CDFD

Annual Report

April 2007 to March 2008

(English version)

CDFD

Center for DNA Fingerprinting and Diagnostics

Hyderabad

CDFD MANTRA

“We dedicate ourselves to achieve world class excellence in basic research and simultaneously endeavour to transfer the benefits of modern biology to every section of society. We view our population not as a problem, but as an advantage, which is unique to India. We believe we can use our extraordinarily large pool of genetic diversity as a “genetic playground” to address a number of questions; questions which will continue to accumulate and will demand answers, as the new millennium is bombarded with increasing arrays of nucleotide sequences as a consequence of global genome projects. Compassion, when coupled with science, can realize its highest ideal, viz., improving the quality of life of the average citizen. This is the lifeline of our activities at CDFD. We are prepared to face the challenging tasks ahead of us with our exceptional human resources. We are confident that we will meet and surpass the expectations and responsibilities reposed in us by the creation of the CDFD”.

CONTENTS

Director's Message

It is my pleasure to present the Annual Report detailing the activities and achievements of CDFD for the year ending 31 March, 2008. Set up just twelve years ago, the Centre has acquired considerable national and international repute and recognition for both its scientific services and research and training activities. This is despite (or perhaps because of !) the fact that we are a relatively small group of about fifteen group leaders or faculty, the majority of whom are under forty years of age. As will be evident from the pages that follow; the present reporting year has also been quite significant and successful and I highlight some of these achievements below.

With respect to one of its service activities i.e., DNA profiling, the Centre is now identified as the premier organization in the country for referrals of complex and sensitive cases from various State Govts. CBI, and the judiciary including the High Courts. A little over 2000 cases have been undertaken by CDFD in the past ten years, of which 172 were received in the current year. The Centre also provides modern diagnostics and counseling services to children and families with genetic and congenital disorders, and around 2500 tests were performed this year in the areas of molecular genetics, cytogenetics and biochemical genetics.

In research, the record of publications in the past year provides a reflection of the excellent work being undertaken by all the groups. Two molecules, Nodular and Gloverin, were identified and studied in silkmoth that confer protection against bacterial infections. Microsatellite markers for the unique Northeast Indian endemic golden muga silkmoth have been developed and are being used to study the phylogeography of this dwindling species. In another genomics approach combined with genetics and linkage mapping, the loci that are associated with some of the unique traits of basmati rice, namely grain length and grain width, have been identified.

In computational biology and bioinformatics, a novel method for protein structure prediction with improved sensitivity and specificity has been developed. A new substitution matric for the prediction and annotation of protein-coding genes in the highly AT-rich genome of *Plasmodium falciparum* was also created. In molecular and structural biology, data were obtained to support the hypothesis that the Rho protein in prokaryotes employs a brute-force mechanism to mediate transcription termination, and the structure of the YefM protein of *Mycobacterium tuberculosis* (*Mtb*) was determined.

In the areas of cancer biology and immunology, evidence was obtained to suggest a role for the DNA methyltransferase DNMT3L in RNA metabolism. Thiadiazolidine derivatives were shown to induce FasL expression and cell death *via* the Akt-FKHR pathway, and azadirachtin (active principle of neem) was found to downregulate TNF receptors and hence TNF-induced inflammation. Presumptive Novel mutations have been identified in colorectal cancers from young patients and in esophageal squamous cell carcinoma.

In the area of biology of infectious diseases, the product of gene RV1168c of *Mtb* has been identified as a potential diagnostic tool for detection of all categories of tuberculosis patients. The PE25/PPE41 protein complex from *Mtb* was shown to induce significant B cell response and to enhance $CD4^+$ and $CD8^+$ T cell populations in the patients. In *Helicobacter pylori*, the mechanism of HP986's action in inducing proinflammatory and apoptotic responses in human macrophages has been investigated.

Several initiatives have been taken up this year which we hope will come to fruition in the coming years. These include preparations to move into an expanded interim campus and to obtain allotment from the Andhra Pradesh State Government of land for our laboratory activities, arranging for a partnership with the Nizam's Institute of Medical Sciences, Hyderabad for establishment of a Medical Genetics Unit, and efforts to outsource the operation of sophisticated equipments in the Centre's possession.

Finally, I wish to acknowledge and thank all my colleagues and staff at the Centre for their efforts through the year, as also the support from the Department of Biotechnology and from distinguished external Members of the Research Area Panel – Scientific Advisory Committee, Finance Committee, Building Committee, Governing Council and Society of CDFD in making possible the Centre's achievements described herein.

March 31, 2008 Dr. J Gowrishankar

MANDATE

Mandate

The objectives for which the Centre for DNA Fingerprinting and Diagnostics (CDFD) was established as enumerated in the EFC documents are:

1. To establish DNA diagnostic methods for detecting genetic disorders and to develop probes for such detection;

2. To use DNA fingerprinting techniques for the authentication of plant and animal cell material, cell lines and to develop new probes where necessary for such purposes;

3. To provide training in DNA fingerprinting techniques and offer consultancy services to medical institutions, public health agencies and industry in the country;

4. To undertake basic, applied and development R & D work;

5. To collaborate with foreign research institutions and laboratories and other international organizations; and establish affiliation with recognized universities and institutions;

6. To acquire or transfer technical know-how from/to entrepreneurs & industries and, to register patents, designs & technical know-how in the interest of the Centre;

7. To carryout DNA profile and related analysis in civil cases like paternity disputes, immigration, and exchange of new-borns in hospitals, for various agencies including private parties, on appropriate payment;

8. To provide DNA fingerprinting and related analysis and facilities to crime investigation agencies;

9. To assist police personnel, forensic scientists, lawyers and the judiciary in understanding the evidential value of the DNA profile analysis and related techniques in crime investigation and family matters.

Laboratory of DNA Fingerprinting Services

Principal Investigator	J Nagaraju	Scientist In-charge
Other members	Madhusudan R. Nandineni	Staff Scientist
	Varsha	Staff Scientist
	SPR Prasad	Senior Technical Examiner
	V N Sailaja	Technical Officer Gr.I
	Ch V Goud	Technical Examiner
	D S Negi	Technical Examiner
	B Ramesh Babu	Technical Assistant
	Md Mahfooz Alam	Junior Assistant-I
	G Rajalingam	Attendant

Objectives

1. To provide DNA fingerprinting services in the cases forwarded by law enforcing agencies of State and Federal Governments and Hon'ble Judiciary, relating to maternity/paternity, murder, rape, immigration, child-swapping, body identification, wildlife identity, seed purity testing, strain identification, etc.;
2. To develop human resources skilled in DNA fingerprinting, to cater to the needs of State and Federal Government agencies;
3. To impart periodical training to manpower involved in DNA fingerprinting sponsored by State and Federal Government agencies;
4. To provide advisory services to State and Federal Government agencies in establishing DNA fingerprinting facility; and
5. To create DNA marker databases of different caste populations of India.

Summary of work done until the beginning of this reporting year

A total number of 172 cases was received for DNA fingerprinting examination during the period under report. Out of these, 83 cases were related to paternity/maternity, 68 cases to identification of

deceased, 11 cases pertaining to sexual assault, 8 cases pertaining to murder and 2 cases involved wildlife poaching. The cases involving paternity/maternity (48%), identification of the deceased (40%) and rape/sexual assault cases (6%) constituted the bulk of the cases received.

Almost all the States of India have availed DNA fingerprinting services of CDFD during this period. Karnataka State forwarded the highest number (39) of cases, followed by Rajasthan (31), Madhya Pradesh (17), Maharashtra (13), Punjab (9), Haryana (9), Andhra Pradesh (8), Chattisgarh (8), Kerala (7), Delhi (7), Uttar Pradesh (4), Goa (3), Pondicherry (3), Jharkhand (2), West Bengal (2) and one case each from Assam, Bihar, Chandigarh, Himachal Pradesh, Jammu & Kashmir, Orissa, Tamil Nadu, Tripura, Uttaranchal. A case from the neighboring country Nepal was also received.

An amount of Rs.27,65,050/- (Rupees twenty seven lakhs, sixty five thousand and fifty only) was received towards DNA fingerprinting analysis charges (which is inclusive of service charge as levied by Government of India) in various cases.

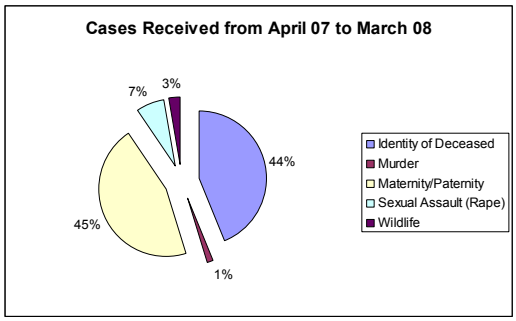
Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

A total number of 148 cases was received for DNA fingerprinting examination during this period. Out of these, 67 cases were related to paternity/maternity disputes, 65 cases were related to identification of deceased, 10 cases were pertaining to rape/sexual assault, 2 cases were pertaining to murder and 4 cases were related to wildlife poaching. Similar to previous year, the cases involving paternity/maternity (45%), identification of the deceased (44%) and rape/sexual assault cases (7%) constituted the bulk of the cases received.

Eighteen States of India have availed DNA fingerprinting services of CDFD during this period. Rajasthan State forwarded the highest number (43) of cases, followed by Karnataka (37), Andhra Pradesh (13), Uttar Pradesh (10), Punjab (8), Haryana (7), Chhattisgarh (6), Maharashtra (5), Kerala (4), Bihar (4), Goa (2), Himachal Pradesh (2), and one case each from Andaman & Nicobar, Assam, Jharkhand, Madhya Pradesh, Manipur, Tamil Nadu. This year too, we have received a case from the neighboring country Nepal. The details of the cases received from different States of India and the

neighboring country, Nepal are given in the following table and the percentage of cases received is shown in the figure.

Name of the State	Identity of Deceased	Murder	Maternity/ Paternity	Sexual assault	Wild-life	Total
Andaman & Nicobar	-	-	1	-	-	1
Andhra Pradesh	-	-	11	-	2	13
Assam	1	-	-	-	-	1
Bihar	1	-	2	1	-	4
Chhattisgarh	2	-	2	1	1	6
Goa	2	-	-	-	-	2
Haryana	5	-	2	-	-	7
Himachal Pradesh	1	-	1	-	-	2
Jharkhand	-	-	1	-	-	1
Karnataka	10	-	27	-	-	37
Kerala	-	-	3	-	1	4
Madhya Pradesh	-	-	1	-	-	1
Maharashtra	-	-	5	-	-	5
Manipur	-	-	-	1	-	1
Punjab	6	-	1	1	-	8
Rajasthan	29	2	6	6	-	43
Tamil Nadu	-	-	1	-	-	1
Uttar Pradesh	7	-	3	-	-	10
Neighboring countries						
Nepal	1	-	-	-	-	1
	65	**2**	**67**	**10**	**4**	**148**



An amount of Rs.19,22,936/- (Rupees nineteen lakhs, twenty two thousand, nine hundred and thirty six only) has been received towards DNA fingerprinting analysis charges (which is inclusive of service charge as levied by Government of India) in various cases.

Deposition of evidence in Hon'ble Courts

During this reporting year, the DNA experts defended their reports in about 20 cases in various Hon'ble Courts throughout the Country.

Training/Lectures/on DNA fingerprinting examination

Training on DNA fingerprinting examination was provided to the scientists from the State Forensic Science Laboratory, Rajasthan, Jaipur.

The lectures were delivered for the benefit of 1) Foreign delegates (from 15 countries) of the National Crime Records Bureau (NCRB); 2) Trainee investigating officers from Central Detective Training School, Ramanthapur, Hyderabad; 3) IPS Officers from Sardar Vallabhabhai Patel National Police Academy, Hyderabad and 4) Air Force Trainee Officers, Pune.

Diagnostics

Principal Investigator	Ashwin Dalal	Staff Scientist
Other Members	Radha Rama Devi	Consultant
	R Angalena	Technical Officer 1
	S M Naushad	Technical Officer 1
	Usha Rani Dutta	Technical Assistant
	Jamal Md Nurul Jain	Technical Assistant
	Ram Prakash Singh	Tradesman
	C Krishna Prasad	Tradesman
	R Sudheer Kumar	Tradesman
	V Murali Mohan	Attendant
	G Srinivas	Attendant
	P Rajitha	Project Associate
Collaborator	M D Bashyam, Molecular Oncology, CDFD	

Objectives

- Clinical evaluation and counseling of patients with genetic disorders.
- Research on single gene disorders
- Providing social awareness in early detection and prevention of genetic disorders
- Training the medical and paramedical students in genetic testing

I. Services provided during the year 2007-2008

Clinical Genetics

A total of 2343 patients attended the clinic for genetic evaluation and counseling, during the year 2007-08. These consisted of patients with chromosomal disorders, monogenic disorders, mental retardation, congenital malformations, multiple malformation syndromes, hemolytic anemias, short

stature, skeletal dysplasias, myopathies, neurodegenerative disorders, ataxia, hypogonadism (male/female), ambiguous genitalia, inborn errors of metabolism, and familial disorders. The details of genetic investigations done during 2007-08 are given in the following tables.

Genetic investigations done during 2007-08

Investigation	**Total cases**	**Positives**
Cytogenetics	799	63 (7.9%)
Proband	765	63 (8.2%)
Prenatal	34	0
Molecular Genetics	503	109 (21.6%)
Proband	492	106 (21.5%)
Prenatal	11	3 (27.2%)
Biochemical Genetics	1229	149 (12.1%)
Proband	1222	142 (11.6%)
Prenatal	7	7 (100%)

Cytogenetics

Disease	**Abnormality**	**Number of cases**
Down syndrome	Trisomy 21	34
	46, XY, rob (21; 21) +21	2
	46,XY, rob (13;21) +21	1
Turner syndrome	Monosomy X (45,X)	6
	Iso X (46,X, i(X))	2
	Mosaic Turner syndrome (46, X / 46,X,i(X))	2
	Deletion X (46,X, del(X)(q22.3 →qter)	1
Sex Reversal	Phenotypic male with 46, XX	2
	Phenotypic female with 46, XY	2
Triple X Syndrome	47,XXX	1
Klinefelter syndrome	47,XXY	1
Structural chromosomal abnormalities		10
	46, XY, del (7) (q33 →qter)	1
	46, XY, inv (10) (p11.2q21.2)	1
	46, XX, del (13) (q22 →qter)	1
	46, XY, t (3; 14) (p12;q12)	1
	46, XX, t(2;22)(q33;q11.2)	1
	46, XX, del (21) (q22)	1
	46,XY,t(18;22)(q21.2;q24)	1

	46, XY, 15p+	2
	46, XY, der (9),t (5;9)(p13.1;p24)mat	1

Molecular Genetics

NAME OF DISORDER	**No of cases**	**POSITIVE**	**NEGATIVE**		
DMD/BMD	42	23	19		
DMD Carrier Analysis	7	6	1		
Spinal Muscular Atrophy	34	7	27		
		Homozygous	**Heterozygous**	**Compound Heterozygous**	**Normal**
Thalassemia and Sickle cell anemia	21	2	14	1	4
Factor V Leiden	77	-	3	-	74
Factor II mutation	29	-	-	-	29
MTHFR (C677T)	66	1 (TT)	14 (CT)	-	51 (CC)
Cystic Fibrosis	69	6	14	1	48
TRIPLET REPEAT DISORDERS		**POSITIVE**	**NEGATIVE**		
Fragile X Syndrome	102	5	97		
Friedrichs Ataxia	9	1	8		
Myotonic Dystrophy	7	-	7		
Huntington Disease	8	6	2		
SCA Panel	11	1	10		
DRPLA	2	-	2		
SRY/DAZ	8	1	7		
PRENATAL DIAGNOSIS		Normal	Homozygous	Heterozygous	Compound Heterozygous
Thalassemia	8	2	-	4	2
Cystic Fibrosis	1	-	-	1	-
DMD	1	1 Positive			
Spinal muscular atrophy	1	1 Normal			

Biochemical Genetics

Disease	**Number of positives (%)**
Biochemical (N = 1229)	**149 (12.1%)**
Amino acid disorders (N=574)	**21+ 2 Follow up**
Phenylketonuria	1 2 (Follow-up cases)
Maple syrup urine disease	5
Non Ketotic Hyperglycinemia	1
Ketotic Hyperglycinemia	2
Citrullinemia	1
Hyperornithinemia	8
Methyl malonic acidemia	2
Alkaptonuria	1
Homocysteinemia (N=197)	***46 (23.4%)***
Mild Hyperhomocysteinemia (15-30 µmol/L)	35
Moderate-Severe Hyperhomocysteinemia (30-100 µmol/L)	11
Lysosomal storage disorders (N=138)	**40 (29%)**
Metachromatic Leukodystrophy	3
Gaucher's	6
GM1-Gangliosidosis	3
Mucopolysaccharidosis (17)	MPS I: 9 MPS VI: 1 MPS VII: 1
Pompe's disease	6
Tay Sach's disease	5
Sandhoff syndrome	4
I-Cell disease	1
Fabry disease	1
Newborn screening (N=81)	Biotinidase deficiency 1 (1.2%)
Galactosemia (N=36)	2 (5.6%)
Biotinidase deficiency (N=46)	3 (6.5%)
High risk pregnancy screening	
Triple Marker screening (N=86)	16 (18.6%)
First Trimester screening (N=64)	9 (14.1%)
Prenatal (7)	**7**
Pompe's	1
Sandhoff	1
I-cell disease	1
MPS I	1
GM1	1
MPS VI	1
MPS VII	1

II. Research

Project 1: Relationship between methionine synthase, methionine synthase reductase genetic polymorphisms and deep vein thrombosis among South Indians

We have examined the relationship between polymorphisms in genes that regulate remethylation of homocysteine to methionine, i.e., methionine synthase (*MTR A2756G*) and methionine synthase reductase (*MTRR A66G*), and the risk of deep vein thrombosis (DVT) in a South Indian cohort (163 DVT cases and 163 controls) as it has been documented that elevated homocysteine level is an independent risk factor for DVT in the same cohort. The plasma homocysteine analysis was carried out by reverse phase HPLC. The *MTR A2756G* and *MTRR A66G* genetic polymorphisms were detected using PCR-restriction fragment length polymorphism method. Statistical analyses was done using Fisher's exact test for categorical variables, and Student's t-test and Analysis of Variance for continuous variables. Our analysis revealed that the *MTRR 66GG* genotype was associated with a 2.74-fold (95% confidence interval (CI): 1.73, 4.34) risk of DVT, while the *MTR A2756G* polymorphism was not a risk factor. *MTRR GG*/*MTR AG* and *MTRR GG*/*MTR GG* genotypes cumulatively were found to increase the risk of DVT by 2.38-fold (95% CI: 1.43, 3.96). A positive association was observed between plasma homocysteine and the *MTRR G* allele, and the *MTR G* allele was shown to have an additive effect. The risk associated with the *MTRR 66GG* genotype was further increased in subjects who are compound heterozygous for methylene tetrahydrofolate reductase (*MTHFR*) (odds ratio (OR): 3.46, 95% CI: 1.38, 8.63). Hence, our studies show that the *MTRR 66GG* genotype is a risk factor for DVT among South Indians and risk is further increased in the presence of the *MTHFR 677CT/1298AC* genotype.

Project 2: Clinical, biochemical and molecular analysis of treatable lysosomal storage disorders

This is a new activity

Lysosomal storage disorders are a heterogeneous group of disorders associated with specific lysosomal enzyme deficiency. Enzyme Replacement Therapy (ERT) is available for at least 5 of these disorders, namely , Gaucher disease, Fabry disease, Mucopolysaccharidosis type I and VI, and Pompe disease. Phase II trials are underway for another such disorder, Niemann Pick disease. Our study aims to characterize the clinical features, biochemical parameters and molecular defects in these treatable disorders. The results would form the basis for revealing the spectrum of mutations for these disorders in the Indian population which will in turn help in better diagnosis of carriers for these disorders and accurate prenatal diagnosis. At present the diagnosis and prenatal diagnosis of these disorders is primarily based on enzyme assay, which has a number of disadvantages. One of which is that there is a large amount of overlap in enzyme levels among carriers and normal people it is very difficult to detect carriers by enzyme assay. Therefore, mutation detection becomes imperative for carrier detection. In addition, it will be helpful in genotype-phenotype studies of these disorders. Further, the knowledge regarding mutations in Indian patients will help in establishing testing for these disorders as a service to the patients.

3. Establishment of EBV transformed cell lines from lymphocytes of patients with rare disorders

This is a proposed new activity

One of the important tasks following mapping & sequencing of the Human Genome is to identify disease-causing genes. It is equally important to resolve genetic heterogeneity & establish genotype-phenotype correlations. For both the above objectives, it is essential to have informative clinical material. The informative index cases & their families are rare, and very often not willing to get investigated, particularly if they have to pay for it. It is important to harness this material as much as possible. The Diagnostics Division of CDFD has a very active clinical and laboratory programme in the field of clinical genetics. It sees over 2000 new cases for various genetic disorders each year, many of which are rare or unique. Since most of the genetic disorders are rare, a significant number of families with any type of disorder need to be accumulated over a period of time. The present

project aims to establish EB virus transformed cell lines from patients with genetic disorders of interest.

III. Publications

1. Naushad SM, Jain Jamal MN, Prasad CK and Rama Devi AR (2008) Relationship between methionine synthase, methionine synthase reductase genetic polymorphisms and deep vein thrombosis among South Indians. ***Clinical Chemistry and Laboratory Medicine*** 46:73-79.

2. Prabhakara K, Damien L Bruno, Priya Padman, Suma Prasad, Sudheer Kumar R, Howard R Slater and Radha Ramadevi A (2008) Prenatal detection of deletion-duplication of chromosome 3 arising from meiotic recombination of familial pericentric inversion. ***Prenatal Diagnosis*** (In Press)

Laboratory of Molecular Genetics

Centre of Excellence on Genetics and Genomics of Silkmoths and Basmati Rice Genetics and Genomics

Principal Investigator	:	J Nagaraju	Staff Scientist
Ph D students	:	V L N Reddy	SRF
		Archana Gandhe	SRF
		Jayendranath Shukla	SRF
		K P Arun Kumar	SRF
		Jyoti Singh	SRF
Other Members	:	V V Satyavathi	Post Doctoral Fellow
		V Satish	Project Associate
		N Mrinal	Research Associate
		E V Subbaiah	Research Associate
		M Muthulakshmi	Technical Officer Gr.I
		A Sobhan Babu	Technical Officer Gr.I

Project 1: Development of RNAi-based baculovirus resistant transgenic silkmoths

Objective

To generate transgenic silkworms resistant to *Bombyx mori* nucleopolyhedrosis virus (BmNPV) using RNAi strategy

Summary of the work done until the beginning of this reporting year

The transgenic silkworm lines that express double strand RNA (dsRNA) for essential baculoviral immediate early gene (*ie-1*), and eight transgenic silkworm lines expressing dsRNA for four essential baculovirus genes simultaneously, were generated and tested for their resistance, if any to *Bombyx mori* nucleopolyhedrosis virus (BmNPV) infection. The transgenic lines that expressed dsRNA for *ie-1* gene were protected against baculovirus infection to the tune of 40% to 60% as against non-

transgenic control lines. On the other hand, the transgenic lines that expressed dsRNA for four essential baculoviral genes were resistant to baculovirus infection to an extent of 90% as against complete mortality in non-transgenic control lines when they were infected per os with 12000 Polyhedral Inclusion Bodies (PIBs)/larva.

Details of progress made in the current reporting year (April 1, 2007 – March 31, 2008)

After confining the transgenicity by reporter assays and baculovirus resistance by viral infection assays, the transgenic lines with single, and multiple baculoviral target genes were characterized for : (i) Copy number and chromosomal location of the integrated trasngenes, (ii) baculoviral viral abundance as quantified by qPCR of the baculoviral gene, and western blot analysis using antibody for baculovirus, coat protein, GP64 of the baculoviral infected transgenics and non-transgenic control lines.

Copy number and chromosomal location of the transgenes in the transgenic lines were determined by transposon element display (TED). Formation of a single Polymerase Chain Reaction (PCR) amplification product after the second PCR reaction indicated the presence of a single copy insertion in the transgenic lines. **(Fig.1A)**. TED was used to target the left arm of the *piggy Bac* transposon. The presence of TTAA sequence near the genomic integration site further confirmed the *piggy Bac-based* transgenisis **(Fig.1B)**.

Quantitative PCR analysis: The relative abundance of baculoviral target genes in the transgenic lines was estimated by real-time PCR on the total RNA extracted from the moths emerged from the transgenic and non-transgenic larvae infected at fourth instar. The accumulation of the baculoviral target gene, *ie-1* transcripts was lower by 60% to 40% compared with the non-transgenic control line.

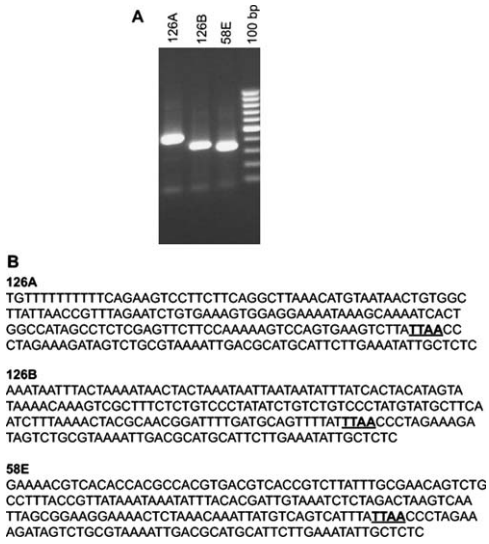


Fig.1(A). Transposable element display shows the single insertion of transgene in the three transgenic lines. PCR amplification using left inverted repeat-specific primer gave a single amplicon in all the three transgenic lines suggesting the integration of one copy of the transgene. These products were sequenced to identify the chromosomal location of the integrated transgene. (**B).** Sequence information of the integrated site showing the flanking region of the left inverted repeat. The characteristic TTAA repeat is highlighted.

Project 2: Molecular Characterization of immune response proteins of silkmoths

Objective

Genetic, biochemical and functional characterization of lepidopteran immune proteins

Summary of the work done until the beginning of this reporting year

Immune transcriptome analysis of the silkworm, *Bombyx mori* and wild silkworm, *Antheraea mylitta* led to the identification of many novel antimicrobial proteins. These include lysozyme like proteins (LLPs) (so designated owing to their partial similarity to lysozymes) but lacked characteristic catalytic amino acid residues essential for muramidase activity. The RNAi mediated knock-down of the LLPs resulted in substantial increase in haemolymph bacterial load suggesting the involvement of LLPs in immunity in silkworm. Antibacterial mechanism was shown to be due to peptidoglycan binding rather than via peptidoglycan hydrolysis or membrane permeabilization as observed with lysozymes and most other antimicrobial peptides. Further, the antibacterial mechanism revealed that LLPs are bacteriostatic rather than bacteriocidal against *E.coli* and *Micrococcus luteus*.

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

Among many new candidate antimicrobial genes identified from the immune transcriptome of the wild silkmoth, *Antheraea mylitta*, three (DFP-1, 2, and 3) had similarity with extracellular matrix

proteins from vertebrates. These genes expressed abundantly in the immune transcriptome suggesting their possible involvement in insect innate immunity.

DFP-1 was expressed in Sf9 cells by baculovirus mediated expression system as a recombinant protein. The molecular weight of the recombinant DFP-1 was 19 kDa. Our initial results indicated a role for this protein in nodulation response of insects and hence we designated it as Noduler. Noduler was present constitutively in the insect haemolymph and was upregulated several fold upon bacterial infection. Immunoblot analysis of bacteria challenged haemolymph samples with Noduler-specific peptide antibody detected Noduler as early as 30 mins post infection and maximum expression was seen at around 4 hrs as shown in **Fig.2**.

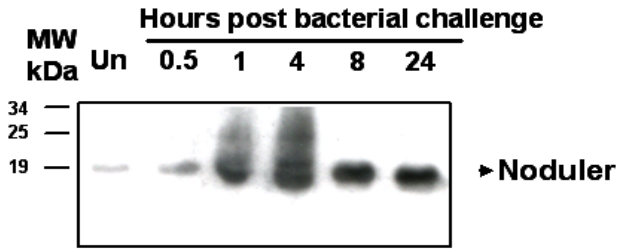


Fig.2. Immunoblot analysis of *Escherichia coli* challenged *Antheraea mylitta* hemolymph samples at different time points post infection using Noduler-specific antibody. Un- Unchallenged.

Noduler possessed a characteristic reeler domain as found in several extracellular matrix vertebrate proteins. The two additional paralogues of Noduler showed high level of sequence similarity at both nucleotide and amino acid level **Fig.3 (A &B)**.

RNA interference mediated knock-down of Noduler resulted in significant reduction in the number of nodules (**Fig.4)**. Nodulation was reduced significantly ($P<0.001$) in larvae injected with Noduler-dsRNA prior to *Staphylococcus aureus* or *Escherichia coli* injection as compared to GFP-dsRNA or

saline injected larvae. The high level of sequence similarity among the Noduler paralogues made the detection of effect of knock-down of each of the three Noduler family proteins very difficult at molecular level. Thus it seems likely that RNA-interference mediated phenotype of Noduler in our study is a result of knock-down of all the three Noduler family proteins.

Earlier reports have shown that insect pattern recognition proteins (PGRPs, lectins, βGRPs, hemolin) bind to microbial cell wall components and trigger responses such as phagocytosis, nodulation and activation of phenoloxidase cascade. Noduler was indeed shown to bind microbial

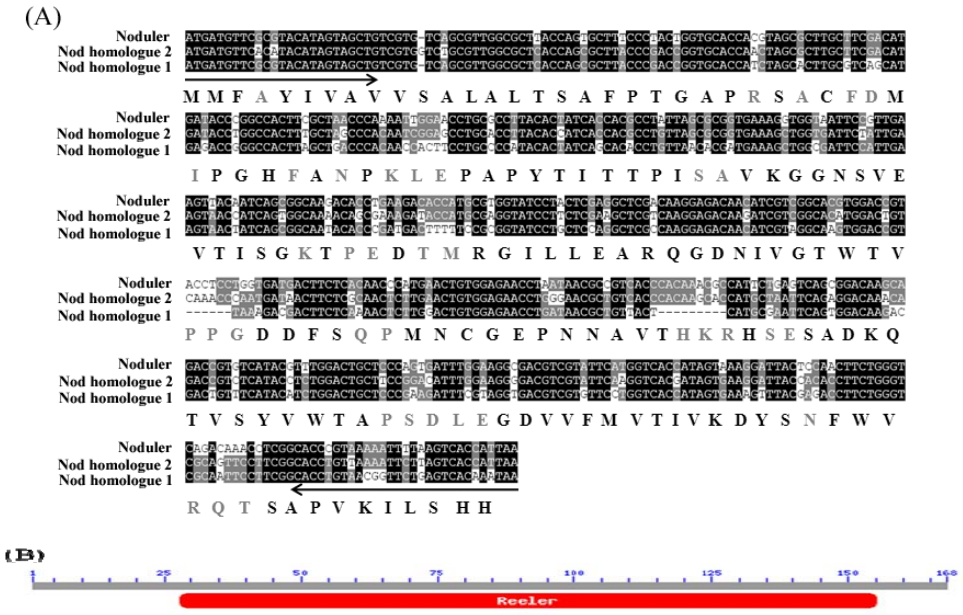


Fig.3. Noduler and its two paralogues in *Antheraea mylitta* (A) The cDNA sequence comparison of Noduler and its two homologues, Nod homologue 1 and Nod homologue 2. The forward and backward arrows indicate the regions used for designing forward and reverse primers, respectively for preparation of double stranded RNA for RNAi experiments. The full length protein sequence of Noduler is shown below the cDNA sequence alignment. The amino acid residues completely conserved amongst all the three proteins are indicated in black colour while the partially conserved residues are shown in grey colour. (B) Schematic representation of the organization of the Noduler from *A. mylitta*.

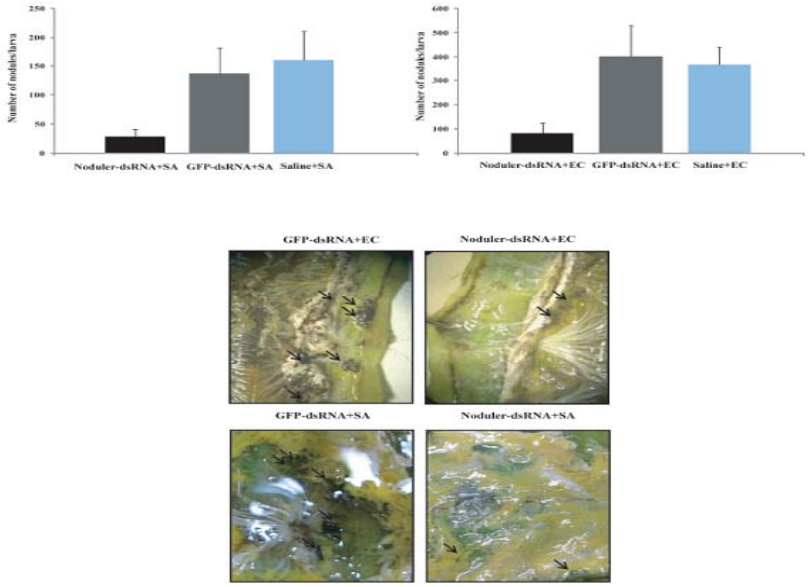


Fig.4. Effect of RNAi mediated Noduler depletion on nodulation response. Nodulation was reduced significantly ($P<0.001$) in larvae injected with Noduler-dsRNA prior to *Staphylococcus aureus* (A) or *Escherichia coli* (B) injection as compared to GFP-dsRNA or saline injected larvae. The average number of nodules per larva (n=8) in each group is plotted on Y-axis and the error bars represent standard deviation of three independent experiments. (C) Formation of melanised nodules in Noduler knockdown (Noduler-dsRNA+ *S. aureus/E. coli*) and control larvae (GFP-dsRNA + *S. aureus/E. coli*). The black arrows indicate the nodules. SA- *S. aureus* EC- *E. coli.*

cell wall components and was involved in nodulation as well as phenoloxidase cascade mechanisms. Noduler specifically binds to lipopolysaccharide, lipotechoic acid and β-1, 3 glucan components of microbial cell walls and also insect hemocytes. Experimental observations like up-regulation of Noduler within minutes of bacterial infection, reduction in number of nodules upon Noduler silencing and subsequent increase in bacterial load, binding of Noduler to bacteria, yeast and insect haemocytes, and the co-occurrence of bacteria, Noduler and haemocytes in the nodules suggest that Noduler mediates nodulation by virtue of its binding to both bacteria as well as haemocytes (**Fig.5).**

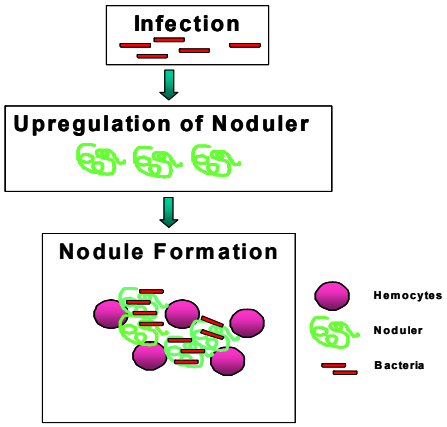


Fig.5. The suggested role for Noduler in the formation of nodules.

