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.

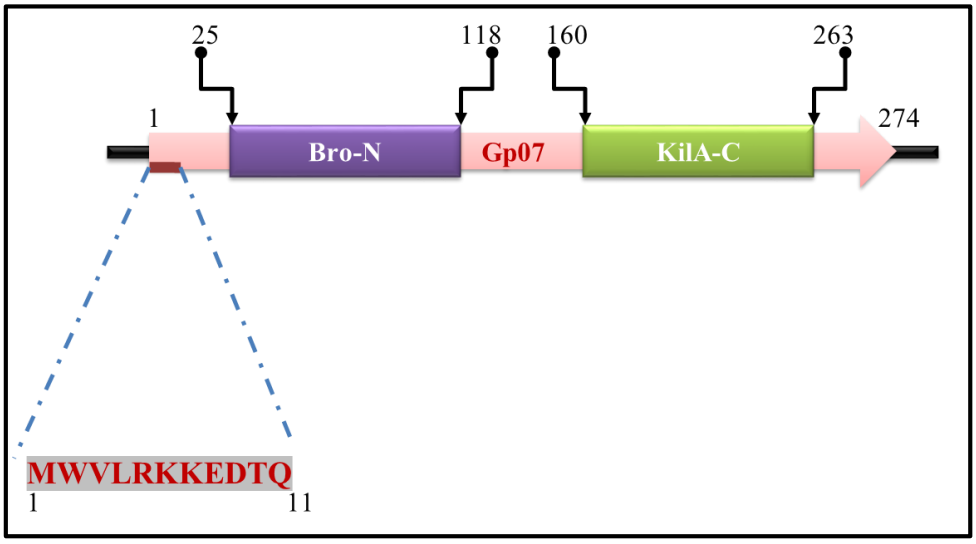


Figure 3.1. || Domain architecture of Gp07. Pfam analysis indicates that Gp07 harbours two domains - the amino terminal Bro domain and carboxy terminal KilA domain; the first eleven unique amino acids are coloured gray.

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.).

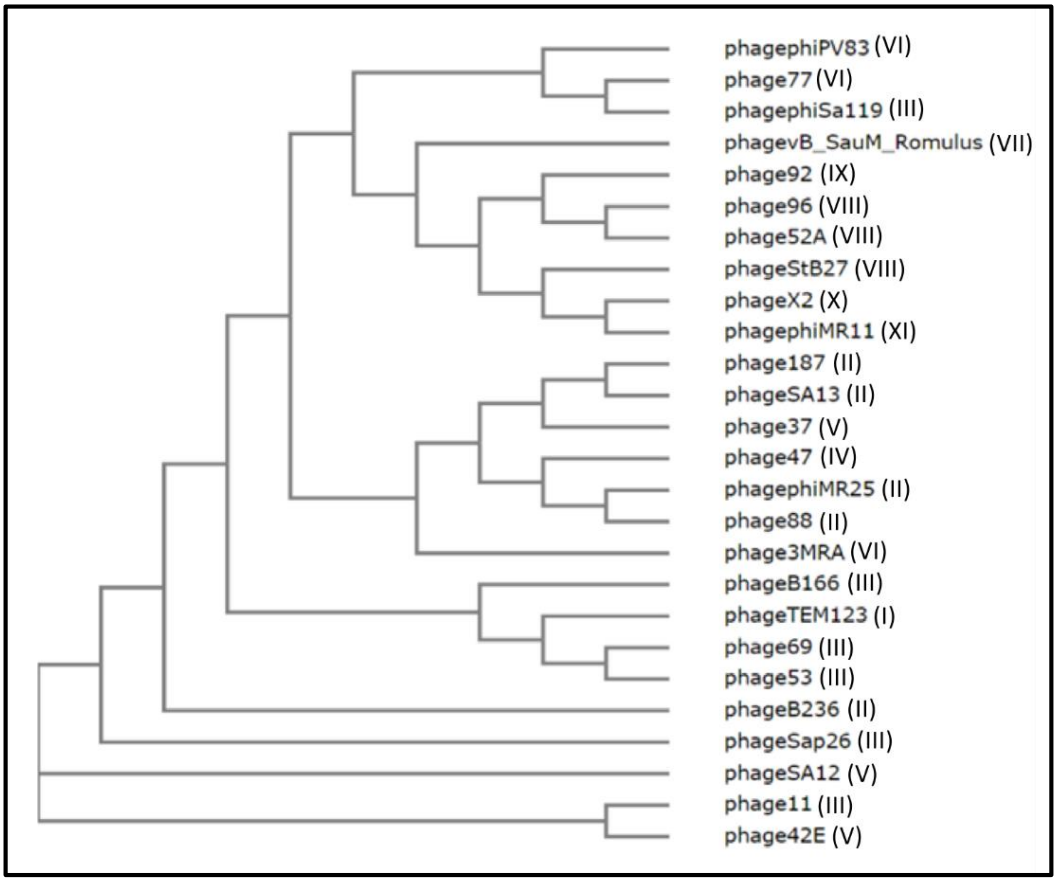


Figure 3.2. || Phylogenetic analysis. Phylogenetic analysis of Phi11 Gp07 has been aligned with ClustalO (See Materials and Methods for details) by neighbour-joining method. The Phi11 is evolutionary closely related with phage 42E. This analysis shows that distantly related phages with same gene organization.

