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° C. *E. coli* BL21(λDE3) harbouring the recombinant plasmids were harvested (CI overexpression was carried out at 14° 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° C for the over-expression of Gp07, ΔGp07 and rCTD. The resulting cell pellets were washed with 0.9% NaCl and stored at -80° 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	Forward primer for synthesis of *cro*	GAATTC**AACATGCAATGGAATTTAATAAAG**	Das et al 2009
PCR4	Reverse primer for synthesis of *cro*	AAGCTT**GCATTTGTTATGTCTCC**	
PCI5	Forward primer for synthesis of *cI*	GAATTC**AAAATGGATAAAAAAGAATTAG**	Das et al 2007
PCI4	Reverse primer for synthesis of *cI*	AAGCTT**AGGCGCTATTAATCAC**	
C-*gp07*-F	Forward primer for synthesis of Gp07 and rNTD	CCATGG**GAATGTGGGTGTTGAGGAAAAAGGAGG**	Chapter 3
C-*gp07*-R	Reverse primer for synthesis of *gp07* and Δ*gp07*	CTCGAG**CGCTCCCCCTAAATTAGCTTCATAAC**	
C-*Bro*-R	Reverse primer for the synthesis of rNTD	CTCGAG**GTCTGGATCTTTTAATGTTTGTTCAATTACATTG**	
N-*KilAC*-F	Forward primer for the synthesis of rCTD	CATATG**TACATCATTACAGTGTTGACTGAGTATAAGAAAG**	
N-*gp07*-R	Reverse primer for the synthesis of rCTD	CTCGAG**TTACGCTCCCCCTAAATTAGCTTCATAACC**	
C-Δ*gp07*-F	Forward primer for synthesis of Δ*gp07*	CCATGG**AAATGCAAGCATTACAAACATTTAATTTTAAAGAGC**	
N-*lexA*-F	Forward primer for synthesis of *lexA*	CAT**ATGAGAGAATTAACAAAACGACAAAGC**	This study
N-*lexA*-R	Reverse primer for synthesis of *lexA*	CTCGAG**TTACATTTCGCGGTACAAACCAATTAC**	
N-*recA*-F	Forward primer for synthesis of *recA*	*CAT^{A}**TGGATAACGATCGTCAAAAAGC**	
N-*recA*-R	Reverse primer for synthesis of *recA*	CTCGAG**CTATTCTTCGTCAAATAATGACTTTGG**	
N-*cro*-F	Forward primer for synthesis of *cro*	CAT**ATGCAATGGAATTTAATAAAGTTGAG**	
N-*cro*-R	Reverse primer for synthesis of *cro*	CTCGAG**TTAAGATGTTTGTTTGCGTTTCG**	
pHC1	Synthesis of *O* DNA	GGATCCTAAATCTTCTTGAGTAC	Das et al 2007
pHC2	Synthesis of *O* DNA	GAATTCTTGGTTCTATAGTATCTG	
p-*gp07*-F	Synthesis of *gp07* promoter	**AGGAGACATAACAAATGCAAGACC**	This study
p-*gp07*-R	Synthesis of *gp07* promoter and 192 bp region of *gp07* promoter	**TCAGCAGACGGTTATCGCAATG**	
p-192*gp07*	Synthesis of 192 bp region of *gp07* promoter	**AACGGTGTTGAAAGCGATAACAAAG**	
p-*gp07*-F′	Synthesis of 145bp region of *gp07* promoter	**AAATTGAAGCTTATGATAAATCACTTAAAG**	
p-*gp07*-R′	Synthesis of 145bp region of *gp07* promoter	**ATAGCACTTCGTAGCTTTTTAGTAATTTC**	
p-*lexA*-F	Synthesis of *lexA* promoter	**CATTTTCACTCCTAGAACATTTGTTTGTATTAG**	
p-*lexA*-R	Synthesis of *lexA* promoter	**TATATAGGCACTCCCTAAATTAATTTAAATACAG**	
p-*recA*-F	Synthesis of *recA* promoter	**ATTGTATTATCGATAAAAATATAAGCACG**	
p-*recA*-R	Synthesis of *recA* promoter	**TTTTTGACGATCGTTATCCAAAGC**	

#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° 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° 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.).

