other known regulatory mechanisms.

5.5. **References**

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Summary of results and discussion

S. aureus is a human and animal pathogen which causes an array of health disorders, such as toxic shock syndrome (TSS), skin diseases as well as staphylococcal food borne diseases (SFD). Random use of antibiotics to curb *S. aureus* infections have led to the emergence of antibiotic resistant *S. aureus.* Hence, the effective antibiotics (e.g. vancomycin and methicillin) can no longer be used to treat *S. aureus* infections. The growing incidence of antibiotics-resistant *S. aureus* is a major concern today. It has now become critical to devise novel therapeutic strategies and phage therapy to combat infections caused by multi-drug resistant (MDR) pathogens. The work on Phi11 Gp07 revealed the inhibitory role of Gp07 on the expressing host cell. Thus Gp07 became an interesting candidate for further studies. The present work deals with in - depth structural and functional characterization of Gp07, its domains and its mutant.

Chapter 2: Changes in the functional activity of Cro repressor is mediated by various ions

- The effect of various ions upon the structure and function of Phi11 Cro repressor was investigated by substituting NaCl in the reaction buffer with different cations and anions.
- Substitution of monovalent cations (Na^+, K^+, Li^+ and NH_4^+) showed approximately similar DNA binding capacity, with no significant changes.
- On the other hand, the divalent ions (Mg^{2+} and CO_3^{2-}) reduced or inhibited the binding of Cro to its cognate operator DNA.
- On the structural front, CD spectroscopic study of Cro in the "far-UV" spectral region (200-240nm) showed maximum α-helical content with less randomness in presence of Mg^{2+} and CO_3^{2-} containing buffers, indicating that Mg^{2+} and CO_3^{2-} does not destabilize the secondary structure of the Cro protein.
- Existence of multimeric forms of Cro in presence of $MgCl_2$ buffer or Na_2CO_3 buffer was observed. However, similar concentrations of Cro repressor when incubated in NaCl buffer showed the presence of the monomeric form of the protein. Possibly, the incubation of Cro in $MgCl_2$ buffer or Na_2CO_3 buffer enhanced its multimerization thereby blocking the operator binding sites in the protein.
- Far-UV CD spectral analysis also indicated that the replacement of Na^+ with K^+, Li^+, NH_4^+ and $C_2H_3O_2^-$ ions maintain the biologically active conformation of Cro, whereas presence of $C_6H_5O_7^{3-}$ ion in the reaction buffer decreased the affinity of Cro towards its cognate operator and also unfolded the protein.

Chapter 3: Expression of Gp07 causes filamentation in *Escherichia coli*

- *gp07* gene (ORF7 of Phi11, annotated to as putative anti-repressor) has been cloned in pET28a followed by overexpression in *E. coli*.
- Some preliminary bioinformatic studies have been carried out on the *gp07* gene product, Gp07.
- Gp07 has two unique domains, namely, an amino terminal Bro-N domain and a carboxy terminal KilA-C domain.
- The sequence alignment analysis revealed extra unique eleven amino acids at amino terminal of Gp07.
- A deletion mutant of Gp07 (called ΔGp07) has been constructed by deleting the unique eleven amino acids.
- ΔGp07, Bro-N and KilA-C domains have been successfully cloned into an overexpression vector, pET28a followed by overexpression in *E. coli*.
- It has been found that over expression of Gp07, ΔGp07 and the KilA-C domain leads to filamentation in *E. coli*.
- Taken together, our results indicate that Gp07 and ΔGp07 exert a growth inhibitory effect upon *E. coli* cells.
- Interestingly it is the KilA-C domain and not the Bro-N domain which is essential for the growth inhibitory activity of Gp07.
- Moreover, the growth inhibition of *E. coli* induced by the Bro-N domain is temporal in nature.