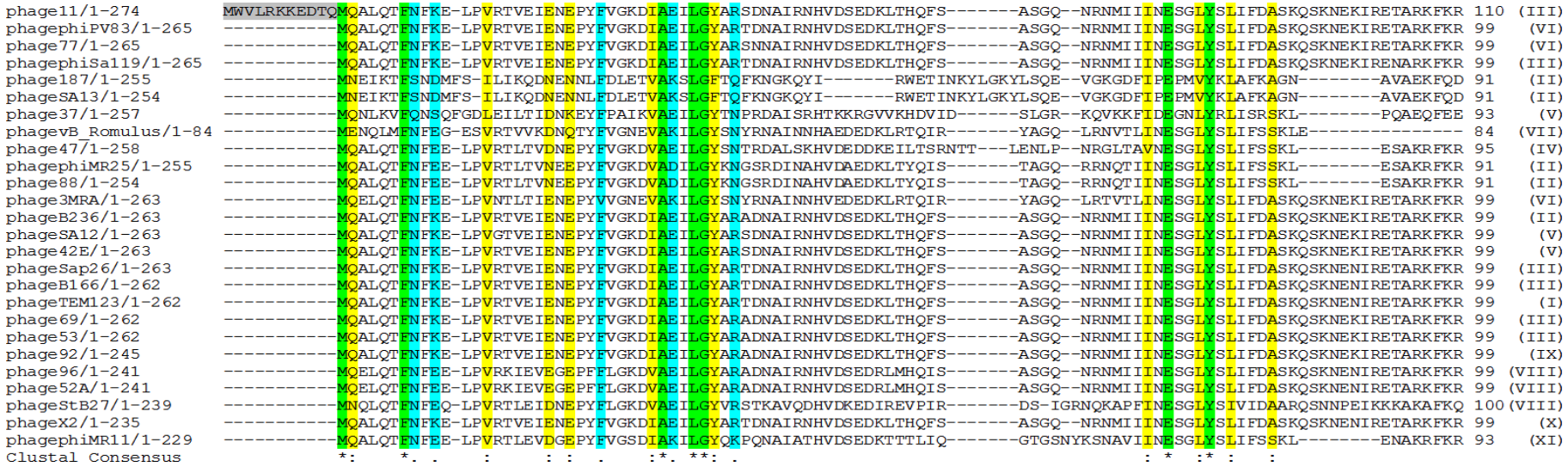


Figure 3.3. || Sequence analysis of the full-length protein of some phages in Caudovirales. Sequences are designated by their phage name, followed by protein length and finally gene organization type (Figure 3.8.). Phi11 Gp07 has been aligned with ClustalO (See Materials and Methods for details). The 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.

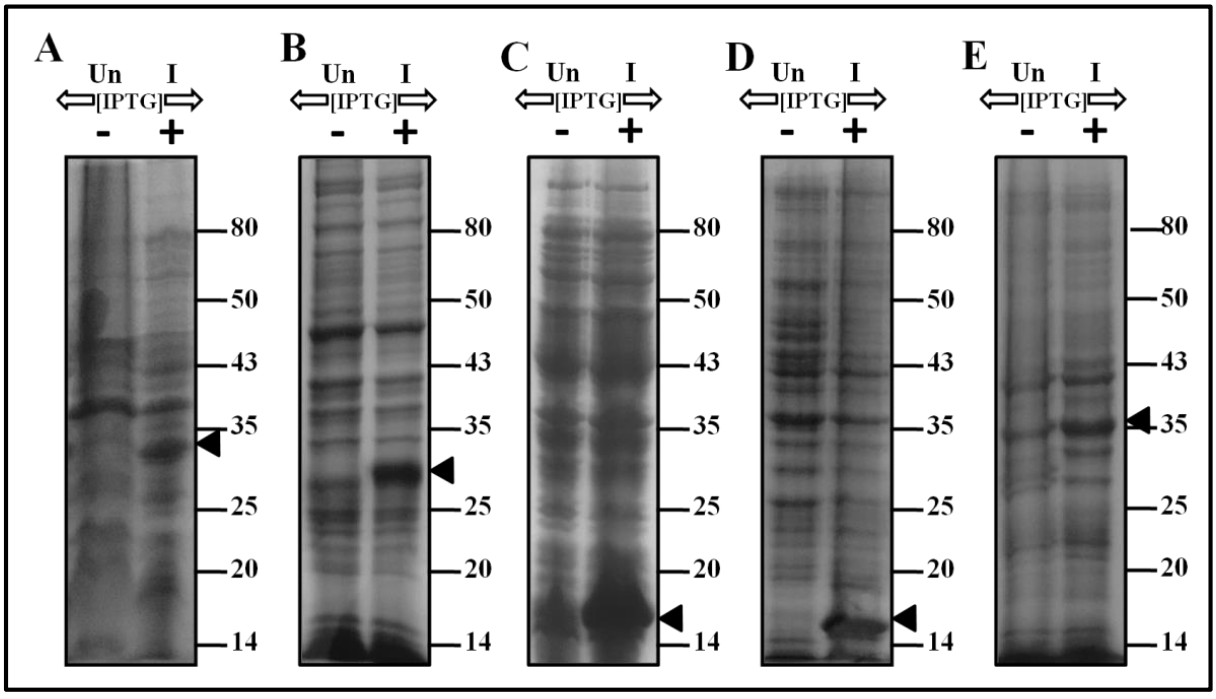


Figure 3.4. || Overexpression of Gp07, its domains (rNTD, rCTD), ΔGp07 and a control protein in *E. coli*. Overexpression of the recombinant proteins (induced with 0.5mM IPTG for 2 h at 37ºC) were analysed on SDS-12%-PAGE. (A) Overexpression of Gp07 (B) Overexpression of ΔGp07 (C) Overexpression of rNTD;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