Basmati rice genetics and genomics

Project 1: QTL mapping in Basmati rice

Objective: To map important Basmati traits like aroma, kernel length, kernel breadth and kernel elongation

Summary of the work done until the beginning of this reporting year

In order to map important Basmati traits, an F_2 population derived from a cross between a traditional Basmati variety, Basmati370 and a semi dwarf variety Jaya was developed. Many important traits such as plant height, panicle no., panicle length, number of filled and chaffy grains per panicle, spikelet no./panicle, spikelet fertility, 1000 seed weight and yield/plant kernel length, kernel breadth, alkali spreading value, amylase content (%), aroma, grain chalkiness, kernel length after cooking, kernel elongation ratio showed normal distribution The parental strains were screened with 502 rice microsatellite markers to identify 203 polymorphic markers. The mapping population was screened with these microsatellite markers to construct QTL map.

Details of progress made in the current reporting year (April 1, 2007 – March 31, 2008)

For mapping QTLs, an F_2 population of 181 plants was derived from a cross betweem a traditional Basmati variety, Basmati370 and a semi dwarf variety Jaya. Phenotypic data for 18 agronomic and quality traits of the parents, hybrids and F_2 mapping population was recorded. The 203 markers that

were polymorphic between the two parental varieties were distributed on all the 12 rice chromosomes. These markers were used for screening the F_2 population. A molecular linkage map comprising 134 microsatellite markers spanning the rice genome at 7.76 cM interval was constructed using MAPMAKER/EXP v 3.0 and MapDisto v 1.7 softwares. A total of 47 QTLs for 16 economically important traits of Basmati was identified employing interval mapping (IM) and composite interval mapping methods of the QTL Cartographer v 2.5. These QTLs were found to be distributed on all the rice chromosomes except 7 and 11. Of the 47 QTLs, 17 contributed to more than 15% phenotypic variance (Table 1). Interestingly, a single region on chromosome 5 at the marker interval of M4-M5 was found to be controlling four important grain quality traits viz., grain length, grain breadth, length-breadth ratio and grain elongation ratio (**Fig.6**). The promising QTLs controlling important economic traits in basmati rice, identified in this study, could be used as candidates for future fine mapping and positional cloning.

Table 1: List of promising quantitative trait loci (QTLs) identified in the present study

S.No.	Trait	Chromosome	LFM (cM)	RFM (cM)	LOD	PVE (%)
1	Plant height (cm)	1	16	10.4	5.138	15.418
2	Filled grains (no.)	1	10	19.55	3.244	22.677
3	Single plant yield (g)	9	48	66	3.154	82.155
4	Grain length (mm)	3	10	1.7	9.217	46.065
5		5	6	5.2	6.603	17.468
6	Grain breadth (mm)	5	4	7.2	3.333	17.149
7	Length-Breadth ratio	3	20	23.13	3.323	74.353
8		3	8	3.7	4.358	22.342
9		3	2	10.21	4.770	24.958
10		5	8	3.2	4.650	46.531
11	Elongation ratio	5	14	4.88	3.706	19.931
12		5	4	7.2	3.711	18.931
13	Alkali spreading value	6	4	8.22	26.746	71.735
14	Aroma	8	8	16	4.998	20.226
15	Chalkiness	3	0	44.07	3.234	15.083
16		4	14	28.62	3.138	63.795
17		4	22	3.15	5.896	25.092

PVE: Phenotypic Variance Explained by each QTL (%); LFM-Left flanking marker (cM), RFM-Right flanking marker (cM); LOD-Logarithm of odd ratio

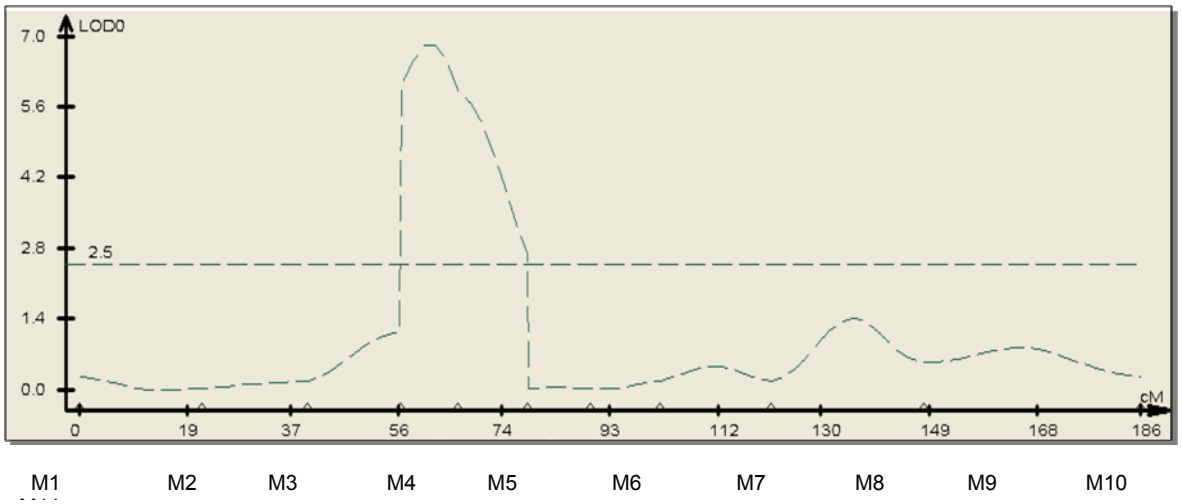


Fig.6: QTL cartographer LOD peak for grain appearance traits (grain breadth, grain length, length-breadth ratio and grain elongation ratio) on chromosome 5. LOD 2.5 taken as threshold for detecting a QTL. X-axis shows the markers and their genetic distances in cM. Y-axis shows the LOD values.

Publications

1. Gandhe AS, Janardhan G and Nagaraju J (2007) Immune upregulation of novel antibacterial proteins from silkmoths (Lepidoptera) that resemble lysozymes but lack muramidase activity. ***Insect Biochemistry and Molecular Biology*** 37: 655-66

2. Kanginakudru S, Royer C, Edupalli SV, Jalabert A, Mauchamp B, Chandrashekaraiah, Prasad SV, Chavancy G , Couble P and Nagaraju J (2007) Targeting *ie-1* gene by RNAi induces baculoviral resistance in lepidopteran cell lines and in the transgenic silkworms. ***Insect Molecular Biology*** 16: 635-44

3. Gandhe AS, John S H and Nagaraju J (2007) Noduler, a novel immune upregulated protein mediates nodulation response in insects. ***Journal of Immunology*** 179: 6943-51

4. Lakshminarayanareddy V, Archak S and Nagaraju J (2007) Capillary electrophoresis is essential for microsatellite marker based detection and quantification of adulteration of Basmati rice (*Oryza sativa*). ***Journal of Agriculture and Food Chemistry*** 55: 8112-17

5. Ravi Kumar A, Sathish V, Balakrish Nair G and Nagaraju J (2007) Genetic characterization of *Vibrio cholerae* strains by inter simple sequence repeat-PCR. ***FEMS Microbiology Letters*** 272: 251-58

6. Sreenu VB, Kumar P, Nagaraju J and Nagarajaram H A (2007) Simple sequence repeats in mycobacterial genomes. ***Journal of Biosciences*** 32: 1-15

7. Archak S and Nagaraju J (2007) Computational prediction of rice (*Oryza sativa*) miRNA targets. ***Genomics, Proteomics and Bioinformatics*** 5: 196-206

8. Archak S, Lakshminarayanareddy V and Nagaraju J (2007) High-throughput multiplex microsatellite marker assay for detection and quantification of adulteration in Basmati rice (*Oryza sativa*). ***Electrophoresis*** 28: 2396-2405

9. Biju SD, Bocxlaer IV, Giri VB, Roelants K, Nagaraju J and Bossuyt F (2007) A new nightfrog, *Nyctibatrachus minimus* sp. nov. (Anura: Nyctibatrachidae): The smallest frog from India. ***Current Science*** 93: 854-58

Laboratory of Genomics and Profiling Applications

Principal Investigator	Madhusudan R Nandineni	Staff Scientist
Other Members	Anita Kumari	Project Assistant
	Deepika Dasyam	Project Assistant
	K Hanuma Kumar	Project Assistant (Until Feb, 08)
	Aruna Devi	Project Assistant
	E. Sadasrinu	Project Assistant
	Jeffrey Pratap	Project Assistant
	Nidhi Upadhyay	Project Assistant
	Ranjit Sagar	Project Assistant
	Smita Sinha	Project Assistant
Collaborators	S P R Prasad, CDFD	Technical Assistant
	Ch V Goud, CDFD	Technical Assistant

Objectives:

1. Development, standardization and validation of DNA markers for genetic fidelity testing of tissue culture raised plants and for phylogenetic studies
2. Referral Centre for detection of genetically modified foods employing DNA-based markers
3. Development of novel strategies/methodologies for enrichment of human DNA from mixtures containing human and non-human DNAs for DNA profiling-based human identification
4. To study the human genetic diversity among various population groups of India

Project 1: Referral Centre for Genetic Fidelity Testing of Tissue Culture Raised Plants Employing DNA Markers.

Summary of work done until the beginning of this reporting year:

This is a new activity.

One of the major problems with tissue culture raised plants is the somaclonal variation in the clones leading to differences in the genome with respect to the mother explant. The National

consultation group on certification of tissue culture raised plants sponsored by Department of Biotechnology has proposed that plant certification is essential for import/export of material as per the rules/procedure laid down in the Plant Quarantine Order 2003, whose primary goal is to ensure production of virus-free plants of assured quality raised through tissue culture. Thus, a 'Referral Centre for the Genetic Fidelity Testing of Tissue Culture Raised Plants Employing DNA Markers' was established at CDFD and was proposed to undertake the true-to-type testing of important crop plants like banana, potato, sugarcane, black pepper and vanilla. The project entails development, standardization and validation of molecular markers to test true-to-typeness of above micropropagated plants.

Details of progress made in the current reporting year (April 1, 2007-March 31, 2008)

Isolation of Microsatellite Markers: Microsatellites or simple sequence repeats (SSRs) are tandemly repeated motifs of 1-6 bases and are widely distributed throughout the eukaryotic genomes, making them the preferred markers for very-high resolution genetic mapping. They are characterized by a high degree of length polymorphism and have proven to be an extremely valuable tool for genome mapping in many organisms. But a major challenge for utilizing SSR markers for genetic fidelity testing is the requirement of prior information of genomic DNA sequence for designing primers to the flanking regions of the repeat motifs. Since genomic DNA sequences are not yet known for potato, banana, sugarcane, black pepper and vanilla crops plants, SSRs would be isolated from them based on the selective hybridization procedure (Glenn and Schable, *Methods in Enzymology*, 2005, 395: 202-222).

The method involved isolation of DNA from the plant, subjecting the DNA to restriction digestion with a frequent cutter (RsaI), followed by attachment of linkers and amplification of the linker ligated DNA. Microsatellite containing DNA fragments were enriched by hybridization of biotinylated SSR oligonuleotides to amplified linker ligated DNA and their subsequent capture by streptavidin coated para-magnetic beads. After extensive washing to remove the DNA

fragments that are non-specifically bound, repeat-containing DNA fragments were eluted, amplified and cloned into suitable vectors.

Subsequently, the clones were screened for microsatellites and the sequences containing microsatellites were contiged and edited to ensure accuracy of the sequence. So far, nine clones for black pepper and three clones for potato have been identified from these enrichments which contain SSRs along with flanking sequences. These SSR markers have to be tested and validated for polymorphism in different varieties of each species. Since it is desirable to isolate multiple SSR markers, which are polymorphic and informative, the above strategy would be adopted to isolate more number of microsatellites in these crop plants for genetic fidelity testing.

Project 2: Referral Centre for Detection of Genetically Modified Foods Employing DNA-based markers.

Summary of work done until the beginning of this reporting year:

This is a new activity.

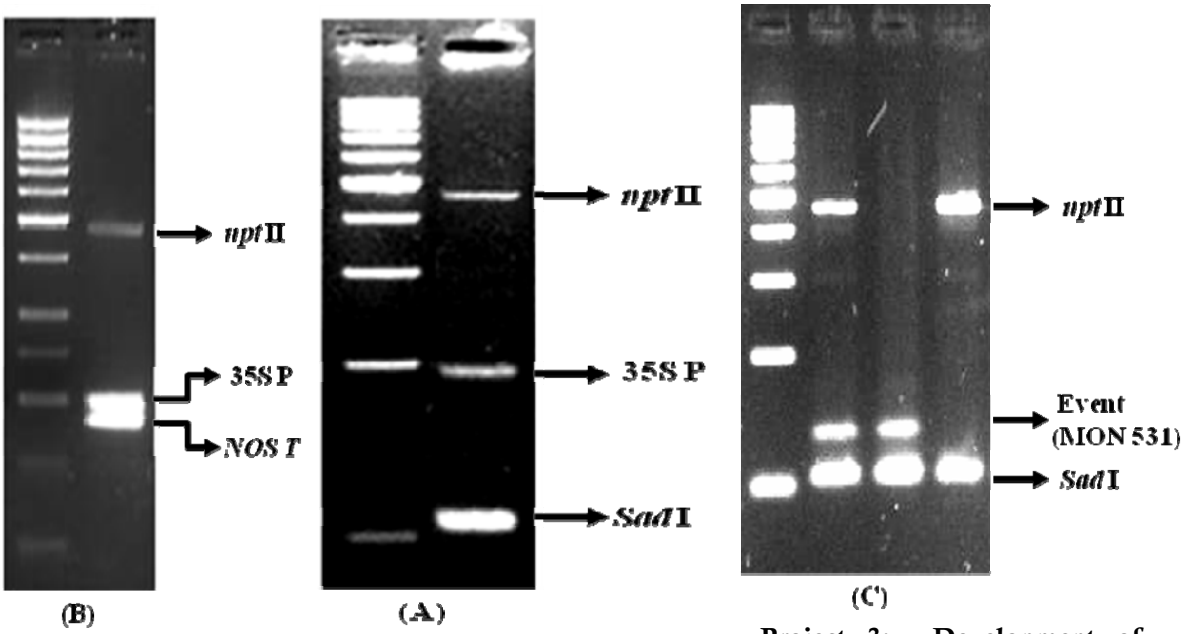
Over the recent years, the cultivation of genetically modified (GM) crop varieties gained an unprecedented rise with both the variety of crops, and the land expanse being cultivated witnessing a steady increase every year. Although GM crops are regarded advantageous over the conventional crop varieties in the increased production, and protection from insect-pests, the safety of these genetically engineered foods pertaining to environment and human health are strongly debated. In order to ensure food security, and biosafety, numerous methods to detect genetically modified foods have been developed in the recent past; and among which, DNA-based detection of genetically modified foods has been recognized one of the reliable and accurate methods to detect the transgenic elements in the genetically engineered foods. In order to develop and employ DNA-based markers for the detection of genetically modified foods, a 'Referral Centre for Detection of Genetically Modified (GM) Foods Employing DNA-based Markers' has been established in CDFD. Protocols for the detection of *cry1A(c)*, *cry2A(b)*, CaMV

35S promoter, NOS-terminator and *nptII* in whole or crushed seed samples of cotton and rice were earlier developed at CDFD by Dr. J. Nagaraju. We are currently developing DNA-based protocols for the detection of various GM cotton varieties grown in India.

Details of progress made in the current reporting year (April 1, 2007-March 31, 2008)

We have standardized a polymerase chain reaction (PCR) based qualitative detection of transgenic elements in the numerous varieties of transgenic cotton (*Gossypium hirsutum*). These cotton varieties, which are either commercially released or under field trials in India, were obtained from different seed producing companies and comprised of four transgenic cotton events viz., MON 531 (*cry1Ac*), MON 15985 (*cry1Ac+cry2Ab*), Event 1 (*cry1Ac*) and GFM event (*cry1Ac+cry1Ab*). Four different DNA extraction methods viz., Guanidium-Chloroform method, Cetyl Trimethylammonium Bromide (CTAB) method, Phenol-chloroform extraction method and Polyvinyl pyrollidone (PVP) method were optimized as per the International Standards Organization (ISO) protocols. PCR conditions for the detection of various transgenes viz., *CaMV 35S* promoter, *NOS* terminator, *nptII* (neomycin phosphotransferase II), *cry2Ab* and *cry1Ac* in the transgenic cassette of GM cotton varieties were standardized. Also, event-specific detection of MON 531 event in cotton was standardized using event-specific PCR primers, that precisely differentiates MON 531 event from other transgenic cotton events based on its unique flanking plant genomic sequences. In addition, duplex and triplex PCR conditions were standardized for simultaneous detection of two and three transgenes, respectively, in the transgenic cassette of GM cotton varieties (Fig). In future, sampling strategies (DNA mixing and seed sampling) for quantitative detection of genetically modified foods using quantitative-competitive PCR and real time PCR would be standardized. Also DNA-based detection protocols for other GM crops would be standardized.

Fig. (A) Multiplex PCR showing amplification of *npt* II, CaMV 35S promoter, and *Sad* I (cotton endogenous reference gene) (B) Multiplex PCR showing amplification of *npt* II, CaMV 35S promoter, and *Nos* T transgenes (C) Triplex and Duplex PCR showing amplification of *npt* II transgene, *Sad* I- cotton endogenous reference gene, and MON531 event specific PCR product.



Project 3: Development of novel strategies/methodologies for enrichment of human DNA from mixtures containing human and non-human DNAs for DNA profiling-based human identification

Summary of work done until the beginning of this reporting year:

This is a new activity.

Numerous strategies have been developed worldwide to identify human skeletal remains after wars, socio-political disturbances and natural or man-made mass disasters. The degraded or decomposed skeletal remains pose challenges for successful DNA-typing because of microbial contamination and environment-induced changes. Owing to its semi-arid tropical environment, which results in robust microbial infestation, obtaining good quality human DNA for body identification by DNA profiling has not been very successful in India. Therefore newer methodologies are needed to circumvent these problems. It was proposed to selectively enrich the human DNA from the mixture of microbial DNAs by employing antibodies directed against

5-methyl cytosine (5-mC), i.e., to specifically 'pull down' human DNA sequences containing 5-mC, followed by whole genome amplification (WGA) to amplify such DNA sequences. Subsequently, human short tandem repeat (STR)-containing fragments would be 'captured' and subjected to STR/mini-STR genotyping analysis to unambiguously identify the body parts/ skeletal remains of deceased persons.

Details of progress made in the current reporting year (April 1, 2007-March 31, 2008)

To test whether the antibodies directed against 5-mC would enrich ('pull-down') human DNA sequences containing 5-mC, immunoprecipitation reactions were performed employing sheep polyclonal antibody directed against human 5-mC on sonicated human genomic DNA. The precipitated DNA when subjected to STR genotyping analysis showed amplification of only few of the 15 loci examined. To increase the efficiency of the STR analysis, the above immunoprecipitated DNA would be subjected to WGA prior to STR genotyping to increase the starting template DNA. In another strategy, 3'-biotinylated oligonucleotides complimentary to the flanking sequences to the STRs were used to 'capture' the human DNA sequences containing the STRs. The repeat containing sequences were isolated employing biotinylated oligos ("probe") and were captured by streptavidin coated para-magnetic beads. In the pilot studies, four such biotinylated oligonuleotides were used for pull down and the precipitated DNAs when subjected to human STR genotyping showed specific amplification of the expected loci, when used either in singleplex or multiplex reactions. It has to be tested whether by employing biotinylated oligonucleotides that are complementary to the flanking regions of all the 15 STR loci, (which are commonly used in human identification), one could selectively enrich those regions and accomplish the human identification purposes from the DNAs isolated from degraded or decomposed human skeletal remains.

Laboratory of Fungal Pathogenesis

Understanding the pathobiology of an opportunistic human pathogen, *Candida glabrata*

Principal Investigator	Rupinder Kaur	Staff Scientist
Ph.D Students	Gaurav	JRF
	Maruti Nandan Rai	JRF

Project 1: Functional genomic analysis of C. glabrata-macrophage interaction

Objectives:

1. Analysis of intra-cellular behavior
2. Screening of the mutant library for altered intracellular survival profiles
3. Identification and analysis of the genes required for survival in-vitro

Summary of the work done until the beginning of this reporting year

New activity started last year

Work done in the current reporting year (April 1st, 2007 – March 31st, 2008)

To study the molecular interactions of *C. glabrata* with macrophages which in conjunction with neutrophils constitute the first line of host defense against this pathogenic yeast, we are trying to establish an *in-vitro* system using human monocytic cell line THP1. THP1 cells were differentiated into macrophages by treating with PMA (Phorbol-12 Myristate 13-acetate) for 24 hours and were then infected with wild-type *(wt) C. glabrata* cells at MOI (multiplicity of infection) ranging from 1:10 to 10:1. About 58-62% phagocytosis of *C. glabrata* was seen after two hours of co-incubation with THP1 cells and viability of *wt* cells was followed over a period of 24 hours by CFU (colony forming unit) assay. Initial infection time course data suggests that wild type *C. glabrata* cells are not killed by activated human monocytes, rather a 5-7 fold increase in CFUs was seen over a period of 24 hours. In contrast, a *C. glabrata* strain disrupted for putative secreted aspartyl proteases was unable to survive in macrophages displaying 1.34% viability after

24 hrs of co-incubation with macrophages. Importantly, both *wt* and protease defective mutant were able to grow in RPMI media under tissue culture conditions. Currently attempts are being made to compare the intra cellular behavior of *C. glabrata* with that of *S. cerevisiae* and next conditions will be standardized to screen the *C. glabrata* mutant library for altered intra cellular profiles.

Project 2: Innate resistance of *C. glabrata* to fluconazole

Objectives:

1. Identification and characterization of fluconazole sensitive mutants
2. To understand the molecular basis of high innate resistance of *C. glabrata*

Summary of the work done until the beginning of this reporting year

New activity started last year

Work done in the current reporting year (April 1st, 2007 – March 31st, 2008)

A major clinical challenge in treating *C. glabrata* infections is the innate resistance of this yeast towards fluconazole which is the most commonly used anti-fungal drug. To better understand the inherent low fluconazole susceptibility of *C. glabrata*, we have screened the fluconazole sensitive mutants (previously identified through a plate screen) for their inability to survive fluconazole stress by CFU assay. Two mutants (named *flv1* and *flv2* (fluconazole loss of viability)) were identified which not only display sensitivity to fluconazole but also lose viability rapidly upon fluconazole exposure as has been previously reported for mutants defective in calcium signaling. Next, the phenotypic characterization of *flv1* and *flv2* mutant was carried out by plate growth assays and their sensitivity towards other stresses such as temperature, salt, cell wall stress *etc* was tested. The mutant *flv1* was found to be sensitive to high temperature (37ºC) and cell wall stress causing agents such as calcofluor white and caffeine but displayed no sensitivity to salt or other drugs. *flv2* mutant also exhibited reduced growth on plates containing Congo Red and calcofluor white, however, no growth inhibition was observed at high temperature (37ºC). These results indicate that *flv1* and *flv2* mutants are not only sensitive to fluconazole but their growth is also retarded by cell wall damaging compounds.

To gain insights into the function of *FLV1* and *FLV2* in survival under fluconazole stress, attempts were made to disrupt these genes in *wt* background. We have disrupted the *FLV1* gene in the *wt* background by both one step gene replacement (labeled as YRK1) as well as two-step gene replacement methodology (labeled as YRK21). Briefly, 500bp of 5' UTR of *FLV1* was PCR amplified from *wt* genomic DNA and cloned upstream of the hygromycin resistance gene (*hph*) (under PGK promoter) in a plasmid containing *URA3* gene as a selection marker. Next, 3' UTR (550bp) of *FLV1* was cloned downstream of *hph* gene so that the *hph* gene was flanked by the 5' and 3'UTR of *FLV1* (plasmid pRK5). For one step strategy, the plasmid pRK5 was digested with Bcg1 (sites engineered during primer design) and the linear fragment carrying HYG cassette (*hph* gene flanked by the 5' and 3'UTR of *FLV1)* was transformed into wild-type strain and transformants were selected for hyrgomycin resistance. Replacement of *FLV1* ORF with *hph* gene was verified by PCR using a primer external to the cloned fragment (both at 5' and 3' end) and one primer that anneals within the *hph* gene. In addition, the lack of amplification with primers that anneal to the region within the ORF of *FLV1* was taken as the final evidence of disruption of *FLV1* with *hph* gene and *flv1*Δ (YRK1) was chosen for further studies.

For two step gene replacement strategy, the plasmid pRK5 was linearized with Bgl*II* (unique site in the cloned 5'UTR of *FLV1*) and then transformed into *C. glabrata* selecting for Ura^+ transformants. Integration at *FLV1* locus was verified by PCR with appropriate set of primers and the correct integrants were streak-purified on minimal media plates lacking uracil (CAA-Ura) followed by overnight growth in YPD. Next, the cells were streaked on CAA plates containing 5-FOA (5-Fluoroorotic acid) to select for recombinants that had lost the integrated plasmid and thus will be uracil auxotrophs (Ura-). The resulting Ura- segregants had either regenerated the wild-type *FLV1* locus or had *FLV1* disrupted with the *hph* gene (*flv1*Δ*::hph*). PCR was carried out on several 5-FOA resistant (uracil auxotrophs) colonies using primers outside the cloned UTR regions to check the genomic configuration of *FLV1* locus in these segregants and two *flv1*Δ mutants (from two independent integrants) were obtained. These deletion mutants were made Ura+ by integrating *URA3* gene back into the genome (YRK21-YRK23)

Surprisingly, the knock-outs made by one step and two step gene replacement methods showed totally opposite phenotype with regard to their sensitivity to fluconazole. YRK1 displayed clear sensitivity to fluconazole while YRK21 was resistant to fluconazole (see figure 1).

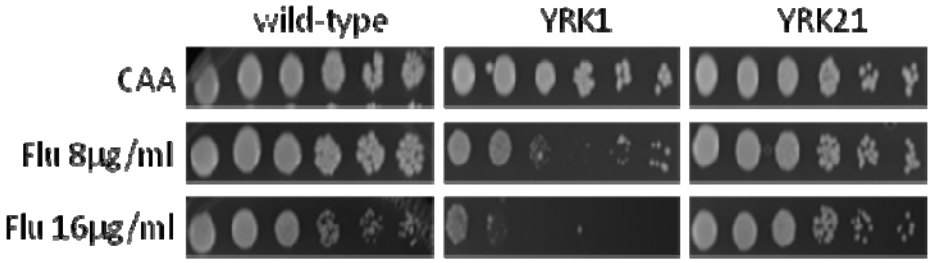


Figure1: Fluconazole susceptibility profiles. Equal numbers of cells were spotted in 10-fold serial dilutions onto minimal media plates with or without fluconazole and the plates were photographed after 48hrs of incubation at 30°C.

This apparent resistance of YRK21 also extended to other azoles such as ketoconazole and and clotrimazole but not to other drugs such as tolnaftate, cycloheximide *etc.* Since one of the mechanisms to acquire fluconazole resistance in *C. glabrata* is to lose mitochondrial function, we checked if the observed resistance of YRK21 towards fluconazole was due to the loss of mitochondrial function by plating assays. However, neither the fluconazole resistant colonies of YRK21 were petites (cells that are unable to utilize glycerol as carbon source indicating the presence of dysfunctional mitochondria) nor YRK21 loses mitochondrial function at a higher frequency than the *wt* cells. These results suggested that YRK21 indeed is a bonafide fluconazole resistant strain and this resistance is unlikely due to the overexpression of multi drug transporters as the YRK21 strain retains sensitivity to cycloheximide.

Since the disruption of *FLV1* in both YRK1 and YRK21 was confirmed by PCR, we are planning to do Southern Blot on these strains to rule out the possibility of multiple integrations in their genomes which might be responsible for their differential phenotypes. Depending upon the results of the Southern, attempts will be made to further characterize these knock-outs for their sensitivity/resistance towards fluconazole.

To disrupt *FLV2*, we have cloned the 5'UTR (550bp) and 3'UTR (600bp) of gene *FLV2* in the plasmid, however efforts to obtain *flv2*Δ had not been successful despite several attempts. A total of 164 colonies had been screened by PCR for the disruption of *FLV2* but the wild-type copy of the *FLV2* was retained in all the segregants after the resolution of integration intermediates suggesting that *FLV2* probably is essential in *C. glabrata*. Currently, we are trying to clone the complete ORF of *FLV2* for transient expression during the resolution of integration intermediates to generate *flv2*Δ deletion mutant.

Publications

1. ***Kaur R, Ma B and Cormack BP (2007) A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. ***Proceedings of the National Academy of Sciences of the USA*** 104: 7628-33

   (*** work done elsewhere)

Laboratory of Virology

Molecular Pathogenesis of lentiviruses

Principal Investigator	S Mahalingam	Adjunct Faculty
Ph.D students	P K Singhal	SRF
	M K Subba Rao	SRF
	Gita Kumari	SRF
	B Neelima	JRF
Other members	P Srilakshmi	Post doctoral fellow
	Y Padward	Post doctoral fellow
	S Chary	Project Assistant

Objectives:

1. To understand the mechanism of human immunodeficiency virus pathogenesis.
2. Functional analysis of putative Nucleolar GTPases.
3. To understand the function of Ras effector proteins.

Summary of work done until the beginning of this reporting year

Ras proteins regulate a wide range of biological processes by interacting with a variety of effector proteins. In addition to the known role in tumorigensis, activated form of Ras also exhibits growth-inhibitory effects by yet unknown mechanisms. Several Ras effector proteins identified as mediators of apoptosis and cell cycle arrest also exhibit properties normally associated with tumor suppressor proteins. Here, we show that Ras effector, RASSF5/NORE-1 binding strongly to K-Ras but weakly to both N-Ras and H-Ras. RASSF5 was found to localize both in nucleus and nucleolus in contrast to the other Ras effector proteins, RASSF1C and RASSF2, which are localized in the nucleus but excluded from nucleolus. A 50-amino acid transferable arginine-rich nucleolar localization signal (NoLS) identified in RASSF5 is capable of interacting with importin-beta and transport the cargo into the nucleolus. Surprisingly, similar arginine-rich signals identified in RASSF1C and RASSF2 interact with importin-alpha and transport the heterologous cytoplasmic proteins to the nucleus. Interestingly, mutation of arginine residues within these nuclear targeting signals prevented interaction of Ras effector proteins with respective transport

receptors and abolished their nuclear translocation. The results provide evidence for the first time that arginine-rich signals are able to recognize different nuclear import receptors and transport the RASSF proteins into distinct sub-cellular compartments. In addition, it suggests that the nuclear localization of RASSF5 is critical for its cell growth control activity. Together, it suggests that the transport of Ras effector superfamily proteins into nucleus/nucleolus may play a vital role in modulating RAS mediated cell proliferation during tumorigenesis

Grn1p from fission yeast and GNL3L from human cells, two putative GTPases from the novel HSR1_MMR1 GTP-binding protein subfamily with circularly permuted G-motifs play a critical role in maintaining normal cell growth. Deletion of Grn1 resulted in a severe growth defect, a marked reduction in mature rRNA species with a concomitant accumulation of the 35S pre-rRNA transcript, and failure to export the ribosomal protein Rpl25a from the nucleolus. Deleting any of the Grn1p G-domain motifs resulted in a null phenotype and nuclear/nucleolar localization consistent with the lack of nucleolar export of preribosomes accompanied by a distortion of nucleolar structure. Heterologous expression of GNL3L in a Deltagrn1 mutant restored processing of 35S pre-rRNA, nuclear export of Rpl25a and cell growth to wild-type levels. Genetic complementation in yeast and siRNA knockdown in HeLa cells confirmed that the homologous proteins Grn1p and GNL3L are required for growth. Failure of two similar HSR1_MMR1 putative nucleolar GTPases, Nucleostemin (NS), or the dose-dependent response of breast tumor autoantigen NGP-1, to rescue deltagrn1 implied the highly specific roles of Grn1p or GNL3L in nucleolar events. Our analysis uncovers an important role for Grn1p/GNL3L within this unique group of nucleolar GTPases.

Details of work done in the current reporting year (April 1, 2007 - March 31, 2008)

The following were the significant findings made by the group in the current reporting year.

RASSF5 is a nucleolar protein: Recent reports suggest that Ras proteins are localized in different intracellular compartments in addition to their known association with the plasma membrane and the function of various tumor suppressor genes (TSGs) depends upon their sub-cellular

localization. Proteins with a molecular mass less than 35-40 kDa can enter the nucleus by passive diffusion rather than by a signal-mediated process. To distinguish between these two possibilities, we evaluated the transport activities of Ras associated TSGs in the context of a chimeric protein designed to exceed the diffusion limit of the nuclear pore. Enhanced Green Fluorescent Protein (eGFP) fusion proteins of RASSF1A, RASSF1C, RASSF2, and RASSF5 were generated and expressed in Cos-7 cells to characterize their cellular localization. Results suggest that RASSF1A is associated with the microtubule assembly whereas RASSF1C, a variant of RASSF1, was localized in the nucleus as punctate pattern in addition to its microtubular association. RASSF2 was localized to the nucleus, and excluded from nucleolus, while RASSF5 was localized both in the nucleus and nucleolus. In contrast, GFP was found to be distributed throughout the cell, consistent with its passive diffusion (data not shown). We generated both N-terminal and C-terminal tagged fusion proteins with Flag or GFP and our results showed that the RASSF5 containing Flag or GFP at the N-terminus localized in the cytoplasm. In contrast, RASSF5 fusion protein containing tag (either GFP or Flag) at the C-terminus localized to the nucleus. Interestingly, we observed the cytoplasmic localization of RASSF5 in lung adenocarcinoma cell line, A549 consistent with the previous report by Moshnikova et al. Surprisingly, GFP-RASSF5 fusion protein translocated to the nucleus when A549 or Cos-7 cells were treated with well-known nuclear export inhibitor, leptomycin B (LMB) suggest that RASSF5 may be a nucleo-cytoplasmic shuttling protein. RASSF5 with C-terminal fusion of GFP was used for further analysis since this fusion protein localized to the nucleus and can be visualized directly in living cells without antibody staining. In conclusion, RASSF1C, RASSF2, and RASSF5 appear to possess specific signals and transported into nucleus by a signal-mediated process. Based on the basal distribution of GFP-fusion proteins, we used GFP as a living tracer to understand the mechanism(s) of RASSF1C, RASSF2, and RASSF5 nuclear transport.

RASSF1C and RASSF2 encode an arginine-rich nuclear import signal: Deletion mutants of RASSF1C and RASSF2 were generated and their sub-cellular localization examined in Cos-7 cells using immunofluorescence microscopy to identify the signal(s) and the mechanism(s) underlying

the nuclear localization. RASSF1C mutants containing amino acids 1-150 were retained in the nucleoplasm similar to full-length protein. In contrast, truncated proteins with deletion of amino acids 100-271 showed either diffuse distribution throughout the cells like GFP or localized only in the cytoplasm. In addition, deletion of amino acids 201-271 from C-terminus abolished microtubular association of RASSF1C. Furthermore, replacement of arginine residues at positions 119 and 120 (F1C-Rm1) as well as at 129-131 (F1C-Rm2) by alanines completely blocked the nuclear transport of mutant RASSF1C proteins. Western blot analysis of the transfected cell lysates suggested that all the RASSF1C deletion mutants containing GFP fusion proteins express correct size polypeptides. Collectively, the results suggest that the amino acids within N-terminal domain 100-150 contain the information sufficient to target RASSF1C to the nucleus, while the C-terminal region is required for its association with microtubule assembly.