Chapter 4: Overexpression and purification of Gp07-a lethal protein

- Purification of Phi11 Gp07 in a heterologous *E. coli* system, as a histidine tagged variant, was carried out with the optimization of the time of induction at which the protein appeared in the soluble extract.
- Interestingly, the results indicated that induction with IPTG for 1hour is a crucial point to stop the recombinant Gp07 from forming inclusion bodies.
- Further, a two stage IMAC purification has been employed which led to the purification Gp07 to homogeneity.

- The size exclusion chromatographic analysis revealed that Gp07 existed as a dimer in solution.
- Secondary structure elucidation of Gp07 by CD spectroscopy revealed that a change in temperature changes the secondary structure of the protein.
- Gp07 carries four tryptophan residues. Bioinformatic secondary structure analysis shows that one of the four tryptophan residues (the second amino acid from the amino terminal) is not a part of either the alpha helix or beta sheet. The other three tryptophan residues are constituents of the alpha helical structure (111th residue) and beta sheets (216th and 250th residues).
- Intrinsic tryptophan fluorescence study shows that the obtained red shift in presence of increasing GdnHCl concentration is possibly due to the exposure of the three tryptophan residues (at 111th, 216th and 250th residues) in the secondary structure of the protein.
- Although the employed purification strategy led to comparatively low protein yield, this has accelerated further biochemical characterization of Gp07.

Chapter 5: The role of Gp07 in the developmental pathway of Phi11

- ΔGp07 and the KilA-C domain (both of which exhibited growth inhibitory effect upon overexpression) have also been purified to homogeneity by employing the purification strategy used in case of Gp07.
- It has been experimentally found that Gp07 helps Cro to bind tightly to its cognate operator at a lower concentration and represses the lysogenic development from *O*3 operator, so the possible functional role of Gp07 could be that of a co-repressor.
- Furthermore, it has been demonstrated that the enhanced binding of Cro to its cognate operator is solely the function of the Bro-N domain.
- KilA-C does not lead to any enhancement in the binding of Cro to its cognate operator.
- Phi11 CI and the host LexA repressor has been found to repress Gp07 expression by binding to the putative promoter of Gp07 at different sites.
- Finally, as a part of the SOS induction, the host RecA protein leads to the inactivation of global repressor LexA and CI repressor by auto-cleavage at their AG sites. This reduces the concentration of LexA repressor and CI repressor and promotes the lytic pathway.
- The regulatory units and regulation pattern involved in the developmental pathway of Phi11 appears to be distinct from the regulatory mechanisms employed by other known bacteriophages.

Conclusion

S. aureus phage Phi11 is a Siphoviridae phage and when compared with the known phages, it is found to possess a different set of regulatory genes. Particularly interesting is the *gp07* gene. Its protein product, Gp07, possesses two novel domains (Bro-N and KilA-C). The work presented in this thesis suggests that this early gene (*gp07*) is crucial for the lysogenic-lytic switch in Phi11 and its mechanism of action is very different from that of other known bacteriophage antirepressors.