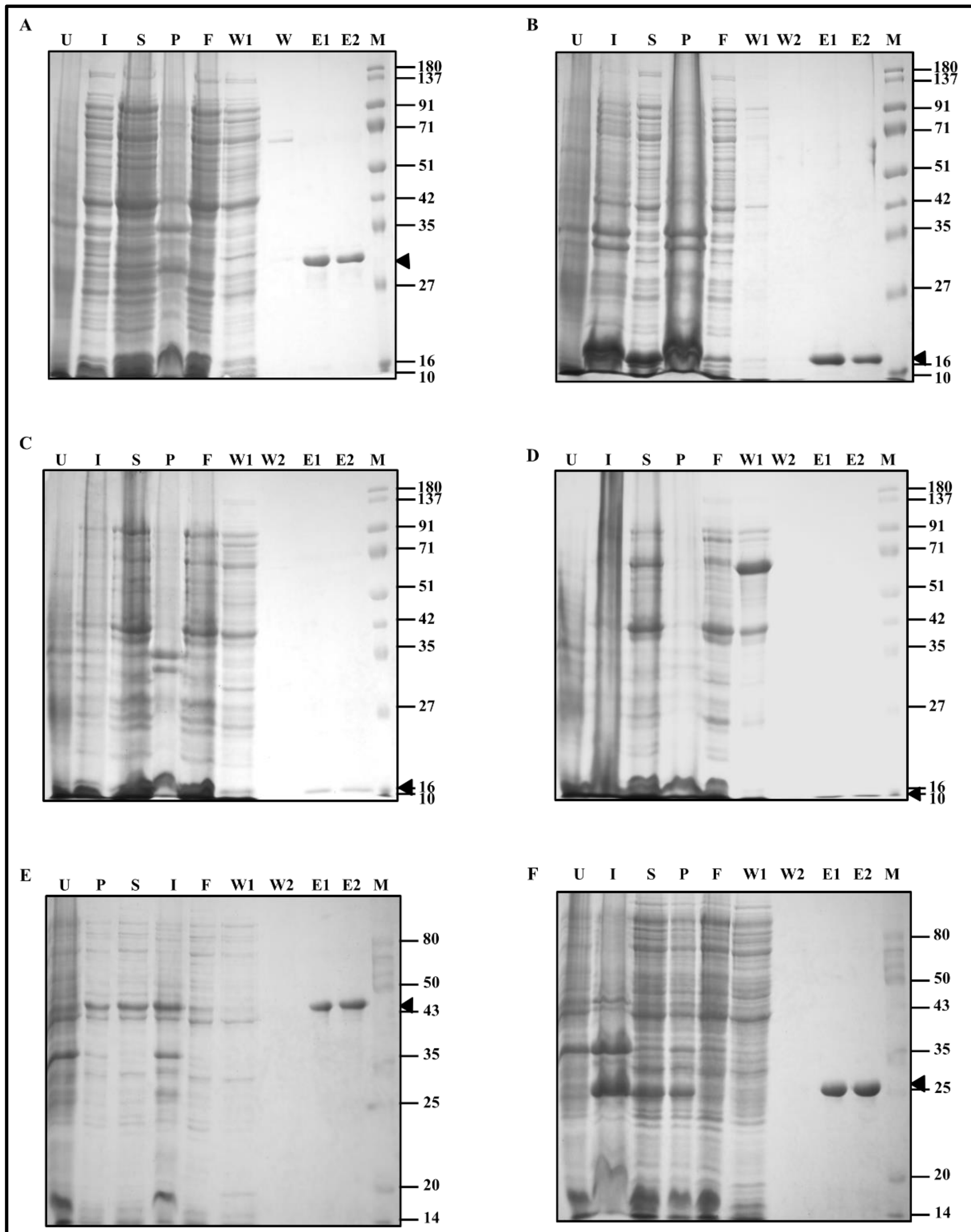

Figure 5.1. || Purification of the constructed recombinant proteins (from Phi11 and *S. aureus*). The purification profile of different histidine-tagged proteins prepared from different cell extracts (See materials and methods for details, Section 5.2.5.), were analyzed by SDS-12% PAGE. Panel A: purification 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.).