Our results indicate that deletion mutants of RASSF2 containing amino acid residues 1-200 retain the wild type nuclear localization pattern. In contrast, deletion of amino acid domain between 100-200 completely abrogated the nuclear retention of mutant proteins suggesting that nuclear transport signal is located within this domain. Exchange of arginine residues at positions 150-152 (F2-Rm1) and 165-166 (F2-Rm2) with alanines resulted in alteration of RASSF2 mutant proteins nuclear translocation. Interestingly, replacement of arginine clusters (positions 150-152 as well as 165-166) completely abrogated the nuclear localization of mutant protein RASSF2-Rm3 (Fig. 3H). The data thus suggest that arginine-rich bipartite transport signal within the amino acid domain 100-200 plays a critical role in translocation of RASSF2 into the nucleus.

Signal residing within amino acids 51-100 of RASSF5 is essential and sufficient for nucleolar targeting of heterologous protein: N-terminal deletion fragments of RASSF5 were generated and tested for the ability to undergo nuclear/nucleolar transport to determine nuclear localization signal (NLS)/nucleolar localization signal (NoLS) of RASSF5. Western blot analysis of the transfected cell lysates suggests that all RASSF5 deletion mutants containing GFP fusion proteins were expressing the correct size polypeptides. Results show that amino acid residues 51-100

represent the smallest fragment that is sufficient to maintain NoLS activity of RASSF5. GFP fusion protein containing amino acids 51-100 of RASSF5 localized to the nucleus with predominant nucleolar staining. In contrast, the deletion of amino acid 51-100 completely prevented the nuclear transport of RASSF5. Thus suggesting that amino acid residues 51-100 contain signal sufficient to target RASSF5 as well as the heterologous cytoplasmic protein, GFP to the nucleolus.

Amino acid sequence analysis indicates that clusters of basic amino acids, particularly arginine residues within 51-100 of RASSF5 might function as NLS/NoLS. Recent reports demonstrate that arginine-rich domains are able to transport the cargo proteins to the nucleus/nucleolus by interacting directly with the nuclear transport receptor, importin-beta in the classical transport pathway. To understand whether the arginine residues present within domain 51-100 play any role in the nucleolar translocation of RASSF5, mutant form of RASSF5 ($RASSF5^{Rm}$) was constructed in which RRR residues at positions 92, 93, and 94 were replaced by alanines and was transiently expressed in Cos-7 cells. This replacement resulted in complete prevention of nuclear/nucleolar localization of RASSF5, thus suggesting that arginine residues within amino acid domain 51-100 play a critical in nucleolar retention of RASSF5.

Confocal scanning microscopy was done to determine the immunolocalization of $RASSF5^{51-100}$-GFP in laser sections of intact cells. Serial sections through positively immunostained cells showed that $RASSF5^{51-100}$-GFP was localized predominantly in the nucleolus and certain amount was also present in the nucleoplasm of transfected cells. The specificity of nucleolar staining was further confirmed by co-expression of human nucleolar GTPase, NGP-1. The results in figure 4E demonstrate that $RASSF5^{51-100}$ co-localized with DsRed tagged NGP-1 in the nucleolus. Together, these data suggest that RASSF5 encode a transferable NoLS, which is sufficient to target the heterologous proteins to the nucleolus.

RASSF family of Ras effectors transported into the nucleus by classical nuclear transport pathways: To understand the mechanism(s) of nuclear transport of RASSF family proteins, we

first determined the receptor with which they interact. RASSF1, RASSF2, and RASSF5 interaction with the known nuclear transport receptors (importin-alpha and importin-beta) was first tested by *in vitro* protein-protein interaction assays. Flag tagged RASSF1A, RASSF1C, and RASSF2, RASSF5, and GFP expression plasmids were transfected in Cos-7 cells and the cell lysates containing fusion proteins were mixed with glutathione-sepharose beads that had been prebound with either GST or GST fused to importin-alpha or importin-beta. Following overnight incubation, the beads were washed, and the bound proteins, as well as a fraction of the input proteins were examined by SDS-12% PAGE followed by Western Blot analysis using anti-Flag monoclonal antibody. An interaction of RASSF1C, RASSF2 and RASSF5 with importin-alpha was readily detected. In contrast, no interaction of RASSF1C and RASSF2 with importin-beta was observed suggesting the specificity of RASSF5 interaction with importin-beta. The inability of GST to interact with RASSF family proteins as well as the lack of interaction of RASSF1A or GFP with importin-alpha and importin-beta served to illustrate the specificity of RASSF1C or RASSF2 interaction with importin-alpha and RASSF5 interaction with both importin-alpha as well as with importin-beta. NLSs of SV40 T antigen and HIV-1 Rev served as positive controls in this assay. Equal amounts of GST, GST-importin-alpha, and GST-importin-beta bound with glutathione-sepharose beads were used in all *in vitro* pull-down assays. It is likely that RASSF1C, RASSF2, and RASSF5 interaction with importin-alpha or importin-beta in these pull-down experiments is a consequence of direct interaction.

To further characterize the interaction of transport receptors with Ras effector proteins, a series of assays were performed in which the interaction between RASSF family proteins and the nuclear transport receptors were tested *in vivo*. Specifically, Cos-7 cell lysates containing RASSF1A, RASSF1C, and RASSF2, and RASSF5 containing Flag tag were immunoprecipitated with anti-Flag monoclonal antibody and T-NLS and HIV-1 Rev NLS with anti-GFP monoclonal antibody followed by Western Blot using polyclonal importin-alpha or importin-beta antibodies. Results suggest that RASSF1C, RASSF2, and RASSF5 interact and co-precipitate with endogenous importin-alpha. Interestingly, co-immunoprecipitation of importin-beta was observed only with

RASSF5 but not with other RASSF proteins. No interaction of GFP with importin-alpha and importin-beta was observed in this assay, suggesting the specificity of RASSF family proteins interaction with importin-alpha and importin-beta *in vivo*. Collectively, these results provide evidence that RASSF5 interacts with both importin-alpha and importin-beta while RASSF1C and RASSF2 interact only with importin-alpha both *in vivo* and *in vitro* and transported into the nucleus.

Evidence of importin-alpha interaction with arginine-rich nuclear transport signal identified in RASSF1C and RASSF2: Deletion mutants of RASSF1C (RASSF1C$^{1-100}$, RASSF1C$^{1-150}$, and RASSF1C$^{1-200}$) and RASSF2 (RASSF2$^{1-100}$, RASSF2$^{1-200}$, and RASSF2$^{1-300}$) were transfected into Cos-7 cells to identify the domain(s) that are required for importin-alpha interaction. The cell lysates containing equal amounts of RASSF1C and RASSF2 mutant proteins were mixed with glutathione-sepharose beads that had been prebound with GST fused to importin-alpha. The bound proteins were resolved by SDS-12% PAGE followed by Western Blot using anti-GFP monoclonal antibody. Our results indicate that between amino acid residues 1-150 of RASSF1C interact with importin-alpha, which is further supported by its nuclear localization. Interestingly, deletion of amino acid residues 100-326 (RASSF2$^{1-100}$) completely abrogated RASSF2 interaction with importin-alpha (Fig. 6A, lane 7). In contrast, RASSF2 mutant proteins retaining amino acid residues 100-200 interacts with importin-alpha as like full length protein suggest that the amino acid residues 100-200 are critical for RASSF2 interaction with importin-alpha. This was further supported by the nuclear accumulation of RASSF2$^{1-200}$, and RASSF2$^{1-300}$. Results in figure 6C indicate that exchange of arginine residues within domain 100-150 of RASSF1C completely prevented its interaction with importin-alpha. To verify the integrity of *in vitro* GST pull-down assay the NLS of SV40 with glutathione-sepharose bound importin-alpha was used as positive control. Importantly, the inability of GST to interact with RASSF1C as well as RASSF2 and the lack of interaction between GFP and importin-alpha served to illustrate the specificity of importin-alpha interaction with Ras effectors RASSF1C and RASSF2. These data provide evidence that

both RASSF1C and RASSF2 interact with importin-alpha through their NLS for efficient nuclear import.

RASSF5 interaction with importin-beta is critical for its transport into the nucleolus: To further understand the mechanism of RASSF5 nucleolar transport, we performed a series of protein-protein interaction assays in which the interaction between RASSF5 and the nuclear transport receptors importin-alpha and importin-beta was examined. The GFP fusion of full length and mutants (RASSF5$^{51-100}$, RASSF5$^{101-418}$, RASSF5$^{201-418}$, and RASSF5$^{301-418}$, and RASF5^{Rm}) of RASSF5 and GFP expression plasmids were transfected in Cos-7 cells. The cell lysates containing equal amount of fusion proteins were mixed with glutathione-sepharose beads prebound with either GST or GST-importin-alpha or GST-importin-beta as described above. Following overnight incubation the beads were washed, and the bound proteins were resolved in SDS-12% PAGE followed by Western Blot using anti-GFP monoclonal antibody. RASSF5 interacts with importin-alpha as well as importin-beta. Interestingly, RASSF5^{Rm} interacts with importin-alpha but not (weak to barely detectable) with importin-beta. Interestingly, we observed the interaction of RASSF5$^{51-100}$ with importin-beta but not with importin-alpha. In addition, RASSF5 mutant proteins (RASSF5$^{101-418}$, RASSF5$^{201-418}$, and RASSF5$^{301-418}$) lacking amino acid residues 51-100 did not interact with importin-beta. Interestingly, lack of interaction of RASSF5^{Rm} with importin-beta was further supported by the cytoplasmic localization of this mutant protein. These data provide evidence that RASSF5 interacts with importin-beta through 50 amino acid NoLS, which is necessary and sufficient for efficient nucleolar retention of RASSF5. Surprisingly, results in figure 7C suggest that full-length and the deletion mutants retaining the C-terminal portion of RASSF5 interact with importin-alpha. Although only a weak interaction was observed with importin-alpha but it is specific. In addition, RASSF5^{Rm} interacts with importin-alpha though the mutant protein localized to the cytoplasmic compartment raises the possibility for the presence of additional nuclear targeting signal in RASSF5. The cytoplasmic appearance of RASSF5^{Rm} mutant protein may be due the presence of dominant nuclear export signal. This was further supported by the nuclear accumulation of RASSF5 in A549 or Cos-7 cells when treated with

leptomycin B. To verify the integrity of the *in vitro* GST pull-down assay, cell lysates containing importin-alpha with glutathione-sepharose bound importin-beta was used as positive control (data not shown). We used the well-characterized NLSs of SV40 T antigen and HIV-1 Rev as add on positive controls. GST, used as a negative control, had no interaction with full length RASSF5. Importantly, the inability of GFP to interact with importin-alpha or importin-beta suggests the specificity of RASSF5 interaction with importin-alpha and importin-beta. Equal amount of GST, GST-importin-alpha, and GST-importin-beta bound with glutathione-sepharose beads were used in all *in vitro* pull-down assays. In summary, these data provide evidence that interaction of RASSF5 with importin-beta plays a critical role for its transport into the nucleolus.

Nuclear translocation of RASSF5 is critical for its cell growth inhibitory activity:

To understand the functional relevance of RASSF5 nuclear transport, both lung adenocarcinoma cell line, and A549 cells were transfected with wild-type and the nuclear defective mutant ($RASSF5^{Rm}$) and determined the growth inhibitory effect following selection with hygromycin. Results in figure 8 show that wild type RASSF5 expression suppressed the growth of A549 cells as evidenced by reduced growth of colonies expressing RASSF5 in soft agar. In contrast, no growth inhibition was observed both in the GFP and nuclear import defective mutant ($RASSF5^{Rm}$) expressing colonies. The results provide evidence that RASSF5 expression modulate the cell growth and nuclear translocation of RASSF5 may play an important role in regulating cell proliferation.

Publications

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Laboratory of Molecular Immunology

Understanding the mechanism of Azadirachtin-mediated cell signaling: role in anti-inflammation and anti-tumorigenesis.

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Objectives

1. Design and detection of novel pathway mediated by novel small molecules to induce anti-inflammatory and anti-tumorigenic responses.
2. Regulation of TNF receptors in different cell types.

Summary of work done until the beginning of this reporting year

It is a common practice to evaluate the efficacy of any compound as a drug in cell-based *in vitro* system followed by *in vivo* murine model prior to clinical trials in human. Cardiac glycosides are very effective to kill human cells, but not murine cells. We had described the comparative molecular mechanism of oleandrin, a cardiac glycoside action in human and murine cells. Treatment with oleandrin facilitated nuclear translocation of FKHR in human, but not murine cells by following events: altering membrane fluidity - inhibiting Na^+/K^+-ATPase activity – increasing intracellular free Ca^{2+} level – increasing calcineurin activity - dephosphorylation of Akt – nuclear translocation of NF-AT – induction of FasL – induction of apoptosis (**Apoptosis, 2007**; **Mol. Immunol. 2007).** Though Oleandrin is known to induce apoptosis in tumor cells, no reports are available on its efficacy in primary cells. We reported for the first time that oleandrin induces apoptosis in tumor cells potently but not in primary cells like peripheral blood mononuclear cells (PBMC) and neutrophils through activation of Fas, thereby inducing apoptosis specifically in tumor cells. Overall, the results suggest

that oleandrin mediates apoptosis in tumor cells by inducing Fas, but not in primary cells indicating its potential anti-cancer property with negligible or no side effect (**J. Clin. Immunol. 2006**). The compound 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (P_3-25) induces cell death in NF-kB-expressing cells through downregulationof NF-kB-dependent genes involved in cell cycle and cell proliferation. It decreases phosphorylation of p65 by inhibiting upstream kinases, such as protein kinase A and casein kinase II. P_3-25 alone induces apoptosis in NF-□B-expressing and doxorubicin-resistant breast cancer cells and it potentiates apoptosis in the presence of other chemotherapeutic agents. Our studies showed that P_3-25 exerts anti-tumorogenic activity by inhibiting phosphorylation of p65, the transcriptional active subunit of NF-□B through inhibiting its upstream kinases, and potentiates apoptosis mediated by chemotherapeutic agents (***Cell Death & Differ. 2007***) and thus, indicate, novel approaches for design of anti-cancer drugs for combination chemotherapy.

Details of progress in the current reporting year (April 1, 2007 - March 31, 2008)

a. Thiadiazolidine derivatives induce cell death by arresting cell cycle and FasL expression via Akt-FKHR pathway

The 1,2,4-thiadiazolidine derivatives have shown to induce cell death but the exact mechanism is not known. In this study, we provide evidence that dichlorophenyl form of thiadiazolidine (designated as P_3-25) is a stronger inducer of cell death than any other form. P_3-25 potentially arrests cell cycle at G1 phase and down regulates cyclin D1 and cyclin E without interfering p16 and p27. It decreases c-Myc level and thereby inhibiting DNA binding ability of Myc-Mad complex. P_3-25 de-phosphorylates retinoblastoma and Akt thereby facilitating nuclear translocation of forkhead transcription factor, which in turn, expresses its dependent gene FasL. Activated FasL inhibits cell proliferation and induces cell death. Our results suggest that P_3-25 derivative exerts anti-tumor activities by decreasing Myc-mediated response and increasing FasL expression, which may have a role in designing such drugs for tumor therapy.

Thiadiazolidine-derivatives induce cell death in U-937 cells.

To detect the role of different Thiadiazolidine–derivatives on cell death, U-937 cells are treated with different concentrations of P_3-21, P_3-22, P_3-23, P_3-24 or P_3-25 for 72 h and then cell death was assayed by multiple methods. U-937 cells treated with P_3-21, P_3-22, P_3-23, P_3-24 or P_3-25 (100 nM each for 48 h) showed 18%, 21%, 29%, 36%, or 58% cell death, respectively when assayed by FACS using annexin V-PE staining (Fig.1A). All these compounds arrest cells at G1-S phase (data not shown) and our data suggests that thiadiazolidine-derivatives potently induces apoptosis.

P_3-25 decreases the amount of c-Myc and DNA binding activity of Myc-Max complex.

DNA binding activity of the Myc-Max was decreased in the nuclear extracts obtained from P_3-25-treated cells, as detected by gel shift assay (Fig.1B). Also the expression of Myc-Max-dependent genes, p21 and Cdc25A decreased with increasing time of P_3-25 treatment (Fig.1B). The results thus suggest that Myc-Max-dependent genes expression is also abrogated by P_3-25 treatment.

Phosphatase or caspase inhibitor partially protects P_3-25-mediated cell death.

Okadaic acid, a phosphatise inhibitor protects P_3-25-mediated decrease of phospho-Rb and cyclin D1 but increases the amount of FasL partially (Fig.1C).

P_3-25 induces FasL expression.

FasL-dependent luciferase gene expression, as detected by luciferase activity, increases kinetically in P_3-25-treated U-937 cells transfected with FasL-luciferase construct (Fig.1C), which is further indicated by increased expression of FasL upon P_3-25 treatment.

We also show that some 5-substitutedarylimino-2-N-substitutedphenyl-3-oxo-1,2,4-thiadiazolidine derivatives inhibit cell cycle by arresting at G1/S phase. Among these derivatives 5-(4-methoxyarylimino) 2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine, designated as P_3-25, is shown to be more potent in inducing cell death. P_3-25 dephosphorylates Akt and thereby inducing nuclear translocation of FKHR. FKHR in turn induces Fas and FasL expression, which induce cell death. It decreases phospho-retinoblastoma level and suppresses c-Myc-mediated transcription, which further induces cell death. This observation may be helpful to design P_3-25 as a novel anti-inflammatory and/or anti-tumor drug.

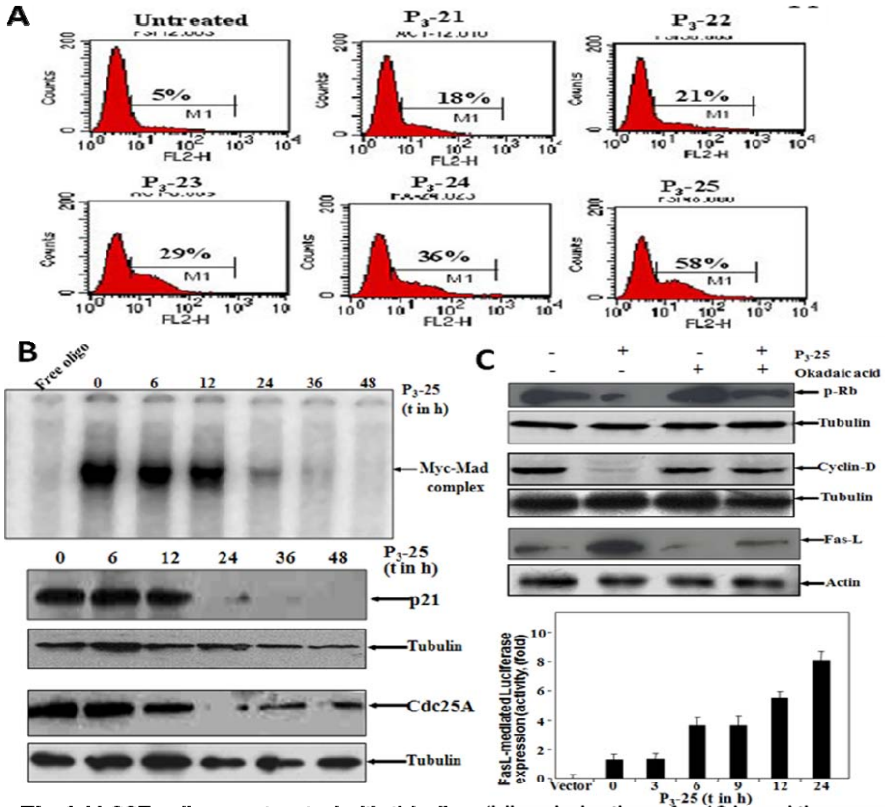

Fig.1. U-937 cells were treated with thiadiazolidine derivatives for 48 h and then cell death (%) was detected by FACS using Annexin-V-PE (A). P_3-25 inhibited Myc-Mad DNA binding activity (B, upper panel) and its dependent gene p21 and Cdc25A (B, lower panels). Phosphatase inhibitor, okadaic acid protected P_3-25-mediated dephosphorylation of pRb thereby protected deregulation Cyclin D and FasL (C).

b. Azadirachtin inhibits TNF-induced inflammatory responses by downregulating TNF receptors.

Azadirachtin, derived from Neem tree has shown to affect insects and molluscs nervous and endocrine systems. Azadirachtin, highly oxygenated C-secomeliacins has been used as traditional medicine for house hold remedy against various human ailments. Oil, bark and leaf extracts of neemtree have been therapeutically used as folk medicine to control leprosy, intestinal helminthiasis, respiratory disorders, constipation and as anti-bacterial, anti-Tuberculosis, anti-viral treatments. In mammalian cells, nuclear DNA is a critical target for azadirachtin as evidenced by the formation of micronuclei, which was investigated. Neem leaf extract has been reported to be non-toxic, non-mutagenic and found to possess immunomodulatory, anti-inflammatory and anticarcinogenic properties. Azadirachtin, is structurally similar to Ecdysone of insect hormone has been shown to block ecdysone-mediated insect moulting. Ecdysone, after binding with ecdysone receptor (EcR)

must heterodimerize with its partner ultra spirical (USP), which is the insect homologue of the mammalian retinoid X receptor (RXR).

Aza inhibits the TNF induced NF-kB.

The activation of NF-**k**B is one of the early signals induced by TNF in most cells. Therefore, we used TNF to induce NF-kB in U937 cells. Cells were treated with different concentrations of azadirachtin and then stimulated with TNF, following which nuclear extracts were prepared and assayed for NF-kB by gel shift assay. Azadirachtin inhibited TNF-induced NF-kB DNA binding activity in a dose-dependent manner (Fig.2A). Azadirachtin inhibited TNF-induced expression of several genes such as ICAM1, Cox2 etc. and also their reporter gene expression (data not shown).

Azadirachtin inhibits TNF induced NF-kB at TNFR, but not TRAF2, TRADD, IKK, or p65 level.

Cells were co-transfected with plasmid of TNFR, TRAF2, TRADD, IKK, and p65k and plasmid bearing NF-kB promoter linked to heat stable secretory alkaline phophatase (SEAP) gene. Some of these cells were further treated with azadirachtin and the others were not. Culture supernatants were assayed for SEAP. We observed that Aza treatment in the cells transfected with TNFR showed inhibition of SEAP activity as compared to untreated (Fig.2B), indicating that TNFR is the target for azadirachtin.

Azadiractin inhibits TNF, but not IL-8, TRAIL, IL-1 or IL-4 binding.

U937 cells were treated with different concentration of azadirachtin at 37ºC for 2 hrs, and then incubated with 4 ng ^{125}I-labeled TNF, IL-1, IL-8, TRAIL, or IL-4 for 2 hr at 4ºC. Labeled ligand binding was detected in gamma counter. Azadirachtin decreased the binding of ^{125}I-TNF, but not other ligands in a dose-dependent manner, suggesting that azadirachtin specifically downregulates TNFRs (Fig.2C).

Azadirachtin downregulates TNFRs as detected by Scatchard analysis.

As azadirachtin inhibited TNF binding, number of TNFRs and their affinity toward TNF was detected by Scatchard analysis using different concentrations of labeled TNF binding at 4ºC in

untreated and azadirachtin-treated cells. Number of TNFRs was determined as 4898 receptors/cell and 988 receptors/cell in untreated and azadirachtin-treated cells ,respectively (Fig.2D).

This study clearly demonstrate that Aza acts as a potent inhibitor of TNF induced NF-kB and NF-kB-dependent genes such as ICAM-1, VCAM and Cox-2. The TNF induced NF-kB dependent reporter gene expression and NF-kB dependent gene expression were completely inhibited by pre treatment of azadirachtin. Azadirachtin inhibits the TNF, which induces NF-kB at the TNF receptor level. This was proved by co-transfecting different clones of the TNF pathway such as *TNFR, TRAF2, TRADD*, *IKK,* and *p65* transfected cell. In this report, we provide evidences that azadirachtin downregulates cell surface TNFRs and thereby inhibiting TNF-induced cell signaling. How azadirachtin downregulates TNFRs needs to be clarified by further studies. However, our studies so far, suggest that azadirachtin can be an important herbal molecule to cure diseases involving inflammation.

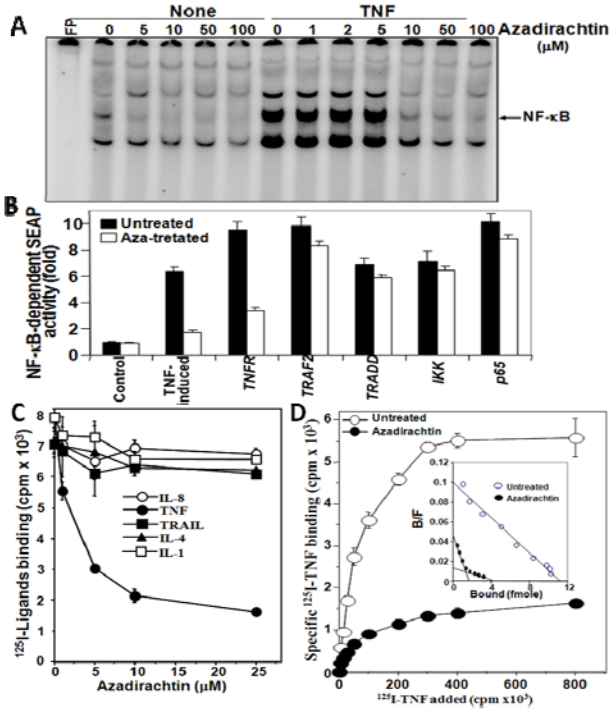

Fig.2. Azadirachtin potentially inhibits TNF-induced NF-κB activation: U-937 cells, were treated with azadirachtin and nuclear extracts were assayed for NF-κB by gel shift assay (A). Azadirachtin interacts TNFRs level, but not TRAF2, TRADD, IKK, or p65 level. Cells were transfected with SEAP and indicated constructs followed by treatment with azadirachtin. NF-κB activity was assayed from SEAP activity (B). Different ligands were Iodinated and cell surface binding was detected (C). Scatchard plot showed the azadirachtin-mediated decreases of TNFRs (D).

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Laboratory of Bacterial Genetics

Studies on gene regulation, transcription termination, and amino acid and ion-transport in *Escherichia coli*

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Objectives

1. To study the ArgP regulon and the mechanism of ArgP-mediated transcriptional regulation of the arginine exporter ArgO
2. To test the model of and mechanisms mediating R-loop formation from nascent untranslated transcripts
3. To investigate an unusual phenomenon of K^+ toxicity in *hns trx* double mutant strains

Summary of work done until the beginning of this reporting year

1. In previous work (Nandineni and Gowrishankar, J. Bacteriol., 2004, 186: 3539-3546; Nandineni et al. J. Bacteriol., 2004, 186: 6391-6399) we had shown that mutations in the *argP* gene confer both an osmosensitive phenotype (in strain backgrounds with additional mutation in the *gltBD* locus encoding glutamate synthase) and a phenotype of increased sensitivity to the arginine (Arg) analogue canavanine (CAN). Mutations in another locus

designated *argO* were also identified to confer a CAN-supersensitive phenotype, and genetic evidence was obtained to support the conclusions that (i) *argO* encodes an exporter for Arg and CAN, whose inactivation leads to intracellular CAN accumulation and hence to CAN-supersensitivity; and (ii) the *argP* gene is involved in transcriptional regulation of *argO,* such that *argO* transcription fails to occur in *argP* mutants and that, in *argP*$^{+}$ strains, it is induced by Arg or CAN and repressed by lysine (Lys). In last year's report, we had presented the evidence from *in vitro* experiments with ArgP, RNA polymerase (RNAP), and the *argO* DNA regulatory region to support a model that Arg-bound ArgP binds the *argO* operator to recruit RNAP and activate transcription, whereas Lys-bound ArgP also binds the *argO* operator to recruit RNAP but restrains the latter at the promoter. We had therefore proposed that the *argO* case is the first known instance of an environmental signal regulating bacterial transcription at the final stage of promoter escape (i.e., after open complex formation) by RNAP.

2. Based on genetic studies with *rho* and *nusG* mutants defective in factor-dependent transcription and the demonstration that these mutants are killed upon transformation with plasmids such as pACYC184 or pUC19 whose replication is dependent upon formation of RNA-DNA hybrids (R-loops), we had proposed a model that the mutants suffer increased chromosomal R-loops from nascent untranslated transcripts that are expected to accumulate in them (Harinarayanan and Gowrishankar, J. Mol. Biol., 2003, 332: 31-46; Gowrishankar and Harinarayanan, Mol. Microbiol., 2004, 54: 598-603). Consistent with the model, the plasmid-mediated lethality in *rho* and *nusG* mutants was suppressed by overexpression of R-loop removing enzymes such as RNase H1, RecG, or (phage T4-encoded) UvsW. The plasmid-mediated lethality phenotype was also suppressed by manipulations that served to reduce the activity of the endoribonuclease RNase E (by either mutation in its structural gene *rne* or overexpression of phage T7-encoded protein kinase which phosphorylates the protein and down-modulates its activity); the RNase E results have been explained on the hypothesis that endonuucleoytic cleavage of the nascent untranslated transcripts is necessary

to overcome a topological constraint for chromosomal R-loop formation in *rho* and *nusG* mutants. We had also found that moderate overexpression of RNase E was lethal in the mutants whereas it was well tolerated in the wild-type strain. A bisulfite-treatment assay was standardized to test for occurrence of single-stranded DNA regions displaced by R-loop formation at chosen target loci.

3. Potassium uptake in *E. coli* is mediated by constitutively expressed Trk transporters and an inducible Kdp transporter. The latter is encoded by the *kdp* operon whose transcription is regulated by potassium availability in the growth medium. We had earlier found that *kdp* transcription in vivo is down-regulated by mutations in *hns* and *trx* affecting, respectively, the nucleoid protein H-NS and the cytoplasmic redox protein thioredoxin (Sardesai and Gowrishankar, J. Bacteriol., 2001, 183: 86-93), and subsequently also that *hns trx* double mutants are killed in ordinary minimal media with ≥100 mM potassium. The potassium-toxicity phenotype was suppressed by mutations in *ahpC*, *metL, ptsP,* and *rpoS*, as well as by constitutive-activating mutations in *oxyR* or supplementation of the medium with hydrogen peroxide.

Work undertaken in the current reporting year (April 1, 2007 - March 31, 2008)

1. ***ArgP regulon and argO transcriptional regulation***

a. With respect to in vitro experiments on ArgP-mediated regulation, it was shown by different approaches that the transcriptionally inactive complex of ArgP and RNAP that is assembled on the *argO* promoter in presence of Lys can be "chased" into a transcriptionally active form upon replacement of Lys by Arg as co-effector. These approaches included both solution- and bead-based experiments to study transcription from *argO*, as well as an exonuclease III-protection assay to demonstrate that the RNAP footprint on the *argO* promoter, which is observed in presence of ArgP and Lys, is lost (because RNAP moves away from the promoter in productive transcription) upon addition of excess Arg to the mixture. These data support the model that Lys-bound ArgP restrains RNAP at the *argO* promoter at a step after open complex

formation, perhaps prior to the step of DNA scrunching, and that this restraint for productive transcription is removed when ArgP is bound to Arg as co-effector.

b. We have also undertaken experiments to test whether other genes in *E. coli* are regulated by ArgP. This protein has been reported previously to bind several DNA sequences including *oriC*, *dnaA,* and *nrdA*, and our results indicate that the binding affinity of ArgP to these sequences is at least ten-fold less than that for *argO*. Other candidate genes such as *gdhA* are also to be tested in future experiments.

2. ***Studies on E. coli rho and nusG mutants and the R-loop model***

a. It has previously been shown by Dreyfus and co-workers using a T7-*lac* construct that the much faster rate at which phage T7 RNAP-directed transcriptional elongation in *E. coli* occurs (as compared to the rate of transcription elongation by *E. coli* RNAP) leads to the uncoupling of transcription and translation, thereby rendering the exposed transcript region susceptible to cleavage by RNase E so as to result in reduced β-galactosidase activity in these cells. We found that the overexpression of UvsW in these cells led to a 7-fold increase in β-galactosidase activity, suggesting that R-loops probably do occur from the nascent untranslated transcripts even under these conditions and that their occurrence perhaps leads to reduced transcription of *lacZ* by succeeding T7 RNAP molecules. (It may be noted that T7 RNAP-directed transcription is insensitive to Rho-mediated termination.)

b. The bisulfite-treatment assay was employed to test for occurrence, in *rho* and *nusG* mutants, of single-stranded DNA regions at two target loci (*lacZ* and *trpE*) carrying chain-terminating mutations. Although bisulfite-reactivity was observed in these cases (detected as multiple C-to-T changes in the target regions), similar reactivity was also obtained in the control wild-type strain. We are in the process of cloning the target loci on plasmids in an attempt to improve the signal-to-noise ratio in these experiments. We are also initiating additional approaches for R-loop detection such as experiments of primer extension and chromatin immunoprecipitation at the target loci in both mutant and wild-type strains.

c. Two recent and interesting findings from other laboratories have been the identification for the first time of a 5′-3′ exoribonuclease in a bacterium (designated RNase J1, in *Bacillus subtilis*) (Mathy et al., Cell 2007, 129: 681-692) and of an RNA pyrophosphohydrolase RppH in *E. coli* which converts the 5′-triphosphate end of mRNA molecules to 5′-monophosphate and thus marks them for endonucleolytic cleavage by RNase E (Deana et al., Nature 2008, 451: 355-359). Experiments have been initiated to test the possible effects of RNase J1 and RppH on the phenotypes related to R-loop formation being studied in our laboratory.

3. ***Potassium toxicity in hns trx mutants***

a. In the current year, we tested whether the mechanism by which *rpoS* mutations suppress potassium toxicity in the *hns trx* double mutants was through the control by RpoS of the catalase gene *katE*. This possibility was considered because of our earlier finding that mutations in *ahpC* encoding alkylhydroperoxidase lead to suppression of potassium toxicity. However, neither *katE* nor *katG* mutations suppressed the potassium toxicity, indicating that *rpoS* was acting through a mechanism unrelated to its control of *katE.*

b. The potassium-toxicity phenotype was suppressed by supplementation of the growth medium with cysteine, cystine, methionine or glutathione but not by dithiothreitol. These observations indicated that at high potassium concentration, the *trx hns* mutants may experience a sulfur assimilation defect and not an intracellular redox imbalance. Thioredoxin is indeed known to be stoichiometrically required for sulfate reduction and assimilation in *E. coli*, and this idea (of a defect in sulfur assimilation in the mutants) is being pursued further.

c. Work with *hns trx* mutants bearing mutations in one or more of the potassium uptake systems has suggested that it might be the extracellular, rather than intracellular, potassium concentration which dictates the toxicity phenotype.

d. During the course of these studies, it was also observed that the phenotype of potassium toxicity conferred by the *hns trx* mutants was manifest in one *E. coli* strain background but not another, suggesting the existence of a spontaneous suppressor allele in the latter. Genetic

experiments have been undertaken to tag and map the suppressor locus to the 82-min region of the *E. coli* chromosome, and efforts for its molecular identification are under way.

