Physicochemical studies on the mutants of the VC0395_0300 protein of *Vibrio cholerae*

THESIS

Submitted in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

by

Om Prakash Chouhan

Under the Supervision of
Dr. Sumit Biswas
and
Prof. Udo Heinemann

BITS Pilani
Pilani | Dubai | Goa | Hyderabad

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN) INDIA
2018

Abstract

Vibrio cholerae, the cause of seven noted pandemics, leads a dual lifecycle which switches between a virulent form in human hosts, and a sessile, non-virulent form in aquatic bodies in surface biofilms. Bacterial infections always proved to be difficult to eradicate when they form a bacterial biofilm, which gives an extra advantage to bacteria to survive in harsh conditions. Research on bacterial biofilm formation and motility has been gaining increasing medical attention because biofilms represent a mode of survival for numerous pathogenic bacteria like *V. cholerae, Salmonella typhimurium,* and *Staphylococcus aureus*. Consequently, biofilm infections are difficult to treat with conventional drugs. Biofilm gives an extra level of protection to the bacteria to fight against all possible detrimental conditions such as change in osmolarity, protozoan predation, radiation, pH and nutrient availability. The formation of the biofilm is associated with the secretion of an exo-polysaccharide, which in turn, has been shown to be under the regulation of c-di-GMP levels in the cell. The c-di-GMP molecule synthesized by the diguanylate cyclase activity of GGD(/E)EF domain proteins, is universally reported in all bacterial groups. Diguanylate cyclases have an important role to play in the lifestyle switching mechanisms of bacteria like *V. cholerae* by involving in the stimulation and maintenance of surface biofilms. The cellular functions regulated by c-di-GMP include cell motility, cell cycle progression, virulence, biofilm formation, antibiotic productions and other unknown functions. The ability to produce c-di-GMP by the utilization of GTP makes GGEEF domain containing enzymes completely essential for the lifecycle of the bacteria. The VC0395_0300 protein from chromosome 1 of the *V. cholerae* classical strain O395, serotype O1 has been established in our ViStA lab to be a diguanylate cyclase with a necessary role in biofilm formation. This thesis work reports the mutations in the central positions of the GGEEF active site of the VC0395_0300 protein by site-directed mutagenesis. The conditions for maximum production of mutated protein have been optimized. All the mutated proteins were biophysically characterized to analyze their structural and biochemical features. For instance, the mutant proteins have been studied using spectrofluorimetry and circular dichroism spectroscopy. Diguanylate cyclase activities for all mutated proteins were determined by HPLC assay method. While there is a significant loss-of-biofilm-forming activity in the mutants, the basis for the same needed an investigation at the structural level. The wild type and mutated proteins have been crystallized to reveal the GGEEF domain structure, understanding the reaction mechanism for c-di-GMP synthesis and steric inhibition for the diguanylate cyclase activity. The overall structure

of the protein does not show significant changes due to the mutagenesis, despite the loss of biofilm formation in the mutants.

In conclusion, the major aspects of the thesis are as follows:

- Functional and biophysical characterization of the mutant proteins of VC0395_0300, compared with the wild type protein.
- Structure elucidation of the GGEEF domain in VC0395_0300.
- Role of mutations and the importance of amino acids in the GGEEF active site.

Gaps in existing research

Based on available research data about GGDEF domain protein we found following gaps in existing research:

- The GGD(/E)EF domain, through ubiquitous in bacteria but it is still a relatively novel domain with very little information about its functional sites (Active site and Inhibitory site) and folding status available.
- With respect to *V. cholerae,* this domain has been found in both the chromosome of *V. cholerae* GGD(/E)EF domain protein. Nobody has ever attempted to mutate any of the signature amino acids in the domain and check for the consequences.
- No one has tried to crystallize this novel protein to find accurate protein structure, its folding status, and arrangement of signature amino acids in GGD(/E)EF domain. In the absence of crystal structure prediction of activity by bioinformatics method is also not feasible.
- In the absence of crystal structure of this novel protein information regarding the binding of substrate molecule GTP to the active site and binding of c-di-GMP as a regulatory molecule to this domain is still not available.