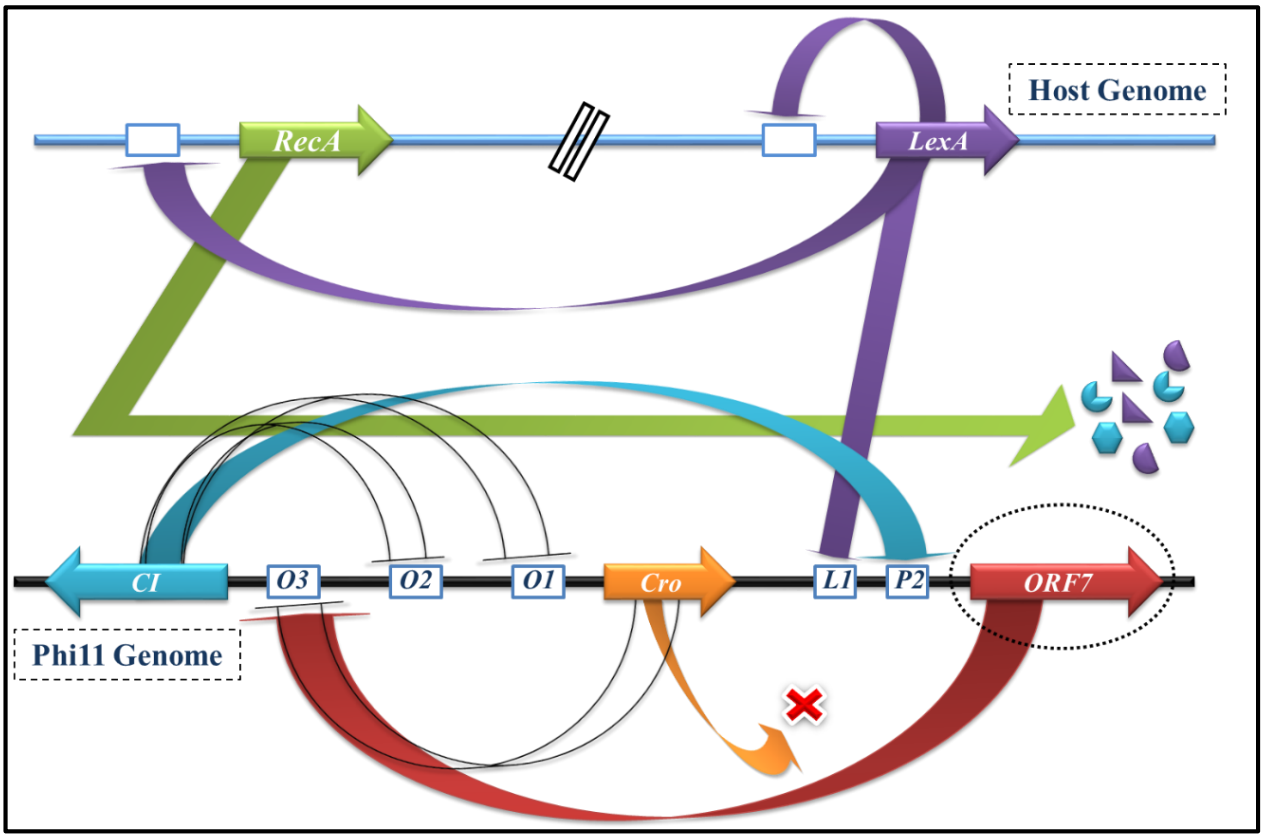

Figure 6.1. || Lytic-lysogenic developmental switch of phage Phi11. In Phi11, the expression of early genes (*cI*, *cro* and *gp07*) start as soon as the phage injects its genome into its host *S. aureus*. To maintain lysogeny, the CI repressor binds to *O*1operator followed by *O*2 and *P*2 to repress the expression of *cro* and *gp07*. Meanwhile, the host transcriptional repressor LexA binds to *gp07* operator (*L*1) to maintain the lysogenic steady state. Simultaneously, LexA also regulates the SOS response genes (like *recA*) and auto regulates its own expression to maintain the cell's normal growth and lysogeny. Due to DNA damage or SOS response induction by environmental changes, RecA gets activated. The resulting condition leads to the activation of SOS response genes, which allow the host cells to repair any DNA damage and arrest cell growth. The activated RecA also acts as a co-protease and enhances the auto-proteolytic activity of LexA and CI repressor, which ultimately lowers the concentrations of LexA and CI in host cells. Auto-proteolysis of LexA further removes the repression imposed upon the *gp07* operator, while auto-proteolysis of CI removes the repression on *cro* synthesis. These incidents finally allow the expression of lytic repressor Cro. Further, the derepression of *gp07* leads to the expression of Gp07, which enhances the binding of Cro to its cognate operator (*O*3). Binding of Cro to *O*3 results in the repression of *cI* expression. The entire cascade of events direct the developmental pathway of Phi11 towards the lytic cycle. All the solid coloured arrows were studied as part this work. Whereas, three transparent arrows were studied earlier (Das et al 2007; Das et al 2009; Ganguly et al 2009).