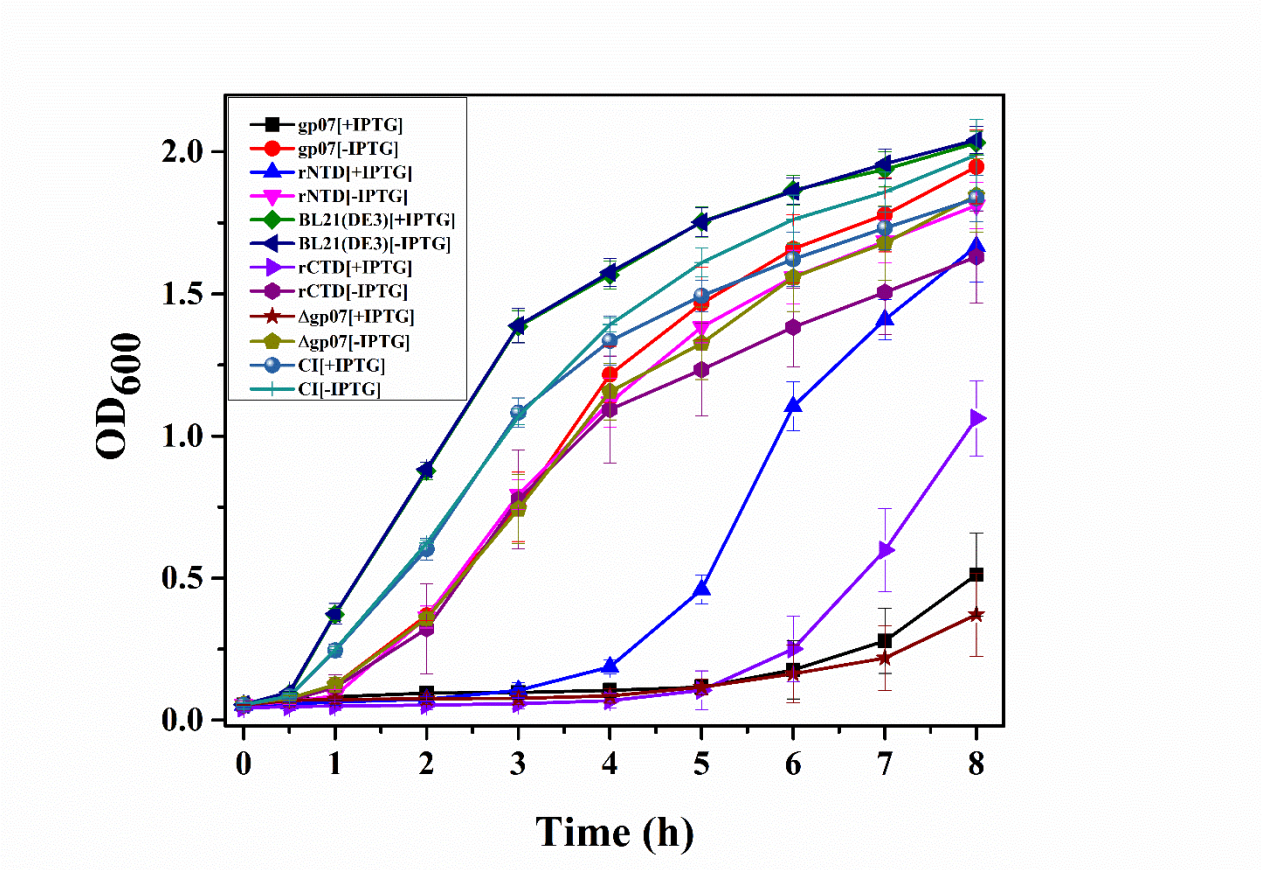
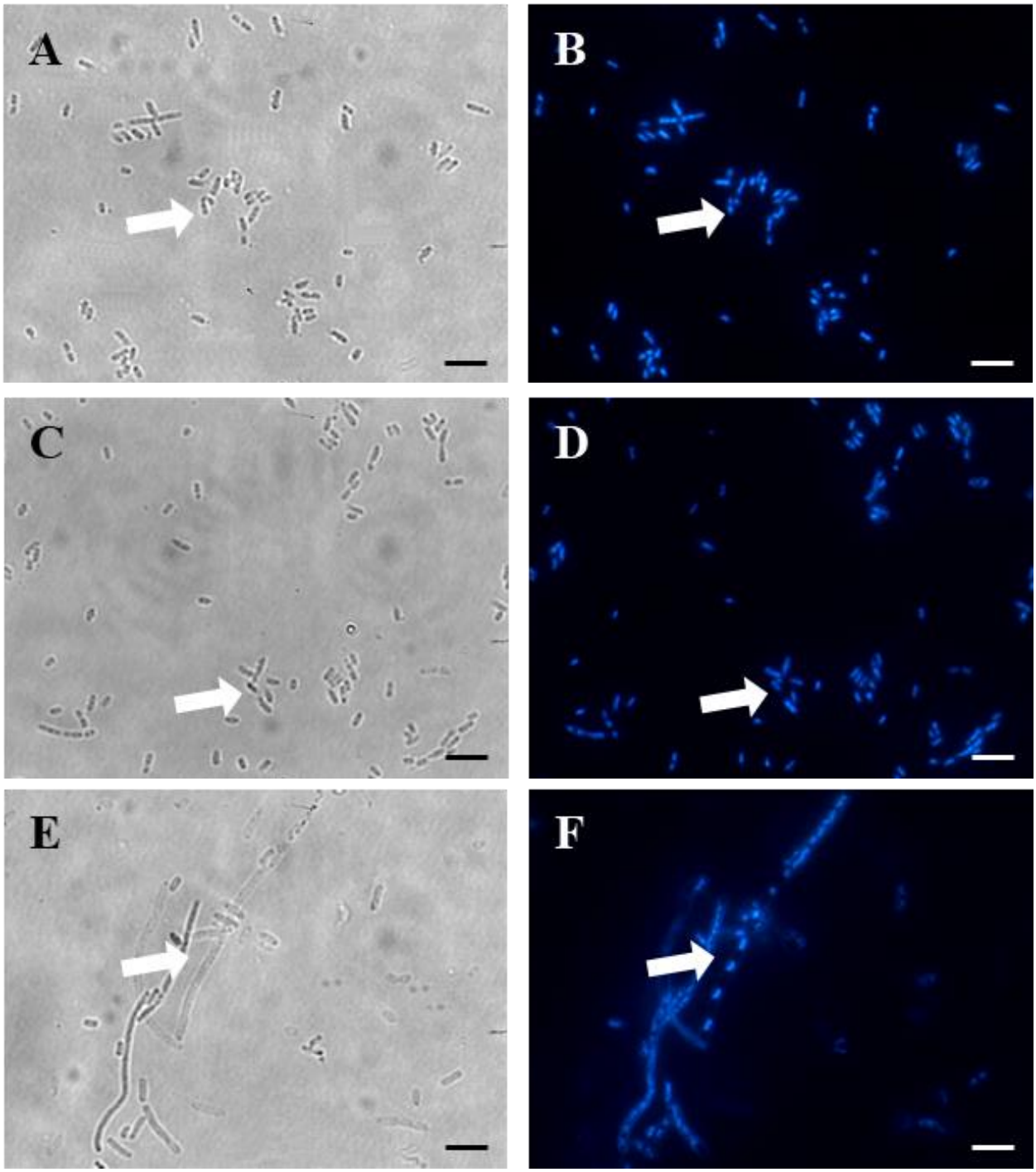


