CHAPTER 35

MICROBIOLOGY

Doctoral Theses

01. BABBAL
Bioprocess optimization studies of recombinant ubiquitin-like proteases (Ulps) from Schizosaccharomyees pombe in E.coli.
Supervisors: Prof. Mandira Varma Basil
Th 26793

Abstract

Small ubiquitin-related modifier (SUMO) protein is efficiently used as a fusion partner to target the soluble production of various difficult-to-express proteins in E. coli. However, the use of the SUMO tag is majorly constrained due to SUMO protease`s high cost and poor stability (Ulp). The Ulp proteases recognize the tertiary structure of SUMO and specifically cleave after the di-glycine motif, thereby generating protein-of-interest with an authentic N-terminus. This study highlighted the identification and characterization of two novel SUMO proteases, i.e., Ulp1 and Ulp2 from Schizosaccharomyces pombe and compared their catalytic efficacy with S. cerevisiae Ulp1. Bioinformatics tools were used to obtain the sequence and to predict the structural features of the SpUlp1 and SpUlp2 catalytic domains. From protein-protein interaction studies, SpUlp1 exhibited a higher affinity towards SUMO than ScUlp1. The SpUlp1 catalytic domain was purified with an 83.33 % recovery yield. In vitro activity data showed the fast-acting nature of SpUlp1SD compared to ScUlp1, where a 90 % cleavage of SUMO from SUMO-GFP was obtained in 1 h, thus indicating the commercial importance of novel SpUlp1SD. Biophysical characterization showed 8.8% α-helices, 36.7% β-sheets in SpUlp1SD, where the Tm was found to be 45 °C. Further, high cell density cultivation using the fed-batch fermentation strategy resulted in 4.8 g/L of SpUlp1SD production with YP/X of 70.588 mg/g DCW and volumetric productivity of 300 mg/L/h. The specific product formation rate reached a maximum value of 37.3 mg/g/h at 3 h post-induction. Thus, the high-level production of the fast-acting SpUlp1 catalytic domain could lead to the economical production of various SUMO fusion proteins. The enzyme immobilization was also targeted to reduce the production cost of recombinant protein. The SpUlp1SD immobilized on chitosan-coated iron oxide nanoparticles displayed high recyclability, stability, and activity for up to 100 repetitive cycles. The FRET assay confirmed the high catalytic efficiency of the enzyme produced in the study with Kcat 9.08 s-1 and Kcat/KM ratio of 1.39 × 106 M-1s-1. The Kcat value was relatively high, confirming the more significant interaction between the SpUlp1 and SUMO. Thus, the highly efficacious SpUlp1SD enzyme developed in the study holds enormous potential as a technology to reduce the production cost of soluble recombinant proteins with authentic N-terminus.

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Immobilization of SpUlpl catalytic domain. 8. FRET assay for determination of SpUlpl kinetic parameters. 9. Summary and Conclusion 10. Bibliography.

02 CHANCHAL KUMAR
Functional analysis of cell intrusion proteins of Mycobacterium tuberculosis as potential target for vaccine development.
Supervisors: Prof. Mandira Varma Basil
Th 26795

Abstract

During the course of infection M. tuberculosis subverts the host immune response through numerous immune-evasion strategies. Gaining entry inside the host cells is crucial for establishing intracellular infection and survival within macrophages. There are several aspects of macrophage-mycobacterium interactions, including binding to macrophage via surface receptors (Schlesinger et al..1994), Phagosome-lysosome fusion (Gutierrez et al., 2008), mycobacterial growth inhibition via free-radical mechanisms (Lamichhane, 2011) and cytokine-mediated mechanisms to recruit accessory immune cells (Raja, 2004). M. tuberculosis has an ability to skip the immune response and invade macrophages. However the primary event in the pathogenesis of mycobacteria, is invasion of host cells. The analysis of the complete sequence of the m. tuberculosis h37Rv genome revealed the presence of genes involved in cell invasion. Four homologs of mammalian cell entry (mce 1-4) genes were observed in the M .tuberculosis genome sequence, all encoded in an operon structure consisting of six genes (mceA, mceB, mceC, mceD, mceE, mceF). There is in increasing evidence showing that these operons are also important for the virulence of the M. tuberculosis complex species) Ahmad et al., 2005; Uchiya et al., 2013) the first indication came from Arruda et al. who described an invasion-like gene, later known as mcelA that conferred on non- pathogenic E. Coli the ability to invade and survive within macrophages and human HeLa cells (Arruda et al., 1993) surprisingly, the disruption of one of these operons, mce1, causes M. tuberculosis to become hypervirulent (Shimono et al;, 2003), whereas the mce3 and mce4operon mutants are attenuated in mice model(senaratne et al., 2008) M. tuberculosis H37Rv mce2 operon deletion had no effect on bacterial growth in 7H9 liquid broth or survival within macrophages (Marjanovic et al., 2010). Previously it has been demonstrated in our laboratory tha mce4A when expressed I non-pathogenic E. coli enabled it to enter and survive within HeLa cells and differentiated THP-1 cells respectively (Saini et al., 2008)