Publications

1 Laishram R S and Gowrishankar J (2007) Environmental regulation operating at the promoter clearance step of bacterial transcription. ***Genes and Development*** 21:1258-72

Patents

1 Gowrishankar J and Harinarayanan R. A method of altering levels of plasmids. US Patent 7220588 B2, issued 22 May 2007.

Laboratory of Computational Biology

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	P Suresh	Project Assistant

Project 1: Deciphering the Structural Dynamics of Proteins with Disease Causing Mutations

Objective:

To study the effect of disease causing mutations on protein structure, dynamics and function

Summary of the work done until the beginning of this reporting year

Molecular modeling and molecular dynamics simulations studies on 'wild-type' (WT) and eight Primary Congenital Glaucoma (PCG) mutant (MTs) forms of human CY1b1 revealed effects of disease-causing mutations on the protein structure (Biophysical J., 2006). Furthermore, essential motions derived from MD simulation data revealed qualitative and quantitative differences in collective dynamical properties between MTs and WT.

Detail of progress in the current reporting year (April 1, 2007 to March 31, 2008)

Our in-depth structural analysis by means of Molecular Dynamics followed by Principal Component Analysis of atomic motions had revealed altered structural and dynamic properties in Heme Binding Region (HBR) and Substrate Binding Region (SBR) in MTs as compared to WT. Since these regions constitute the site of the catalytic activity of the enzyme, a comparative docking study of the binding of the substrate in the active site of WT and MTs, was, therefore, undertaken. Estradiol was considered as the ligand and docking was performed using GOLD software. The receptor structures used for docking calculations were selected from the clustering

of the MD trajectories. The three cluster centers selected for docking are the three most populous conformations of the active site of the molecule during the simulation. Table 1 gives the volume of the SBR, the GOLD-fitness scores of the best docking solution, the number of hydrogen bonding interactions and the number of hydrophobic contacts between the ligand and the protein.

Table 1: The Volume of SBR of the three clusters of WT and MT structures. The Gold Fitness Scores of the docking calculations, the number of HB interactions, number of non-bonded contacts and the Distance of Ligand atoms from Heme FE.

	Cluster No.	Volume of SBR (Å^3)	GOLD Fitness Score	Ligand-FE Average Distance (Å)	Ligand 2OHsite-FE Distance (Å)	Ligand 4OHsite-FE Distance (Å)	Ligand-Receptor H-bonds	Ligand-Receptor NB-contacts
WT	1	275	38	10	8	11	1	53
	2	261	46	12	11	12	1	85
	3	432	46	12	11	13	0	66
A115P	1	279	44	11	7	8	0	65
	2	280	41	11	7	7	0	73
	3	649	46	9	13	12	0	84
M132R	1	695	40	7	3	5	0	69
	2	324	46	6	8	7	1	94
	3	165	49	7	11	10	0	86
Q144P	1	308	45	6	10	9	0	90
	2	260	44	7	10	10	1	84
	3	310	42	7	10	11	2	70
P193L	1	139	39	7	10	10	0	67
	2	683	42	14	15	14	0	81
	3	434	38	14	18	18	0	56
E229K	1	235	42	7	5	6	1	68
	2	356	39	13	17	17	1	57
	3	784	44	11	13	11	1	74
S239R	1	236	49	8	4	3	0	73
	2	274	51	6	10	9	0	94
	3	188	61	6	3	3	0	127
R368H	1	215	44	5	5	5	2	139
	2	216	40	13	15	14	0	81
	3	241	33	12	12	14	0	106
G466D	1	598	42	6	8	6	0	78
	2	306	39	9	9	6	2	69
	3	319	40	8	3	4	0	87

Our studies revealed the following:

a) Binding of the ligand in the active site of human CYP1b1 seems to be predominantly dependent on the non-bonded or hydrophobic interactions that the ligand makes with the protein. There were only occasional occurrences of 1 or 2 hydrogen bonds between the ligand and the receptor. CYP1b1 active site region has a cluster of side chains of aromatic amino acid residues which stack with the ligand molecules.

b) As compared to the WT, ligand-protein interactions in MTs are different as a consequence of changed geometry at SBR and also due to a change in the position of the heme relative to the active site. In WT, the ligand does not make non-bonded contacts with heme, but in most of the MTs, the ligand makes non-bonded contacts with heme.

c) The interaction with residue F261 of G-helix, which is found in WT is absent in all the MTs. An important difference in ligand-protein interactions between the WT and MTs is the presence of stacking interaction with phenyl residues in WT and its absence in the MTs.

In conclusion, our study revealed the nature of differences between ligand-protein interactions in WT and MTs. The differences in geometry at the active site region in WT and MTs preclude favorable protein-ligand interactions in the MTs and this perhaps results in compromised catalytic activity.

Project 2: Knowledge based approach for protein fold-recognition

Objective:

To develop a new fold-recognition tool with a sensitivity and specificity better than the existing tools

Summary of the work done until the beginning of this reporting year

We investigated several sequence and structural properties of proteins for their fold-discrimination capabilities using a Support Vector Machine (SVM) based approach. The training and testing of SVM was carried out on a benchmark dataset comprising of about 300 proteins for training and about the same number of proteins for testing. Of the several features tested secondary structural and solvent accessibility state frequencies of amino acids and amino acid pairs collectively gave rise to the best fold-discrimination.

Detail of progress in the current reporting year (April 1, 2007 to March 31, 2008)

Having found a novel set of fold-discriminatory features we continued to test its performance by means of a five-fold cross-validation study. This study was performed on a benchmark dataset comprising of about 2500 proteins. The five fold-cross validation study further confirmed our

earlier results by giving rise to an overall prediction accuracy of 70%, which is ~8% higher than the best published fold-recognition method (Fig. 1). We also tested, for the first time, an all-together multi-class method known as *Crammer and Singer* method for protein fold-classification. Our studies reveal that the three multi-class classification methods, namely *one versus all*, *one versus one*, and *Crammer and Singer* method, yield similar predictions (Fig. 1).

In conclusion, the new SVM-based method developed by us outperforms other fold-recognition methods. Currently, we are training SVM on all permissible protein folds including many of the so called 'orphan' folds to eventually build a web-server. We would also use this tool for genome-wide protein fold-recognition in the genomes of interest.

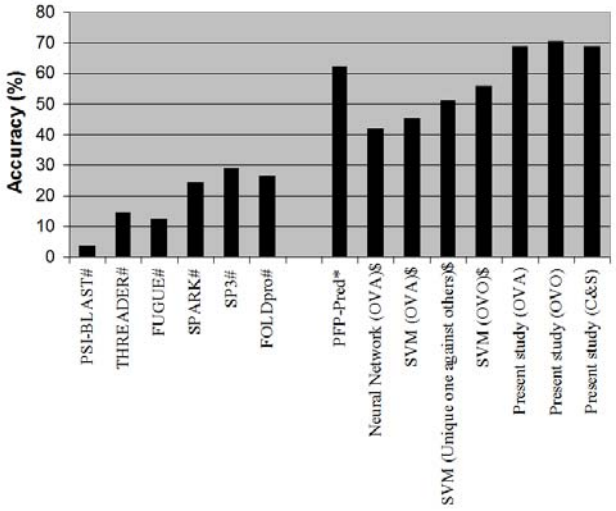

Figure1 The best prediction accuracy (Q) for protein fold-recognition reported by different fold-recognition methods. The Q value for the template-based methods (#) corresponds to the % of top 1 hits match the correct folds. #Cheng and Baldi, 2006; *Shen and Chou, 2006; $Ding and Dubchak, 2001.

Project 3: Analysis of simple sequence repeats in prokaryotic genomes

Objective

Analysis of frequencies, abundance and polymorphism of simple sequence repeats (SSRs) in fully sequenced prokaryotic genomes

Summary of the work done until the beginning of this reporting year

The whole genome sequences of five different strains of *Yersinia pestis* and its ancestor *Yersinia pertuberculosis* (YPTB) were surveyed for distribution and abundance of perfect SSRs. Our survey revealed that these each of Yersinia genomes harbor about a million SSRs with a frequency of one SSR every 4bp. Tract density profiles revealed some regions significantly enriched with SSRs

and some others with a very poor presence of SSRs. Comparison of motif-wise counts in real and stochastically modeled genomes revealed surprisingly, enrichment of mono, di and penta nucleotide SSRs. Preliminary cross-genome comparisons led to the identification of about 400 SSRs exhibiting length polymorphism.

We also developed a new tool called IMEx to extract imperfect SSRs in whole genome sequences (Bioinformatics, 2007). We also showed that this new tool is more sensitive and specific as compared to other known tools.

Details of progress in the current reporting year (April 1, 2007 – March 31, 2008)

We refer to the SSRs exhibiting length polymorphism across related genomes as PSSRs (Polymorphic SSRs). We studied distribution of PSSRs in relation to coding regions in Yersinia genomes (Fig. 1(a) and (b)). In essence, genes harboring PSSRs encoded proteins involved in cellular processing and signaling. On the other hand, genes harboring PSSRs in their upstream regions, encoded proteins involved in metabolism (house-keeping genes).

In our earlier study we had used a simple homogeneous stochastic model of genomes for computing genome-wide and motif-wise enrichment of SSRs. Since, genomes are, by nature, heterogeneous we, therefore, re-analyzed enrichment of SSRs with the help of robust heterogeneous stochastic models of genomes. Our analysis revealed that hexa nucleotide SSRs which are usually reported to be enriched in prokaryotic genomes occur less than expected in Yersinia genomes. Tetra nucleotide SSRs which were earlier found to be enriched by the simple homogeneous stochastic model were found to be occurring less than expected.

We also carried out a detailed study of some of the PSSRs and analyzed their effect on coding regions as observable across genomes. For example, one of the PSSRs which is a poly G tract ((G)5)) is in the functional UreD gene which encodes urease enzyme in YPTB. YPTB as it lives in soil and water requires urease enzyme for the degradation of nitrogenous product for its nitrogen requirements. In *Yersinia pestis* genomes the SSR tract has been expanded to (G)6 thereby introducing a frame-shift mutation in *UreD* leading to its premature termination. Hence,

it seems that the SSR mutation which led to loss of UreD is correlated to the life style of *Y.pestis* which does not require urease activity. It can be surmised that while evolving from its ancestor *Y. pestis* may have selected for loss of the redundant urease activity. This selection has been made possible due to the SSR tract expansion. This is an example of involvement of SSR in rendering an optimum adaptive capability to a pathogen.

Publications:

1. Sreenu VB, Kumar P, Nagaraju J and Nagarajaram H A (2007) Simple sequence repeats in mycobacterial genomes ***Journal of Biosciences*** 32: 1-15

2. Suresh B M and Nagarajaram H A (2007) IMEx: Imperfect Microsatellite Extractor. ***Bioinformatics*** 23:1181-1187

3. The NMITLI-BioSuite Team (…..Nagarajaram HA….) (2007) BioSuite: A comprehensive bioinformatics software package (A unique industry academia collaboration) ***Current Science*** 92: 29-38

4. Shamim MTA, Anwaruddin M and Nagarajaram H A (2007) Support Vector Machine-based classification of protein folds using the structural properties of amino acid residues and amino acid residue pairs. ***Bioinformatics*** 23: 3320-3327

Laboratory of Cellular and Molecular Biology

Molecular pathogenesis and functional genomics of *Mycobacterium tuberculosis*

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Objectives

1. Characterization of genes involved in DNA replication
2. To study the host immune response against the individuals and complex PE/PPE proteins
3. To understand the role of conserved hypothetical protein (CHP) in modulation of macrophage effector function

Project 1: Characterization of genes involved in DNA replication

Summary of work done until the beginning of this reporting year

Mycobacterium tuberculosis (*M.tb*), the pathogen that causes tuberculosis, is capable of staying asymptomatically in a latent form. While the region between *dnaA* and *dnaN* gene is capable of autonomous replication, little is known about the interaction between DnaA and oriC and their negative effectors of replication. We have used $KMnO_4$ footprinting assay to map the unwinding of *oriC* region of *M.tb* and identified the nucleotide position from where *oriC* starts melting. Our results demonstrate that in *M.tb* duplex opening begins near position – 500 with respect to start of *dnaN* gene. Contrary to *E. coli,* in *M.tb* the DnaA bound to non-hydrolysable analogue of ATP (ATPγS) is incompetent for helix opening suggesting that ATP hydrolysis is critical. We have

also showed that recombinant Rv1985c (IciA) inhibits *in vitro* helix opening mediated by DnaA protein, provided the open complex formation has not initiated, perhaps by specifically binding to A+T rich region of *M.tb oriC,* as confirmed by electrophoretic mobility shift assay. Gel filtration assay showed that rIciA is a dimer. These results have a bearing on the functional role of an important regulator of *M.tb* chromosomal replication belonging to the LysR family of bacterial regulatory proteins.

Details of progress made in the current reporting year (April 1, 2007 - March 31, 2008)

DnaA protein shows higher ATPase activity in the presence of supercoiled template:

ATPase assay was carried out either in the absence of DNA, or presence of linear DNA, or supercoiled pUC OriMtb and non specific supercoiled template pBSK II. As could be seen, ATPase activity in the absence of DNA (Figure 1A, lane 1-4) or in the presence of DnaA box (lane 5-8) is almost equal. However, ATPase activity increases significantly in the presence of supercoiled pUC_OriMtb (lane 9-12) and pBSK II (lane 13-16). The results while confirming that the refolded rDnaA protein is functionally active, also confirm that DnaA has very weak intrinsic ATPase activity which is increased in the presence of supercoiled DNA.

Open complex is formed near the A+T rich repeat: For the helix-opening assay increasing amounts of DnaA protein (0.025-0.3µg) were incubated with supercoiled pUC_OriMtb as described. Primer extension reaction was carried out both from top (upstream) (Figure 1b,C) and bottom (downstream) of the template (Figure 1B, a and b). Primer SeqOriR1 annealed between position – 292 to – 320 of template strand, primer SeqOriR2 annealed between positions – 402 to – 420 of the template strand and primer SeqOriR3 annealed at position of -40 of pUC18. Thus, SeqOriR1 and SeqOriR2 were used to analyze from bottom and SeqOriR3 was used to analyze from top. The extension products were then fractionated on a standard (6% or 15% as shown in the legend) urea sequencing gel (Figure 1B, a, b and c). Helix opening could be detected only in the presence of 0.075µg or higher amount (Figure 1B, a, lane 3) of rDnaA protein but not when 0.025µg or 0.050µg (Figure 1B, a, lane 1-2) of rDnaA was used and this was evident from the presence of extension products of 199bp and 200bp (lane 3) corresponding to -500 and -501 from

the start of the *dnaN* gene. To further pinpoint the extent of helix opening another primer SeqOriR2 was utilized and the extension products were again analyzed on 15% urea gel. As can be seen (Figure 1B, b, lane 2-6) extension products corresponding to 98, 99, 113 and 115 nucleotides could be observed which represent position -500, -501, -515, -517 from start of *dnaN* gene. Primer SeqOriR3 annealed at position of -40 of pUC18 and generates extension products (Figure 1B, c, Lane 2-5) of 63, 65, 66, 76, 77 and 79 nucleotides which represent position -517, -515, -514, -504, -503 and -501 from start of *dnaN* gene. The results together reveal that an 18bp stretch of *M.tb oriC* becomes sensitive to $KMnO_4$ (Figure 1B, d). They further provide evidence for the first time that in *M.tb* the duplex opening occurs near position – 500 to – 517 (from start of *dnaN* gene) which lies within the A+T rich region.

IciA inhibits helix opening: IciA, in addition to other functions, is a known inhibitor of chromosome replication initiation in *E. coli*. *M.tb* ORF *Rv1985c* displays 35.8% sequence identity to *iciA* of *E. coli*. Analysis of secondary structure also demonstrated that both IciA of *E. coli* and the putative *M.tb iciA* (*Rv1985c*) could be functionally identical. Therefore, we analyzed the inhibitory effect of *Mtb iciA*, if any, on open complex formation. Helix opening reaction was carried out in the presence of increasing concentrations of recombinant purified IciA protein. 0.2µg of rDnaA protein was used. The appearance of primer extension products of 98, 99 and 113 nucleotide using downstream primer SeqOriR2 (Figure 1C, a, lane 1), or six extension products of 63, 65, 66, 76, 77 and 79 nucleotides using upstream primer SeqOriR3 (Figure 1c, B, lane 1), is a reflection of helix opening. These bands disappear as a direct function of increasing IciA concentration (Figure 1C, a, lanes 2-4; Figure 1C, b, lane 2-4). IciA protein could only inhibit if it was included in the helix opening reaction prior to the opening of the helix thereby suggesting that once the helix opening has been initiated by the binding of DnaA protein to *oriC* and the 13- mer region has opened, IciA protein can not block formation of the open complex. These results demonstrate that IciA protein can block open complex formation by possibly binding directly to the *oriC* sequences.

IciA inhibits DNA replication: Having shown the ability of rIciA to inhibit helix opening *in-vitro,* experiments were designed to monitor the ability of rIciA to actually inhibit DNA replication using a reconstituted replication system. *M. bovis BCG* fraction II which supports *in-vitro* replication of DNA from *M.tb oriC* was utilized. It could be seen (Figure 1D, a) that maximal DNA synthesis occurs in the presence of 80µg of fraction II. This concentration of fraction II was selected to test whether rIciA could inhibit DNA replication *in-vitro* in the presence of increasing amounts of rIciA. In this assay rIciA was added before the addition of fraction II. As seen in Figure 1D, b, rIciA inhibited DNA replication as a direct function of its concentration and the results directly point to the ability of rIciA to act as an inhibitor of DNA replication.

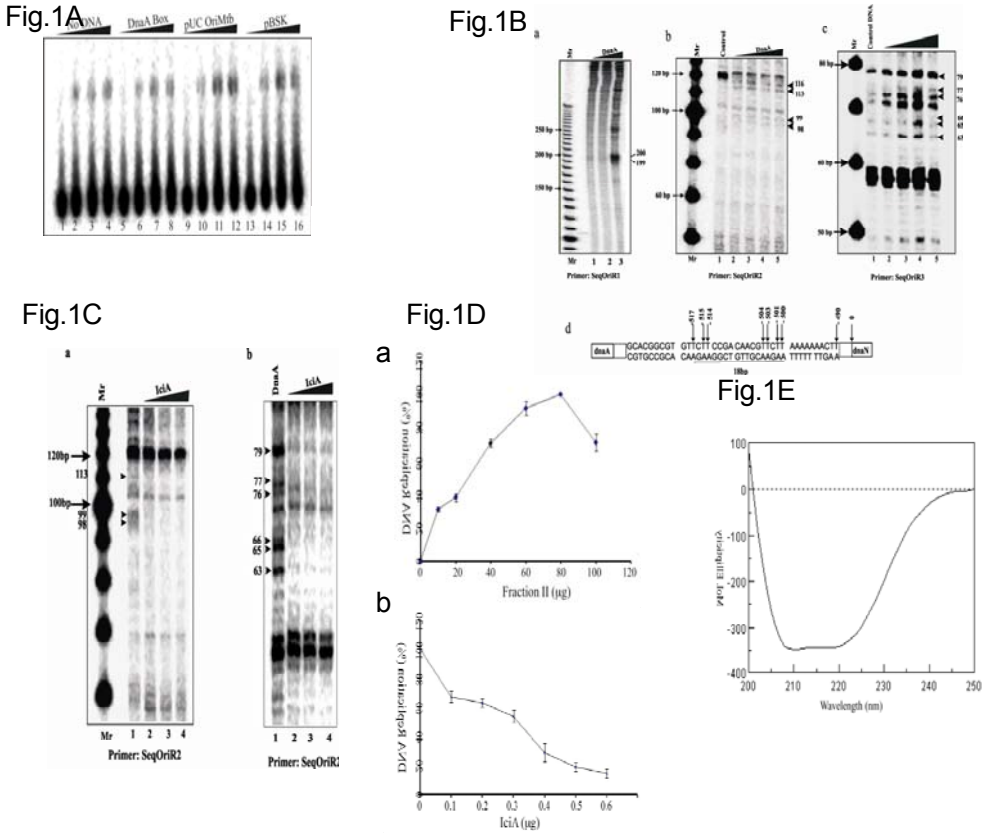

Fig1. (B) Helix unwinding mediated by recombinant DnaA protein is inhibited by IciA like protein. (A) rDnaA exhibits ATPase activity. (B) *M.tb* helix opening by rDnaA occurs near position -500 to -517 within the A+T rich region. (Ba, Bb, Bc)pUC_OriMtb was used as a template for helix opening in the presence of increasing amounts of rDnaA with $\gamma^{32}P$ labeled SeqOriR1 and the product was fractionated on 6% or 15% Sequencing gel. (Bd) About 13bp stretch of pUC_OriMtb becomes sensitive to $KMnO_4$ modification. (C) rDnaA mediated helix opening is inhibited by rIciA. (Da) DNA replication was carried out with ammonium sulphate fractionated crude cell lysate. (Db) DNA replication mediated by *M. bovis BCG* extract (80 µg) was assayed in the presence of increasing concentration(0 to 0.6 µg) of rIciA (E) CD Spectromatic analysis of rIciA reveals its secondary structure.

Secondary structure analysis of rIciA reveals almost equal α helix and β sheet content: CD spectrum of recombinant IciA was recorded in a range of 200 to 250nm (Figure 1E). The recombinant native protein showed 27.4% α helical content, 30.8% β sheet, 13.0% turn and 28.8% random coil, thus revealing that the rIciA has an almost equal α helical and β sheet content.

Project 2: To study the host immune response against the individuals and complex PE/PPE proteins

Summary of work done until the beginning of this reporting year

We have been focusing on the genomic organization of PE/PPE genes, interaction among themselves and interaction with the host. It was observed that the PE/PPE genes are scattered throughout the genome in a unique fashion in such a way that PE genes are situated upstream to the PPE genes in most cases and are predicted to be in operon. With a representative study with one of the PE/PPE pair, PE25 (Rv2431c) and PPE41 (Rv2430c), we found that the two genes are indeed in operon. The PE25 protein and PPE41 protein when expressed in *E. coli* alone go into inclusion bodies and when co-expressed they come into soluble form, indicating that the two proteins help each other at the translational level to bring into soluble form. Further experiments showed that the two proteins interact with each other and the PE25/PPE41 complex protein exists as hetero-tetramer and the individual proteins as oligomers.

Details of progress made in the current reporting year (April 1, 2007 - March 31, 2008)

The PE25/PPE41 protein complex induces significant B cell response in TB patients: Having earlier shown that the highly immunodominant PPE41 protein discriminates TB patients from healthy individuals and the genes encoding PPE41 and PE25 are co-operonic, we were interested to know how the host humoral system reacts to the three different form of proteins, individually and when present as a complex. We found that both PPE41 and PE25/PPE41 complex elicited significantly higher antibody response ($P<0.0001$) in TB patients as compared to the PE25 protein alone (Fig. 2Aa). We then checked for the number of individuals eliciting B cell response to the protein/complex. As could be seen in Fig. 2A, b; the number of patients showing higher

antibody response (O.D > 0.55) to PE25/PPE41 is more (75%) compared to PPE41 (45%) or PE25 protein (9%) alone ($p<0.0001$). The higher stability and solubility of the PE25/PPE41 complex, could possibly explain why the complex protein showed superior antigenicity compared to PE25 or PPE41. The absence of any significant antibody response in sera from healthy individuals suggests that these proteins are mostly expressed during active infection with *M tb.*

Mice immunized with PE25 elicit low B cell response while PPE41 and PE25/PPE41 complex show increased IgG response: The low antibody response in TB patients suggests that either the PE25 protein is not antigenic in nature or the antibodies raised against PE subunit of the PE25/PPE41 complex fail to recognize recombinant PE25 protein due to incorrect conformation. To specifically address this issue, mouse mice were immunized with PE25, PPE41 and the PE25/PPE41 complex without any adjuvant. Sera collected from mice after four weeks of immunization, with a booster at the second week, were used in ELISA to score for antibody response against the recombinant PE25, PPE41 and PE25/PPE41 complex. It can be seen that the PE25 protein generated a significantly lower antibody response than that of PPE41 or PE25/PPE41 complex (Fig. 2B). This indicates that the PE25 protein is a weak antigen compared to PPE41 and PE25/PPE41 complex.

The PE25/PPE41 complex protein induces high splenocyte proliferation: Further studies were carried out to investigate the potential of these recombinant proteins to elicit T cell response. Splenocytes isolated from mice immununized with PBS buffer or 20μg of PE25 or PPE41 or PE25/PPE41 complex were stimulated *in vitro* with the respective recombinant protein. The proliferation activities of splenocytes were measured by thymidine incorporation assay. As could be seen in Fig. 2C, the PE25/PPE41 complex induces higher proliferation as opposed to PPE41 or PE25 protein. No proliferation was seen when splenocytes isolated from PBS-treated mice were activated with these recombinant proteins. These results once again show that the PE25 protein is a weaker antigen and the high proliferative activity of the PE25/PPE41 complex could be due to higher solubility and stability as compared to the individual proteins.

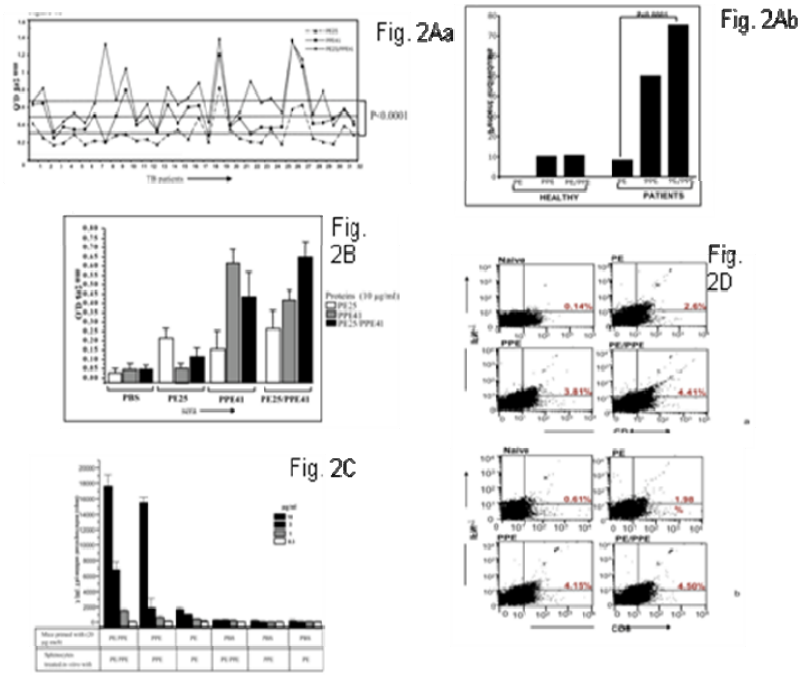

Fig. 2. (A) The PE25/PPE41 protein complex induces a much stronger antibody response in sera derived from TB patients. a) antibody response against the three proteins PE25, PPE41 and PE25/PPE41 complex using sera from 32 TB patients. b) the percentage individual reactivity to the recombinant proteins. (B) The PE25 protein induces low B cell (IgG) response whereas the PPE41 and PE25/PPE41 complex proteins induce high B cell response in mice. (C) The PE25/PPE41 protein complex and PPE41 show high splenocyte proliferation activity in mice. The proliferation activity was measured by Thymidine incorporation assay. (D) The PPE41 and PE25/PPE41 complex proteins enhance $CD4^+$ and $CD8^+$ T cell activity. Splenocytes from naive mice or mice immunized with each of the recombinant proteins (PE25, PPE41 and PE25/PPE41) were stimulated with different concentration of the corresponding proteins *in-vitro*. The intracellular IFN-g secreted by $CD4^+$(a) and $CD8^+$ T cells (b) specific to the PE25, PPE41 and PE25/PPE41 complex proteins was measured by flow cytometry

The PPE41 and PE25/PPE41 protein complex enhance $CD4^+$ and $CD8^+$ T cells population: IFN-γ is extremely important for the induction of protective immunity against *M.tb.* Interestingly, we found that both the PPE41 and the PE25/PPE41 could induce higher levels of IFN-γ. We therefore examined the type of T cells responsible for the secretion of IFN-γ. Splenocytes isolated from mice immunized with either the PE25 or PPE41 or PE25/PPE41 were stimulated *in-vitro* with the respective protein for 24 h and stained for the CD4 and CD8 T cells inducing IFN-γ by flow cytometry. It could be seen (Fig. 2D, a) that immunization with the PE25/PPE41 complex generates the maximum number of $CD4^+$ (4.41%) T cells which were positive for IFN-γ as compared to PPE41 (3.81%) or PE25 (2.6%) protein. Similarly, immunization with the PE25/PPE41 complex generates a larger number (4.5%) of $CD8^+$ T cells which were positive for

IFN-γ (Fig. 2D, b) as compared to the PPE41 (4.15 %) or PE25 (1.98%) protein. This suggests that both $CD4^+$ and $CD8^+$ T cells produce higher levels of IFN-γ specific to the complex protein.

Project3: To understand the role of conserved hypothetical protein (CHP) in modulation of macrophage effector function

Summary of work done until the beginning of this reporting year

The present study was undertaken to characterize one of the secretory proteins of *M.tb.* The Rv2626c is shown to be highly immunogenic for B cell response as reflected by presence of antibodies in active TB patients. This study further reinforced the belief that it may be involved in influencing the host immune response and accordingly this study was carried out in analyzing the host immune response to this protein. The results described in the previous year show that rRv2626c increases iNOS induction and NO production in RAW 264.7 as well as in J774 macrophages and this effect was specific since the proteinase K treatment of rRv2626c abrogated this effect.

Details of progress made in the current reporting year (April 1, 2007 - March 31, 2008)

The rRv2626c activates pro-inflammatory response in RAW 264.7 cells*:* A recent study has shown that DNA vaccination with *Rv2626c* in infected mice increases the Th-1 type cytokines like IFN-γ and IL-2 levels along with increased cytotoxic activity *in vivo*. Th-1 responses are regulated at the level of IL-12 and both IL-12 and TNF-α are protective cytokines against *M. tuberculosis*. It was therefore checked whether rRv2626c actually activates the macrophages to induce a Th-1 response. TNF-α as well as IL-12 production was measured by ELISA in culture supernatants 48 h after treatment of macrophages with different concentrations of rRv2626c. It could be observed that treatment of macrophages with rRv2626c increases production of TNF-α and IL-12 as a function of protein concentration. Thus, rRv2626c can act as an immunomodulator by activating pro-inflammatory cytokines.

rRv2626c promotes Th-1 response in PBMCs from active TB patients: The immunomodulatory role of Rv2626c on PBMCs harvested from active TB patients was next

investigated by monitoring the levels of various Th-1 type cytokines like IFN-γ, TNF-α and IL-12 by EIA using culture supernatants of PBMCs treated with rRv2626c (5μg) for 72h. It could be observed that rRv2626c was able to increase IL-12, TNF-a and IFN-γ secretion in PBMCs cultures of TB patients as compared to the healthy controls. These results demonstrate the involvement of rRv2626c as an immunomodulator in active TB patients. rRv2626c also increases expression of co-stimulatory molecules like B7-1, B7-2 and CD40 indicating *Rv2626c* can influence the antigen presenting activity of macrophages to prime T-cells by directly activating expression of the co-stimulatory molecules.

Publications

1. Akhter Y, Tundup S and Hasnain S E (2007) Novel biochemical properties of a CRP/FNR family transcription factor from *Mycobacterium tuberculosis*. ***International Journal of Medical Microbiology*** 297: 451-457

2. Ahmed N, Saini V, Raghuvanshi S, Khurana JP, Tyagi AK, Tyagi AK and Hasnain SE (2007) Molecular analysis of a leprosy immunotherapeutic bacillus provides insights into Mycobacterium evolution. ***PLoS ONE*** 2:e968

3. Banerjee S, Nandyala A K, Raviprasad P, Ahmed N and Hasnain S E (2007). Iron-dependent RNA-binding activity of *Mycobacterium tuberculosis* aconitase. ***Journal of Bacteriology*** 189: 4046-4052

4. Boddupalli CS, Ghosh S, Rahim SS, Nair S, Ehtesham NZ, Hasnain SE and Mukhopadhyay S (2007) Nitric oxide inhibits interleukin-12 p40 through p38 MAPK-mediated regulation of calmodulin and c-rel. ***Free Radical Biology and Medicine*** 42:686-697

5. Duggirala, A., Kenchappa, P., Sharma, S., Peeters, J. K., Ahmed, N., Garg, P., Das, T. & Hasnain, S. E. (2007). High-resolution genome profiling differentiated *Staphylococcus epidermidis* isolated from patients with ocular infections and normal individuals. ***Investigative Ophthalmology and Visual Science*** **48**, 3239-3245

6. Khan N, Ghousunnissa S, Jegadeeswaran SM, Thiagarajan D, Hasnain SE and Mukhopadhyay S (2007) Anti-B7-1/B7-2 antibody elicits innate-effector responses in macrophages through NF-κB-dependent pathway. ***International Immunology*** 19:477-86

7. Rasheedi S, Ghosh S, Suragani M, Tuteja N, Sopory S K, Hasnain S E and Ehtesham N Z (2007) *Pisum sativum* contains a factor with strong homology to eIF5B. ***Gene*** 399: 144-151

8. Rasheedi S, Ramachandran A, Ehtesham N Z and Hasnain S E (2007) Biochemical characterization of Sf9 Sp-family-like protein factors reveals interesting features. ***Archives of Virology*** 152 :1819-1828

9. Yusuf A, Smanla T and Hasnain SE (2007) Novel biochemical properties of a CRP/FNR family transcription factor from Mycobacterium tuberculosis, ***International Journal of Medical Microbiology*** 297:451-457

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11. The NMITLI-BioSuite Team (…… Hasnain SE, Mande S, Nagarajaram, H A, Ransan A….) (2007) BioSuite: A comprehensive bioinformatics software package (A unique industry academia collaboration) ***Current Science*** 92: 29-38

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13. Akhter Y, Yellaboina S, Farhana A, Ranjan A, Ahmed N and Hasnain SE (2008) Genome scale portrait of cAMP-receptor protein (CRP) regulons in mycobacteria points to their role in pathogenesis. ***Gene*** 407:148-158

14. Ahmed N, Dobrindt U, Hacker J and Hasnain SE (2008) Genomic fluidity and pathogenic bacteria: applications in diagnostics, epidemiology and intervention. ***Nature Reviews Microbiology*** (In Press)

15. Farhana A, Kumar S, Rathore S S, Ghosh P C, Ehtesham N Z, Tyagi A K and Hasnain S E (2008) Mechanistic insights into a novel exporter-importer system of *Mycobacterium tuberculosis* unravel its role in trafficking of iron. ***PLoS ONE*** (In Press)

16. Khan N, Alam K, Mande S C, Valluri V L, Hasnain S E and Mukhopadhyay S (2008) *Mycobacterium tuberculosis* heat shock protein 60 modulates immune response to PPD by manipulating the surface expression of TLR2 on macrophages. ***Cellular Microbiol*ogy** (In Press)

17. Demuth A, Aharonowitz Y, Bachmann T T, Blum-Oehler G, Buchrieser C, Covacci A, Dobrindt U, Emody L. van der Ende A, Ewbank J, Fernandez L A, Frosch M, Portillo F G, Gilmore M S, Glaser P, Goebel W, Hasnain S E, Heesemann J, Islam K, Korhonen T, Maiden M, Meyer T F, Montecucco C, Oswald E, Parkhill J, Pucciarelli M G, Ron E, Svanborg C, Uhlin B E, Wai S N, Wehland J and Hacker J (2008) Pathogenomics: An updated European Research Agenda. ***Infection, Genetics and Evolution*** (In Press)

Laboratory of Molecular and Cellular Biology

Macrophage biology and host-pathogen interaction in tuberculosis

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Project 1: Understanding the role of *Mycobacterium tuberculosis* heat shock protein 60 as Th1/Th2 immunomodulator

Objective:

To study whether heat shock protein 60 of M. tuberculosis triggers a Th2 response

Summary of work done until the beginning of this reporting year

Last year we reported that the anti-PPD T cells' responses were skewed to Th2 type by heat shock protein 60 of M. tuberculosis (MtbHsp60) when macrophages were used as antigen presenting cells. Since IL-12 can directly influence the T cell effector functions as Th1/Th2, we explored the possibility whether Mtbhsp60 influenced the PPD-induced IL-12 signaling in macrophages to establish a Th2 response. Therefore, the macrophages were stimulated with PPD in the absence or presence of Mtbhsp60. The results indicate that MtbHsp60 inhibits PPD-induced IL-12 p40 in a dose dependent manner (maximum inhibition was noticed with 3 μg/ml concentration of MtbHsp60). While studying the transcription factor(s) involved in IL-12 p40 downregulation, it was observed that MtbHsp60 protein mainly inhibited the nuclear c-rel

transcription factor but not the p50- or the p65 NF-κB in PPD-activated macrophages. This was further confirmed by over-expressing c-rel transcription factor in the macrophages co-treated with PPD and MtbHsp60. The results indicate that overexpression of c-rel significantly restored IL-12 p40 induction in response to PPD in the macrophages evenafter being treated with Mtbhsp60 indicating that mycobacterial Hsp60 protein targets the c-rel transcription factor to downregulate IL-12 p40 induction in PPD-stimulated macrophages.