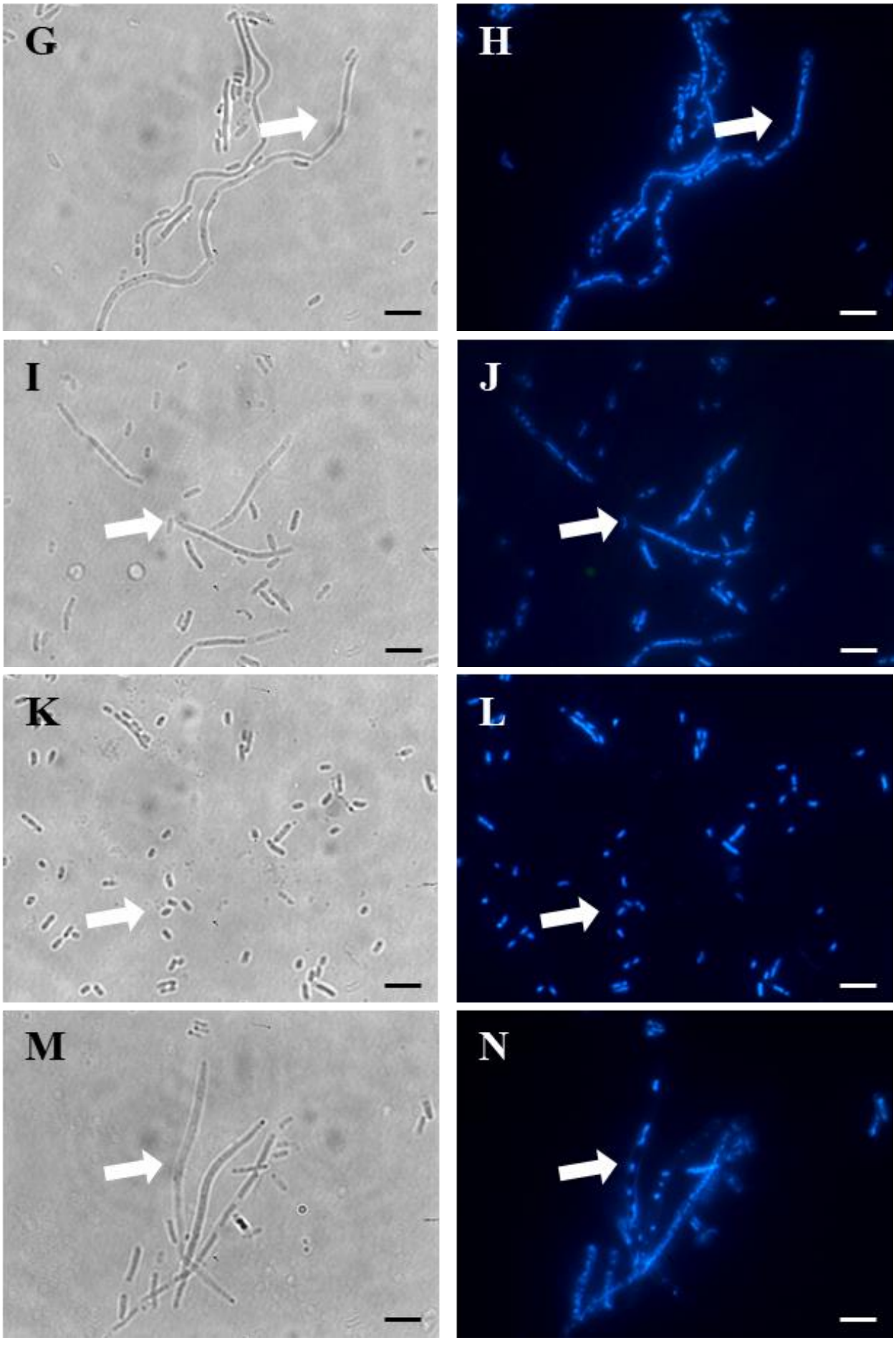
Figure 3.5. || Effect of Gp07, its domains and ΔGp07 upon the growth of *E. coli*.
Overexpression of Phi11 Gp07, rCTD as well as the ΔGp07 inhibited growth of the host cells. Overexpression of rNTD initially repressed host cell