Future scope of work

- Site directed mutagenesis of *cro* and *cI* will provide information regarding the amino acids involvement for the DNA-protein interaction.
- Site directed mutagenesis of Gp07 to identify the crucial amino acids involved in its active site.
- Gp07 will be further studied for its growth inhibitory action.
- Co-crystallization of Gp07-Cro-*O* DNA complex will provide in depth information about their mode of interaction.
- Tightly regulated vectors for the *S. aureus* system will be designed by utilizing the regulatory elements so identified.

Appendix A

Buffer and reagent composition

- 30% acrylamide and bis-acrylamide solution (100ml)

Acrylamide	29.2gm
Bis-acrylamide	0.8gm
Makeup the volume to 100ml with autoclaved miliQ water.	

- 10X SDS-PAGE running buffer (100ml)

Tris base	3gm
Glycine	14.44gm
SDS	1gm
Makeup the volume to 100ml with autoclaved miliQ water, no need to adjust pH.	

- Coomassie staining solution (100ml)

Coomassie brilliant blue R250	0.1gm
Methanol	50ml
Glacial acetic acid	10ml
Makeup the volume to 100ml with autoclaved miliQ water.	

- 6X SDS sample loading dye (10ml)

1M TrisHCl (pH 6.8)	2.4ml
20% SDS	3ml
100% Glycerol	3ml
β-mercapitalethanol	3.2ml
Bromophenol blue	12mg
Makeup the volume to 10ml with autoclaved miliQ water.	

- 1X transfer buffer (100ml), western blot

Tris base	0.3gm
Glycine	1.44gm
Methanol	20ml[#]
Makeup the volume to 100ml with autoclaved miliQ water, no need to adjust pH. # Depend on protein size amount may vary.	

- Destaining solution (100ml)

Methanol	20ml
Glacial acetic acid	10ml
MiliQ water	70ml

- 50X TAE buffer (100ml)

Trisbase	24.2gm
Glacial acetic acid	5.7ml
0.5 M EDTA (pH 8.0)	10ml
Makeup the volume to 100ml with autoclaved miliQ water.	

- 5X TBE buffer (100ml)

Tris base	5.4gm
Boric acid	2.75ml
0.5 M EDTA (pH 8.0)	2ml
Makeup the volume to 100ml with autoclaved miliQ water.	

- 8% urea-PAGE (60ml), footprinting gel

Urea	30gm
5X TBE	12ml
20% acrylamide	24ml
Makeup the volume to 60ml with autoclaved miliQ water.	

- 5X Bradford reagent

Coomassie Brilliant Blue G-250	100mg
100% methanol	47ml
85% phosphoric acid	100ml
Makeup the volume to 200ml with autoclaved miliQ water.	

- STET buffer

TrisHCl (pH 8.0)	10mM
NaCl	100mM
EDTA (pH 8.0)	1mM
Triton X-1000	5%
Makeup the volume with autoclaved miliQ water.	

- 6X DNA loading dye

Glycerol	30% (v/v)
Bromophenol blue	0.25% (w/v)
Makeup the volume with autoclaved miliQ water.	

- Ethidium bromide solution

Ethidium bromide	10% (w/v)
Makeup the volume with autoclaved miliQ water.	

- BCIP/NBT color development substrate, western blot

NBT	33µl
BCIP	16.5µl
BCIP/NBT substrate buffer	5ml
Add the NBT first, mix, add the BCIP, and mix again.	

Appendix B

List of publications

B1. List of publications related to the thesis

- **Das, A.,** Mondal, S., and Biswas, M. Studies on the gene regulation involved in the lytic-lysogenic switch in *Staphylococcus aureus* temperate bacteriophage Phi11. (***Communicated*; 2019**).

- **Das, A.,** and Biswas, M. (2019). Cloning, overexpression and purification of a novel two-domain protein of *Staphylococcus aureus* phage Phi11. *Protein Expression and Purification*, 154, pp.104-111.