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1. Introduction. 2. Review of Literature. 3. To explore the cell surface intrusion proteins of Mycobacterium tuberculosis through bioinformatics analysis. 4. To study the change in expression of in Silico selected genes involved in Establishment of Mychobacterium tuberculosis infection in Macrophage. 5. To study the effect of a panel of the candidate cell intrusion proteins on bacterial invasion and survival using and over expression approach. 6. To study immunogenicity of a panel of the candidate cell intrusion proteins of M. tuberculosis Ex vivo. 7. Summary and Conclusion. References, Appendix and List of Publications.

03. CHAUDHARI (Yash)
Role of peste des petits ruminants (PPR) virus coded Nucleocapsid (N) protein in the modulation of Autophagy pathway.
Supervisors: Prof. Rajeev Kaul
Th 26798

Abstract

Viruses have evolved to escape from or modulate the host defense mechanisms for the establishment of an infection. Similarly, Peste des petits ruminants virus (PPRV) induces autophagy by interacting with components of defense system of the recipient host. In India, PPRV causes economically important livestock disease in small ruminants with high mortality and morbidity. Therefore, a better understanding of the virus role in disease pathogenesis is essential to control and prevent the spread of the disease. In the present study, the proteomics studies through LC-MS analysis indicated the interaction of PPRV-N protein with the multiple core components of cellular autophagy pathway i.e. VPS34, BECN1 and VPS15. These were further analyzed by STRING and CYTOSCAPE online databases. These interactions were validated by co-immunoprecipitation assays and GST-pulldown assays. Co-immunoprecipitation data clearly showed that the viral N protein and PI3K complex-I proteins interact with each other stably in whole cell lysates. However, GST-pulldown assay showed that PPRV-N protein interacts specifically with VPS34 of the PI3K complex-I. Furthermore, the co-localization studies using confocal microscopy indicated that the viral protein co-localizes with these cellular proteins in the cytoplasm. Their interaction with PPRV-N protein did not affect the sub-cellular localization of the host proteins. The functional significance of these interactions was analyzed by detecting the presence of autophagosomes, by monitoring the autophagic activity, and transmission electron microscopy (TEM). Immunoblot analysis of SQSTM1/62 and LC3B indicated that the expression of N protein of PPRV resulted in increase in the cellular autophagic activity. The formation of typical autophagosomes with double membranes was identified morphologically by TEM that confirmed induction of autophagy. Together, our findings provided the evidence that N-protein of PPR virus has a critical role in the induction of autophagy mediated by PI3K complex of the host.

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04. GULATI (Pallavi)
Characterization of Picrophilus torridus Type I modification methylase M.Ptol.
Supervisors: Prof. Mandira Varma Basil
Th 26794

Abstract

DNA methylation is a widespread epigenetic modification that is universal among all domains of life. Methylation is carried out by group of enzymes called DNA methyltransferases that utilize S-adenosyl methionine (SAM) as methyl group donor. In prokaryotes, role of DNA methylation has been implicated in a myriad of cellular processes including DNA replication, DNA repair, gene regulation etc. Apart from this DNA methylation is also known to play a vital role in the protection of host DNA from the cognate restriction endonuclease. Restriction modification systems are one of the survival strategies used by bacteria and archaea to protect themselves from the bacteriophage attack. The general phenomenon of Restriction Modification system involves antagonistic activity by pair of two opposing enzymes – restriction endonuclease and methyltransferase that recognise specific DNA sequences. Restriction endonuclease cleaves the incoming foreign DNA of bacteriophage whereas, the host genome is protected from cleavage by restriction endonucleases with the aid of cognate