growth but resumption of cell growth was seen after a few hours. The overexpressed 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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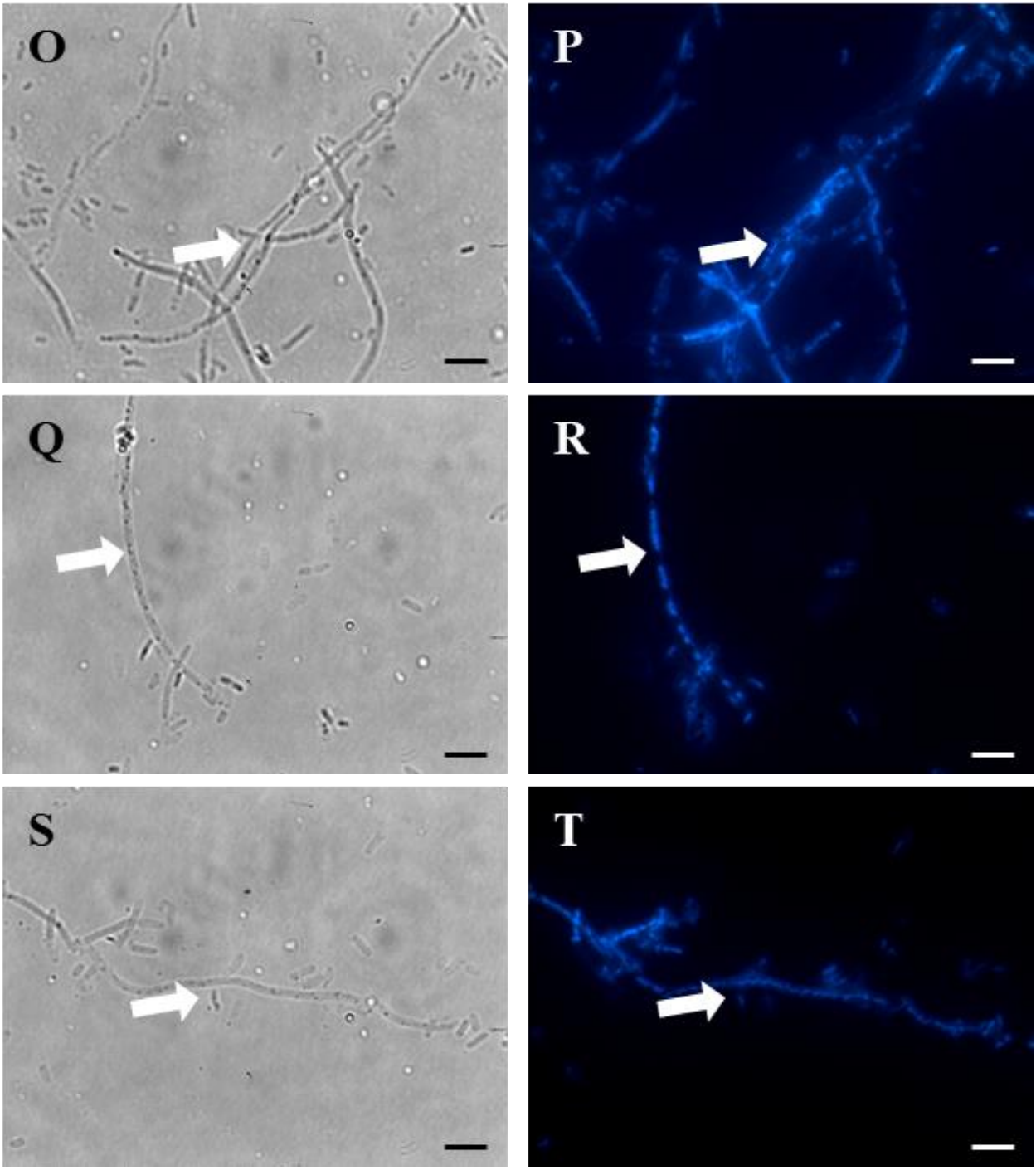
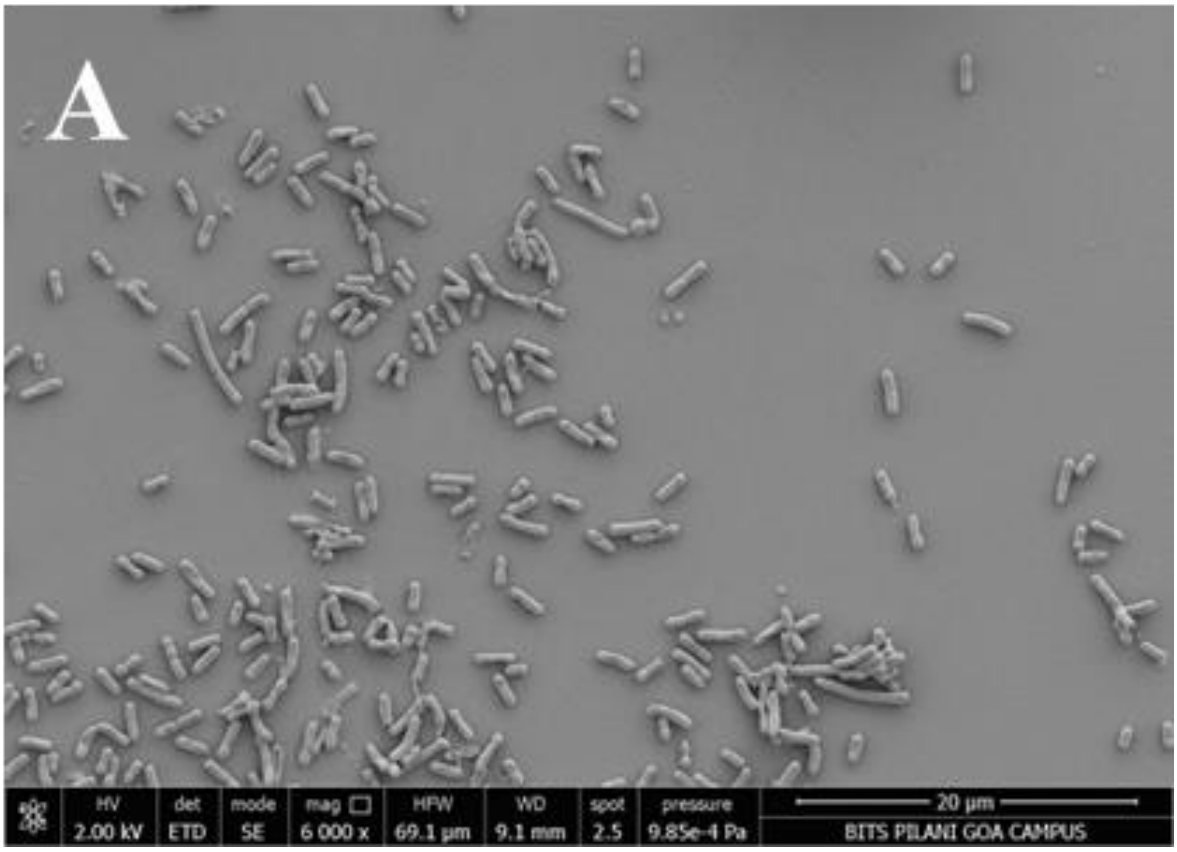


