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

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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INDIA
2019

Abstract

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

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

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

rate and morphology of the host cells. However, the growth inhibition and filamentation induced by the amino-terminal domain of Gp07 is temporal in nature.

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

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

Hence the main objectives of the research proposal are:

A. Cloning, overexpression and purification of the putative antirepressor gene (ant), the Bro-N domain and the KilA-C domain.
B. Characterization of the putative antirepressor gene of Phi11.
C. Generation of deletion mutants of the Antirepressor protein.
D. Identification of the host genes involved in regulation of the putative antirepressor.

E. Studies on the toxic effects exerted by the overexpression of Phi11 ORF7 upon the host cells.
F. Deciphering the interplay between the different Phi11 proteins and host genes/proteins involved in determining the developmental pathway of Phi11.

Gaps in existing research

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

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

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 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.

Future scope of work

- Site directed mutagenesis of *cro* and *cI* will provide information regarding the amino acids involvement for the DNA-protein interaction.
- Site directed mutagenesis of Gp07 to identify the crucial amino acids involved in its active site.
- Gp07 will be further studied for its growth inhibitory action.
- Co-crystallization of Gp07-Cro-*O* DNA complex will provide in depth information about their mode of interaction.

- Tightly regulated vectors for the *S. aureus* system will be designed by utilizing the regulatory elements so identified.

List of publications related to the thesis

- **Das, A.,** Mondal, S., and Biswas, M. Gp07: A novel protein involved in the genetic switch of bacteriophage Phi11. (***Communicated*; 2019**).

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

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

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

Conferences attended

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

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

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

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

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

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

Workshops attended

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

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

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

Brief Biography of the Candidate

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

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

Brief Biography of the Guide

Dr. Malabika Biswas completed her Ph.D. in Bose Institute, Kolkata, under the supervision of Prof. Subrata Sau, as an Institute Fellow and finally as a CSIR-SRF in 2008. During her doctoral work she analysed the genetic switch involved in the developmental pathway of bacteriophage Phi11. She went on to work as a postdoctoral researcher at the Department of Biochemistry, Bose Institute, Kolkata from April 2008 to July 2009 with Prof. B. Bhattacharyya. Dr. Malabika Biswas joined the Department of Biological Sciences of K. K. Birla Goa campus as an Assistant Professor in January 2012.She has since been involved as the Principal Investigator of two research projects

funded by BRNS, and DST, as well as the co-Investigator of a DST project. Her research interests include studies on the molecular biology of temperate phages, specifically aureophages. She further studies the genes of pathogenic bacteria which are essential for host invasion. Dr. Biswas has 14 publications in reputed journals and several conference publications to her name.

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