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

Involvement of the toll-like receptors in the regulation of IL-12 p40 by Mtbhsp60: The Hsp60 is known to interact strongly with the toll-like receptors (TLRs), namely the TLR2 and the TLR4. Several studies indicate that induction of specific cytokine profile upon recognition of microbial pathogens depends to a great extent on specific TLRs being triggered. We, therefore, hypothesized that the nuclear c-rel and IL-12 p40 regulation by Mtbhsp60 could be TLR dependent. The THP-1 macrophages which express higher levels of both TLR2 and TLR4 upon PMA activation were pretreated with either neutralizing anti-human TLR2 monoclonal antibody (mAb) or neutralizing anti-human TLR4 mAb to block TLR2/TLR4 receptors and then stimulated with PPD in the presence of Mtbhsp60 or GST. The control group received the isotype-matched Ab (IgG2a). Results reveal that PPD-induced IL-12 p40 (Fig. 1A) and nuclear c-rel (Fig. 1B, panel 1) were suppressed by Mtbhsp60 in the group treated with isotype-matched Ab, which was expected. When anti-TLR4 mAb was used, both the IL-12 p40 (Fig. 1A) and the nuclear c-rel levels (Fig. 1B, panel 2) were downregulated. In contrast, when macrophages were pretreated with anti-TLR2 mAb, Mtbhsp60 augmented IL-12 p40 induction (Fig. 1A, compare lane 10 with lane 2, $p < 0.001$ and lane 12 with lane 4, $p < 0.001$). The increase in IL-12 p40 in the anti-TLR2 mAb-treated macrophages was correlated well with an increase in the nuclear c-rel level (Fig. 1B, panel 3). The results (Fig. 1) confirm the Mtbhsp60-mediated downregulation of IL-12 is dependent predominantly on TLR2 population present on the macrophage surface via c-rel signaling downstream of TLR2.

Mtbhsp60 increases TLR2 expression on macrophages: Since TLR4 positively regulates IL-12 secretion and we observe a net negative effect of Mtbhsp60 in PPD-stimulated macrophages, it is likely that TLR2 is the predominant molecule by which IL-12 expression is downregulated. The net negative effect on IL-12 expression could be brought upon either by preferential signaling by Mtbhsp60 through TLR2 or by modulating TLR2 expression in such a way that surface TLR2 population is much higher than that of TLR4. Since, Mtbhsp60 interacts with both the TLR2 and TLR4 (Ohashi *et al.*, 2000; Vabulas *et al.*, 2001), it is likely that Mtbhsp60 increases TLR2 expression in the macrophages. To test this hypothesis, the THP-1 macrophages were either left untreated or activated with PMA surface expression profiles of TLR2 and TLR4 were examined upon stimulation with Mtbhsp60. Interestingly, we observed that Mtbhsp60 upregulated surface expression of TLR2 in both PMA-differentiated (Fig. 1C) and undifferentiated THP-1 macrophages. However, there were no significant changes in the surface expression of TLR4 in both the cases. When Mtbhsp60 was added to human blood-derived monocyte-macrophages, again an upregulation of TLR2 but not TLR4 was noticed.

To categorically document that Mtbhsp60-mediated suppression of IL-12 p40 was indeed TLR2 dependent, we next silenced the TLR2 by siRNA approach. When compared with the negative control siRNA-transfected THP-1, the group containing TLR2 siRNA did not exhibit substantially increased levels of TLR2 upon treatment with Mtbhsp60. The TLR2 deficiency in the TLR2 siRNA group led to an increase in IL-12 p40 induction during treatment with Mtbhsp60 (Fig. 1D, compare lane 8 with lane 4, $p < 0.001$). These results demonstrate that Mtbhsp60-mediated downregulation of IL-12 p40 in activated macrophages is dependent predominantly on the TLR2 population. The Mtbhsp60 significantly increases TLR2 expression without affecting TLR4 population and thereby makes TLR2 to be the predominant signaling gateway to suppress IL-12 p40 production in activated macrophages. Downregulation of IL-12 production by Mtbhsp60 possibly results into suppression of anti-PPD Th1 immune responses. This could well constitute an important point of regulation by which the tuberculosis bacilli can

modulate a confronting Th1 environment to more favorable Th2 and probably Mtbhsp60 plays an important role in such regulation.

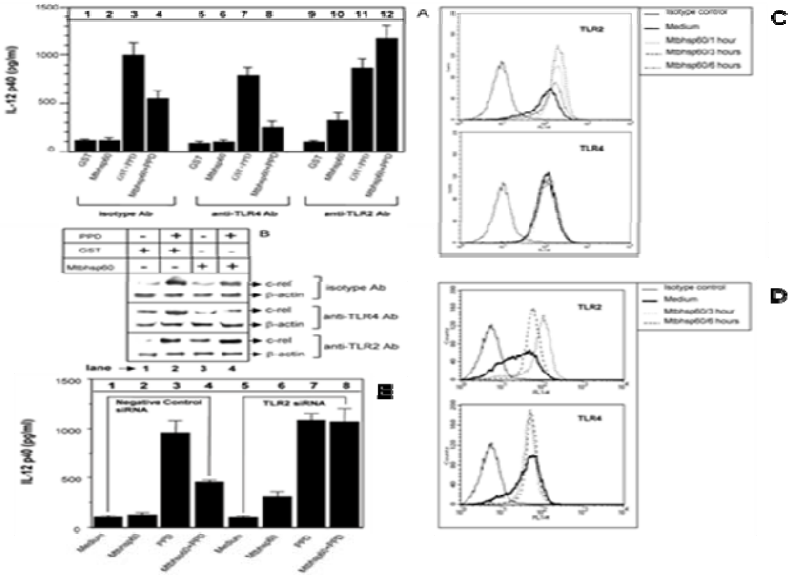

Fig. 1. The regulation of IL-12 p40 by Mtbhsp60 is TLR dependent. The IL-12 p40 (A) and nuclear c-rel in response to PPD+Mtbhsp60/GST were measured in PMA-differentiated THP-1 macrophages pretreated with anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control Ab. Surface expression of TLR2/TLR4 was measured in PMA-treated (C) or untreated (D) THP-1 exposed to Mtbhsp60. IL-12 p40 induction was compared between negative control siRNA- and TLR2-mRNA-transfected THP-1 macrophages stimulated with PPD in the presence of Mtbhsp60. Results shown are representative of three different experiments.

Project 2. **Understanding roles of the PE/PPE family of proteins in modulation of immune responses in host**

Objectives

In this proposal, we focus to study i) whether the PE/PPE proteins interfere with the macrophage-signaling cascades and whether such interference leads to suppression in macrophage-immune functions and ii) whether the PE/PPE proteins (that are overexpressed in macrophages during infection) can be used as diagnostic antigens to detect TB patients with higher sensitivity

Summary of work done until the beginning of this reporting year

We have shown earlier that the PE/PPE family proteins with SH3 binding domain can play important roles in modulation of immune responses. One of these, the Rv1168c (PPE protein) was found to associate with ESAT-6 gene cluster region 5 (ESX-5) (19), which is predicted to encode a novel secretory apparatus. It has also been shown that this cluster is conserved among

the various pathogenic mycobacteria, but not in the saprophytic species like M. smegmatis. Further, a BLAST analysis suggested that no genes that are strongly homologous to Rv1168c are present in the non-TB mycobacterial species that have been sequenced. We showed earlier that Rv1168c binds to macrophages and modulates IL-12 p40 signaling by specific activation of IL-10 indicating that Rv1168c could play important role in the pathogenicity of M. tuberculosis. Based on its predominant expression during the conditions that mimic in vivo phagosomal environment and high antigenicity index as calculated by Kyte-Doolittle algorithm, we expected Rv1168c to induce a strong B-cell response in people having active tuberculosis (TB) infection. Therefore, in the present study we tested whether Rv1168c is highly sensitive to detect patients with active TB.

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

We examined specific antibody reactivity in response to Rv1168c protein in sera from TB patients and compared with the BCG-vaccinated healthy controls. The sensitivity and specificity of Rv1168c immunoreactivity was compared with the responses elicited by ESAT-6 and Hsp60 well-known immunodominant antigens of M. tuberculosis and PPD which is routinely used in tuberculin test. In a conventional enzyme immunoassay (Fig. 2A) the recombinant Rv1168c protein displayed stronger immunoreactivity (mean absorbance value at 492 nm [OD492] ± SD = 1.05 ± 0.381) against the sera obtained from clinically active TB patients (n = 109) compared to PPD (OD492 ± SD, 0.415 ± 0.184), Hsp60 (OD492 ± SD, 0.571 ± 0.230) and ESAT-6 (OD492 ± SD, 0.612 ± 0.264), and could distinguish TB patients from the BCG-vaccinated controls (OD492 ± SD, 0.373 ± 0.066; $p < 0.0001$). When the proportion of highly reactive sera (antibody levels greater than or equal to the mean OD492 of BCG-vaccinated control sera plus 6 SD) among responders to each antigen was calculated (Fig. 2B), it was observed that Rv1168c elicited high level antibody responses in the majority (75.2%) of responders as compared to PPD (14%), Hsp60 (24%) and ESAT-6 (33.1%). Thus, it appears that Rv1168c is more immunodominant and serologically more sensitive than PPD, Hsp60 and ESAT-6.

Due to limitations of current array of diagnostic methods, diagnosis of extrapulmonary cases and the smear-negative pulmonary cases (since they are mostly sputum negative) is more difficult than the smear-positive pulmonary TB. Therefore, a diagnostic method with potential to identify patients with extrapulmonary/smear-negative pulmonary TB would be highly valuable. When the immunogenicity of Rv1168c over ESAT-6, Hsp60 and PPD was compared, the mean reactivity of Rv1168c was found to be significantly ($p < 0.001$) higher in comparison to that of ESAT-6, Hsp60 and PPD in both smear-negative pulmonary and extrapulmonary patient sera. When expressed as percentages of high-level responders showing antibody levels greater than or equal to cutoff values (mean OD492 of BCG-vaccinated control sera plus 6 SD), the majority of the extrapulmonary (81.3%) individuals showed antibody levels greater than the cutoff value against Rv1168c antigen whereas only 21.9% responders had higher levels against ESAT-6, 16% against Hsp60 and 12.5% against PPD, respectively. Like in extrapulmonary cases, Rv1168c was found to be more sensitive than the ESAT-6, Hsp60 and PPD to detect the smear-negative pulmonary TB patients. In smear-negative TB patients (n = 24), serum samples from 75% of the patients had antibodies to Rv1168 whereas only 45.8% of patients had antibodies to ESAT-6, 25% of patients had antibodies to Hsp60 and 20% of patients had antibodies to PPD. In the cohort of smear-positive TB patients (n = 53), 71.6% possessed Rv1168c, 3 4% had ESAT-6, 28.3% had Hsp60, and only 9.4% had PPD specific antibodies. The results thus indicate that Rv1168c can potentially detect all the categories of TB patients like the extrapulmonary, smear-negative pulmonary and smear-positive pulmonary TB cases with higher sensitivity than compared to ESAT-6, Hsp60 and PPD.

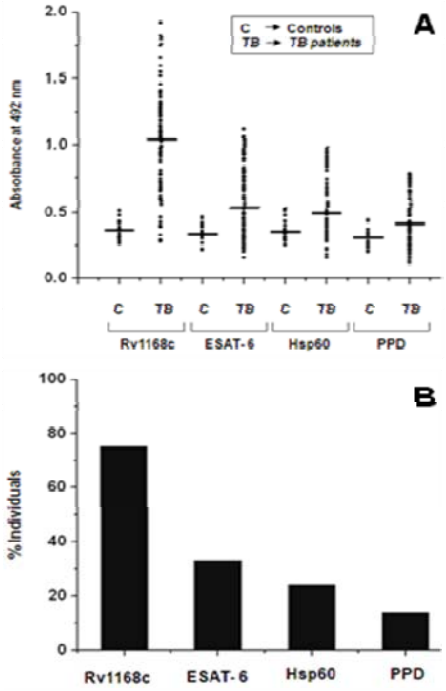

Fig. 2. The PPE protein Rv1168c is more sensitive to discriminate TB patients from the BCG vaccinated controls. Scattered plot of the sera cross reactivities to the Rv1168c, EGAT 6, Hsp60 and PPD of *M. tuberculosis* is shown in sera of either active tuberculous patients or BCG-vaccinated controls by EIA. The horizontal line indicates the mean of the absorbance values (A). Responders to Rv1168c was compared with that of ESAT 6, Hsp60 and PPD by calculating percentage of TB patients showing absorbance value greater than or equal to the cutoff value calculated as mean OD. of control sera plus 6 (B) SD. Mean OD. (SD) used for cutoff determinations were as follows. Rv1168c, 0.376 (0.066); EGAT 6, 0.343 (0.07); Hsp60, 0.359 (0.08) and PPD, 0.395 (0.071). Statistical significance was determined by student's t test.

Publications

1. Khan N, Ghousunnissa S, Jegadeeswaran SM, Thiagarajan D, Hasnain SE and Mukhopadhyay S (2007) Anti-B7-1/B7-2 antibody elicits innate-effector responses in macrophages through NF-κB-dependent pathway. ***International Immunology*** 19:477-486

2. Boddupalli CS, Ghosh S, Rahim SS, Nair S, Ehtesham NZ, Hasnain SE and Mukhopadhyay S (2007) Nitric oxide inhibits interleukin-12 p40 through p38 MAPK-mediated regulation of calmodulin and c-rel. ***Free Radical Biology and Medicine*** 42:686-697

3. Mukhopadhyay S, Nair S and Hasnain SE (2007) Nitric oxide: Friendly rivalry in tuberculosis. ***Current Signal Transduction Therapy*** 2:121-28

4. Hussain MA, Naveed SA, Sechi LA, Ranjan S, Alvi A, Ahmed I, Ranjan A, Mukhopadhyay S and Ahmed N (2008) Isocitrate dehydrogenase of *Helicobacter pylori* potentially induces humoral immune response in subjects with peptic ulcer disease and gastritis. ***PLoS ONE*** 3:e1481

5. Khan N, Alam K, Mande S C, Valluri V L, Hasnain S E and Mukhopadhyay S (2008) *Mycobacterium tuberculosis* heat shock protein 60 modulates immune response to PPD by manipulating the surface expression of TLR2 on macrophages. ***Cellular Microbiol*ogy** (In Press)

6. Khan N, Alam K, Nair S, Valluri V L, Murthy K J R and Mukhopadhyay S (2008) Strong immune responses to the PPE protein Rv1168c are associated with active tuberculosis disease. ***Clinical and Vaccine Immunology*** (In Press)

Pathogen Evolution Laboratory

Genomics and Biology of important bacterial pathogenises

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Objectives

Analysis of diversity, evolution and functional biology of pathogenic Mycobacteria*, Helicobacter pylori* and Leptospira in the context of evolution of survival mechanisms, acquisition and optimization of virulence, and their impact on dissemination dynamics, invasion, persistence, signaling events, molecular pathogenesis, strain evolution and prevention measures.

Summary of work done until the beginning of this reporting year

Last year we reported research progress pertaining to several projects encompassing *Helicobacter pylori* evolution in India, identification and characterization of new virulence markers from the genomic plasticity region of *H. pylori* and pathogenic mechanisms of *M. avium paratuberculosis* (MAP). We presented findings related to ancestral European roots of *H. pylori* in India (Devi *et al.*, 2007). Also, we have discussed *in vivo* evolution of *H. pylori* T4SS encoding genomic regions in a single patient observed for 10 years and discussed its implications on understanding acquisition and optimization of virulence in pathogenic bacteria in a changing host (Alvi *et al.*, 2007). In addition, leads obtained on putative roles of two of the novel *H. pylori* proteins that might be

involved as apart of survival mechanism at the base of chronic gastritis – a precancerous sequel of *H. pylori* infection (Rizwan *et al*., 2008) were also discussed

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

1. Mechanistic insights into the role of HP986 as an inducer of proinflammatory and apoptotic responses in human macrophages

Helicobacter pylori induces cytokine mediated changes in gastroduodenal physiology, wherein the activated macrophages at the submucosal space play a central role in mounting innate immune response against *H. pylori*-antigens. The bacterium gains niche through persistent inflammation and local immune-suppression causing peptic ulcer disease or chronic gastritis, the latter being a serious risk factor for the development of gastric adenocarcinoma. What favors persistence of *H. pylori* in the gastric niches is not clearly known. We previously reported characterization of a functionally unknown gene (hp986) linked to patient isolates associated with peptic ulcer and gastric carcinoma. Expression and purification of rHP986 revealed a novel, ~29kDa protein in biologically active form which recognized significant levels of humoral immune responses in diseased individuals. Also, it induced very high levels of TNF-α and Interleukin-8 in cultured macrophages concurrent to the translocation of nuclear transcription factor kB (NF-kB). Similarly, the rHP986 also induced apoptosis of macrophages through TNF-α mediated pathway. Dissection of the underlying signaling mechanism revealed that rHP986 induces both TNFR1 and Fas expression to cause apoptosis. We further demonstrated direct interaction of rHP986 with TNFR1 and its role in apoptosis. Interaction of rHP986 with TNFR1 was validated by immunoprecipitation using anti-TNFR1 antibodies. The receptor was detected in the eluate treated with rHP986; however no signals corresponding to the cell lysate treated with either LPS or recombinant ICD were detected. Exclusive interaction of rHP986 with TNFR1 was further confirmed in competition with neutralizing antibodies against TNF α; the amount of immune complex detected was less as compared to the eluate amount obtained in the absence of TNF-α. This suggested a direct interaction between TNFR1 and rHP986 and ruled out any possible role of endogenous TNF-α. To further confirm or negate the possible role of TLR4 in the induction

of cytokines binding of rHP986 to TLR4 was tested although no co-precipitation of the two was obtained.

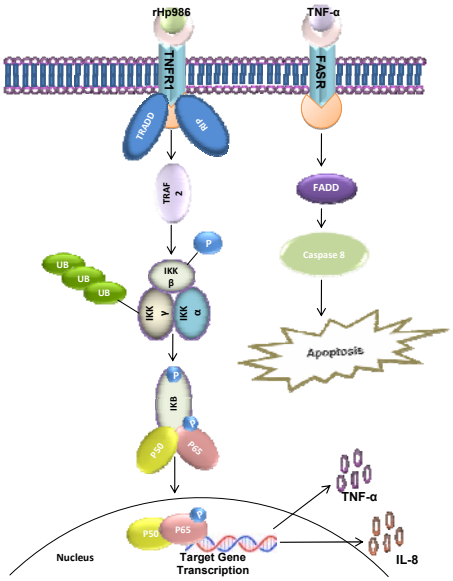

Figure 1: Schematic portrayal of HP986 induced apoptosis in cultured human macrophages

rHP986 upregulates Fas expression: Considering the interaction of rHP986 with TNFR1, its involvement in inducing Fas expression was tested, as the latter is known to function in synergy with TNFR1 and constitutively regulate downstream signaling cascade leading to apoptosis. Concomitant expression of Fas and TNFR1 as evident from competition binding assays was also observed. Pretreatment of PMA differentiated cells with neutralizing antibody against TNFR1 significantly downregulated Fas expression. Having established the role of rHP986 in the induction of Fas expression, our analysis was extended to determine its association in selectively regulating Fas expression. rHP986 effectively regulate Fas expression in a time and dose dependent manner. Comparative expression analysis with increasing protein concentration at different time points revealed significant and proportionate increase in Fas expression upto 24 hours. Nonetheless, expression levels declined after 12 hrs in cells subjected to higher protein dose (5.0µg/ml), which could be due to increased cell death. Immunocytochemical staining also

showed an increased expression of Fas on the surface of rHP986 stimulated cells as compared to unstimulated ones.

rHP986 induces apoptosis of cultured macrophages: rHP986 triggered apoptosis in cultured macrophage cells in a dose and time dependent fashion. A substantial and proportionate increase in cell death was observed when the cells were treated with increasing concentration of rHP986 (0.5μg/ml-10μg/ml) for varied time interval, up to 48 hours (36.65% ±3.25% to 41.0%± 4.2%), when compared to untreated cells (10.65% ± 1.85%). Our studies validated the pro-apoptotic property of rHP986 by blocking its interaction with TNFR1 using neutralizing antibodies. Following this, the rate of apoptosis declined significantly, indicating the involvement of rHP986 in TNFR1 mediated cell death. Similar results were obtained when the cells were stimulated in the presence of neutralizing antibody against TNF-α, suggesting that the effect was not secondary to endogenous TNF-α. Parallel to this, His-tagged ICD protein from *H. pylori* failed to induce apoptosis (data not shown). In addition, a comparative analysis between Oleandrin (a known inducer of apoptosis) and rHP986 also confirmed the latter being an equally potent inducer of apoptosis. Collectively, all these findings confirm rHP986 to be a potent apoptosis-inducing agent.

Independent proinflammatory and apoptotic responses triggered by rHP986 as shown in this study point to its role, possibly in the form of a survival strategy to gain niche through inflammation and to counter the activated macrophages to avoid clearance.

2. Molecular analysis of pathogenic Leptospira species - development of new diagnostic and epidemiological tools

a) Genetic affinities within a large global collection of pathogenic leptospira

Leptospirosis is an important zoonosis with widespread human health implications worldwide. Biology of the underlying organisms has been the fascination of infection biologists and evolutionists also since the genome sequence based data for multiple species are getting accumulated. Non-availability of accurate identification methods for the individualization of different leptospirae for outbreak investigations poses problems in disease control arena.

We applied fluorescent amplified fragment length polymorphism analysis for Leptospira and investigated its potentials in individualization of 272 isolates in the context of species level assignments of the members of a precious global collection of strains obtained from an extremely diverse array of animal hosts. Also, this method was compared to an in house multilocus sequence typing (MLST) method based on polymorphisms in 3 housekeeping genes, the *rrs* locus and 2 outer membrane proteins. Phylogenetic relationships were deduced based on bifurcating neighbor joining trees as well as median joining network analyses incorporating FAFLP and MLST data.

FAFLP was found to be an important method for outbreak investigation and for clustering of isolates based on their geographical descent rather than by genome species types, though it was not able to convey any taxonomical utility which could ultimately replace the highly ambiguous serotyping techniques. MLST on the other hand was found to be highly robust and efficient in segregating the outbreak associated strains or otherwise according to their genome species status and therefore, could unambiguously be applied for investigating phyletics of leptospirae in the context of taxonomy as well as gene flow. For example, MLST as compared to FAFLP method was able to more efficiently cluster strains from the Andaman island of India with their counterparts from mainland India and Sri Lanka, implying that such strains share genetic relationships and that leptospiral strains might be frequently circulating between the islands and the mainland.

b) Identification of conserved genetic loci for accurate identification of pathogenic leptospira

Genomic plasticity and phenotypic versatility of pathogenic bacteria are two major forces that stratify potentially virulent and benign species. Our understanding of leptospiral evolution is based on this paradigm where pathogenic forms are thought to have evolved from saprophytic species. While the gene pools of such bacteria are highly fluid, there may be certain regions that serve as static 'anchors' in genomes that experience frequent recombination events. We have analyzed genomic features of one such static anchor, the *S10-spc-α* locus of *Leptospira.* Comparative analyses revealed that genetic organization of this locus is well conserved within

Leptospira. Although the entire locus is broadly conserved, a 245 bp region provides some discriminating potential useful for diagnostic development and therefore, reliable species identification of pathogenic *Leptospira* is possible only through comparative analysis of this region (Victoria *et al.*, 2008). Another diagnostic marker, haemin binding protein A (hbpA) was tested through PCR and Southern hybridization and it was observed that hbpA was conserved in all *L. interrogans* serovars and was notably absent in the non-pathogenic *L. biflexa* serovar Patoc and *L. meyeri* serovar Ranarum. We then extended the PCR based detection of the *hbpA* gene to clinical isolates. The *hbpA* amplicon was demonstrated in all the *L. interrogans* serovars out of a total of 95 *Leptospira* clinical isolates obtained from different geographical areas. Also, MAT-positive serum from human patients with leptospirosis showed presence of the anti-HbpA antibodies. Further studies on these lines will be continued.

3. Roles of *Mycobacterium avium* subsp. *paratuberculosis* bacilli in type-1 diabetes mellitus

The role of pathogenic mycobacteria in diabetes has been a focus of speculation since a decade without any meaningful insights into the mechanism of diabetes causation vis a vis mycobacterial factors. *M. avium* subsp. *paratuberculosis* is a zoonotic pathogen whose association with autoimmune diseases such as Crohn's disease in humans is now established. The objective of our study in this case was to investigate any association of MAP with other chronic diseases such as type 1 diabetes mellitus (T1DM), where the involvement of a persistent pathogen such as *M. avium* subsp. *paratuberculosis* could be the trigger. For this purpose, 59 diabetic patients and 59 healthy controls reporting at the diabetology service of the University of Sassari, Italy were investigated for the presence of antibodies against two recombinant proteins of *M. avium* subsp. *paratuberculosis* and the whole-cell lysate. Extremely significant humoral immune responses to recombinant heparin binding hemagglutinin and glycosyl transferase proteins and the whole-cell lysates of *M. avium* subsp. *paratuberculosis* bacilli were observed in T1DM patients and compared to those of healthy controls (Sechi *et al*., 2008).

Presence of MAP in the blood of T1DM patients was also confirmed by a PCR method based on IS900 element in the genome of MAP (Sechi *et al.*, 2008a). The observations were further confirmed by the use of a phage based ELISA method that identified live, circulating MAP bacilli through the detection of a cell envelope protein mptD by specific M13 phage, fMptD. Our results demonstrated fMptD ELISA assay to be highly accurate and sensitive to detect MAP bacilli in a large fraction of T1DM patients as compared to non-diabetic controls.

Next, we sought to investigate if or not type 2 diabetes (T2DM) patients harbor humoral responses to MAP, for which Humoral antibody profiles were estimated for 57 T2DM patients and 57 healthy controls. Statistical analysis was performed with the Chi-square test with Yates' corrections. It was found that no obvious association of MAP with the incidence of T2DM in Sardinian patients (Rosu *et al.*, 2008).

Evidence to *M. avium* subsp. *paratuberculosis* involvement in T1DM is an important finding that might serve as a foundation stone in establishing an infectious etiology for T1DM. Rapid identification of infectious agent such as MAP in diabetic patients at the level of clinics might be helpful in deciding timely initiation of therapeutic interventions, such as insulin administration.

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14. Sechi LA, Paccagnini D, Salza S, Pacifico A, Ahmed N and Zanetti S (2008) *Mycobacterium avium* subspecies *paratuberculosis* bacteremia in type 1 diabetes mellitus: an infectious trigger? ***Clinical Infectious Diseases*** 46:148-149

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Laboratory of Structural Biology

Structural and Biochemical Characterization of some *M. tuberculosis* Proteins

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Objectives

1 Identification of important proteins of *Mycobacterium tuberculosis* for crystallographic and biochemical analysis

2 Expression and biochemical characterization of the chosen proteins. X-ray structural analysis of the chosen proteins

Summary of work done until the beginning of this reporting year

Three broad categories of proteins from *Mycobacterium tuberculosis* were chosen for biochemical and structural work, They are:

Redox proteins:	• Thioredoxins (A, B and C) • Thioredoxin reductase
Heat Shock Proteins:	• Chaperonin-60 family (Cpn60.1, Cpn60.2 and Cpn10) • Heat shock protein 70 family (Hsp70, Hsp40)
Other Protein including Proteins involved in transcription processes:	• HrcA and HspR: transcriptional repressors of the Hsp60 and Hsp70 operons respectively • YefM:YoeB toxin-antitoxin complex

Thioredoxins and Thioredoxin reductase:

The three thioredoxins (Trx's A, B and C) encoded by the Rv1470, Rv1471, and Rv3914 were cloned and expressed in *E. coli*. All the three proteins were purified to homogeneity. Size exclusion chromatography showed that TrxA, TrxB and TrxC are 1 dimeric proteins. Insulin reduction assays showed that the three proteins are capable of reducing insulin in the presence of a non-physiological reductant, DTT. TrxA was seen to be much slower in this assay than the other two Trx's. Further it was also tested if all the three Trx's are substrates of TrxR by reduction of insulin in the presence of physiological reductants, NADPH and TrxR. It was observed that TrxB and TrxC are the substrates of TrxR, however, TrxA is not a substrate of TrxR. Multiple sequence alignments showed that although all the three Trx's possess the conserved CXXC motif, TrxA does not possess a Trp residue preceding this motif, which is highly conserved among all thioredoxins. Moreover, some of the other residues known to be important for the activity of Trx's, such as Asp26 (*E. coli* nomenclature), were also replaced in TrxA. Thus, subtle sequence variations were ascribed to the lack of functional activity of TrxA. These results suggest that trxA might be a pseudogene, while TrxB and TrxC might be the canonical thioredoxins.

Chaperonins:

We had demonstrated earlier using heterologous complementation and biochemical studies that the loss of chaperoning ability of the mycobacterial GroEL-1 is indeed due to its inability to form canonical tetradecamers. A chimeric *groEL-1* ORF bearing the DNA sequence corresponding to the equatorial domain of *E. coli groEL,* unlike the unmodified *groEL-1* ORF is able to complement the loss of cell viability phenotype conferred by a conditional allele of *groEL*, in *E. coli*. The corresponding polypeptide, *in vitro* is capable of existing, principally in a tetradecameric state, a canonical feature of *E. coli* GroEL. Complementary studies show that an *E. coli* GroEL variant, displaying properties similar to that of *Mtb* GroEL-1 can be obtained provided it bears the amino acid sequence corresponding to *Mtb* GroEL-1 equatorial domain. Analyses of various *groEL* alleles obtained via DNA shuffling and error prone PCR starting with the *Mtb groEL-1* and *groEL-2* DNA as templates suggested that the basic property of a functional GroEL molecule appears to be oligomerization, to facilitate formation of the cavity for sequestration of substrate polypeptides. The substrate-recognizing apical domain may be fairly plastic and is capable of tolerating deletions or insertions. These experiments provided direct evidence for the importance of oligomerization in biologically relevant GroEL function.

Interestingly we observed that GroEL-1 is capable of binding to DNA without any sequence specificity. The affinity of DNA recognition by GroEL-1 is sufficiently high in the range of 100-200 nM suggesting that the protein has naturally evolved to bind DNA. Testing a range of oligonucleotide sequences of varying lengths revealed that GroEL-1 binds DNA in a sequence-non-specific manner. The *groEL-1* gene of *M. tuberculosis* was suggested to have acquired a nucleoid association function through loss of oligomerization, by utilizing its fundamental property as a non-specific substrate binding protein.

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

Project1: Characterization of Redox proteins

Thioredoxins and thioredoxin reductase

During the period under review, further functional characterization of the Trx's was carried out by RT-PCR, and by measurement of redox potential of the different disulfide linkages in the three thioredoxins. *M. tuberculosis* H37Rv was treated with different oxidative stress conditions, such as in presence of H_2O_2, diamde and cumene hydroperoxide. Under all these conditions, the *trxB* and *trxC* genes were seen to be upregulated. However, no effect was found on the *trxA* gene expression. Moreover, even under normal conditions of growth, *trxA* gene was not expressed. These results suggest that the *trxA* gene might not be functional as a thioredoxin. The measured redox potential shows that TrxB and TrxC have similar redox potential as the *E. coli* thioredoxin.

SigH and anti-SigH (RshA):

Understanding the regulation of oxidative stress response in *Mycobacterium tuberculosis* has remained intriguing, especially due to mutations in the OxyR repressor, which is the principal regulator of oxidative stress in other bacteria. One possible mechanism is by the control of gene expression of the oxidative defense genes through Extra Cytoplasmic Function (ECF) sigma factors. Indeed, the ECF sigma factors, SigE and SigH, are known to control gene expression of many redox genes in *M. tuberculosis*. The activity of SigH is further controlled by the anti-sigma factor, RshA. In this study we characterized the *M. tuberculosis* SigH-RshA complex and have suggested a possible pathway for RshA mediated redox regulation. We have been able to show that RshA remains tightly bound to SigH under reducing conditions to form a homogenous complex. The complex was seen to disassociate under strongly oxidizing conditions. Furthermore, limited proteolysis followed by MALDI-TOF analysis of fragmented peptides showed that, the region where RshA binds to SigH, is involved in binding of SigH to the RNA polymerase. Thus, RshA when bound to SigH occludes the binding of SigH to the RNA polymerase, thereby exerting control over gene expression of the redox genes. Though, SigH

interacts with RshA only through a small domain (residues 28-99), the other regions of the protein were seen to protect RshA from oxidation. Characterization of Cysteine mutants shows the critical role that the Cysteine residues of RshA play in mediating the redox transition. Thus, these studies pave way for understanding the molecular basis of redox response in *M. tuberculosis*.