Objectives of the proposed research:

Therefore, to fulfill the existing gaps in research about the GGDEF domain protein, the following research objectives are proposed for this study:

- Cloning of mutants of *VC0395_0300* gene in *E. coli*.
- Expression and purification of mutant proteins.
- Biophysical characterization of VC0395_0300 mutant proteins.
- Structure elucidation of VC0395_0300 mutant proteins.

Summary of the results

The work from this thesis therefore, highlights the importance of the previously putative protein VC0395_0300 from *V. cholerae* and its mutant entities. I proceeded with my research work using VC0395_0300 protein and its mutant clones in *E. coli.* These were subsequently analyzed for functional and structural features of the protein along with comparisons of mutant proteins. The conclusions of this study are summarized here:

Chapter1: Introduction and review of literature

In the first chapter, I described about *V. cholerae,* cholera disease history, route of transmission, its life cycle and why vaccinization for cholera disease is difficult. Biofilm formation in *V. cholerae* was intensely described with all steps during bacterial biofilm formation. The intricate details about the c-di-GMP molecule, history, role in biofilm formation, the effect on bacterial virulence and effect on microbial physiology have been deliberated. The *V. cholerae* genome, with its multiplicity of GGEEF domains was also highlighted.

Chapter2: Site-directed mutagenesis of *VC0395_0300* gene and cloning in *E. coli.*

In this chapter, I explained experiments about site-directed mutagenesis of *vc0395_0300* gene. Genomic DNA of *V. cholerae* serotype O1 classical strain O395 was isolated and served as a template DNA in PCR reaction. Four point mutations were individually made at the GGEEF active site of *VC0395_0300* gene using PCR based site-directed mutagenesis. In each of the mutations, one amino acid at the GGEEF sequence was replaced by another amino acid. All four mutated insert DNAs were separately ligated into cut pGEX-6P1 vector and four mutated clones were constructed. Mutated clones were effectively transformed into host *E. coli* strain DH5α and expression host BL21 (DE3).

Chapter3: Expression and purification of mutant proteins

Here, I described experiments for protein expression methods, and small-scale overexpression of mutant proteins. The optimizations for overexpression of all proteins were achieved by checking at different IPTG concentrations and induction time. It was observed that the VC0395_0300 gene was not able to express as a soluble protein with pET-28a (His-Tag containing) vector, but with pGEX-6P1, soluble protein coule be obtained. Various protein constructs for VC0395_0300 protein were also prepared by systematic

truncations (based on secondary structure predictions) and conditions for maximum soluble protein overexpression was optimized.

Chapter4: Biophysical characterization of VC0395_0300 mutant proteins

This chapter deals with comparative studies of all mutant proteins versus the wild-type VC0395_0300 protein. For functional characterizations, all mutated strains along with wild-type were analyzed for biofilm formation ability and bacterial motility. All mutant strains showed significantly decreased biofilm formation and increase in bacterial motility (predominantly in $VC0395\_0300_{(G237R)}$ and $VC0395\_0300_{(E238K)}$). Diguanylate cyclase activity results showed that mutations in active site amino acids do not abolish product formation completely, but reduce the activtity significantly. All mutated proteins were therefore able to produce c-di-GMP, but with a much-reduced intensity of product peak. This was indicative of a partial loss-of-function for the protein. Secondary and tertiary structures of the VC0395_0300 mutant proteins do not alter significantly as seen from overall similar structural features in fluorescence spectroscopy and CD spectroscopy.