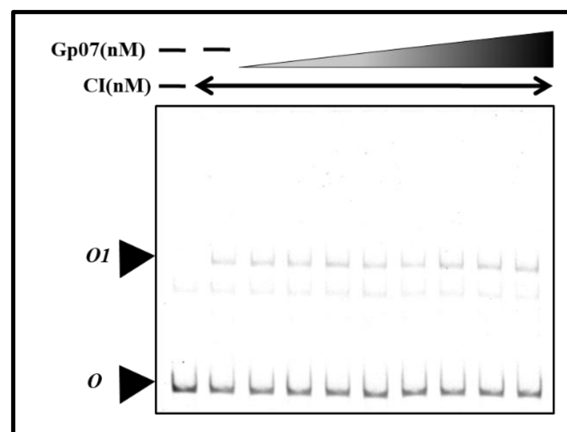

Figure 5.2. || Analysis of the effect of Gp07 upon the binding of CI to *O* DNA. 6% non-denaturing PAGE showing the interaction between operator DNA(*O*), Gp07 and CI. Here a fixed concentration of CI is allowed to bind to *cI-cro* intergenic region (*O* DNA), in presence of increasing concentrations 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.).

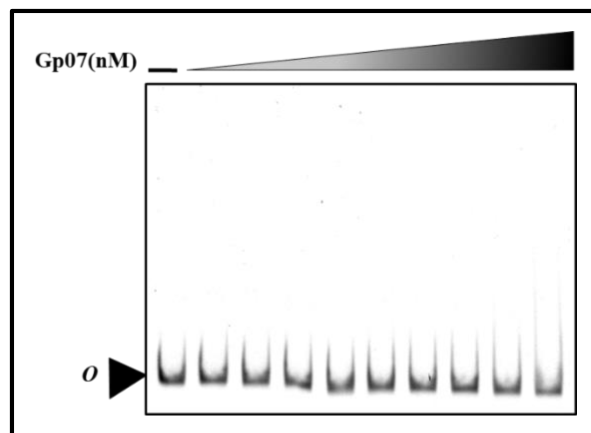

Figure 5.3. || Analysis of interaction of Gp07 with 269 bp *cI-cro* intergenic region (*O* DNA). Gp07 was incubated with 90ng operator DNA(*O*) and analysed on a 6% non-denaturing PAGE. Arrowhead represents the free *O* DNA. The concentration of Gp07 ranges from 0.1 µM - 2.5 µM. Negative symbol indicates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

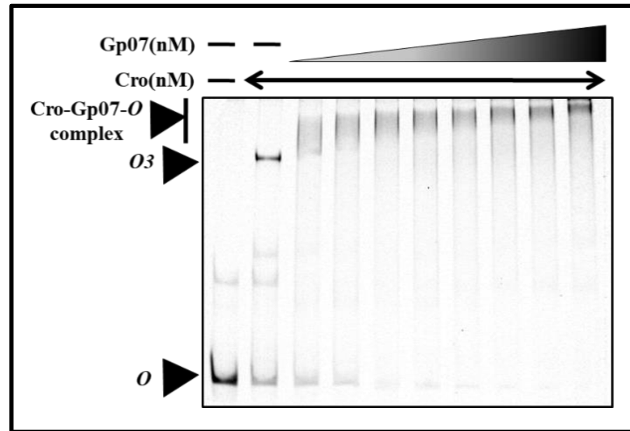

Figure 5.4. || Analysis of Cro and *O* DNA binding enhancement by Gp07. 6% non-denaturing PAGE showing the interaction between 90ng operator DNA(*O*) and Phi11 Cro (400nM) in presence of increasing concentrations of Gp07. The concentration of Gp07 ranges from 0.5 µM - 1.5 µM. Arrowheads with bar represent 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).