Project 2: Molecular characterization of Chaperonins

We had earlier reported a detailed sequence analysis of the available GroEL sequences. This was to address the paradoxical observation that many genome sequences show occurrence of duplicate or multiple copies of *groEL* genes in certain bacteria. During the period under review, extensive mutagenesis of the groEL genes has been carried out, and the mutants were tested for their ability to complement Ts phenotype of *E. coli*. It was established that if chimera are constructed such that hybrid proteins contain oligomerization domain of *E. coli* GroEL, and substrate-binding domain of *M. tuberculosis* GroEL, the hybrid protein is functional in *E. coli*. However, the reverse is not true. This observation supports the idea that oligomerization is the most important property of a functional chaperonin.

In the previous report, we had also shown that *M. tuberculosis* GroEL-1 possesses an unusual property as a DNA-binding protein. We had hypothesized that this property might be due participation of GroEL-1 in nucleoid formation in *M. tuberculosis*. In the period under review, we purified the nucleoids of *M. smegmatis*, and probed if these are likely to contain GroEL-1. The results are currently being verified, also with the purified nucleoids of *M. tuberculosis*.

Project 3: Structural studies on toxin-antitoxins

The *yefM:yoeB* toxin-antitoxin gene was PCR amplified during the period under review. The protein was purified as a complex and was crystallized under two different conditions.

Although the complex crystallized in two different crystal forms, the structure determined using Se-methionine SAD phasing showed the presence of only the antitoxin molecules in the crystals (Figure 1). The antitoxin is observed to be a tetramer, which is a dimer of a dimer. The disassociation of the complex is hypothesized to be due to low pH of the crystallization buffer.

Each of the dimer has identical structure, except at the 20 residues of the C-terminus. These residues are highly flexible in solution. We propose that by virtue of this flexibility, the antitoxin is able to reach out to the toxin molecules, and thereby latch on to form a stable complex.

Future plans and direction

We plan to undertake analysis of the heat shock repressor proteins of *M. tuberculosis*, as well as study properties of *M. tuberculosis* GroELs. These will also be undertaken for crystallographic analysis.

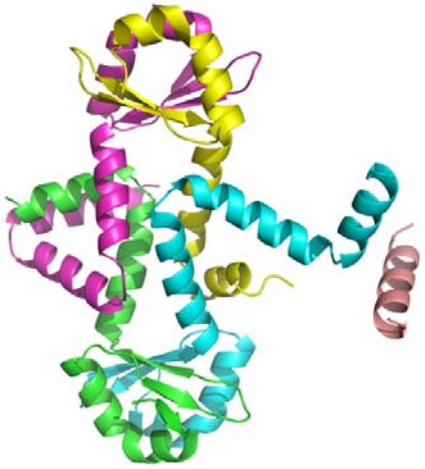

Figure 1: Structure of the tetrameric antitoxin, YefM

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Laboratory of Mammalian Genetics

Epigenetic mechanisms underlying developmental pathways

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Objectives are to study:

1. Specialized chromatin structures as epigenetic imprints to distinguish parental alleles
2. *DNMT3L*: epigenetic correlation with cancer
3. Role of *Dnmt2* in mammalian cells
4. Epigenetic mapping of the human Y-chromosome

Project 1: Specialized chromatin structures as epigenetic imprints to distinguish parental alleles

Summary of work done until the beginning of this reporting year:

Previously, we had reported methylation-restricted protein binding to a GC-rich motif within the transcription-independent, parental-allele-specific DNase I hypersensitive site present in the second intron of the imprinted mouse *neuronatin* gene. Our last report described the identification of GC-rich motif binding proteins using yeast One-hybrid assay. Furthermore, we had initiated experiments to examine whether the 250 bp second intron of *neuronatin* has any regulatory effect on the transcription of a reporter gene using P-element transposition strategy in Drosophila.

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

Identification of GC-binding proteins: To supplement the list of GC-binding proteins that we had identified using yeast One-hybrid assay, affinity chromatography using Biotin-tagged GC motif in conjugation with peptide fingerprinting (mass spectroscopy) of bound proteins was performed. The table below lists various proteins that were identified in the assay. Interestingly, several of the proteins identified were capable of binding to both RNA and DNA and most of them contain the RRM domain. Further studies are underway to test the methylation-restricted GC-binding of these proteins (recombinant).

S.No.	Gene Symbol	Protein Name	Motifs
1.	***Sfpq***	***splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)***	**RRM, RRM1, NOPS**
2.	***HnrnpR***	***Heterogeneous nuclear ribonucleoprotein R***	**RRM**
3.	***Nono***	***Phf7***	**RRM, RRM1, NOPS**
4.	***Ewsr1***	***Ewing sarcoma breakpoint region 1***	**RRM, zf-RanBP**
5.	***Parp1***	***poly (ADP-ribose) polymerase family, member 1***	**parp_like, ligA, zf-PARP, WGR, PADR1**
6.	***Parp3***	***poly (ADP-ribose) polymerase family, member 3***	**parp_like, WGR**
7.	***HnrnpU***	***heterogeneous nuclear ribonucleoprotein U***	**SAP, SPRY, ATPase**

Functional analysis of neuronatin's second intron: For analyzing the functional role of the second intron of *Neuronatin* we examined its effect on transcription of *mini-white* reporter transgene in *Drosophila*. This 250 bp intron, flanked by *lox*P sites, was inserted in both orientations upstream of the *hsp*70 promoter driven *mini-white* reporter gene containing P-element vector pCaSpeR. Several independent transgenic lines were generated on different chromosomes and these lines had the intronic region either in the positive orientation or negative orientation with respect to the *hsp*70 promoter and *mini-white* gene. To investigate whether the observed effect on the expression of the *mini-white* gene was because of the presence of *Neuronatin* intron or due to the chromosomal location where the transgene was located, the intronic region was flipped out by

crossing the transgenic lines to flies containing *cre* recombinase and the flipped out version of each transgenic line was established. Comparison of eye color showed that many of the transgenic lines had eye color that was darker than their respective flipped out counterparts (figure 1) indicating that the putative ICR functions as an activator of transcription in Drosophila. This was also confirmed by quantification of eye pigments.

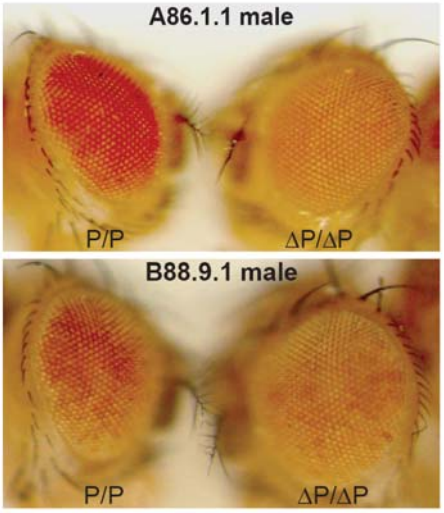

Figure 1: ***Neuronatin's second intron acts as a transcriptional activator in Drosophila. Transgenic Drosophila lines having Neuronatin's second intron inserted in positive (A) or negative orientation (B) upstream of reporter mini-white gene (P/P) showed darker eye color than their counterpart lines where the intron had been removed (ΔP/ΔP).***

Project 2: *DNMT3L*: epigenetic correlation with cancer

Summary of work done until the beginning of this reporting year

Previously, we had reported comparison of DNA methylation profile of a few reprogramming genes like DNA methyltransferases, histone modifiers, etc and stem cell specific genes like *Oct4*, *Nanog*, etc. between normal and cervical cancer samples. Our pilot study had shown interesting DNA methylation profile for *DNMT3L,* a reprogramming molecule belonging to the family of DNA methyltransferases but lacking the catalytic domain. We had observed that more than 90% of the cancer cervical samples showed loss of DNA methylation at the promoter of *DNMT3L* gene in comparison to normal samples which showed promoter hypermethylation.

Details of progress made in the current reporting year (April 1, 2006- March 31, 2007)

Correlation of DNA methylation changes at the DNMT3L promoter with expression level: DNA methylation of a gene promoter is usually associated with transcriptional regulation of gene expression (hypomethylation is correlated with expression and hypermethylation with repression). As reported last year, this correlation was found to exist for the promoter of *DNMT3L* in cervical cancer cell lines. HeLa cell line, which had lost methylation at several CpG's within the *DNMT3L* promoter, showed 8 fold more expression than SiHa cell line (promoter is predominantly methylated). To corroborate these findings we used the inhibitor of DNA methyltransferase (5-azacytidine). As the difference in DNA methylation may not be the only epigenetic modification correlated with *DNMT3L* expression, we also looked into the effect of histone acetylation on *DNMT3L* expression using the drug Trichostatin A, which is an inhibitor of Histone Deacetylase. Use of both the inhibitors, individually as well as in combination, was found to increase *DNMT3L* expression.

Characterization of epigenetic signatures at the DNMT3L loci: Above mentioned loss of DNA methylation was observed for a 300 bp region within the promoter and 1st exon of *DNMT3L*. To analyse if the difference in DNA methylation was limited to this specific region or encompassed a large region within the *DNMT3L* locus two other CpG islands within this locus were analysed for their DNA methylation status in the two cervical cancer cell lines, Hela and SiHa. No difference was observed for the CpG island present downstream to the promoter region. Similar to the promoter CpG island, the upstream CpG island also showed loss of methylation in Hela cells in comparison to SiHa. We are at present analysing the histone modifications that are associated with these regions.

DNMT3L and Nuclear Reprogramming: To examine if *DNMT3L* has any effect on cellular functions, it was overexpressed in cancer cell lines HeLa and SiHa. Comparison of *DNMT3L* overexpressing cells with appropriate vector controls suggested that *DNMT3L* can effect genome-wide reprogramming. Not only does *DNMT3L* overexpression stimulate cellular proliferation in HeLa and SiHa cells, as judged by clonogenic (figure 2) and soft agar assays, but it also changes

the cell cycle progression profile of the transfected cells. In addition, morphological changes and wide-spread gene-expression changes were also observed. The mechanism by which this nuclear reprogramming was achieved by *DNMT3L* overexpression in HeLa and SiHa cells is being investigated.

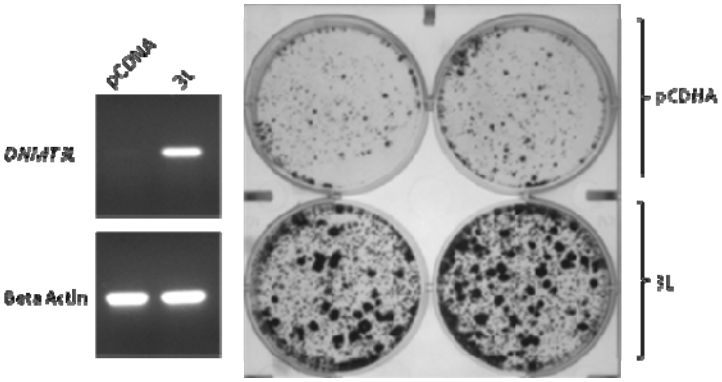

Figure 2: Clonogenic assay on *DNMT3L* expressing HeLa cells. HeLa cells were either transfected with vector alone (pCDNA) or *DNMT3L* cDNA construct (3L). Selected transfected cells (using G418) were pooled and grown for further 14 days and stained with crystal violet. *DNMT3L* expressing HeLa cells form larger colonies indicating proliferative advantage.

Project 3: Role of *Dnmt2* in mammalian cells

Summary of work done until the beginning of this reporting year

Though it has all the domains specific for methyl transferases, *Dnmt2* has failed to show significant DNA methylation *in vitro* and *in vivo* conditions. Recently, *Dnmt2* was shown to methylate tRNA in mice but its role in mammals remains a mystery as *Dnmt2* Knock-out mice are viable and do not show any phenotypic abnormalities. As reported in the last report we had initiated experiments to investigate the role of *Dnmt2* through the functional capabilities of its interacting partners.

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

Identification of Dnmt2-interacting partners: As many of the proteins that were identified in the co-immunoprecipitation experiment were part of the RNA processing machinery, we performed Co-IP or GST pull down assay with known members of this machinery. Our experiments showed that the proteins from the ribonuclear protein family like *HnrnpK* and *HnrnpU* can also interact with *Dnmt2*.

Role of Dnmt2 in RNA metabolism: As has been reported by other groups, sub-cellular localization studies for *Dnmt2* showed that it is predominantly localized to the cytoplasm. However, careful examination of the live-cell imaging data revealed that it is also localized to certain foci in the nucleus but only for a limited duration of time. Interestingly, the localization of *Dnmt2* within the cytoplasm is also not uniform but is concentrated in specific foci (figure 3). The position and size of these foci are not fixed but dynamic. Data-mining through previous literature showed that proteins localizing to RNA processing bodies, also known as Processing bodies or Stress granules, also show similar localization. Dnmt2 was found to co-localize with proteins that are present in P-bodies. The possibility therefore, exist that *Dnmt2* has a role to play in P-bodies. Alternatively, it is also possible that the presence of *Dnmt2* in P-bodies is casual. In the light of fact that many of the interacting partners of *Dnmt2* are a part of RNA processing machinery, work is underway to examine the various possibilities and understand the role of that *Dnmt2* in RNA metabolism.

Project 4: Epigenetic mapping of the human Y-chromosome

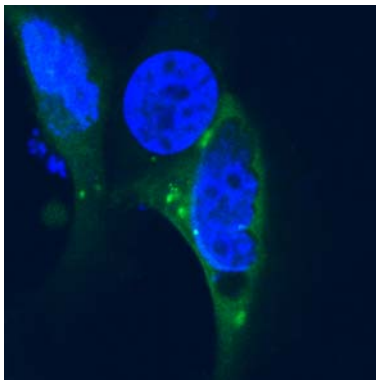

Figure 3: Sub-cellular localization of *Dnmt2*. GFP-tagged *Dnmt2* (green) transfected in to HeLa cells localizes to foci within the cytoplasm. Nuclei are stained by DAPI (blue).

Summary of work done until the beginning of this reporting year

In collaboration with CCMB, Hyderabad and CHG, Bangalore, a pilot project was initiated to determine DNA methylation patterns as well as chromatin modification patterns (histone H3-lysine 9 di methylation and histone H3-K9 acetylation) of all genes on human Y chromosome (~320 genes). Within this project, our laboratory's focus is on determining the DNA methylation status of the promoters of 160 genes present on the Y chromosome.

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

For DNA methylation analysis of Y chromosome gene promoters, the human Y chromosome sequence from NCBI (Build 36.1) was used for designing primers for the various regions. The first 160 genes in the database (including the pseudoautosomal region) were selected for analysis at CDFD. Methylation analysis was done by bisulfite treatment on two blood samples. Correct PCR for 120 of the 144 genes (excluding the genes present in the pseudoautosomal region) was obtained using primers against the converted sequences. The PCR products were cloned and 10-12 clones for each region were sequenced. We were able to obtain good methylation profile for 95 genes based on sequencing results. For the remaining regions for which either the PCR had not worked or the PCR product did not give the correct sequence are reanlaysed using new primer sets. PCR and cloning for the remaining genes has been completed. Preliminary analysis of DNA methylation profile data for the 95 genes showed that most of the gene promoters on Y chromosome were completely methylated for all the examined CG's in blood cells. Since methylation of a promoter is correlated with repression of gene expression, this would suggest that most genes on Y chromosome are transcriptionally silent. Only 8 genes showed hypomethylation and 3 showed a combination of CG's with hyper and hypomethylation (figure 4). Interestingly, SRY gene which is involved in sex determination and plays a role in germ cells was found to be hypomethylated. Dr. Rakesh Mishra's laboratory has generated a map for histone modifications on Y chromosome. We are now in the process of collating the data on DNA methylation and histone modifications to analyse correlation between the various epigenetic modifications at respective loci. We are also collating the data from the three laboratories in to a database to chart out exact combinations of epigenetic modifications at each loci on Y chromosome.

Figure 4: A snapshot of DNA methylation profiles for some of the Y-chromosome genes. Gene ID and positions are based on Y-chromosome sequence available from NCBI (build 36.1). Methylation profiles are shown by colored circles. Each circle represents one CG. Red- >70% methylation, Yellow: 30-70% methylation, Green: < 30% methylation. % methylation is calculated as no. of clones for the respective locus that show methylation for the particular CG.

Publications

1 Gokul G, Gautami B, Malathi S, Sowjanya A, Poli U, Jain M, Ramakrishna G and Khosla S (2007) DNA methylation profile at the *DNMT3L* promoter: a potential biomarker for cervical cancer. ***Epigenetics*** 2: 80-85

2 Nyamath P, Alvi A, Habeeb A, Khosla S, Khan AA and Habibullah CM (2007) Characterization of hepatic progenitors from human fetal liver using CD34 as a hepatic progenitor marker. ***World Journal of Gastroenterology*** 13:2319-2323

Laboratory of Molecular Oncology

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Project 1: Molecular genetic analyses of sporadic colorectal cancer occurring in the young

Objectives

1. Screening young colorectal cancer (CRC) patients for chromosomal instability and microsatellite instability and compare with older patients.

2. Identifying recurrent copy number alterations (CNAs) as well as important genes located within the CNAs from non-familial young CRC patients and compare with older patients.
3. Identification of pathways that might distinguish young non-familial CRC patients from elder patients.

Summary of the work done until the beginning of this reporting year

Only about 20% of young patients appeared to have an activated Wnt signaling pathway in their tumours as compared to about 80% of older patients, which is a significant difference (P=0.00007; Fisher's exact test). In addition, we detected novel mutations in the MCR region of the APC gene in four samples. Preliminary results revealed no significant difference between the two age groups in frequency of microsatellite instability and BRCA1 LOH.

Details of progress in the current reporting year (April 1, 2007- March 31, 2008)

The project is now funded by a Fogarty International Research Collaboration Award (FIRCA) of the National Institutes of Health, USA. We have extended the analysis of status of Wnt signaling in tumours from the two groups of patients to one hundred and three samples. Only 40% of young patients appeared to harbor an activated Wnt signaling pathway in their tumours as compared to 75% of older patients; a significant difference (P=0.007; Fisher's exact test). We identified several novel mutations (in addition to those identified last year) in the APC gene including nt4372 (-C), p.H1349R (CAC to CGC), p.V1414I (GTA to ATA), p.G1365S (GGT to AGT), p.Q1378ter (CAG to TAG), and p.K1370Q (AAA to GAA); the mutations were confirmed by bi-directional sequencing and cloning in plasmid vectors, and, were not present in the matched normal samples. A significant proportion of mutations (60%) were novel (which is surprising given that more than 1000 mutations have been reported worldwide), indicating perhaps a distinct mutation spectrum in the Indian population. We are currently analyzing the possible effects of the mutations on gene/protein function. In order to validate the oligonucleotide array (HEEBO) platform, we carried out aCGH and gene expression arrays on the pancreatic cancer cell line AsPC1, for which aCGH and gene expression data had been generated previously using the cDNA array platform. The data obtained from HEEBO arrays

were consistent with that obtained earlier (fig. 1a) indicating that the HEEBO arrays could faithfully detect localized DNA amplifications and deletions at a high resolution. So far we have analyzed six CRC samples using aCGH; we detected a novel localized amplification in a young female patient located at 17p11.2 (fig. 1b). Analysis of the amplified region revealed oncogenes including MAP2K3 and MAPK7. Analyses of genome-wide Copy Number Alterations and gene expression profiles might reveal the biological pathway(s) that drive(s) tumour initiation/progression in the young sporadic CRC patients.

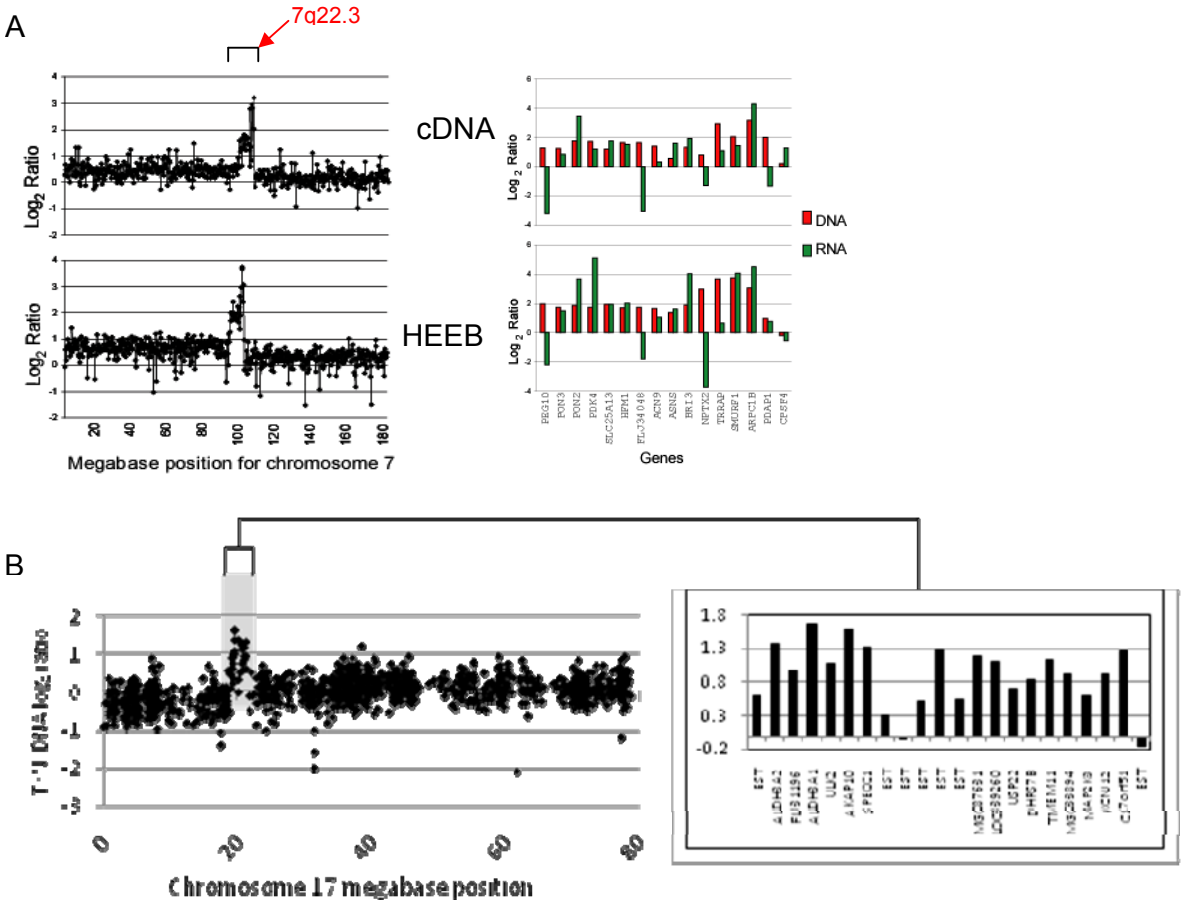

Fig. 1A. aCGH performed on the pancreatic cancer cell line AsPC1 using whole genome human cDNA array (top panel) and whole genome human oligonucleotide-based HEEBO array (bottom panel) yield similar results. The left panel shows log_2 ratios for chromosome 7 probes indicating a high level localized amplicon at 7q22.3. The right panel shows log_2 ratios for DNA and RNA for genes/ESTs located at 7q22.3, indicating the overexpression of genes located within the amplicon. 1B, aCGH carried out on a young female colorectal cancer sample from India. The left panel shows DNA ratios in log_2 scale for all probes from chromosome 17. The right panel shows DNA ratios for probes located within the localized

Project 2: Identification of novel esophageal squamous cell carcinoma (ESCC) genes by using a combination of array based CGH and gene expression microarrays.

Objectives

1. To determine molecular differences between adenocarcinoma and squamous cell carcinoma occurring in Indian patients
2. To identify novel esophageal cancer genes by using array-based CGH
3. To validate selected genes by gene expression microarrays/real time PCR
4. To identify candidate oncogenes (within amplicons) and tumor suppressor genes (within deletions).

Summary of work done until the beginning of this reporting year

This is a new activity sponsored by the DBT.

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

Esophageal Squamous Cell Carcinoma (ESCC), although a common and highly aggressive cancer in India (as against adenocarcinoma, which is more common in the West), is poorly studied. We have chosen to characterize status of four important genes/pathways in order to dissect molecular differences between adenocarcinoma and squamous cell carcinoma of the eshophagus namely p53, EGFR, Wnt signaling and Microsatellite Instability. We have standardized the conditions for detection of genetic lesions from both, paraffin embedded blocks of archived and fresh esophageal cancer samples (collected from AIIMS, N Delhi; IMS, BHU, Varanasi; Apollo Hospitals, Hyderabad; Nizam's Institute of Medical Sciences (NIMS), Hyderabad; Indo American Cancer Institute and Research Centre (IARC), Hyderabad and MNJ Institute of Oncology and Regional Cancer Center, Hyderabad) following ethical committee clearance from each hospital, and informed consent.

i) Screening for CIN/Wnt activation:

We have carried out IHC to elucidate intracellular localization of β-catenin in 54 esophagous cancer samples. Interestingly, 36% of adenocarcinoma samples (5 of 14) as opposed to only 0.1% (3 of 40) squamous cell carcinoma samples exhibited an activated Wnt signaling, indicating

thereby a possible molecular difference between squamous and adenocarcinoma of the esophagous. However, we need to analyze a larger number of samples to come to a definite conclusion.

ii) MSI screening:

So far, we have completed MSI screening for 5 samples so far, out of which one was MSI-H (MSI-high), two were MSI-L (MSI-Low) and two were MSS (microsatellite stable). We have also used the MSI assay to detect Loss Of Heterozygosity (LOH) in specific tumour suppressor genes including p53, p16, BRCA1 and APC. Representative electropherograms are shown in figure 2a. Two out of five ESCC samples exhibited LOH at the BRCA1 locus.

iii) Screening for p53 mutations and intracellular localization:

We have screened exons 5, 6, 7 and 8 of the p53 gene for presence of mutations. So far, we have completed the screening for 8 samples; mutations were detected in 6, out of which three were novel mutations. Interestingly, one sample harbored two mutations. All mutations were confirmed by bidirectional sequencing and were not present in the corresponding matched normal sample. We are planning molecular studies to characterize the novel mutations. All the mutations detected in our study were represented by only one peak in the electropherogram. Although it is possible that the mutations were present in both alleles, a more likely explanation would be deletion of wild type allele. To confirm this possibility, we are carrying out LOH analysis using primers specific for polymorphic microsatellite markers located in the p53 loci. Wild type p53 protein has a very short half life; and an important fallout of p53 mutation is stabilization of the protein in the nucleus which can be detected by IHC. Out of 43 samples analyzed so far, 20 (47%) exhibited significant nuclear staining. Barring one, all samples that harbored a mutation in the p53 gene also exhibited stabilization of the p53 protein in the nucleus.

iv) Array-based CGH:

Sixty freshly resected esophagous cancer samples were collected from the collaborating centres including Sher I Kashmir Institute of Medical Sciences (SKIMS), Srinagar, with the help of a surgeon, and snap frozen in liquid nitrogen for isolation of nucleic acids. A section of the tumor

was also collected in formalin for archiving in paraffin embedded blocks, to be used for determination of percentage tumor epithelial cells and tumor grade. The normal samples for the corresponding tumor were collected 5 cm away from the tumor margin, and confirmed to be tumor-free by the collaborating pathologist. Samples which contained a minimum of 60% tumor epithelial cells were used for array analyses. As of now, aCGH has been carried out on six ESCC samples. Interestingly, we identified a localized high level DNA amplification at 10q21 in two samples (fig. 2b). It would be interesting to screen additional samples to determine whether the 10q21 amplification could be a recurrent event in ESCC. In addition, another amplicon harboring the CCND1 gene was also identified in both samples.

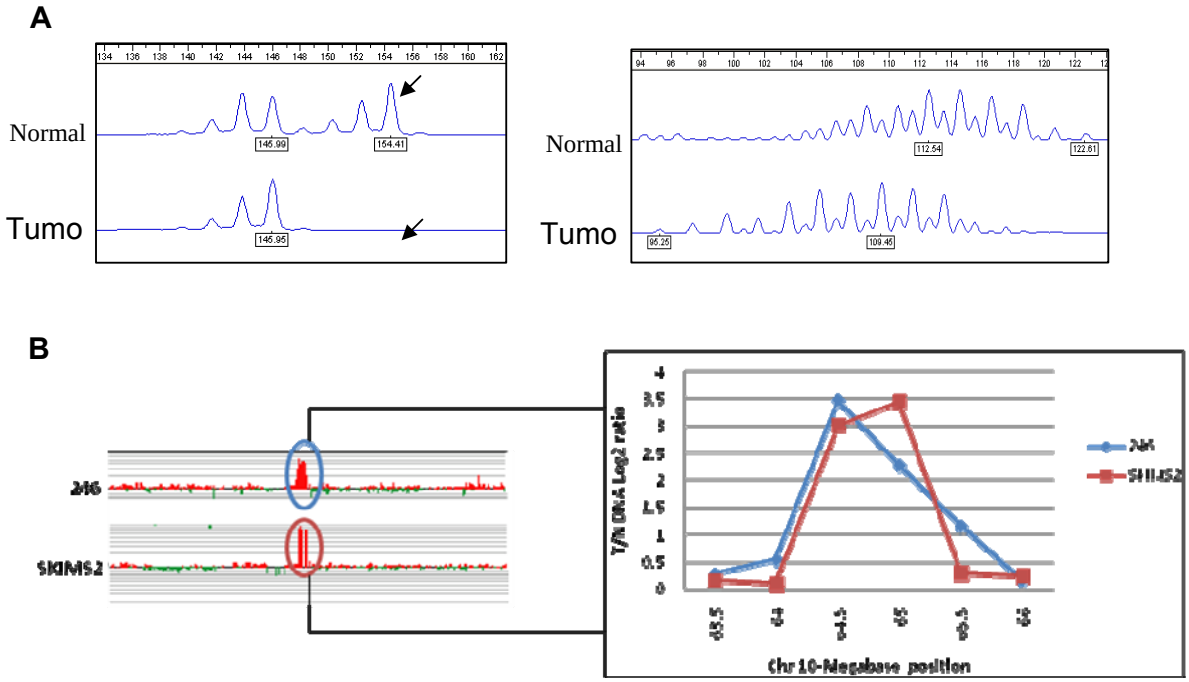

Fig. 2. Molecular analyses of esophagous cancer. A, Representative electropherograms depicting results of genotyping; left panel shows Loss of Heterozygosity at the BRCA1 locus and the right Panel shows microsatellite instability at the p16 locus. B, aCGH performed on two ESCC samples reveal a recurrent amplicon at 10q21. The left panel indicates aCGH ratios for the two samples in $\log_{10}$ scale for probes located on the 10th chromosome; red bars located over the baseline indicate copy number gain and green bars below the baseline indicate copy number loss. The right panel indicates DNA ratios (in $\log_2$ scale) for the probes located within the amplicon, highlighting the overlap between the two

Project 3: Molecular genetic analyses of hypohidrotic or anhidrotic ectodermal dysplasia (HED)

Objectives: **Identification and characterization of disease-causing mutations in HED**

This is a new activity.

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

Hypohidrotic or anhidrotic ectodermal dysplasia (HED) is a rare congenital disorder that results in abnormalities in the structures of ectodermal origin, viz. teeth, eccrine sweat glands, hair and nails. The X-linked form of the disease, called X-linked HED (XLHED) (the most common form of HED), is caused by mutations in the ectodysplasin-A (EDA) gene. Autosomal recessive form of the disease is caused due to mutations in the EDA receptor (EDAR) and both autosomal recessive and dominant forms are caused due to mutations in other components of the downstream signaling pathway. In collaboration with the Diagnostics Division, at CDFD we have carried out the first molecular characterization of HED patients from India. Out of a total of eight patients studied, four harbored novel mutations, two in the EDA gene (p.Y304C and p.M279R), and two in the EDAR gene (nt478delC and IVS6+5 G to A); one harbored a known mutation in the EDA gene (p.R155C) and three harbored a known mutation in the EDAR gene (p.G382S). We have characterized the affect of the two novel EDA mutations (p.Y304C and p.M279R) at the protein level. Using multiple alignments and the PAM 260 substitution matrix, we first confirmed the high extent of evolutionary conservation of the mutated amino acids (EDA Y304 and M279) among various species. Following this, we modeled the mutations on the available structure of the wild type protein and the results revealed that the p.Y304C mutation results in destabilization of the functional EDA trimer, whereas the p.M.279R mutation disturbs the electrostatic surface charge in the protein (fig. 3). Based on this work, genetic counseling was provided by the Diagnostics Division to all the affected families.

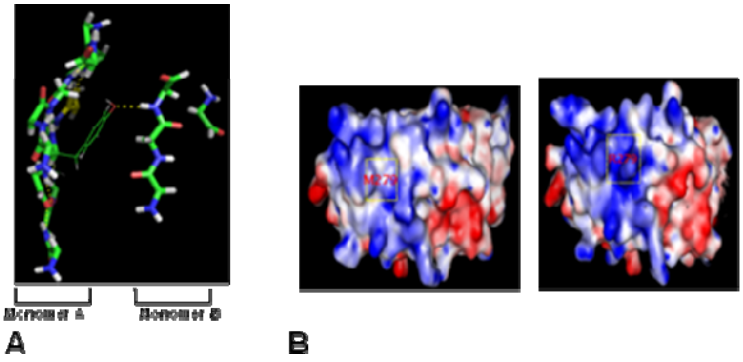

Fig. 3. Structural analysis of the EDA p.Y304C and p.M279R mutations. Panel A shows residues surrounding 0.4nm region of EDA Y304. The hydrogen bond between the side chain of Y304 (monomer A) and the backbone of V351 of the other monomer (monomer B) is indicated by a broken line. Panel B shows surface electrostatic potential of the wild type (M279) and mutated (R279) EDA protein. +5kT is depicted in blue colour, neutral in white colour and

Project 4: Molecular genetic analyses of Familial Hypertrophic Cardiomyopathy (FHC)

Objectives: Identification and characterization of disease causing mutations in FHC

Summary of work done until the beginning of this reporting year

We had initiated this DBT-sponsored study last year to profile mutations that cause FHC in Andhra Pradesh. Data from 64 patients were collected and the patients exhibited a wide heterogeneity in clinical presentation, emphasizing the variations in the disease as previously reported. In four patients, we detected an identical mutation (p.R787H) in the MYH7 gene. Interestingly, the mutation appeared to be sporadic in two of the four families screened. Sequence and structure analysis revealed that the mutation might compromise binding of the myosin heavy chain to the myosin essential light chain.

Details of progress made in the current reporting year (April 1, 2006- March 31, 2007)

We have now extended the study to a total of 90 samples and included the Myosin binding protein C3 gene (MyBPC3) in the screening. So far we have detected mutations in ten probands, four had the identical p.R787H mutation (reported last year) and the other six harboured mutations in the MyBPC3 gene; p.C1124ter in one sample; p.G757D in two samples, IVS28+1 (G to A) in one sample and a novel mutation IVS19+7 (G to A) in two samples. As per reports available from the western population, a majority of patients harbour mutations in the MYH7 gene; interestingly, we detected mutations in the MyBPC3 gene in a significantly large proportion of patients (6 out of 10). Patients harbouring mutations in the MyBPC3 gene usually harbour a milder form of the disease, with less likelihood of sudden cardiac death, whereas

mutations in the MYH7 or the Troponin T2 gene are strongly associated with sudden cardiac death. The fact that the sample patients of our study are those who are reporting to the hospital (thereby probably excluding those who suffer sudden cardiac death due to a 'malignant' mutation) may explain the larger proportion of patients harbouring a mutant MyBPC3 gene. However, the true picture is expected to emerge after we complete the screening for all the samples.