Chapter5: Structure elucidation of VC0395_0300 and its mutant proteins

In this chapter, I elucidated the protein structural features by X-ray crystallography. The full-length VC0395_0300 protein proved difficult to crystallize, due to the presence of a flexible region in the N-terminal end. So, I attempted to generate truncates of the full-length protein by shortening of the N-terminal flexible part and generate four different protein constructs of VC0395_0300 protein. The $VC0395\_0300_{(161-321)}$ truncated protein was successfully crystallized, which showed the arrangement of α1- α2- β1- α3- α4 - β2- β3- α5- β4- β5- β6- α6- β7 sheet and helices in their active sites, with 11% of the total accessible solvent area. One asymmetric unit of $VC0395\_0300_{(161-321)}$ protein possesses four polypeptide chains in it. Two mutant proteins $VC0395\_0300_{(G237R)}$ and $VC0395\_0300_{(E238K)}$ were also crystallized with similar architecture, but showing the presence of arginine at 237^{th} position and lysine in the 238^{th} position in the A-site. Both the mutant proteins show a steric obstruction due to the extension of the extra side chain in the active site for binding the substrate GTP molecule.

The work from this thesis therefore, highlights the importance of the previously putative protein VC0395_0300 from *V. cholerae.* The central amino acids of the inherent GGEEF domain of this protein have an immense importance in the functional activity of this

protein. Mutations in the amino acids G (237) and E (238) seemed to have the maximum effect, resulting in a significant loss of diguanylate cyclase activity of this protein. Though the loss of activity was not associated with a complete structural rearrangement of the entire protein (as predicted by indirect structural methods like spectrofluorimetry and CD spectroscopy), the mutations appeared to have created some subtle changes which debilitated the protein. Since the protein does not have a I-site or inhibitory site to regulate c-di-GMP formation, the mystery was further deepened.

The answers came to light when the crystal structure of the wild type and mutant proteins were solved. The presence of an obstacle in the postulated path-of-entry of GTP to bind the GGEEF signature motif pointed to a mechanism for the loss of activity in the mutants. The steric hindrance may also inhibit the activity of the protein by not synthesizing c-di-GMP. More about the mechanism could be elucidated if the attempted crystallizations with GTP were successful, but the crystals with GTP proved to be always elusive. Nevertheless, the work done in this thesis presents a novel finding of the function and structure of the VC0395_0300 protein from *V. cholerae*.

Future Scope of the work:

- Crystallization of the wild type and mutant proteins with the GTP and c-di-GMP in their respective sites.
- Design of diguanylate cyclase deficient *V. cholerae* and insertion of mutated *vc0395_0300* inserts.
- Examination of the roles of the PAS and PAC domains in the functional activity of the protein.
- Response of deletion mutants to environmental stimuli.

Publications from PhD thesis

Subtle changes due to mutations in the GGDEF domain result in loss of biofilm forming activity in the VC0395_0300 protein from *Vibrio cholerae*, but no major change in the overall structure.
Protein Peptide Letter (2018), DOI: 10.2174/0929866525666180628162405
Om Prakash Chouhan, Sumit Biswas

Putative protein VC0395_0300 from *Vibrio cholerae* is a diguanylate cyclise with a role in biofilm formation.
Microbiological Research (2017), DOI: 10.1016/j.micres.2017.05.003
Divya Bandekar, **Om Prakash Chouhan**, Swati Mohapatra, Mousumi Hazra, Saugata Hazra, Sumit Biswas.

Effect of Site-Directed Mutagenesis at the GGEEF Domain of the Biofilm Forming GGEEF Protein from *Vibrio cholerae*.
AMB Express (2016), DOI: 10.1186/s13568-015-0168-6
Om Prakash Chouhan, Divya Bandekar, Mousumi Hazra, Ashish Baghudana, Saugata Hazra and Sumit Biswas

Structural studies of the active GGEEF domain of VC0395_0300 from Vibrio cholerae. **(In revision)**.
Om Prakash Chouhan, Divya Bandekar, Yvette Roske, Udo Heinemann, Sumit Biswas

Workshop and Conferences

INTERNATIONAL CONFERENCE on "Trends in Biochemical & Biomedical Research – Advances and Challenges (TBBR-2018) organized by Banaras Hindu University, Varanasi, INDIA in Feb 2018.