- **Das, A.**, Biswas, S., and Biswas, M. (2018). Expression of Phi11 Gp07 Causes Filamentation in *Escherichia coli*. *The open microbiology journal*, 12, 107.

- **Das, A.,** and Biswas, M. (2016). Changes in the Functional Activity of Phi11 Cro Protein is Mediated by Various Ions. *The protein journal*, 35(6), 407-415.

B2. Other publications

- Hemmadi, V., **Das, A.**, Chouhan, O.P., Biswas, S. and Biswas, M., (2019). Effect of ions and inhibitors on the catalytic activity and structural stability of *S. aureus* enolase. *Journal of Biosciences*, *44*(4), p.90.

- Kumar, V., Naik, V.G., **Das, A.**, Bal, S.B., Biswas, M., Kumar, N., Ganguly, A., Chatterjee, A. and Banerjee, M., (2019). Synthesis of a series of ethylene glycol modified water-soluble tetrameric TPE-amphiphiles with pyridinium polar heads: Towards applications as light-up bioprobes in protein and DNA assay, and wash-free imaging of bacteria. *Tetrahedron*, *75*(27), pp.3722-3732.

- Naik, V. G., Hiremath, S. D., **Das, A.**, Banwari, D., Gawas, R. U., Biswas, M., Banerjee, M. and Chatterjee, A., (2018). Sulfonate-Functionalized Tetraphenylethylenes for Selective Detection and Wash-Free Imaging of Gram-positive bacteria (*Staphylococcus aureus*). *Materials Chemistry Frontiers*, 2(11), pp.2091-2097.

- Bhutia, Z. T., **Das, A.**, Biswas, M., Chatterjee, A., and Banerjee, M. (2018). 7-Oxa-4-thia-1-aza-bicyclo [3.2.1] octane 4, 4-Dioxides: Mechanochemical Synthesis by Tandem Michael Addition–1, 3-Dipolar Cycloaddition of Aldoximes and Evaluation of Antibacterial Activities. *European Journal of Organic Chemistry*, 2018(4), 506-514.

- Bhutia, Z. T., Prasannakumar, G., **Das, A.**, Biswas, M., Chatterjee, A., and Banerjee, M. (2017). A Facile, Catalyst-Free Mechano-Synthesis of Quinoxalines and their In-Vitro Antibacterial Activity Study. *ChemistrySelect*, 2(3), 1183-1187.

B3. Conferences attended

- **Avijit Das** and Malabika Biswas (2019). "Studies on the gene regulation involved in the lytic-lysogenic switch in *Staphylococcus aureus* temperate bacteriophage Phi11". 43rd Indian Biophysical Society (IBS) Meeting, Kolkata, India on 15th to 17th Mar 2019.

- Malabika Biswas and **Avijit Das** (2018). "The role of *gp07* in the developmental pathway of Phi11". Trends in Biochemical and Biomedical Research: Advances and Challenges, Varanasi, India on 13th to 15th Feb 2018.

- Malabika Biswas and **Avijit Das** (2017). "The ORF7 of Phi11 and its bacteriostatic effect on *E. coli* cells". Microbiology in the New Millennium: from Molecules to communities, Kolkata, India on 27th to 29th Oct 2017.

- **Avijit Das** and Malabika Biswas (2015). "The Antirepressor protein of Phi11 and its effect upon the host cell". 56th Annual Conference of Association of Microbiologists of India (AMI) & International Symposium on "Emerging Discoveries in Microbiology on 7-10th Dec 2015.

- **Avijit Das** and Malabika Biswas (2014). "The putative Antirepressor protein of *Staphylococcus aureus* phage Phi11 has a killing effect on the host cells". The 83rd Society of Biological Chemist (SBC) & "Haldane memorial symposium on evolutionary biology", Bhubaneswar, India on 18th to 21st Dec 2014.

