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.

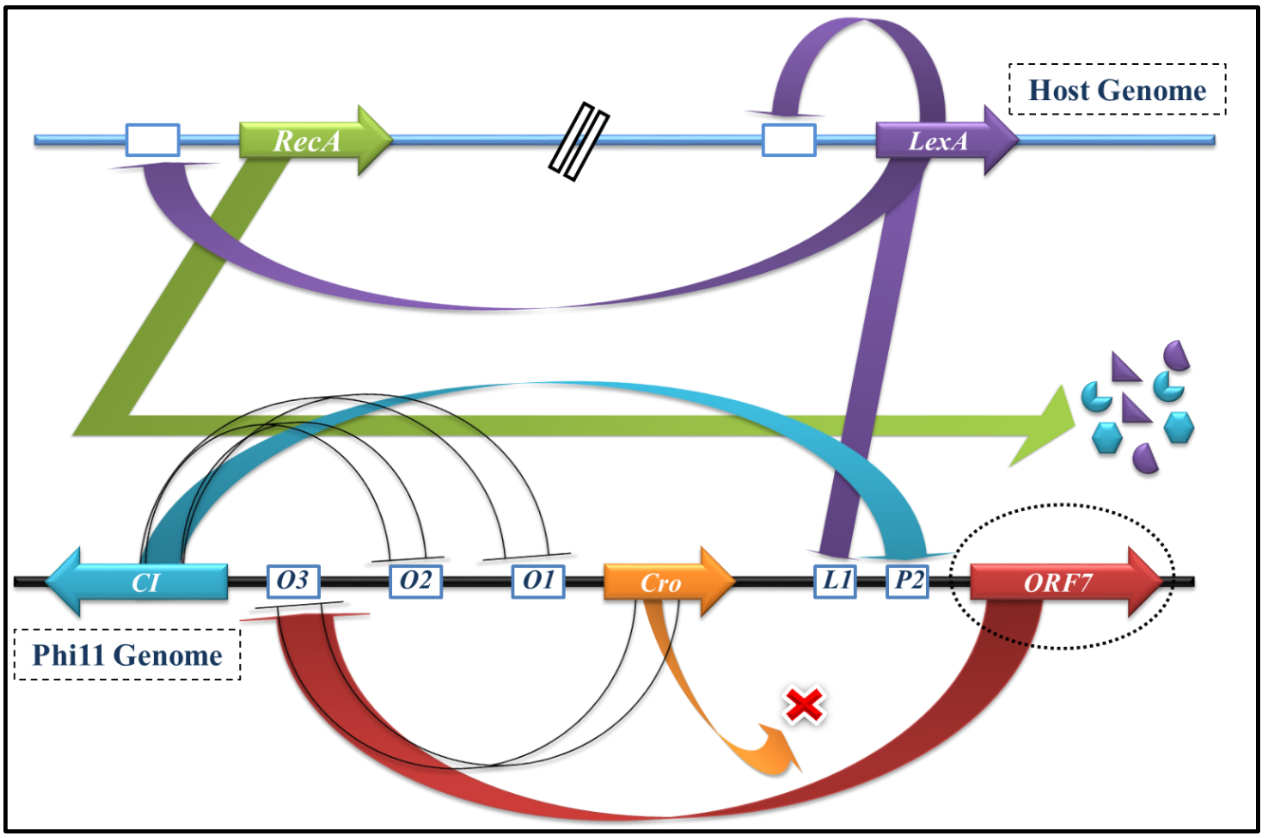

Figure 6.1. || Lytic-lysogenic developmental switch of phage Phi11. In Phi11, the expression of early genes (*cI*, *cro* and *gp07*) start as soon as the phage injects its genome into its host *S. aureus*. To maintain lysogeny, the CI repressor binds to *O*1operator followed by *O*2 and *P*2 to repress 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).