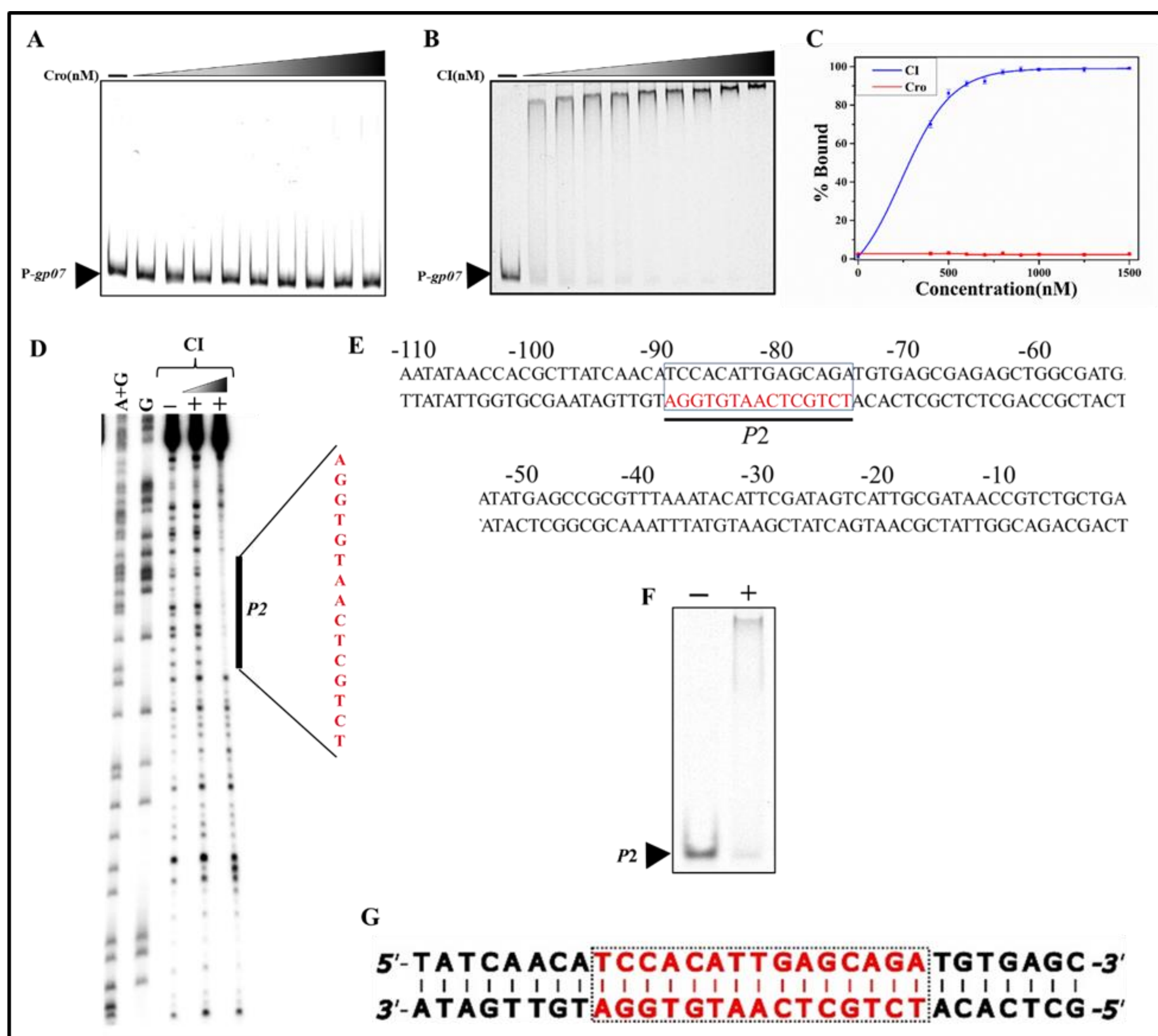

Figure 5.5. || CI and Cro repressors binding to p-*gp07*. A 6% non-denaturing PAGE showing the interaction between 90ng of p-*gp07* and increasing concentrations of Cro (A) or CI (B). Arrowheads represent the free p-*gp07*. The concentration of Cro and CI ranges from 0.4 μM - 1.5 μM. Negative symbol 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).