Publications

1. Rama Devi AR, Reddy C K, Ranjan S and Bashyam M D (2007) Molecular genetic analysis of patients from India with hypohidrotic ectodermal dysplasia reveals novel mutations in the EDA and EDAR genes. ***British Journal of Dermatology*** 158:163-67
2. Bashyam MD and Hasnain SE (2007) Array-based comparative genomic hybridization: applications in cancer and tuberculosis. In: ***Bioarrays***, Edited by K Appasani, Humana press, pp 107-121
3. Bashyam MD, Savithri GR, Gopikrishna M and Narasimhan C (2007) A p.R870H mutation in the beta cardiac myosin heavy chain 7 gene causes familial hypertrophic cardiomyopathy in several members of an Indian family. ***Canadian Journal of Cardiology*** 23:788-790
4. Bashyam M D and Ratheesh R (2008) Genetic instability in colorectal cancer. ***The ICFAI Journal of Biotechnology*** 2:13-26

Laboratory of Molecular Oncology

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	NCI, NIH	

Project 1: Understanding the biology of Ras-mediated signaling events

Objectives:

(a) To delineate the signaling pathways between various point mutants of Ras

(b) To understand cell-type specific differences between fibroblasts and epithelial cells using both an *in vitro* and in *vivo* approach.

Summary of work done until the beginning of this reporting year

1. We evaluated deregulation in Ras-mediated signaling events in human hepatocellular carcinomas (HCC) and found an unusual downregulation in the expression of Ras protein in HCC. However varied protein expression profiles of downstream Ras effectors (Raf, MAPK and AKT) and the cell cycle regulated proteins (p21, P27, Cylin D1) indicated heterogeneity of hepatocellular cancer samples.
2. Using *in vitro* based approach we showed that in both fibroblast and epithelial cells, wild type K-*ras* can induce senescence.

Details of progress made in the current reporting year (April 1, 2007- March 31, 2008)

Earlier we had generated both fibroblasts and epithelial cell lines expressing the tetracycline trans-activator as an initial step to make K-ras inducible Tet-off cell lines to understand the functioning of Ras. Using various conditionally expressing mutant forms of K-*ras* viz., codon 12

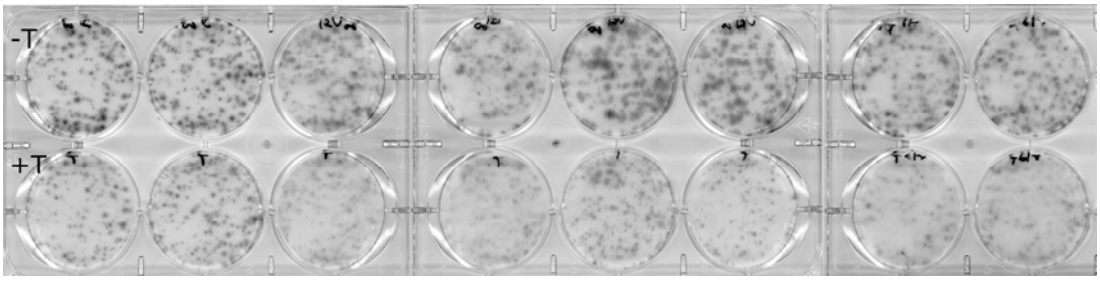
mutant (12V), codon 13 mutant (13V) and codon 61 mutant (Glu 61), we evaluated the growth parameters as well as the downstream signaling events.

1. Comparative growth properties of K-*ras* conditional mutants in epithelial cell line (E10):

   The proliferative efficiencies of different point mutants were compared by the following three different assays (a) colony formation, (b) BrdU incorporation and (c) soft agar assay. For colony formation, assay cells were seeded in six well plates in the absence and presence of tetracycline and the colonies formed after 2 weeks were stained with crystal violet stain. As compared to the control cells (vector alone) the number of colonies was significantly more in the K-*ras* mutants in the absence of tetracycline when the gene is in the inducible state. Amongst the three different *ras* mutants maximum growth was observed in K-*ras* 13V mutant followed by 61R and 12V mutants (Fig. 1 and 2). However, in spite of the increased proliferative rate of the 13V mutant there was no appreciable change in the percentage of the S-phase cells either by cell cycle analysis by FACS or S-phase analysis by BrDU incorporation (data not shown). Although 13V mutant provides a better growth advantage compared to other mutants on tissue culture coated plates , it was, however, not sufficient to confer anchorage independent growth when plated on soft agar.

2. Activation of downstream signaling events:

   Both the Tet-off fibroblasts and the epithelial cells were analysed for the differences, if any, in the activation of the two classical Ras-MAPK and AKT pathways under (a) basal conditions and (b) following serum starvation. Intriguingly, while there occurs an activation of both MAPK and AKT activation following serum stimulation, there was no appreciable difference in the activity when compared amongst the three different point mutant spectra both in fibroblast and epithelial cells.



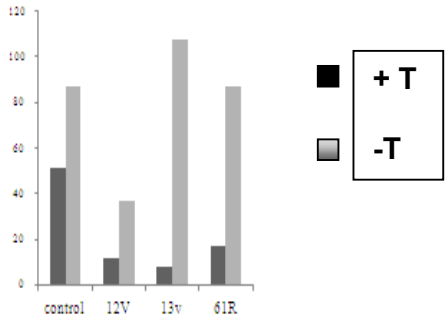


Fig.1 Colony formation assay in presence and absence (-T) of Tetracycline (+T) in control and different point mutants of *ras* (12Vm 13V, 61R). Note the number of colonies are more when the gene is expressed in absence of tetracycline.

Conclusions

It appears that even though Ras-p21 is induced in the absence of tetracycline in all the Tet-off inducible K-*ras* mutant clones we did not find any appreciable difference amongst the different mutants with respect to activation of signaling pathways. The only appreciable change observed was, an increased proliferative rate of 13V *ras* mutant but without significant activation of either MAPK or AKT pathways. Our results using the conditional Tet-off system comes to us by surprise as despite the fact that oncogenic Ras is dominant over the wild type form we did not find increased tumorogenicity or signaling by MAPK and AKT pathways following the expression of mutant Ras especially in fibroblasts. In view of these facts we speculate that (a) the inducible level of mutant K-Ras protein is not sufficient to drive tumorigenesis and (b) the endogenous wild type *ras* in the mutant clones may be acting in a inhibitory manner to suppress the effect of the mutant *ras*.

Work is under progress to specifically knockdown the wild type Ras by RNAi based approaches to check if mutant K-*ras* shows any gain of function in near absence of its wild type counterpart.

Project 2: Persistent infection with human papilloma virus, 3q chromosomal gain and presence of circulating RNA component of the telomerase (TERC) in cervical neoplasia

Objectives

In an ongoing collaborative project with a rural hospital based in outskirts of Hyderabad an attempt is being made to (a) understand the prevalence and genotype distribution of high risk HPV types in and (b) identify new biomarkers which can be used as an adjunct to HPV testing.

Summary of work done until the beginning of this reporting year

From the start of the project in 2004 we had screened close to 1900 women till March 2007 and found a 10% prevalence of HPV infectivity. Majority of the women showed a clearance on follow up. Also we had reported that it is feasible for the women to collect vaginal samples for cervical cancer screening programme.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

1. Till date we have screened a total of 2374 women attending the rural HPV screening and cancer detection clinic of Mediciti hospital at Medchal Mandal. Of these a total of 232 women were positive for HPV indicating HPV prevalence close to 11%. Of these a total of 88 women have till date been screened for the presence of various HPv types using a PCR absed Line blot assay. The major HPV type appears to be HPV-16 as has been reported world wide. However unlike other studies where HPV 18 prevalence always follows HPV-16, in our study other high risk types like 58, 31, 35, 39, 52, 53 were comparatively more prevalent than HPV18. We have to screen all the other positive samples before a conclusion can be made on the order of prevalence of various HPV types. Interestingly the pattern of HPV-type distribution is similar between the clinician collected and self collected sample (Fig.1)

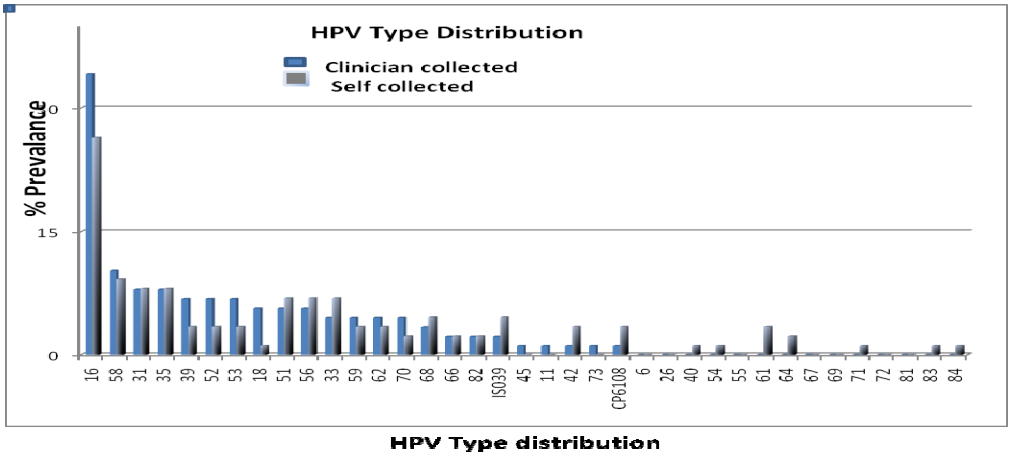
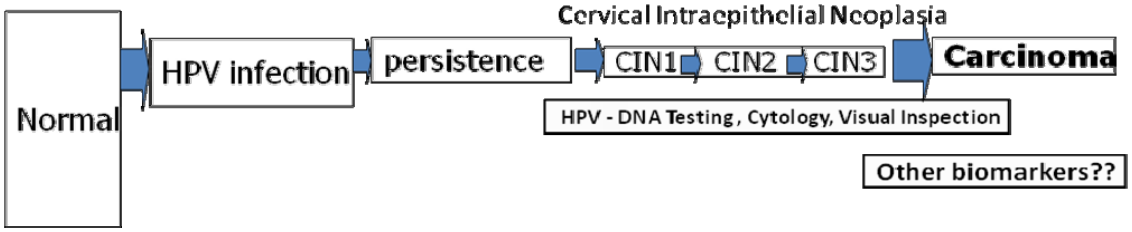


Fig. 1 HPV Type distribution in the cervical samples obatined from clinician assisted or self-collected samples.

2. New activity was started in the current reporting year to identify the newer biomarkers that can be used in adjunct with HPV screening. Infection with high risk HPV-types is a major etiological risk factor for cervical neoplasia. Majority of the women infected with HPV show effective clearance of the virus, however in a subset of women with persistent infection the high risk HPV types lead to neoplastic changes of cervical epithelium. HPV alone is insufficient for causation of cervical disease and other molecular events are essential for progression towards invasive cancer. Chromosomal aberrations and copy number changes are regular features during neoplastic transformation of cervical epithelium. It was known that activation of telomerase is a key event in progression of cancer and interestingly there occurs a gain of chromosomal arm 3q26 which infact harbors the RNA component of telomerase viz, TERC. An initiative was taken in this regard to check if TERC can be used as a potential biomarker for cancer cervix. Therefore recently a study was undertaken to evaluate (a) amplification TERC (present on chromosome 3) during cancer cervix progression and (b) if TERC-RNA can be detected in the circulating blood in women diagnosed with squamous cell carcinomas (SCC) with HPV infection.



Briefly we carried out two different studies in order to evaluate

Study I: In a collaborative study done with National Cancer Institute we evaluated HPV integration with gain in chromosome 3q by Fluorescent In situ Hybridization (FISH) on cervical epithelium cells obtained from different grades of cervical neoplasia viz, CINI, CINII,CIN III and carcinoma

Study II: We screened for presence of major HPV-types in squamous cell carcinomas from women undergoing surgery in a city-based hospital of Hyderabad and used matched serum samples from women with SCC to evaluate the presence of circulating TERC-RNA by PCR based method.

Using the 3q26 bac-probe (harboring TERC) obtained from a collaborative laboratory at NIH we have performed FISH on Thin-Prep cervical smears earlier diagnosed as cancer cervix. We found a gain in mean copy number of TERC in the cancer samples compared to the normal. Further we find an incremental increase in TERC copy number with progression from CIN3 to squamous cell carcinoma.

There are reports existing in literature in other cancer types where tumor derived circulating DNA and RNA have been detected in plasma or serum from cancer patients. In view of this we tested if we can detect RNA subunits TERC in the circulating serum samples obtained from cancer patients. Except for one sample we could not detect any TERC-RNA in the plasma obtained from the normal patient however all of the cancer samples showed the presence of circulating TERC levels. While the study is still on the data from FISH analysis and circulating RNA levels, of TERC indicates the utility of TERC as a important biomarker both for cervical screening strategies.

Laboratory of Computational and Functional Genomics

Computational and Functional Genomics of Microbial Pathogens

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	Mr E Ashwanth Kumar	Project Assistant
	Mr M Jayavardhan Reddy	Project Assistant
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Project 1: Characterization of transcriptional regulators from Mycobacterium tuberculosis

Objectives

Transcription regulators are important molecular players that regulate cellular physiology. Large number of these transcriptional regulators, including GntR family, from *M. tuberculosis* are not

characterized. Previous *in silco* analysis revealed that five genes encode FadR-like transcriptional regulators. Present study deals with the identification of operator site of Rv0586 (Mce2R), one of the FadR-like transcriptional regulator, from *M. tuberculosis.*

The objectives of the project are as follows:

- To Identify Operator site for Rv0586, a FadR-like transcriptional regulator from *M. tuberculosis*
- Experimental validation of computationally identified operator site.

Summary of the work done until the beginning of this reporting year:

Mycobacterium smegmatis is a fast growing non-pathogenic mycobacterium. This organism has been widely used as a model organism to study the biology of other virulent and extremely slow growing species like *Mycobacterium tuberculosis*. Based on the homology of the N-terminal DNA binding domain, the recently sequenced genome of *M. smegmatis* has been shown to possess several putative GntR regulators. A striking characteristic feature of this family of regulators is that they possess a conserved N-terminal DNA binding domain and a diverse C-terminal domain involved in the effector binding and/or oligomerization. Since the physiological role of these regulators is critically dependent upon effector binding and operator sites, these regulators were classified into their specific subfamilies and their potential binding sites were identified. The sequence analysis of *M. smegmatis* putative GntRs has revealed that FadR, HutC, MocR and the YtrA-like regulators are encoded by 45, 8, 8 and 1 genes, respectively. Further out of 45 FadR-like regulators, 19 were classified as FadR group and 26 belong to VanR group. All these proteins showed similar secondary structural elements specific to their respective subfamilies except MSMEG_3959, which showed additional secondary structural elements. Using the reciprocal BLAST searches, the orthologs of these regulators in *Bacillus subtilis* and other mycobacteria were identified. Since the expression of many regulators is auto-regulatory, potential operator sites were identified for a number of these GntR regulators.

Details of progress in the current reporting year (April 1, 2007 – 31, March 2008):

We have characterized one of the seven GntR-like transcriptional regulators, Rv0586 (mce2R) that was classified as FadR-like transcriptional regulator.

Putative orthologs of Rv0586 in the genome of other sequenced mycobacteria and closely related non-mycobacterial species like *Nocardia farciana* were identified. Orthologs in most of the mycobacterial genomes were identified except *Mycobacterium leprae, Mycobacterium gilvum PYR-GCK* and *Mycobacterium ulcerans Agy99* (Table 1). The presence of this gene in genomes of multiple mycobacterial species highlights the importance of this regulator in mycobacterial physiology.

Multiple sequence alignment of Mce2R protein sequences showed a conserved N-terminal DNA binding domain compared to the C-terminal ligand-binding domain across all the *mce2R* genes (Data not shown).

All upstream *mce2R* regions were aligned to find conservation in their operator sites in case they are recognized by a similar DNA binding domain. This operator site conservation was revealed from alignment of all the upstream DNA sequences from the translational start site (Figure 1A, Table 1). Amongst all the identified operator sites, DNA motifs for Rv0586 and Mb0601; Mkms_2771 and Mjls_2751 were found to be identical in sequence (Table 1). The sequence comparison was quite convincing as we expected to find conservation in the operator sites. A consensus sequence logo was produced, using web logo, to show the relative frequency of each base at each position of the motif (Figure 1B)**.** The figure shows that the positions 4 to 9 in the consensus are nearly an inverted palindrome of positions 13 to 18 (Figure 1B).

Table 1: List of identified operator sites of putative orthologs of Rv0586.

ORF	Organism	Potential operator site
Rv0586	*M. tuberculosis*	GGTGTCGGTCTGACCACTTGA
Mb0601	*M. bovis*	GGTGTCGGTCTGACCACTTGA
MAP4081	*M. avium*	GCCGGTGGTCTGACCACCTGA
Mkms_2771	*M. KMS*	GCTAACTGGTCAGACCACTTGAC
Mjls_2757	*M. JLS*	GCTAACTGGTCAGACCACTTGAC
MSMEG_3527	*M. smegmatis*	ACCACTGGTAAGACCACTTGA
Mvan_2942	*M. vanbaalenii PYR*	CACACTGGTCTGACCACTTGA
Nfa1630	*N. farciana*	ACGATTGGTCTTACCACTTGA

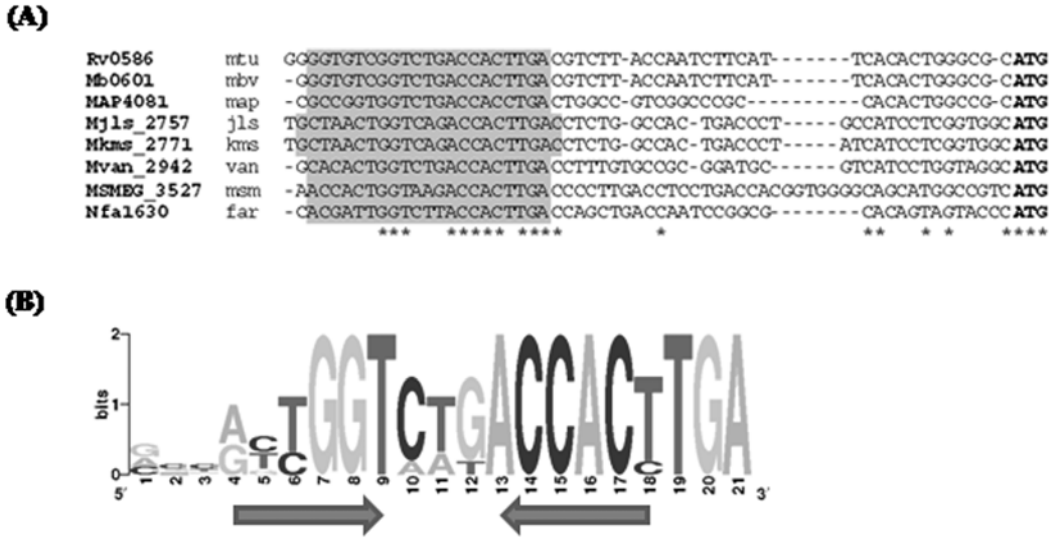

Figure 1. Diagram showing identified operator sites in the upstream sequences of the putative *mce2R* gene in relation to the translations start sites. A. Upstream sequence alignment of the putative *mce2R* ORF showing the position of identified DNA operator site in light gray background. Translational start sites in all the sequence are printed in bold. **B.** A consensus logo was drawn using identified operator (21bp). (Abbreviations: mtu - *M. tuberculosis*; mbv - *M. bovis*; map – *M. avium subsp. paratuberculosis*; van - *M. vanbaalenii PYR*; msm – *M. smegmatis;* kms – *M. sp* KMS; jls – *M. JLS;* far – *N. farciana*)

Mce2R binds to the identified operator site across the mycobacteria

To examine binding of Rv0586 to the predicted operator site, the protein was expressed as His-tagged recombinant protein in *E. coli* (Figure 2A). The protein was subjected to electrophoretic mobility shift assays (EMSA), to validate the identified operator site in the upstream region of ORF Rv0586. Purified protein showed clear binding with increasing concentration to the synthesized double stranded DNA motif (Table 1). The binding was abolished with increasing concentration of unlabeled DNA as a specific competitor (10x, 25x and 50x molar excess),

whereas similar excess of non-specific competitor DNA did not affect the DNA protein complex (Figure 2B). The non-specific DNA was also chosen from the upstream region of the ORF Rv0586 (CAACTTAGCCCGATAACTGCG).

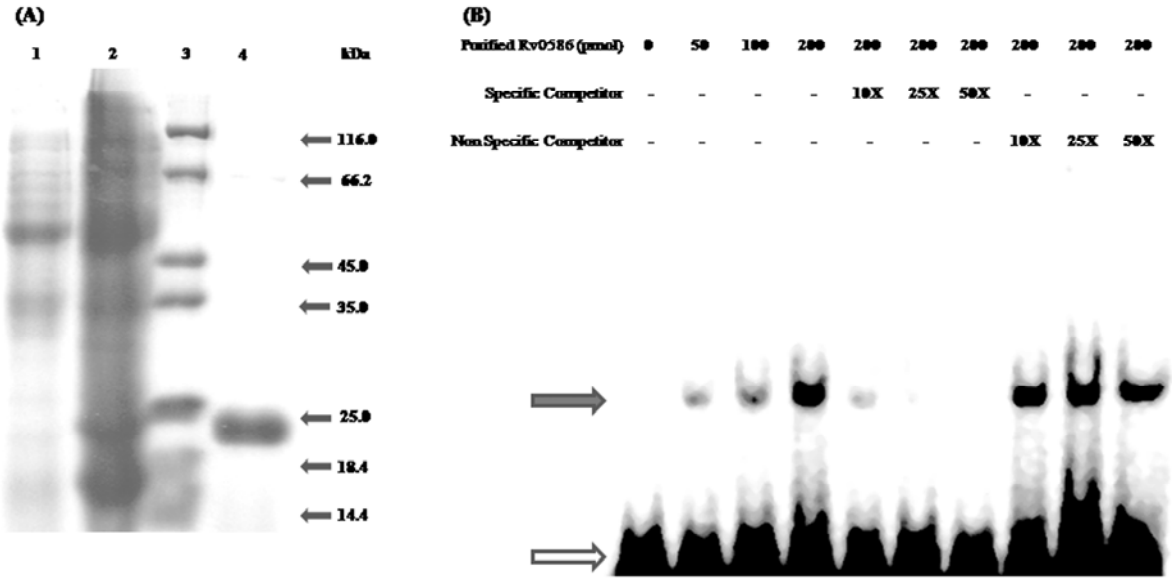

Figure 2. Binding of the Rv0586 protein to the operator DNA from the upstream region of Rv0586 (or Mb0601).

A. Expression and purification of Rv0586. Lane 1, IPTG induced *E. coli* M15 cell lysate harboring pQE30 as a control; lane 2, IPTG induced *E. coli* M15 cell lysate harboring recombinant pQE30 vector cloned with ORF Rv0586 ; lane 3, protein marker; lane 4, Rv0586 purified protein. All samples were loaded on 12% SDS-PAGE followed by Coomassie blue staining. **B.** Lane 1, labeled fragment; lanes 2 to 4, labeled fragment with 50 pmol, 100 pmol, 200 pmol of purified His_6-Rv0586 protein; Lanes 5 to 7 contain an increasing amount of cold specific dsDNA oligonucleotide competitor (10-, 25- and 50-fold molar excess); Lanes 8 to 10 contain an increasing amount of cold nonspecific competitor (10-, 25- and 50- folds molar excess). The positions of DNA protein complex and free probe are shown with solid and open arrows respectively.

Additionally, Rv0586 protein was analyzed for DNA interaction with the predicted operator sites in the upstream region of the orthologs (Table 1). Using EMSA, DNA binding was observed to these predicted DNA motifs in presence of increasing Rv0586 protein concentration. Binding was shown to be specific because the complex was not abolished by non-specific competitor DNA while specific unlabeled DNA successfully abolished the DNA-protein complex at 50-fold excess (Data not shown). The observations suggested that Rv0586 binds specifically to its upstream region to a conserved motif across the related species.

Project 2: Improved Genome Annotation of P. falciparum

Objectives

P. falciparum is a highly AT- rich genome that offers great challenges to identify and annotate the encoded genome. The objectives of this project are to:

1. Develop a computational approach, such as identification of novel *Plasmodium* specific amino acid substitution pattern, that can overcome this problem
2. Characterize functional proteins such as Acyl co A binding protein encoded by the genome.

Summary of the work done until the beginning of this reporting year:

We had proposed to formulate an exclusive substitution matrix for *Plasmodium falciparum* that reflects substitutions from a set of completely annotated proteins of *Plasmodium falciparum* and its distant orthologs. The matrix was calculated from protein blocks obtained from our dataset based on the formalism of Henikoff. We anticipated that using the substitution matrix thus generated might increase the threshold of alignment scores for database searches with *Plasmodium falciparum* leading to better annotation of the *Plasmodium falciparum* hypothetical proteins.

Details of progress in the current reporting year (April 1, 2007 – 31, March 2008):

Nucleotide bias can lead to an overall bias in the amino acid composition of a protein (Fig. 3). We examined the amino acid substitution in *P. falciparum* and found significant differences compared to other genomes (data not shown). Since the use of standard matrices for sequence analysis of biased organisms would not be appropriate, we constructed new substitution matrices in the context of this genome. Three different series of matrices; symmetric, non-symmetric and a specialized non-symmetric matrix were generated at different clustering percentages of the protein blocks (that was used to build the matrices). These blocks were generated from the annotated proteins of *P. falciparum* and its distant orthologs, with a view that the substitutions

captured in these known proteins would be helpful in bridging the differences in the target and background frequencies, usually a matter of concern with standard matrices.

We tested the performance of our matrix series and found significant differences. The symmetric matrix (Smat80) performed the best for database searches e.g. Table 2, and the specialized non-symmetric matrix (PfFSmat60) showed the best performance for pair-wise alignments e.g. Table3. In all the cases tested, the performance of the new matrices was better than the standard matrices.

Further, protein sequence alignments were generated for *P. falciparum* proteins with a prior evidence of their functional role with orthologs using the specialized non-symmetric matrix. We observed an increase in the alignment score & length spanning important motifs of known proteins (Fig. 4c). However, the standard matrices failed to produce meaningful alignments for these instances (Fig. 4a, 4b). The results have important implications on annotation of *P. falciparum* proteins that are of experimental interest but give poor sequence alignments with standard conventional matrices.

The high alignment score with our matrix compared to BLOSUM raised several important issues that we could reason. Usually, optimized parameters and low gap opening & extension penalties would give high scores. However, we have used moderate gap opening and extension penalties (12, 2). We had tested alignments at various parameters, and observed that even at the best-optimized parameter for BLOSUM; the alignment score with our matrix was higher. Secondly, alignment score would increase linearly for a positive matrix where all the matrix values are greater than zero. Our scaling for PfFSmat60 was however stringent; the matrix values were scaled only to the range of BLOSUM values and it was not a positive matrix. The only plausible reason was the dataset of annotated orthologs used for matrix generation. The background

frequencies calculated in this manner were probably more consistent with the target frequencies thus solving the problem of twilight regions. Investigating into our results, we thus conclude that our present approach of substitution matrix calculation to tackle a genome bias may supersede the general method of matrix calculation for an organism specific search.

It was further interesting to apply this approach genome wide to identify novel proteins of pharmacological interest. Presently, we are doing whole genome analysis of *P. falciparum* and our initial results are promising. We are also trying to look at some of the missing metabolic enzymes of this parasite.

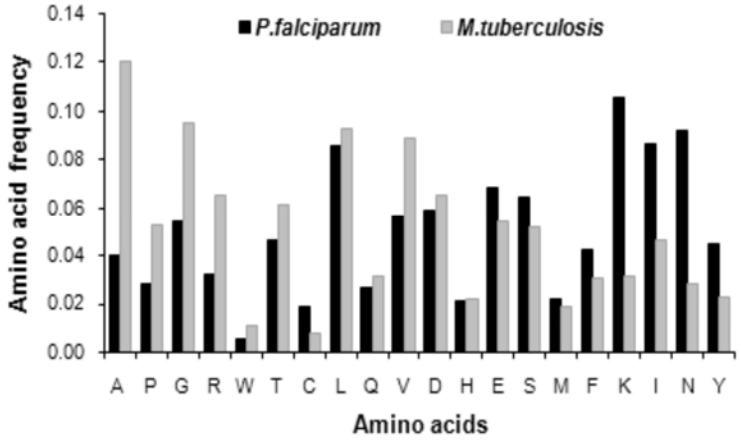

Figure. 3 - Amino acid compositional bias in the ortholog proteins of two diverse genomes

Table 2. SSEARCH scores for the *P. falciparum* cyclin and the yeast cyclin (first hit)

Clustering %	NSmat	NSSmat	Smat	SSmat	PfFmat	PfFSmat
50	105.1 (124)	37.2 (190)	116.4 (124)	74.4 (128)	106.3 (124)	37.0‡, 35.6
60	104.9 (124)	40.9* (186)	116.0 (124)	75.2 (128)	107.7 (124)	36.7‡, 35.3
70	105.6* (124)	36.9 (190)	116.2 (124)	75.3 (128)	108.2* (124)	37.7‡, 36.1
80	105.1 (124)	37.7 (190)	117.8*† (124)	79.6 (128)	103.2 (122)	37.9 (190)
90	102.8 (124)	39.3 (185)	114.2 (124)	90.1* (128)	98.9 (124)	39.4* (190)

The values represented with symbol '‡' are the cyclin family first hits obtained from a different organism and the values following it are the yeast cyclin hits which take up a second position. Blosum score: 113.3 bits

Table 3. FASTA alignment scores for the *P. falciparum* Cyclin and *yeast* Cyclin protein

Clustering %	NSmat	NSSmat	Smat	SSmat	PfFmat	PfFSmat
50	101.7 (124)	234.5 (190)	101.1 (124)	136.8 (128)	98.7 (124)	238.0 (190)
60	102.8* (124)	211.1 (186)	98.7 (124)	135.0 (128)	100.5* (124)	239.8* † (190)
70	102.3 (124)	238.0* (190)	98.7 (124)	135.0 (128)	100.5* (124)	238.0 (190)
80	102.8* (124)	233.3 (190)	101.7* (124)	137.9* (128)	97.0 (122)	226.3 (190)
90	101.1 (124)	225.1 (185)	99.9 (124)	136.2 (128)	97.6 (124)	221.6 (190)

Blosum score: 119.8 bits

Footnote for Table2 and Table3: *NSmat = Non Symmetric matrix; NSSmat = Non-Symmetric Scaled matrix; Smat = Symmetric matrix; SSmat = Symmetric Scaled matrix; PfFmat = Plasmodium falciparum Fixed matrix; PfFSmat = Plasmodium falciparum Fixed Scaled matrix. The term 'scaled' signifies the scaling achieved by the addition of a positive constant to all the matrix values. The first column of the table represents the clustering % at which the matrices (represented in the subsequent columns along the first row) were calculated. Columns 2-7 represent the alignment scores obtained for each matrix series at different clustering percentages. The values in the closed brackets are the amino acid overlap for the alignment.*

* The highest obtained scores for the respective columns;

† The score for the best performing matrix series;

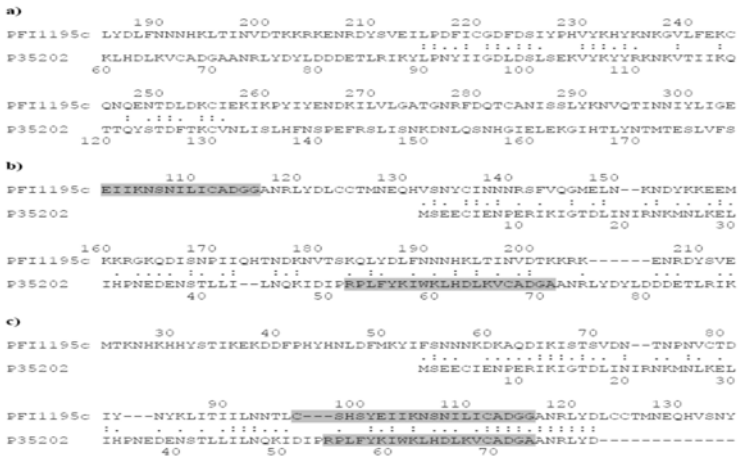

Figure 4: Alignment of the Thiamine pyrophosphokinase protein of yeast and a probable ortholog of *P.falciparum.*The shaded regions are the pyrophosphokinase motifs.

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2. Vindal V, Suma K and Ranjan A (2007) GntR family of regulators in *Mycobacterium smegmatis*: a sequence and structure based characterization. ***BMC Genomics*** 8: 289

3. Saxena V, Garg S, Ranjan S, Kochar D, Ranjan A and Das A (2007) Analysis of elongation factor Tu (tuf A) of apicoplast from Indian *Plasmodium vivax* isolates. ***Infection, Genetics and Evolution*** 7:618-626

4. The NMITLI-BioSuite Team (…, Hasnain SE, Mande S, Nagarajaram, H A. Ranjan A, …) (2007) BioSuite: A comprehensive bioinformatics software package (A unique industry academia collaboration) ***Current Science*** 92: 29-38

5. Akhter Y, Yellaboina S, Farhana A, Ranjan A, Ahmed N, Hasnain SE (2008) Genome scale portrait of cAMP-receptor protein (CRP) regulons in mycobacteria points to their role in pathogenesis. ***Gene*** 407:148-158

6. Hussain MA, Naveed SA, Sechi LA, Ranjan S, Alvi A, Ahmed I, Ranjan A, Mukhopadhyay S and Ahmed N (2008) Isocitrate dehydrogenase of *Helicobacter pylori* potentially induces humoral immune response in subjects with peptic ulcer disease and gastritis. ***PLoS ONE*** 3:e1481

7. Vindal V, Ashwantha Kumar E and Ranjan A (2008) Identification of operator sites within the upstream region of the putative mce2R gene from mycobacteria. ***FEBS Letters*** (In press)

8. Rajesh V, Singamsetti VK, Vidya S, Gowrishankar M, Elamaran M, Tripathi J, Radhika NB, Kochar D, Ranjan A, Roy SK and Das A. (2008) *Plasmodium falciparum*: genetic polymorphism in apical membrane antigen-1 gene from Indian isolates***. Experimental Parasitology*** (In press)

Laboratory of Transcription

Mechanism of transcription termination and antitermination in *Escherichia coli*.

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	Rajesh Sashni	JRF
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	V Jisha	Project Assistant
	Nanci R Kolli	Project Assistant
	Gowresh	Lab Attendant

Objectives

Transcription must terminate at the end of each operon; In E.coli, ends of 50% of the operons consist of intrinsic termination signal that codes for a hairpin followed by a U-rich stretch in mRNA. While, the rest of the operons does not have any signature sequence and it is possible that termination of these operons depends on a factor called Rho. On the other hand, the termination signals can be overcome in response to certain types of modifications in the elongation complex and the process is termed as antitermination. Mechanisms of the termination and antitermination processes is still not very clear and thus offers an exciting subject for study. In our laboratory, studies are underway to understand the following.