- **Avijit Das** (2013). "3rd annual conference of the toxicological society of India and 1st international conference on biology of natural toxins", BITS Pilani K K Birla Goa Campus, India on 19th to 21st Dec 2013.

B4. Workshops attended

- "Healthcare Data Analytics: Underlying Foundations & Perspectives" at BITS Pilani K K Birla Goa Campus, India on 12th Apr 2019.

- "Practical protein crystallography using PX beamline at Indus-2 synchrotron" at Raja Ramanna Centre for Advance Technology Indore, India on 27th to 28th Mar 2018.

- "Bio-entrepreneurship grant-writing and intellectual property management" at BITS Pilani K K Birla Goa Campus, India on 18th to 19th Feb 2016.

B5. Scientific community membership

- Life Member, Society for Bacteriophage Research and Therapy (SBRT), India. 2019 – Present (Id. No.- SBRTLM00012).

- Affiliate Membership, Microbiology Society, United Kingdom. 2018 - Present. (Membership No: C020855).

- Basic Member, International Society for Viruses of Microorganisms (ISVM). 2018 - Present.

- Life Member, Association of Microbiologists of India (AMI), India. 2015 - Present. (Id. No: 4196-2015).

B6. Scholarships

- Awarded Council of Scientific & Industrial Research (CSIR) - Senior Research Fellowship (SRF). [May, 2018 - Present] (File No. 09/919(0033)/2018EMR-I).

- Awarded BITS Pilani, Institute Fellow (IF). [Apr, 2016-Apr, 2018].

- Awarded Board of Research in Nuclear Sciences (BRNS) - Junior Research Fellowship (SRF) [Sep, 2013 - Sep, 2015], Senior Research Fellowship (SRF) [Sep, 2015 - Mar, 2016]. (File No. BRNS:BSC/RP37B.12/BRNS).

Appendix C

Brief Biography of the Candidate

Avijit Das received his Master of Science (M.Sc.) degree in Microbiology from Department of Microbiology, Vidyasagar University in 2012. For his dissertation (M.Sc.), he worked on "Factors Influencing the Synonymous codon and Amino Acid Usage Bias in Giant Phage 201phi2-1" under the supervision of Professor Keya Sau at Department of Biotechnology, Haldia Institute of Technology. Avijit Das has been enrolled in the PhD program of the Department of Biological Sciences, BITS Pilani K K Birla Goa campus. During this period, he worked as a junior research fellow and upgraded to senior research fellow on a BRNS funded project entitled "Cloning and characterization of the promoters of *Staphylococcus aureus* temperate bacteriophage Phi11". Also, he received Institute fellowship from BITS Pilani. Later in 2018, he was awarded with senior research fellowship from Council of Scientific & Industrial Research (CSIR), Govt. of India.

Avijit has co-authored six international publications and has presented his work at six conferences so far.

Appendix D

Brief Biography of the Guide

Dr. Malabika Biswas completed her Ph.D. in Bose Institute, Kolkata, under the supervision of Prof. Subrata Sau, as an Institute Fellow and finally as a CSIR-SRF in 2008. During her doctoral work she analysed the genetic switch involved in the developmental pathway of bacteriophage Phi11. She went on to work as a postdoctoral researcher at the Department of Biochemistry, Bose Institute, Kolkata from April 2008 to July 2009 with Prof. B. Bhattacharyya. Dr. Malabika Biswas joined the Department of Biological Sciences of K. K. Birla Goa campus as an Assistant Professor in January 2012.She has since been involved as the Principal Investigator of two research projects funded by BRNS, and DST, as well as the co-Investigator of a DST project. Her research interests include studies on the molecular biology of temperate phages, specifically aureophages. She further studies the genes of pathogenic bacteria which are essential for host invasion. Dr. Biswas has 14 publications in reputed journals and several conference publications to her name.

Presently, Dr. Biswas has three registered Ph.D. students under her tutelage and numerous thesis dissertation and project students working with her.