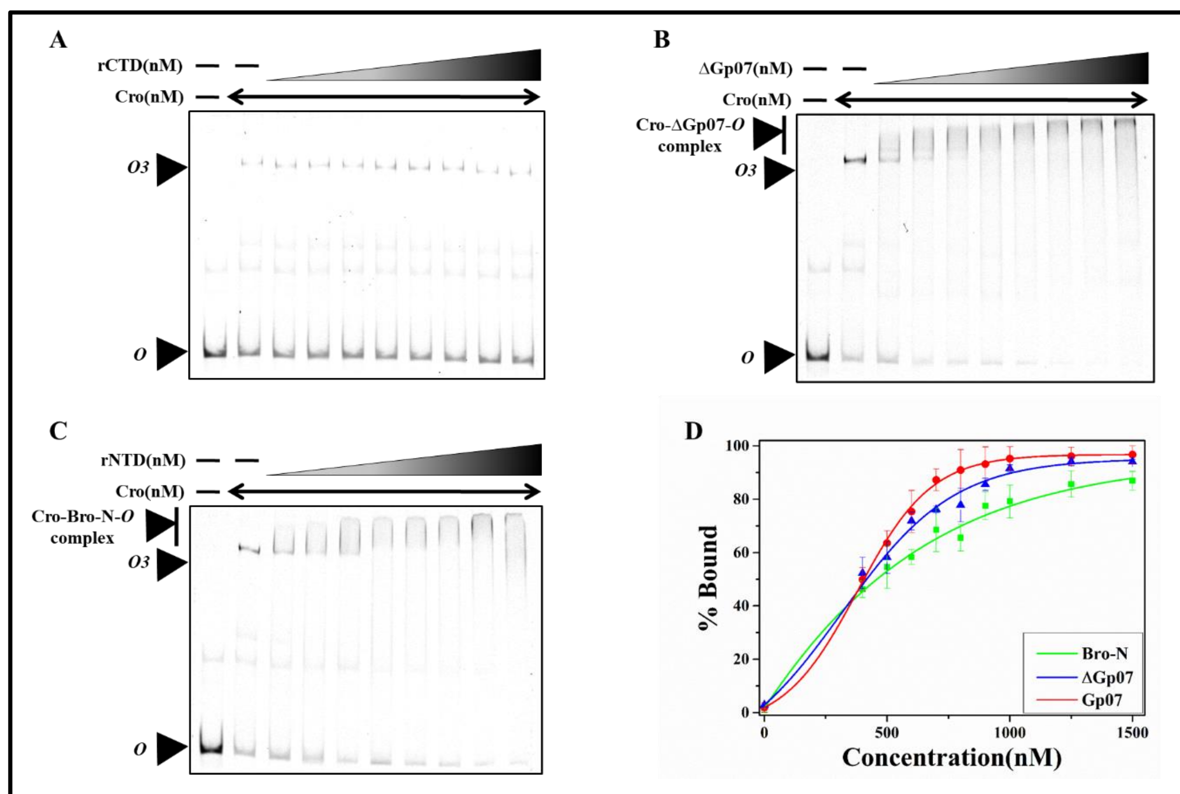

Figure 5.6. || Analysis of the interaction between Cro and *O* DNA in presence of rCTD (KilA-C), ΔGp07 and rNTD (Bro-N). 6% non-denaturing PAGE showing the interaction between 90ng operator DNA(*O*) and Phi11 Cro (400nM) in presence of increasing concentrations of rCTD (A) or ΔGp07 (B) or rNTD (C). The 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.

p-*gp07*	CGAAGACGCATTTG
parE(SACOL1389)	CGAACGTACGTTTG
uvrB(SACOL0823)	CGAACAAACGTTTG
lexA(SACOL1374)	CGAACAAATGTTTG
SACOL2162	CGAAAATATGTTTC
recA(SACOL1304)	CGAACAAATATTCG
SACOL1400	CGAACACGTGTTCT
SACOL1986	CGAACATGTGTTCT
SACOL0436	CGAACATATGTTCT
SACOL1999	CGAACATATGTTCT
sbcD(SACOL1381)	CGAACAAATGTTCT
Clustal Consensus	**** **

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.