NATIONAL WORKSHOP on "DBT BIRAC Workshop Bio-Entrepreneurship – Grant Writing & Intellectual Property Management" organized by BITS Pilani KK Birla Goa Campus, INDIA in Feb 2016.

NATIONAL WORKSHOP on "An Introduction to Practical NMR Spectroscopy" organized by National Chemical laboratory (NCL) Venture Center, Pune, INDIA in Aug 2015.

NATIONAL CONFERENCE on "Gel-based Proteomics" organized by "PROTEOMICS SOCIETY, INDIA (PSI)" held at Indian Institute of Technology (IIT) Mumbai, INDIA in Dec 2014.

INTERNATIONAL CONFERENCE on "Biology of natural toxins", annual conference of the toxinological society of India, organized by BITS Pilani KK Birla Goa Campus, INDIA in Dec 2013.

Brief Biography of the Candidate

Om Prakash Chouhan joined BITS Pilani K K Birla Goa campus after qualifying the PhD examination as a PhD student or research scholar in the Department of Biological Sciences in the year of 2013. Before he joined PhD, he completed his Master degree in microbiology from MDS University Ajmer (Rajasthan) in the year 2008. After completing his master degree he finished his M.Phil from same department and joined one project as a junior research fellow funded by the ministry of environment and forest New Delhi. He has also cleared CSIR NET (2010), ICAR NET (2010) and GATE (2011) national level exam. Om Prakash was awarded DAAD fellowship in year of 2016. He has spent the 15 months in MDC, Berlin under a DAAD Scholarship program. He is also assisting the faculty for various labs as a teaching assistant. He has worked in protein structural biology and X-ray crystallography. He has successfully crystallized three difficult proteins and solved their protein structures. He has published three research articles in peer review journal.

Biography of the Supervisor

Dr. Sumit Biswas completed his Ph.D. in Bose Institute, Kolkata, under the supervision of Prof. Pinak Chakrabarti, as a CSIR fellow in 2008. His doctoral work elucidated the interfaces of protein-nucleic acid interactions, as well as the structure determination of two very important proteins. He went on to work as a DBT Research Associate in the DBT initiative, “Setting up of National Facility on Interactive Graphysics Computer System for Biomolecular Modelling, Molecular Dynamics & Structures” till 2009. Dr. Biswas joined BITS, Pilani, K K Birla Goa Campus as a faculty in 2009. He has since been involved as the Principal Investigator of four research projects funded by BRNS, DAE, DBT and DST, as well as the co-Investigator of a UGC project. His work in the institute involves the molecular mechanism and biology of the Vibrio life cycle, bioinformatics of non-coding RNA and protein-nucleic acid interactions, and therapeutic biology of natural products. Dr. Sumit Biswas has 16 publications in reputed journals and several conference publications to his name. He is also working on a book on Biophysics sanctioned by Prentice Hall of India.

Dr. Biswas has acted as the convenor for 4 meetings/symposia and workshops funded by DST, DBT, DSTE, Goa and BRNS. He is a life member of the Indian Crystallography Association, and a member of CholdInet (a WHO initiative for cholera research) and the

Proteomics Society of India. He has received several awards and honours, the most recent being the prestigious EMBL Scholarship for presenting paper at EMBL Conference on Cancer Genomics, held at Heidelberg. Besides, he has delivered invited talks at different international conferences as well as institutes of repute like IIT, Kharagpur, IIT-BHU, etc. He has been actively involved as a reviewer of international journals from OUP, Elsevier, etc., as well as a question setter for DBT. Presently, he has three registered Ph.D. students under his tutelage and numerous thesis dissertation and project students working with him. He has also been the certified Radiological Safety Officer for the Institute, and is instrumental in setting up biosafety practices in BITS.