methyltransferase that methylase the host DNA. We commenced our study with the examination of *P. torridus* genome for the presence of DNA methylation. The genome of *P. torridus* lacked m5C modification, however m6A methylation was present. We further searched the genome sequence of *P. torridus* on KEGG database for the possible DNA adenine methyltransferases responsible for this m6A methylation. Only one DNA adenine methylase was annotated: PTO1076. This was originally identified as the Dam methylase based on sequence homology with other Dam methylases. However, we found that *P.torridus* genome did not carry GATC methylation despite of presence of putative Dam methylase. Annotation of the whole genome sequence of *P. torridus* showed that it harbour one Type I, three Type II and one Type IV RM system along with one orphan DNA methyltransferase. Among these, the recognition sequence of one Type II system designated as PtoORF585P has been decoded as 5′-CGCG. Till date all Type I modification methyltransferases are known to methylate adenine residue. In the light of these facts, the work embodied in this thesis aims to characterize Type I modification methylase viz. M.PtoI from the thermophilic archaeon *P. torridus*. This is the first study investigating a DNA adeninemethyltransferase in *Picrophilus torridus*. The growth of *P. torridus* was studied at different pHs at 55°C and optimal growth was recorded at pH 0-1. Upon analysis of the amino acid sequences, we found that both the subunits of M.PtoI (PtS and PtM) carried the motifs that typify conventional methylation (hsdM) and specificity subunits (hsdS). The structural details of both the PtS and PtM subunits were examined via homology modelling was done by Phyre 2 tool. PtM carried all the motifs conserved in M subunits including Motif I and Motif IV that are critical to AdoMet binding and catalysis espectively. PtM also harboured α/β folds which is the signature of the classical Rossmann fold present in all AdoMet dependent methyltransferases. PtS was found to be structurally similar to the hsdS subunit of the Type I Restriction Modification system omprising two globular target recognition domains (TRDs) bifurcated by two conserved helical regions. In pursuit of the possible zone of interaction between PtS and PtM subunits in M.PtoI, template-based docking was done via structural superimposition using EcoR124I as the template. PtM was found to interact via C terminal region to the central conserved region (CCR) of PtS subunit. These findings were experimentally verified by clipping off the C terminal region of PtM and replacing 12 amino acids from the conserved region of PtS subunit. Removal of C terminal region of PtM hampered its interaction with PtS subunit. Similarly, replacement of 12 amino acids from the conserved region of PtS also led to compromised interaction between PtS and PtM subunits. The enzymatic properties of M.PtoI were examined via *in vitro* methylation assays.M.PtoI displayed optimal activity at pH 5 between 55-60°C, at low salt conditions. This enzyme was more or less equivalently active over 5-25 mM magnesium acetate and 25-500 µM AdoMet concentration. With the aim to study the role of Motif I and Motif IV of PtM subunit in AdoMet binding and catalysis respectively, both the motifs were mutated by site directed mutagenesis using overlap PCR and thereafter methylation activity of the mutant enzymes was examined. We observed abrogated activity of M.PtoI wherein Motif I and Motif IV of PtM was mutated as compared to that of the wild type. Mutation of Motif I also affected the AdoMet binding ability of M.PtoI as we observed loss of activity at low AdoMet concentration. The findings presented in this thesis suggest that M.PtoI contribute to the adenine methylation in *Picrophilus torridus*.

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05. JAIN (Juhi)
Role of *Peste des Petitis ruminants* (PPR) Virus coded C and V Proteins in the Modulation of NF-kB Signalling.
Supervisor: Prof. Rajeeev Kaul
Th 27254

Abstract

Peste des petits ruminants, a contagious and economically vital disease of sheep and goats caused by an RNA virus PPRV (Peste des petits ruminant virus). The virus belongs to the family Paramyxoviridae under the genus Morbillivirus. The disease impacts the lives of the farmers, whose livelihood depends on these ruminants. The OIE and FAO together launched an eradication program to eradicate the virus by 2030. Peste des petits ruminants virus (PPRV) is known to induce transient immunosuppression in infected small ruminants by modulating several cellular pathways involved in the antiviral immune response. Our study confirms the interaction between NF-κB p65 subunit and PPRV non-structural proteins. The PPRV-V protein interacts with the Rel homology domain (RHD) of the NF-κB p65 subunit while the PPRV-C protein interacts with the transactivation domain (TAD). Both the non-structural proteins have been found to suppress the NF-κB transcriptional activity and NF-κB-mediated transcription of cellular genes. Moreover, PPRV-V protein expression can significantly inhibit the nuclear translocation of NF-κB p65 upon TNF-α stimulation, whereas PPRV-C does not affect it. The NF-κB-mediated pro-inflammatory cytokine gene expression is significantly downregulated in cells expressing PPRV-C or PPRV-V protein. Our study provides evidence suggesting the role of PPRV non-structural proteins V and C in the modulation of NF-κB signalling through interaction with the NF-κB p65 subunit.