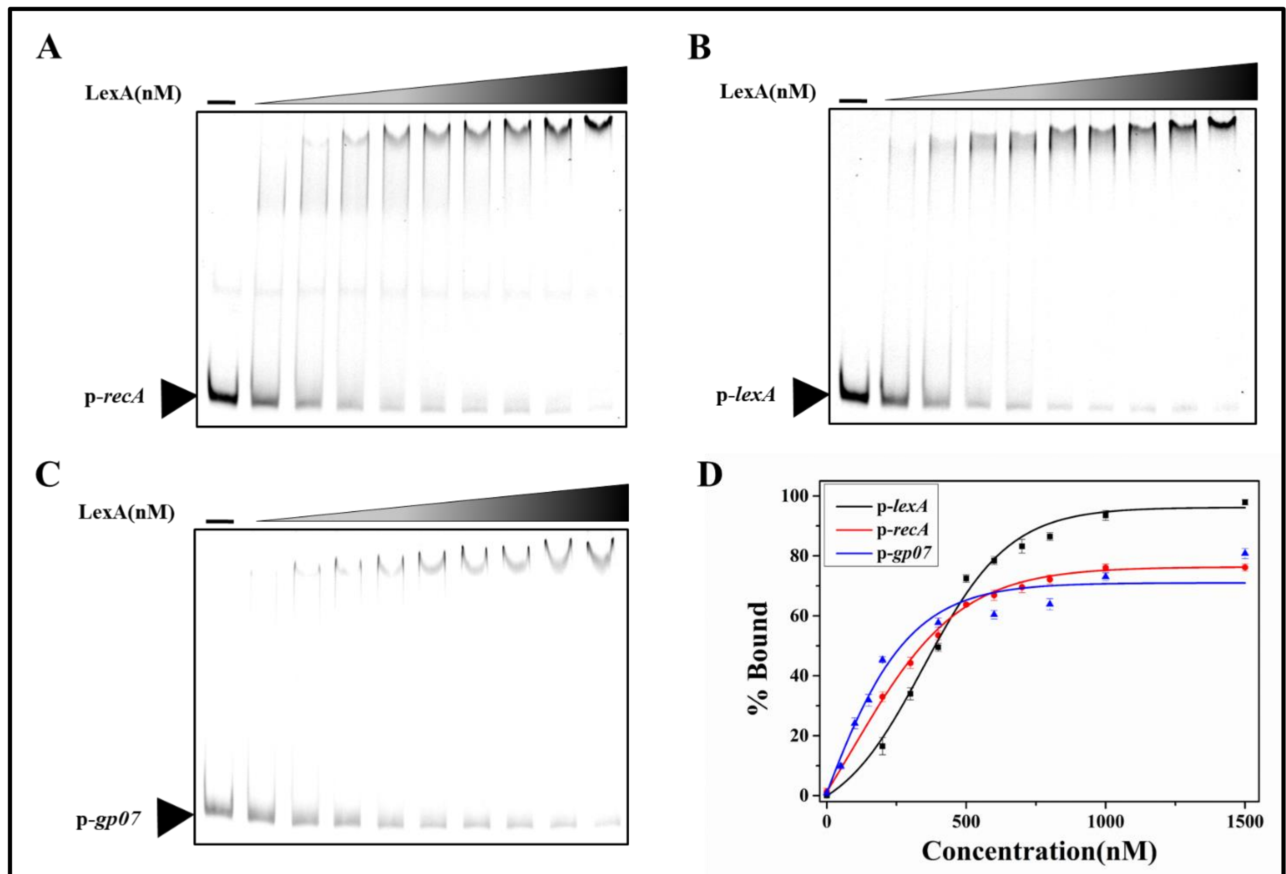

Figure 5.9. || LexA mediated repression of *recA*, *lexA* and *gp07* expression. 6% non-denaturing PAGE showing the interaction between *S. aureus* LexA and 90ng putative promoter DNA of (A) *recA*(p-*recA*), (B) *lexA*(p-*lexA*) and (C) *gp07*(p-*gp07*). Arrowheads represents the free promoter DNA. 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.).

