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.

Acknowledgements

Firstly, I want to convey my heartiest gratitude to my PhD supervisor Dr. Malabika Biswas for giving me this opportunity and for her support, guidance, advice, patience and encouragement during the entire period. Her guidance helped me during my research including manuscripts writing and writing of this thesis.

In the course of my research tenure, she has constantly granted me complete freedom of thought that helped me immensely to foster my scientific attitude in a proper orientation.

Besides my advisor, I would like to express my sincere thanks to my DAC members Professor Sumit Biswas and Professor Angshuman Sarkar for immense help and support whenever required. They are always there for me, whenever I face any problem during my research study. Likewise, their immense help and review helps my research work to improve to a greater extent. I am thankful to them for giving me the access in their laboratory.

A special thanks to Professor Subrata Sau (Professor, Department of Biochemistry, Bose Institute) for allowing me in his lab and all the mental support I received from him. I also thank his research scholar Sukhendu da and Sohom for their help.

I am also very much grateful to Professor Amrita Chatterjee and Professor Mainak Banerjee from Department of Chemistry, BITS Pilani K K Birla Goa Campus for their suggestion and help with different chemicals and synthesized compounds. I profusely thank Zigmee and Viraj for their help.

I am also thankful to all the faculty members of Department of Biological Sciences, BITS Pilani K K Birla Goa Campus for their suggestion and help. I would like thank to the Director of BITS Pilani K K Birla Goa Campus for allowing and helping me to work in BITS Pilani K K Birla Goa Campus. Also, thanks to Vice-Chancellor of BITS Pilani.

I am also thankful to the Board of Research in Nuclear Sciences (BRNS), Govt. of India for providing me junior research fellowships and senior research fellowships (File No. BSC/RP 37B.12/BRNS) and financial support from DST (File No. EMR/2015/002229). BITS Pilani for the institute fellowship. Besides, I am too grateful to the Council of Scientific & Industrial

Research (CSIR), Govt. of India for awarding me with the senior research fellowship (File No. 09/919(0033)/2018 EMR-I).

My sincere gratitude to Dr. Keshab Chandra Mondal (Associate Professor & Head, Department of Microbiology, Vidyasagar University), Dr. Bikash Ranjan Pati (Professor, Department of Microbiology, Vidyasagar University), Dr. Pradeep Kumar Das Mohapatra (Assistant Professor, Department of Microbiology, Vidyasagar University) and Dr. Keya Sau (Associate Professor, Department of Biotechnology, Haldia Institute of Technology) for motivating me to take up research as a career.

I would like to thank all the research scholar of Department of Biological Sciences, BITS Pilani K K Birla Goa Campus for providing me with endless inspiration and assistance. They were always really passionate, caring, friendly and helpful. They are the reason behind every molecular biology and biophysical techniques that I learned.

I would love to express my heartiest gratitude to all my friends and especially to my friends for inspiring and motivating me. A special thanks to my wife Suhasini for always being there by my side.

Last but not the least, I would like to thank my family for their continuous psychological and motivational encouragement and unconditional love. I would never forget the love, the affection and the education provided by my parents to me which helped me to become a good person. They are the real strength of my life. This thesis would never be possible without their sacrifice and help which they did during my doctoral study.

Thank you all for making this journey memorable.

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Summary of results and discussion

Conclusion

Future scope of work

Buffer and reagent composition (Appendix A)

List of publications (Appendix B)

Brief biography of the candidate (Appendix C)

Brief biography of the supervisor (Appendix D)

List of abbreviations

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

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

Appendix A

Buffer and reagent composition

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

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

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

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

- Coomassie staining solution (100ml)

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

- 6X SDS sample loading dye (10ml)

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

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

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

- Destaining solution (100ml)

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

- 50X TAE buffer (100ml)

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

- 5X TBE buffer (100ml)

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

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

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

- 5X Bradford reagent

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

- STET buffer

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

- 6X DNA loading dye

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

- Ethidium bromide solution

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

- BCIP/NBT color development substrate, western blot

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

Appendix B

List of publications

B1. List of publications related to the thesis

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

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

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

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

B2. Other publications

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

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

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

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

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

B3. Conferences attended

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

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

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

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

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

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

B4. Workshops attended

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

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

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

B5. Scientific community membership

- Life Member, Society for Bacteriophage Research and Therapy (SBRT), India. 2019 – Present (Id. No.- SBRTLM00012).
- Affiliate Membership, Microbiology Society, United Kingdom. 2018 - Present. (Membership No: C020855).
- Basic Member, International Society for Viruses of Microorganisms (ISVM). 2018 - Present.
- Life Member, Association of Microbiologists of India (AMI), India. 2015 - Present. (Id. No: 4196-2015).

B6. Scholarships

- Awarded Council of Scientific & Industrial Research (CSIR) - Senior Research Fellowship (SRF). [May, 2018 - Present] (File No. 09/919(0033)/2018EMR-I).
- Awarded BITS Pilani, Institute Fellow (IF). [Apr, 2016-Apr, 2018].
- Awarded Board of Research in Nuclear Sciences (BRNS) - Junior Research Fellowship (SRF) [Sep, 2013 - Sep, 2015], Senior Research Fellowship (SRF) [Sep, 2015 - Mar, 2016]. (File No. BRNS:BSC/RP37B.12/BRNS).

Appendix C

Brief Biography of the Candidate

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

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

Appendix D

Brief Biography of the Guide

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

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