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06. NANDI (Tanusri)
Study of Innate Immune Mechanism through Small Molecules against Influenza A virus Replication.
Supervisors: Prof. Madhu Khanna and Prof. Nirupama Trehanpati
Th 27124

Abstract

Influenza A virus (IAV) an enveloped, segmented, negative strand RNA virus belongs to the family of *Orthomyxoviridae*, a major respiratory pathogen and responsible for causing number of outbreaks globally, which have high morbidity and mortality rates. Presently, there are three classes of anti-influenza drugs with different antiviral mechanisms, including M2 ion channel blockers, viral RNA synthesis inhibitors and neuraminidase (NA) inhibitors are approved. Oseltamivir, a neuraminidase (NA) inhibitor, is widely used to treat influenza A virus infections. However, influenza A virus that are resistant to oseltamivir are gradually emerging, thus limiting the utility of NA inhibitors. During IAV infection, viral and host proteins interact and utilizes an array of innate immune signaling pathways for its replication and immune evasion. Hence, it is important to develop an alternate therapeutic option and its ability to modulate host cellular pathway. The present study involves *in-vitro* assessment of small molecules for anti-influenza activity and to evaluate the expression of innate

immune pathways like autophagy, NFκB and MAP kinase in response to treatment. Suramin and thymol was chosen for the based on *in-silico* binding with HA protein of influenza A/Puerto Rico/8/1934 (H1N1). Molecular docking using CB-DOCK showed that both suramin and thymol demonstrated superior binding with HA subunit of IAV and having binding energy of -8.3 kcal/ mol (suramin) and -5.9 kcal/mol (thymol) respectively. Treatment with suramin showed significant downregulation of viral RNA and protein at 250 µg/ml and 125 µg/ml. Similarly, thymol also showed inhibition at 50 and 25 µg/ml. As suramin demonstrated better anti-viral activity compared to thymol, hence, suramin was chosen to evaluate the modulation of innate immune pathways. Suramin also demonstrated improved efficacy compared to oseltamivir. We further analyzed translocation of NF-κB and expression of pro-inflammatory cytokines and ISGs in response to suramin treatment. Restricted translocation of P65 subunit of NF-κB was observed at 250 µg/ml and 125 µg/ml of suramin treatment. Similarly, expression of pro-inflammatory cytokines and ISGs was reversed to that of control upon treatment with suramin. Activation of autophagy, an important pathway in viral pathogenesis, was analyzed upon treatment with suramin. Autophagic markers like LC3-II, ATG5, ATG7 and P62 was validated. We also observed that influenza induced autophagy is a ATG5 dependent process. Suramin reversed the expression of influenza induced autophagy markers. Similar results were obtained when treated with rapamycin, an autophagy inducer. Another important pathway, mitogen-activated protein kinase (MAPK) pathway has role in IAV pathogenesis. Infection with IAV lead to activation and phosphorylation of P38 and JNK. Treatment with suramin inhibited phosphorylation of P38 and JNK pathway at 250 µg/ml and 125 µg/ml. However, no changes were observed on ERK expression. Overall, the data showed that suramin had a strong anti-viral response against A/Puerto Rico/8/1934 (H1N1) in A549 cells. Suramin was capable of modulating autophagy, NF-κB and MAP kinase pathway indicating that suramin could be potential anti-influenza molecule.

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07. SINGH (Praveen Kumar)
Role of Myeloid derived suppressor cells (MDSCS) in regulation of T cells in pemphigus vulgaris.
Supervisors: Dr. Shukla Das
Th 26797