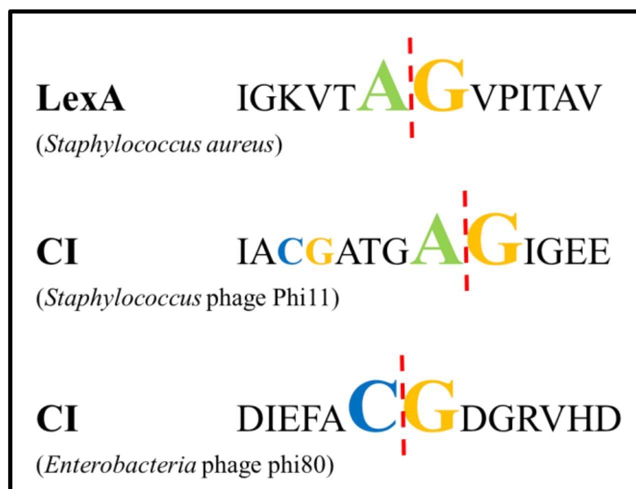

Figure 5.10. || RecA mediated cleavage sites of CI and LexA repressor. *S. aureus* LexA has Ala-Gly (AG) site, whereas CI repressor of phage Phi11 has both Cys-Gly (CG) and AG sites. Our amino terminal sequencing of CI fragments (Figure 5.11.) indicate that CI is cleaved at AG site and not at CG site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