Figure 3.6. || Effect of overexpression of Gp07, its domains and ΔGp07 on the morphology of host cell.
(A) Morphology of wild type host cells (*E. coli*) under phase-contrast microscope at 100X magnification. (C) Morphology of *E. coli* cells overexpressing the control protein. (E,I,M,Q) Morphology of *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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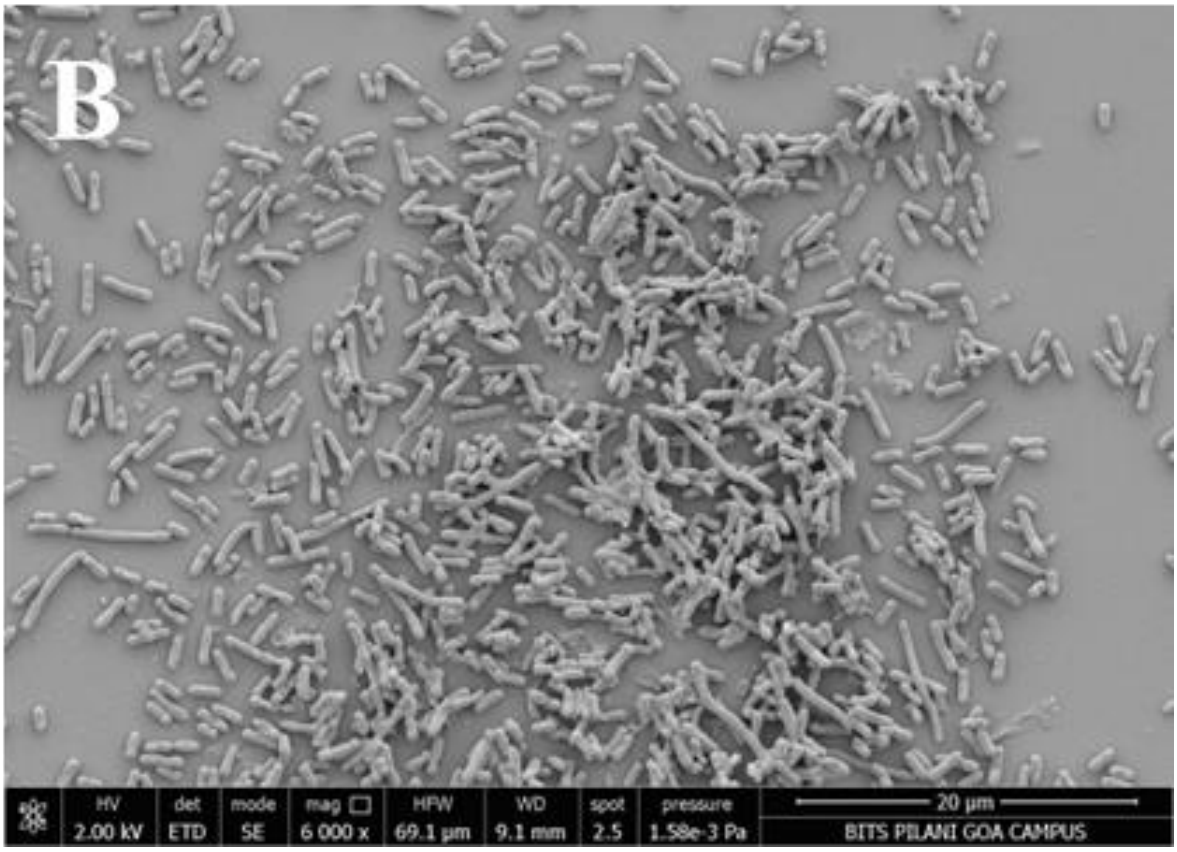
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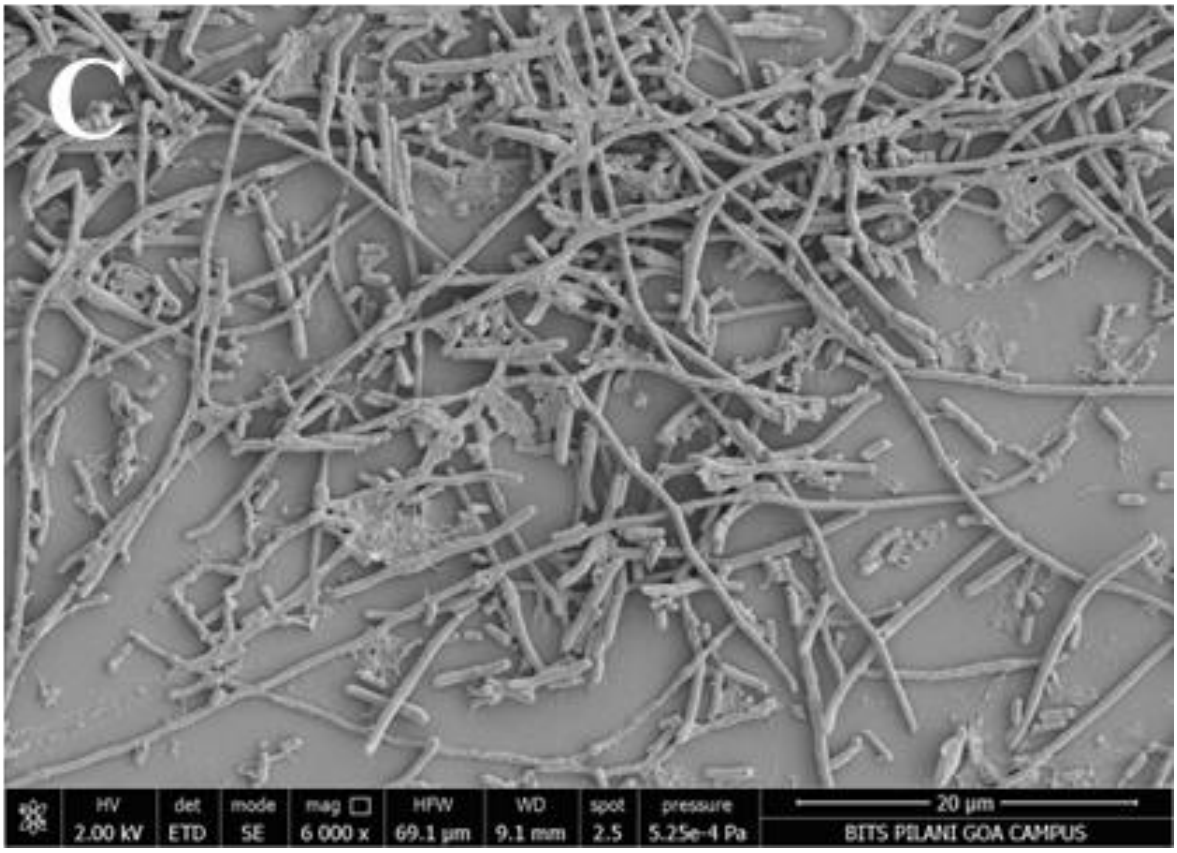
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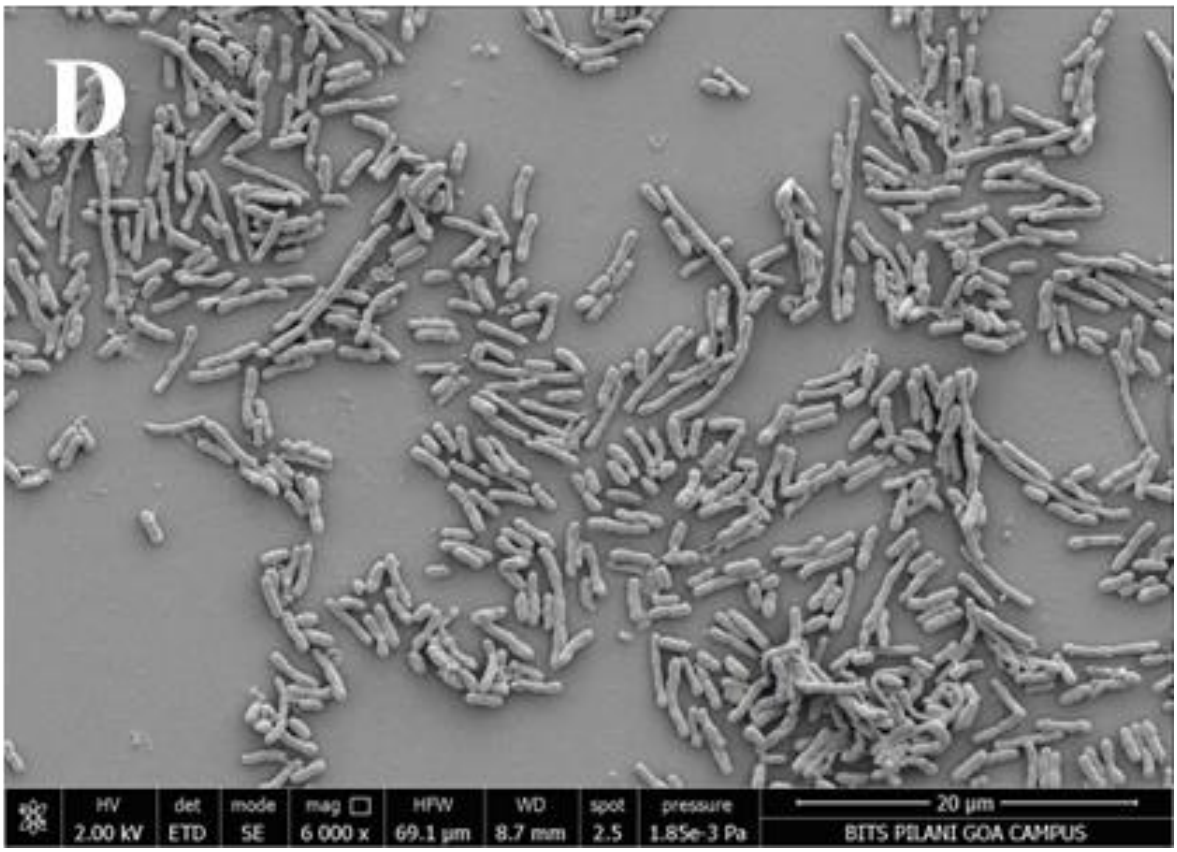
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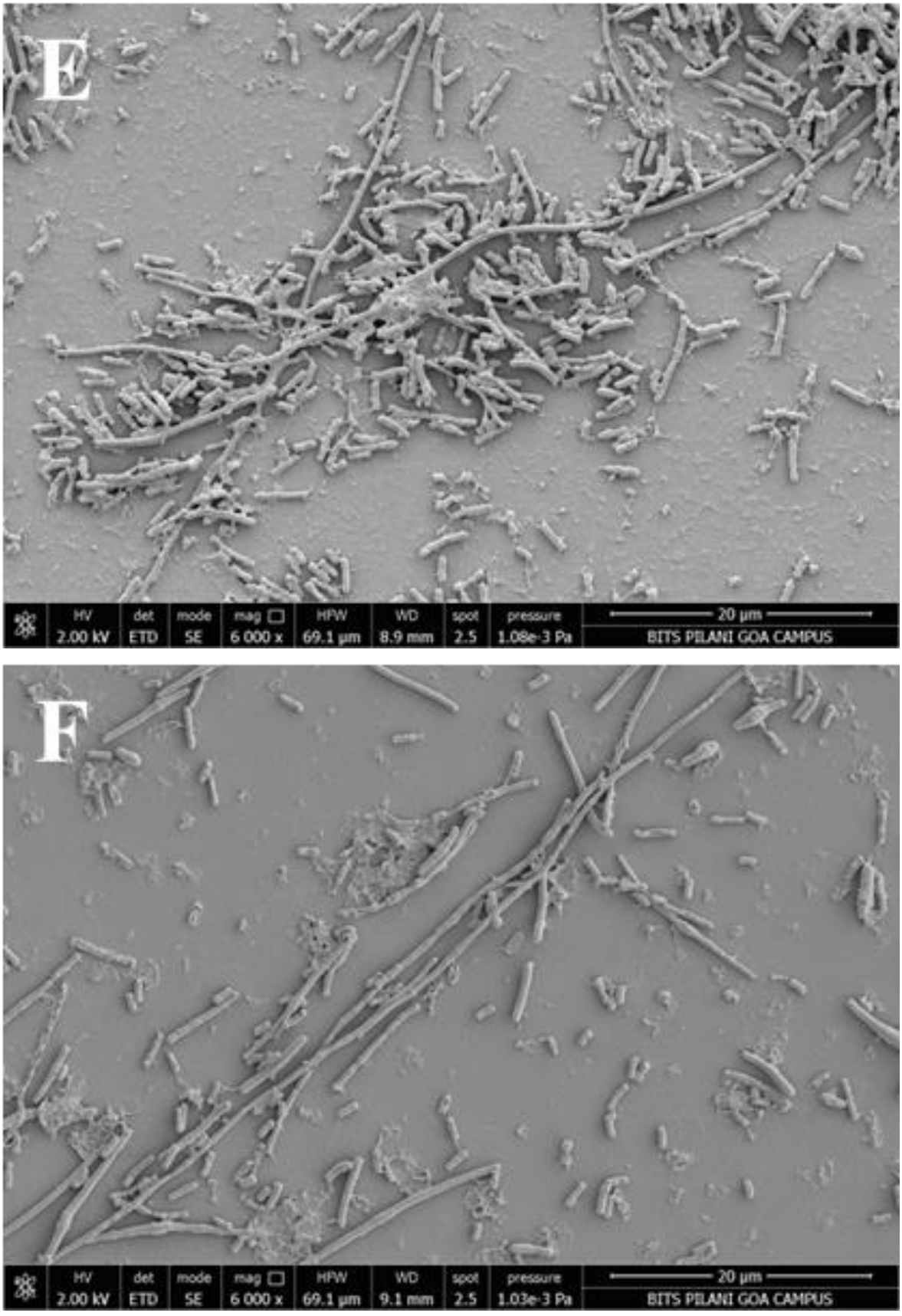