Abstract

Over the course of several years, we have achieved great breakthroughs in the advancement of immunology domains. MDSCs are critical players in the immunosuppression associate with various inflammatory and autoimmune diseases. MDSCs regulate the T cell response via various mencanism, especially through the Arg1 and iNOS *enzyme* production and also by secretion of various soluble mediators like IL-10 GM-CSF and TGF-β. (Groth et al., 2019). The soluble mediators play role in the development, recruitment and expansion of MDSCs. Researchers have investigated MDSCs in various diseases like infractions, GVHD, cancer and autoimmune diseases. MDSCs originated from IMC which can be differentiated in to two type of cells mMDSCs and gMDSCs subset frequency was not elevated in our study. In various autoimmune diseases, the elevation of the MDSCs Population has been reported. When we examined the, mechanism by which MDSCs regulate T cells we found in pemphigus the Arg1 mRNA transcription level was elevated significantly. Arg-1 enzyme depletes arginine by using it as a substrate to create urea and

ornithine. Arginine is the essential amino acid required for the T Cell development and proliferation. We also examine the expression level of iNOS, TGFβ and il10. The expression level of these genes were not elevated significantly. Additionally, we found at a variety of chemical inhibitors as well as blocking antibodies in vitro, we found at greater MDSCs cells ratio to t cell did immunosuppression effectively Based on our observation of greater transcript levels of arg-1 we imply that arg-1 is one of the primary inhibitory mechanisms involved in MDSC- mediated inhibition of T-cell proliferation in Patients with PV.

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08. SHARMA (Varshni)

Investigating the role of SET29 Protein in the Protozoan Pathogen *Leishmania donovani.*

Supervisors: Prof. Swati Saha

Th 27253

Abstract

The SET domain protein family is characterized by evolutionarily conserved SET domain with the inherent ability to methylate histone and non-histone substrates, which provides cells a stringent control over various cellular processes including DNA replication, gene transcription and DNA repair. The human pathogens, trypanosomatids like Leishmania, have atypical cellular biology as compared to other eukaryotes and to find new drug targets, their cellular biology is being studied by various research groups. There are 29 SET domain proteins which have been identified in Leishmania spp. This study presents some of the first data examining the functional role of these proteins in L. donovani the causative agent of visceral leishmaniasis (kala azar). The work presented in this thesis was performed to elucidate the role of the LdSET29 protein. We have adopted the strategy of creating genomic knockouts of the set29 gene, followed by analysis of the resultant phenotypes. We were able to successfully knockout two alleles of set29 from the the L.donovani genome. However, a copy of set29 gene remained in the parasites's genome, suggesting that the gene may have gotten inserted elsewhere in the genome after being resected by homologous recombination. This also suggests that this gene is essential for the cell. Current efforts are being directed towards creating a set29-null in cells expressing SET29 ectopically, to confirm the essentiality of the gene. The depletion of LdSET29, modulated the parasite's response towards oxidative stress, as the LdSET29-depleted parasites were able to endure in vitro oxidative stress better than the wild-type parasites. This behaviour was also reflected in host macrophage's in vivo oxidative milieu, where the LdSET29-depleted parasites were able to thrive and propagate better than the wild type parasites. We also discovered that LdSET29 interacts with another SET domain protein, LdSET1, which is also involved in the parasite's response towards oxidative conditions. This interaction was identified in in vitro conditions, and confirmed to occur in vivo as well. These results suggests that by regulating the parasite's response to an oxidative environment, LdSET29 controls parasite survival in host cells such that infection can be established and be maintained appropriately, without eradicating the host cell population, it needs for survival. The findings from this thesis work underscore the several layers of regulation involved in maintaining host and parasite in infection.

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09. SHRIVASTAVA (Kamal)
Evaluation of an array of *pe/ppe* Genes for Potential Use in a Diagnostic Assay to Identity *Mycobacterium Tuberculosis.*
Supervisors: Prof. Mandira Varma Basil
Th 26796

Abstract

Accurate diagnosis of the causative organism in individuals with TB or TB-like symptoms is essential for successful treatment and transmission prevention Misdiagnosis can lead to unnecessary therapy, poor health, and higher medical expense (Kambashi et al., 2001). TB and be diagnose, particularly In active pulmonary infection, Primarily by suspected clinical symptoms, chest radiography, evaluation of sputum smears for AFB, culture of bacteria from sputum or pulmonary secretions and through immunological tests (Grange et al., 1990; Brodie et.al., 2005) the emergence of molecular tools such as the Xpert MTB/RIF, LPA, and L AMP test, as well as the availability of whole genome sequencing methods for these organisms, now allows for more sensitive and quick diagnosis and distinction among the numerous mycobacterial species. However the expense and specialized infrastructure needs of tis cutting-edge technology have precluded it from reaching the world's more remote locations. This has prompted the hunt for better, more reaching the world's more remote locations. This has prompted the hunt for better, more user-friendly, and economically feasible molecular assays that may be employed in regions of the world a high prevalence of mycobacterial infections.

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