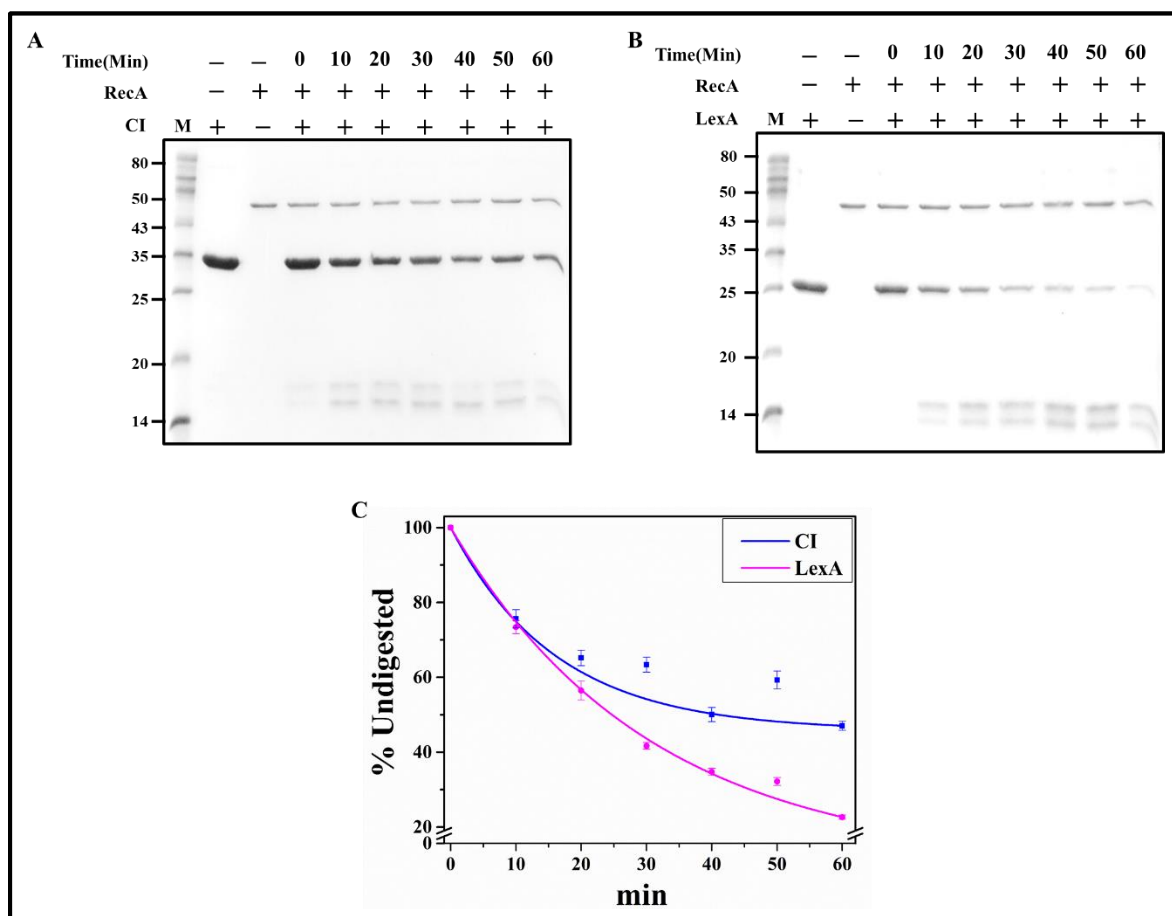

Figure 5.11. || RecA mediated cleavage of CI and LexA. RecA in presence of ssDNA (N-*gp07*-R, Table 5.1.) induce self-cleavage of LexA and CI repressor. RecA induced CI (A) and LexA (B) cleavage at different time (0 to 60 min) points at 37 °C. Left side of the gel indicates the protein marker (M) in 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.

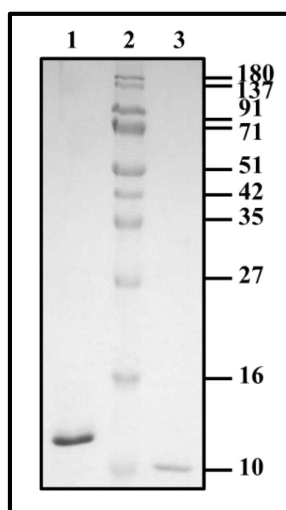

Figure 5.12. || Purification of tag-less Cro. Purification of the histidine tagged Cro (Lane 1) by Ni-NTA affinity chromatography. After TEV protease digestion, the tag-less Cro (Lane 3) was purified and the highly purified protein fractions were analysed by Tris-glycine SDS-15% PAGE. Molecular masses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

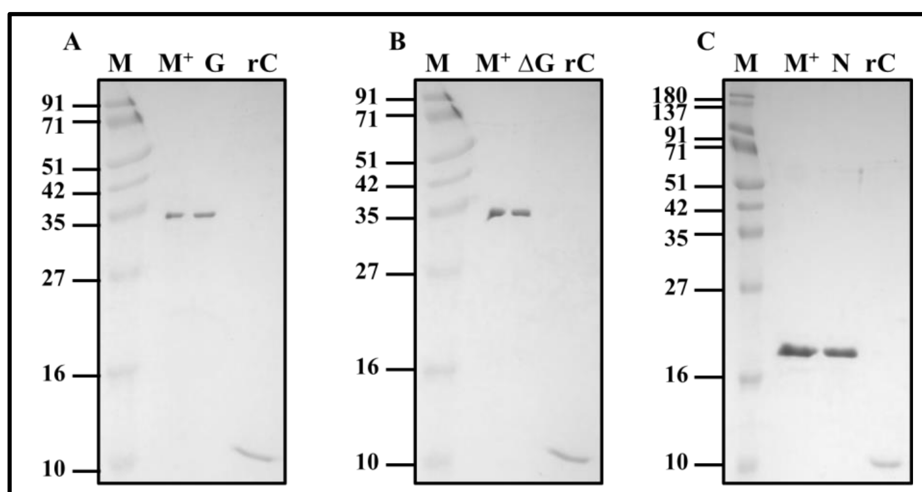

Figure 5.13. || Pull down assay. Cro with Gp07(A), ΔGp07(B) or rNTD(C). M represent the marker lane (kDa are indicated left side of the gels), M^+ represent the reaction mix (Gp07 with Cro, ΔGp07 with Cro or rNTD with Cro), G represent the Gp07, ΔG represent the ΔGp07, N represent the rNTD and rC represent 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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