Figure 3.7. || Scanning electron micrographs of Wild type (WT), Control protein, Gp07, rNTD, rCTD and ΔGp07 expression in *E. coli*. (A) Wild type (WT), (B) Control protein (C) Gp07, (D) rNTD, (E) rCTD, (F) ΔGp07; all scale bars are 20 µm.

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.

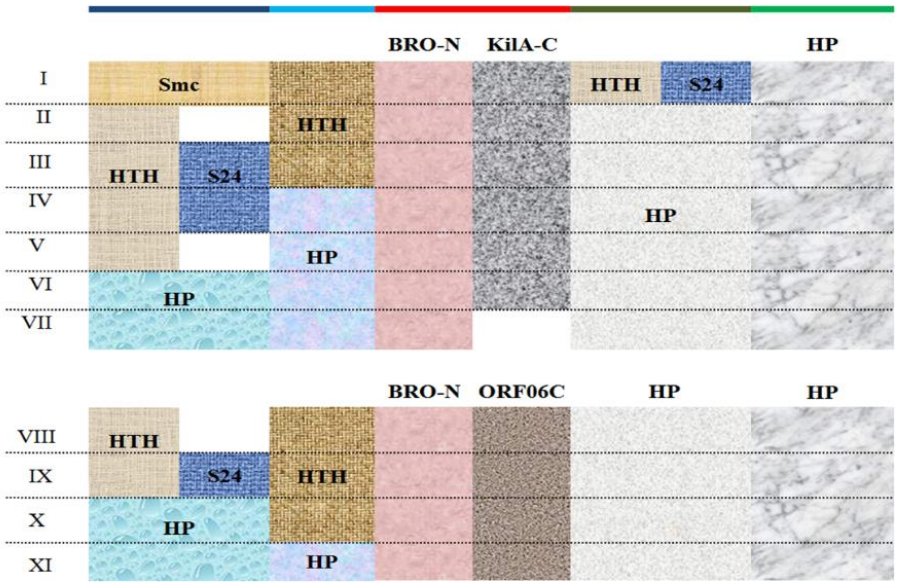

Figure 3.8. || Gene organization analyses of Caudovirales phage protein contain Bro-N domain. Polymorphisms associated with the gene organization of Gp07 and their neighboring genes found in 26 *Staphylococcus* phages. Eleven variations of gene organizations were found (Type I-XI). Top most color bar 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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