1) Mechanism of action of transcription termination factor, Rho.

2) Molecular basis of Rho-NusG interaction.

3) Mechanism of transcription antitermination by N protein at Rho-dependent terminators.

4) Mechanism of action of transcription antititermination of Rho-dependent termination by an anti-rho factor Psu.

Summary of the work done until the beginning of this reporting year

1) Using several protein footprinting techniques we had identified the region of transcription elongation complex (EC) which comes close to the C-terminal domain of the antiterminators protein N. Further, we had also assayed the elongation behavior of N-modified EC at different pause sequences, which are either RNA hairpin dependent or RNA: DNA hybrid dependent (JBC, 2007).

2) Termination defective mutants in Rho were isolated by random mutagenesis. The mutants were mapped on to the structure and further functional and conformational properties were characterized and correlated the involvement of different regions in the process of termination (JMB 2007).

Details of the progress in the current reporting year (April 1, 2007 - March 31, 2008)

Transcription termination factor Rho prefers catalytically active elongation complexes for releasing RNA.RNA polymerase (RNAP) pauses at different sequences during transcription elongation and the pausing is associated with distinct conformational state(s) of the elongation complex (EC). Transcription termination by the termination factor Rho, an RNA-dependent molecular motor, requires pausing of the EC in the termination zone of Rho-dependent terminators. We hypothesized that the conformational state(s) of the EC associated with this pausing would influence the action of Rho. Analyses of the pausing behavior of the EC at the termination points of two well-known Rho-dependent terminators revealed that Rho prefers actively transcribing complexes for termination.

RNA release kinetics from stalled ECs showed that the rate of RNA release by Rho was reduced if the EC was irreversibly backtracked, either by modification of RNA exit channel by an RNA

hairpin or when the bridge helix/trigger loop movement in its active site was perturbed. These defects were overcome significantly by enhancing the rate of ATP hydrolysis either by increasing the concentration of ATP or by using a Rho mutant with higher ATPase activity. Thus, we propose that the force generated from ATP hydrolysis of Rho is the key factor in dislodging the EC through its molecular motor action, and this process is facilitated when the EC is in a catalytically competent state, undergoing rapid "Brownian ratchet" motion (Figure 1).

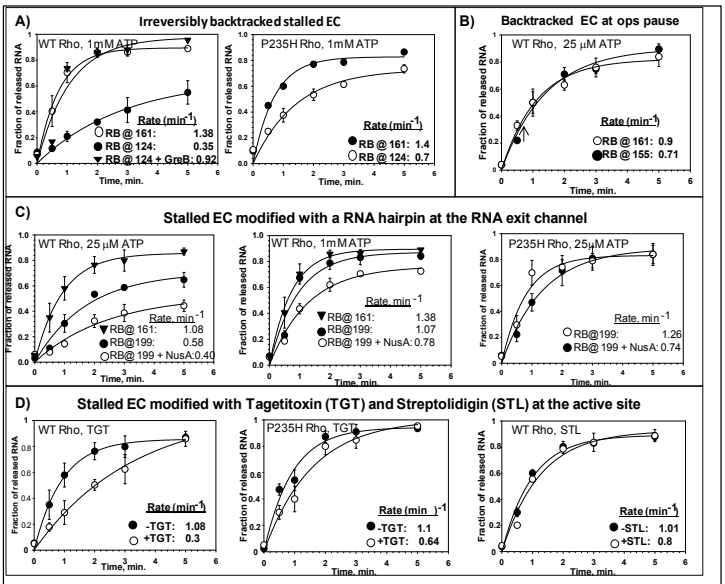

Figure 1. RNA release by transcription termination factor Rho (WT and P235H mutant) from different stalled elongation complexes under indicated conditions.

1. ***Molecular basis of interaction of Rho with its inhibitor Psu.***Bacteriophage P4 derived Psu, a 21kD protein, is a specific inhibitor of Rho-dependent transcription termination in *E.coli.* We have earlier shown that Psu interacts specifically with

2. Rho and slows down its translocation speed along the nascent RNA and thereby makes the later less efficient to exert its action on the elongating RNA polymerase (JBC, 2006). In order to understand the molecular basis of Rho-Psu interaction,

Table 1: *In vivo* activities of WT and Psu mutants:

Psu **alleles**	**β-galactosidase activity (arbitrary units)** $+T_{R1}$	$-T_{R1}$	**%RT** $(+T_{R1}/-T_{R1})$
WT	1020.5 ± 108.5	3046 ± 130	33.5
L21P	456 ± 8	3231 ± 25	14
E56K	197.5 ± 4	3022 ± 77	6.5
S72L	280 ± 9	3161 ± 57	9
P157S	181 ± 9	3017 ± 30	6
P157L	174 ± 5	3250 ± 65	5
R166C	197.5 ± 13	3085 ± 94	6
R166P	219 ± 7	3282 ± 101	7
Δ(95–98)	218 ± 9	3139 ± 79	7
vector	199 ± 9	2878.5 ± 245	7

Average of three to six independent measurements were done either on P_{lac}-H 19B T_{R1}-LacZAY or P_{lac}-LacZAY constructs.

Several Psu-mutants defective in inhibiting the Rho action were generated and characterized. All the mutants exhibited severe *in vivo* defect to prevent Rho-dependent termination and did not show significant association with Rho. S72L Psu, although did not show any *in vivo* activity, was found to be associated with Rho and also exhibited weak anti-rho activity in vitro (Figure 2 and Table 1). Clustering of mutations both in N and C-terminal ends and the intra subunit crosslinking between these two regions suggest that they may come close in the tertiary structure and the location of the mutations may define the interacting region of Psu with Rho. Further characterization of the complimentary interacting surface on Rho is in progress.

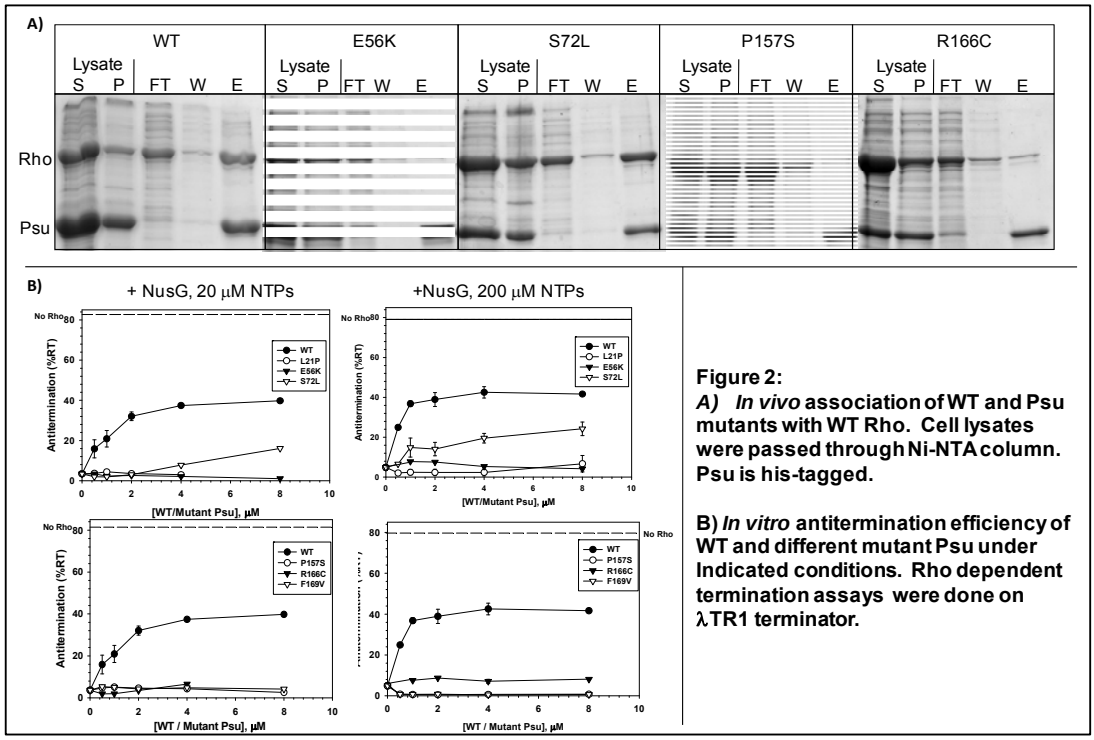

Figure 2:
***A) In vivo* association of WT and Psu mutants with WT Rho. Cell lysates were passed through Ni-NTA column. Psu is his-tagged.**

B) *In vitro* antitermination efficiency of WT and different mutant Psu under Indicated conditions. Rho dependent termination assays were done on λTR1 terminator.

Publications

1. Chalissery J, Banerjee S, Bandey I and Sen R (2007) Transcription termination defective mutants of Rho: role of different functions of Rho in releasing RNA from the elongation complex. ***Journal of Molecular Biology*** 371: 855–872

2. Cheeran A, Kolli N and Sen R (2007) The Site of Action of the Antiterminator Protein N from the Lambdoid Phage H-19B. ***Journal of Biological Chemistry*** 282: 30997-31007

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Laboratory of Cell Signalling

Understanding the role of inositol purophosphates in eukaryotic physiology and metabolism

Principal Investigator	:	Rashna Bhandari	Staff Scientist
Other Members	:	P. Srilakshmi	Post-doctoral Fellow
Collaborators	:	Satish Kumar, CCMB, Hyderabad	
		Rajan Dighe, IISc, Bangalore	

Objective

To understand the role of protein pyrophosphorylation by inositol pyrophosphates in regulating cell metabolism and animal physiology.

Inositol pyrophosphates, exemplified by diphosphoinositol pentakisphosphate (PP-IP_5, $InsP_7$, or IP_7) and bis-diphosphoinositol tetrakisphosphate ($[PP]_2$-IP_4, $InsP_8$, or IP_8), are a class of inositol phosphates that contain pyrophosphate or diphosphate moieties (Fig. 1). They are implicated in diverse biological functions, including growth, vesicular trafficking, apoptosis, telomere length maintenance and insulin secretion. The beta phosphate group of inositol pyrophosphates can be transferred to pre-phosphorylated serine residues on proteins to form pyrophosphoserine (Fig. 2; Science, 2004, 306: 2101-5; Proc. Natl. Acad. Sci., 2007, 104: 15305-10). This novel modification, *pyrophosphorylation*, occurs on several proteins within the cell, including proteins involved in ribosome biogenesis and vesicular trafficking.

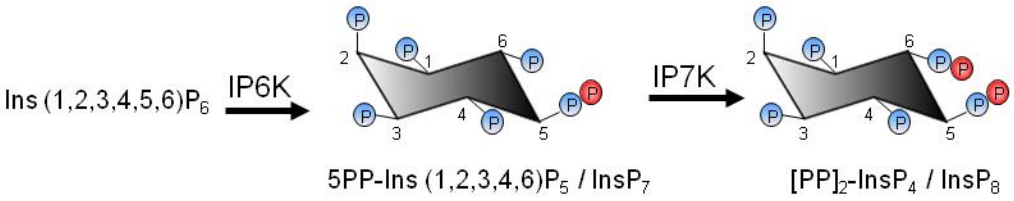

Figure 1. Schematic representation of inositol pyrophosphates. The enzymes involved in the pathway are indicated alongside the arrows. The structures of the inositol pyrophosphates are depicted with the C atoms numbered to indicate the positions of phosphate groups around the inositol ring.

5PP-IP_5 is synthesised from inositol hexakisphosphate (IP_6) and ATP by IP_6 kinases, three isoforms of which are present in mammals (IP6K1, IP6K2 and IP6K3). The recently identified

IP_7 kinases convert 5PP-IP_5 to IP_8. *Saccharomyces cerevisiae* strains lacking inositol pyrophosphates display defects in growth, telomere length maintenance, vesicular trafficking, stress response, and phosphate homeostasis. We have recently characterised IP6K1 knockout mice, and shown that they have low body weight compared with wild type mice, display low insulin levels and defective spermatogenesis (Proc. Natl. Acad. Sci. USA, 2008, 105: 2349-53). Mouse embryonic fibroblasts (MEFs) derived from IP6K1 knockout mice display a 60-70% decrease in the levels of IP_7 compared with wild type MEFs. It is now believed that inositol pyrophosphates mediate diverse biological functions either by specific binding to proteins, or *via* protein pyrophosphorylation.

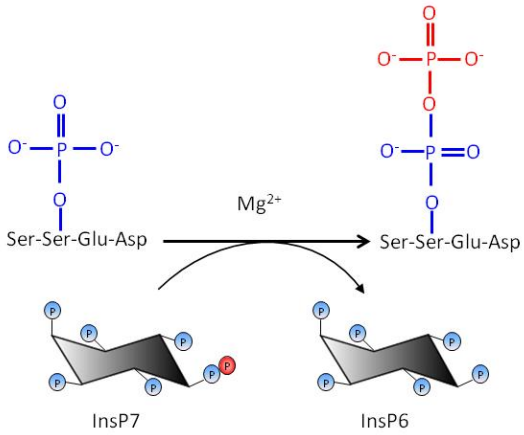

Figure 2. Protein pyrophosphorylation. Inositol pyrophosphates such as IP_7 transfer their beta phosphate group to a pre-phosphorylated serine residue to generate pyrophosphoserine. Pyro-phosphorylation occurs in the presence of divalent magnesium ions, and only on serine residues flanked by acidic amino acids.

Our aim is to understand the biochemical link between protein pyrophosphorylation and cellular phenomena regulated by inositol pyrophosphates. We have begun by utilising *S. cerevisiae* and mammalian cell lines as model systems to investigate the signalling pathways that are altered when inositol pyrophosphate levels are perturbed.

Project 1. Regulation of cell growth and proliferation by inositol pyrophosphates

This is a new activity

Knockout experiments reveal that inositol pyrophosphates regulate cell proliferation in yeast and body size in mammals. The 'slow growth phenotype' of IP_6 kinase knockout (*kcs1*Δ) *S. cerevisiae* and the smaller size of IP6K1 knockout mice could be attributed to decreased pyrophosphorylation of key proteins. IP_7 substrate proteins identified to date include NSR1 and

SRP40 and their mammalian homologues nucleolin and Nopp140, which are involved in rRNA processing and ribosome biogenesis. We will use proteomics-based screening strategies to identify additional IP_7 substrates that may be involved in regulating cell growth. To study the biochemical link between protein pyrophosphorylation and the regulation of cell growth and proliferation by inositol pyrophosphates, we will utilise two model systems, (a) *S. cerevisiae* (strain BY4741) wild type *vs kcs1*Δ, which lacks inositol pyrophosphates, and (b) Mouse embryonic fibroblasts (MEFs) derived from wild type and IP6K1 knockout mice. We will compare cell proliferation, cell cycle progression and protein synthesis in wild type *vs* knockout cells. Site directed mutagenesis strategies will be utilised to abolish pyrophosphorylation of specific serine residues in particular proteins, and the effects of these mutations on protein localisation and function will be examined.

Project 2. Phenotypic and molecular changes in IP6K1 knockout mice: A Functional Genomics Perspective.

This is a new activity.

IP6K1 knockout mice have 20% lower body weight than their wild type litter mates. They display a dramatic decrease in the levels of circulating insulin, yet have no signs of diabetes or glucose intolerance. Instead, they show an increase in insulin tolerance, i.e. they display faster insulin-stimulated uptake of glucose by peripheral tissues compared with wild type mice.

To understand the molecular basis for this and other physiological alterations in the IP6K1 knockout mice, we will perform a functional genomics analysis of these mice. To begin with, we will establish an IP6K1 knockout mouse colony from two founder pairs of heterozygous ($IP6K1^{+/}$) mice, in collaboration with Dr. Satish Kumar at the Centre for Cellular and Molecular Biology, Hyderabad. The tissue distribution of IP_6 kinase isoforms will be examined, to select those tissues where IP6K1 is expressed at high levels. Selected tissues, including brain, pancreas, liver and testes, harvested from age-matched wild type ($IP6K1^{+/+}$) and knockout ($IP6K1^{-/-}$) littermates will be utilised for gene expression profiling using DNA microarrays. Tissues as well as primary cell lines derived from wild type and knockout mice will be used to examine changes

in protein expression and phosphorylation by standard proteomic methods. The results of these analyses will provide clues regarding the signalling or metabolic pathways that are altered in the IP6K1 knockout mice, and perhaps provide the first biochemical links between protein pyrophosphorylation and physiological outcomes.

Project 3. Regulation of spermatogenesis by inositol pyrophosphates

This is a new activity

IP6K1 knockout male mice are sterile, and histological examination of the testes and epididymis reveal the complete absence of mature spermatozoa. The defect appears to lie in the final stage of sperm maturation, spermiogenesis, when the haploid round spermatid loses the majority of its cytoplasm, and matures to form an elongated spermatozoon with a tail. In collaboration with Dr. Rajan Dighe at the Indian Institute of Science, Bangalore, we will examine the differences in the gene expression profile of cells at different stages of spermatogenesis, in wild type and knockout mice. This may give us clues regarding the mechanisms by which inositol pyrophosphates regulate spermatogenesis. Furthermore, a screen for chemical inhibitors of IP_6 kinase activity, or antibodies directed specifically against IP6K1 may provide leads for a male contraceptive, although potential side effects such as lowering of insulin secretion will need to be addressed.

Publications

1. *Draskovic P, Saiardi A, Bhandari R, Burton A, Ilc G, Kovacevic M, Snyder SH and Podobnik M (2008) Inositol hexakisphosphate kinase products contain diphosphate and triphosphate groups. **Chemistry and Biology** 15: 274-286

2. *Bhandari R, Juluri KR, Resnick AC, Snyder SH (2008) Gene deletion of inositol hexakisphosphate kinase 1 reveals inositol pyrophosphate regulation of insulin secretion, growth, and spermiogenesis. **Proceedings of the National Academy of Sciences of the USA** 105: 2349-2353

*Work done elsewhere

National Genomics and Transcriptomics Facility

Principal Investigator	Murali Dharan Bashyam	Staff Scientist
Staff	Ajay Chaudhari	Technical assistant
	C K Reddy	Project assistant

Objectives:

To provide services to scientists both within and outside CDFD in the areas of DNA Sequencing, Genotyping, Real Time PCR and Microarrays.

Summary of the work done until the beginning of this reporting year

During the previous reporting year, sequence data amounting to a total of 5.7 million nucleotides was generated at NGTF. In addition, 13,200 genotyping reactions and 105 real time PCR reactions were also performed. Whole genome microarray services were provided for aCGH and gene expression studies on pancreatic, colorectal and esophagous cancer.

Details of progress in the current reporting year (April 1, 2007- March 31, 2008)

NGTF continues to provide services to scientists both within and outside CDFD in the areas of DNA sequencing, genotyping, real time PCR and microarrays. A total of 7.4 million nucleotides were sequenced during the reporting period. In addition, a total of 8600 genotyping reactions were performed. We also performed 80 real time PCR reactions for several laboratories within CDFD. Microarrays using the Human Exonic Evidence Based oligonucleotides (HEEBO) were procured from the Stanford Functional Genomics and Transcriptomics Facility and array-based comparative genomic hybridization was carried out for esophagous and colorectal cancer samples.

Future plans/directions:

To provide services for mutation detection using the Wave System (dHPLC).

Sun Centre of Excellence in Medical Bioinformatics and Informatics Services

Section Head	**:**	M Kavita Rao	Staff Scientist
Other members	:	G Thanu	Technical Officer (till 2007)
		S Swaminathan	Technical Officer (till 2007)
		M N Pavan	Technical Assistant
		P Prashanthi	Junior Assistant
		A Imam	Project Assistant (till 2007)

Collaboration	:	European Molecular Biology Network (EMBnet)
		SUN Microsystems, Inc, California, USA
		C-DAC, Pune, India
		University of Hyderabad, Hyderabad

Objectives

The "Centre of Excellence in Medical Bioinformatics" was created with an MOU signed by the SUN Microsystems, a leading hardware manufacturer for high end scientific computing, with CDFD to setup one of its CoEs at the bioinformatics facility of CDFD, Hyderabad. The CoE has a major focus on the Medical Bioinformatics. This Centre is a joint venture between M/s Sun Microsystems, CDFD and the Government of Andhra Pradesh. The Sun CoE is equipped with all requisite facilities and infrastructure back-bone to facilitate carrying out high impact research and training in Bioinformatics.

The Bioinformatics services and activities of the section are overseen by an Advisory Committee with Dr S C Mande, Staff Scientist, CDFD as Chairman and includes Drs Akash Ranjan and Nagarajaram, both Staff Scientists, CDFD as Members.

The CoE continued to provide services to the Bioinformatics user groups at CDFD. The hardware environment offered at Sun CoE scalability from desktop to teraflop with binary compatibility across architectures offering mainframe class like availability and superior balanced performance. The servers support features like fault isolated Dynamic System Domains

(Dynamic Hard partitions), Dynamic reconfiguration, full Hardware redundancy and hot CPU upgrades. The infrastructure includes

1. Sun E20K with 1.2 GHz Ultra 36 Sparc IV architecture (dual threaded with chip multi-threading) CPUs and 128 GB memory.
2. SE 6320 Enterprise Storage with 10 TB expandable up to 20 TB
3. 2 L25 LVD LTO Tape Libraries each with 2 tap drives
4. Sun Fire V440 server with 4X1.28 GHz. Ultra Sparc IIIi
5. 2 Sun Fire V240 servers with 2X1.28 GHz. Ultra Sparc IIIi
6. Sun V20z Linux Grid Cluster consisting of 17 nodes each with 2X2.2 GHz. AMD Opteron processors and 4X512 MB memory
7. 25 Sun Ray Thin Clients
8. 10 Sun Blade 1500 3D Graphics Workstations

The specific objective of this centre is

1. To provide Bioinformatics platform and knowledge services to the scientific community.
4. To provide Informatics services to the institute.
5. To maintain the CDFD website, to provide web based services and e-mail services.
6. To maintain Compute Servers, PCs, Printers and other peripheral devices of the centre.
7. To maintain the internet connectivity of the institute and institute-wide Local Area Network.

Summary of the work done until the beginning of this year:

Last year Sun CoE participated in training MTech Bioinformatics students from University of Hyderabad and M.Sc. Bioinformatics students from Jamia Milia Islamia. Dr Akash Ranjan, has organized an Indo German Workshop at CUBIC, Cologne. He was also invited as Resource Faculty at Late summer school on Structural Genomics that was held at University of Wuerzburg, Germany.

Further the informatics services unit looked after E-mail, Internet, web at Nacharam and Gandipet campuses. Installation, administration and maintenance of servers which provides various services, databases and computational jobs were taken care of at both the campuses.

Details of progress in the current reporting year (April 1, 2007 – 31, March 2008):

SUN-Centre of Excellence and its activity in Bioinformatics knowledge sharing

One of the major mandates of Sun CoE is to enable scientists to pursue their interest in Bioinformatics Research. A large number of research groups have used the facility and/or the knowledge services to advance their research. This is reflected in rising number of publications that are having bioinformatics component in them.

In addition the Sun CoE has been actively involved in knowledge sharing in the field of Bioinformatics. It provided six months project training to M.Sc Bioinformatics students from Jamia Milia Islamia University, New Delhi. This was attended by 7 students of Jamia Milia Islamia University, New Delhi.

Sun CoE also participated in University of Hyderabad's M.Tech. Bioinformatics Program and provided lecture and practical training in the field of Bioinformatics to 32 students of M.Tech Bioinformatics program of University of Hyderabad.

The Sun CoE also hosted 4 M.Tech. Bioinformatics students for carrying out M.Tech. Bioinformatics research leading to MTech thesis.

Sun CoE Coordinator/Head, Dr. Akash Ranjan, was invited as resource faculty for a number of workshop and training programs. These include DST sponsored Bioinformatics workshop at Centre for Cellular and Molecular Biology (CCMB), Hyderabad; bioinformatics workshop at Institute of Life Science, Bhubaneswar; bioinformatics workshop at National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra; and DST sponsored workshop at Administrative Staff College of India, Bella Vista, Hyderabad.

Informatics Services

Informatics services such as E-mail, Internet, web were taken care of at Nacharam and Gandipet campuses. Installation, administration and maintenance of servers which provides various services, databases and computational jobs were taken care of at both the campuses.

Installation, administration and maintenance of PCs, Printers and Scanners were also done. High-end PCs (55 Nos.), HP workstation (31Nos.), laptops (2 Nos.) Scanner (1 Nos.) and printers (22 Nos.) were procured and installed.

Several copies of new software were also installed. These include 35 copies of Adobe Suite CS2, 70 copies of Symantec Anti-virus and 200 copies of MS Office 2007, and copies of AMBER software on linux Grid cluster at Gandipet. We have also upgraded some of the Sun systems from Solaris 9 to Solaris 10 operating system

Few of the existing AMC were also renewed. These include AMC for SGI Fuel machines and Symantec Anti spam for mail gateway system. We have also entered into an agreement for remote monitoring and managed services for Sun servers located at Gandipet.

As a part of web services we have reconfigured a JRF online application system and provided information through web as per RTI Act.

Instrumentation Facility

Facility In-Charge:	J Raghavendrachar	Staff Scientist
Other Members:	R N Mishra	Technical Officer I
	S Pavan Kumar	Technical Officer I
	M Laxman	Technical Assistant
	R M K Sathyanarayana	Technical Assistant
	N P Sharma	Junior Assistant-II

Objectives

To maintain, repair and service all the equipment in the laboratory and also to provide pre-installation requirements for new instruments and to coordinate with the manufacturers/their agents in Installation and warranty service of the new instruments. Further, to provide the reports on the newly arrived instruments and to follow up with the suppliers for short shipped items.

Summary of work done until the beginning of this reporting year:

We have performed over 76 new installations of various equipment, including Confocal Microscope, HPLC System, LPLC System, PCR Machines, Water Purification Systems, BOD Incubator, Nanodrop Spectrophotometers, Refrigerated Table Top Centrifuges, Shaking Water baths, Orbital Shaker, Tissue Homogenizer, -860C Freezer, -200C Freezers, Refrigerators, Micro centrifuges, Electrophoresis Apparatus etc.

A total of 440 work orders for repair & maintenance of various laboratory equipments were attended. We were also actively involved in reorganizing the Laboratory for additional space and also the newly leased space in the Ground Floor; got the Confocal Microscope Facility functional; and created the NGTF DNA Sequencing facility in the Ground floor. In addition, we were actively involved in getting the instruments installed at the APEDA facility on the Second Floor.

The instrumentation division has been entrusted with the additional responsibility of complete maintenance of the CDFD Nacharam Campus, including Electrical, Refrigeration, EPABX &

Telephones, UPS Systems, House Keeping, and the same was successfully monitored in addition to procuring the Laboratory tables & erecting the partitions for various group leaders & facilities.

Details of progress made in the current reporting year (April 1, 2007 – March 31, 2008)

During the current year also, over 42 new installations were performed of various equipment, like dHPLC, MegaBace 500 DNA Sequencer of Amersham, Hybridization Oven, CO_2 Incubators, Gel Documentation Systems, PCR Machines, Water Purification System, Nanodrop Spectrophotometers, Refrigerated Table Top Centrifuges, Shaking Water baths, Orbital Shaker, Tissue Homogenizer, -86^{0}C Freezer, -20^{0}C Freezers, Refrigerators, Micro centrifuges, Electrophoresis Apparatus etc.

A total of 395 work orders for repair & maintenance of various laboratory equipments were completed. We were actively involved in reorganizing the Laboratory for additional space and also the newly leased space in the Ground Floor.

In addition, we were involved in organizing the audio & visual requirements for presentations in various seminars, workshops, Foundation day lectures and Distinguished Scientist Lectures held in CDFD both at Nacharam and Gandipet Auditorium. Most of the centers equipment is maintained by instrumentation division with maximum uptime in the Laboratory. The maintenance thus done by instrumentation staff, is saving on the expensive AMCs and done with very little downtime of the equipment.

Instrumentation division is also successfully taking care of complete maintenance of the CDFD Nacharam Campus, including Electrical, Refrigeration, EPABX & Telephones, UPS Systems, and House Keeping.

Research Papers Published (2007)

1. Ahmed N, Majeed AA, Ahmed I, Hussain MA, Alvi A, Devi SM, Rizwan M, Ranjan A, Sechi LA, and Megraud F (2007) genoBASE *pylori*: A genotype search tool and database of human gastric pathogen *Helicobacter pylori*. ***Infection Genetics and Evolution*** 7:463-468

2. Ahmed N, Saini V, Raghuvanshi S, Khurana JP, Tyagi AK, Tyagi AK and Hasnain SE (2007) Molecular analysis of a leprosy immunotherapeutic bacillus provides insights into Mycobacterium evolution. ***PLoS ONE*** 2:e968

3. Akhter Y, Tundup S and Hasnain S E (2007) Novel biochemical properties of a CRP/FNR family transcription factor from *Mycobacterium tuberculosis.* ***International Journal of Medical Microbiology*** 297: 451-457

4. Alvi A, Devi SM, Ahmed I, Hussain MA, Rizwan M, Lamouliatte H, Mégraud F and Ahmed N (2007) Microevolution of *Helicobacter pylori* type IV secretion systems in an ulcer disease patient over a ten-year period. ***Journal of Clinical Microbiology*** 45:4039-4043

5. Archak S and Nagaraju J (2007) Computational prediction of rice (*Oryza sativa*) miRNA targets. ***Genomics, Proteomics and Bioinformatics*** 5: 196-206

6. Archak S, Lakshminarayanareddy V and Nagaraju J (2007) High-throughput multiplex microsatellite marker assay for detection and quantification of adulteration in Basmati rice (*Oryza sativa*). ***Electrophoresis*** 28: 2396-2405

7. Banerjee S, Nandyala A K, Raviprasad P, Ahmed N and Hasnain S E (2007). Iron-dependent RNA-binding activity of *Mycobacterium tuberculosis* aconitase. ***Journal of Bacteriology*** 189: 4046-4052

8. Bashyam MD and Hasnain SE (2007) Array-based comparative Genomic Hybridization: applications in cancer and tuberculosis. In: **Bioarrays**, Edited by K Appasani, Humana press, pp 107-121

9. Bashyam MD, Savithri GR, Gopikrishna M and Narasimhan C (2007) A p.R870H mutation in the beta cardiac myosin heavy chain 7 gene causes familial hypertrophic cardiomyopathy in several members of an Indian family. ***Canadian Journal of Cardiology*** 23:788-790

10. Biju SD, Bocxlaer IV, Giri VB, Roelants K, Nagaraju J and Bossuyt F (2007) A new nightfrog, *Nyctibatrachus minimus* sp. nov. (Anura: Nyctibatrachidae): The smallest frog from India. ***Current Science*** 93: 854-858

11. Boddupalli CS, Ghosh S, Rahim SS, Nair S, Ehtesham NZ, Hasnain SE and Mukhopadhyay S (2007) Nitric oxide inhibits interleukin-12 p40 through p38 MAPK-mediated regulation of calmodulin and c-rel. ***Free Radical Biology and Medicine*** 42:686-697

12. Chalissery J, Banerjee S, Bandey I and Sen R (2007) Transcription termination defective mutants of Rho: role of different functions of Rho in releasing RNA from the elongation complex. ***Journal of Molecular Biology*** 371: 855–872

13. Cheeran A, Kolli N and Sen R (2007) The Site of Action of the Antiterminator Protein N from the Lambdoid Phage H-19B. ***Journal of Biological Chemistry*** 282: 30997-31007

14. Devi SM, Ahmed I, Francalacci P, Hussain MA, Akhter Y, Alvi A, Sechi LA, Mégraud F and Ahmed N (2007) Ancestral European roots of *Helicobacter pylori* in India. ***BMC Genomics*** 8:184

15. Duggirala A, Kenchappa P, Sharma S, Peeters J K, Ahmed N, Garg P, Das T and Hasnain, SE (2007) High-resolution genome profiling differentiated *Staphylococcus epidermidis* isolated from patients with ocular infections and normal individuals. ***Investigative Ophthalmology and Visual Science*** 48: 3239-3245

16. Gandhe AS, Janardhan G and Nagaraju J (2007) Immune upregulation of novel antibacterial proteins from silkmoths (Lepidoptera) that resemble lysozymes but lack muramidase activity. ***Insect Biochemistry and Molecular Biology*** 37: 655-666

17. Gandhe AS, John S H and Nagaraju J (2007) Noduler, a novel immune upregulated protein mediates nodulation response in insects. ***Journal of Immunology*** 179: 6943-6951

18. Gokul G, Gautami B, Malathi S, Sowjanya A, Poli U, Jain M, Ramakrishna G and Khosla S (2007) DNA methylation profile at the DNMT3L promoter: a potential biomarker for cervical cancer. ***Epigenetic***s 2: 80-85

19. Goyal K, Mohanty D and Mande SC (2007) PAR-3D: A server to predict protein active site residues. ***Nucleic Acids Research*** 35:W503-505

20. Kanginakudru S, Royer C, Edupalli SV, Jalabert A, Mauchamp B, Chandrashekaraiah, Prasad SV, Chavancy G , Couble P and Nagaraju J (2007) Targeting ie-1 gene by RNAi induces baculoviral resistance in lepidopteran cell lines and in the transgenic silkworms. ***Insect Molecular Biology*** 16: 635-644

21. ***Kaur R, Ma B and Cormack BP (2007) A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of ***Candida glabrata***. ***Proceedings of the National Academy of Sciences of the USA*** 104 : 7628-33
(***Work done elsewhere)

22. Khan N, Ghousunnissa S, Jegadeeswaran SM, Thiagarajan D, Hasnain SE and Mukhopadhyay S (2007) Anti-B7-1/B7-2 antibody elicits innate-effector responses in macrophages through NF-κB-dependent pathway. ***International Immunology*** 19:477-486

23. Kumari G, Singhal PK, Rao MR and Mahalingam S (2007) Nuclear Transport of Ras-associated Tumor Suppressor Proteins: Different Transport Receptor Binding Specificities for Arginine-rich Nuclear Targeting Signals. ***Journal of Molecular Biology*** 367: 1294-1311

24. Laishram R S and Gowrishankar J (2007) Environmental regulation operating at the promoter clearance step of bacterial transcription. ***Genes and Development*** 21:1258-1272

25. Lakshminarayanareddy V, Archak S and Nagaraju J (2007) Capillary electrophoresis is essential for microsatellite marker based detection and quantification of adulteration of Basmati rice (*Oryza sativa*). ***Journal of Agriculture and Food Chemistry*** 55: 8112-8117

26. Manna SK, Aggarwal RA, Sethi G, Aggarwal BB and Ramesh GT (2007) Morin (3,5,7,2',4'-pentahydroxyflavone) abolishes NF-kB activation induced by various carcinogens and inflammatory stimuli, leading to suppression of NF-kB-regulated gene expression and upregulation of apoptosis. ***Clinical Cancer Research*** 13: 2290-2297

27. Manna SK, Manna P and Sarkar A (2007) Inhibition of RelA phosphorylation sensitizes chemotherapeutic agents-mediated apoptosis in constitutive NF-kappaB-expressing and chemoresistant cells. ***Cell Death & Differentiation*** 14:158-170

28. Mukhopadhyay S, Nair S and Hasnain SE (2007) Nitric oxide: Friendly rivalry in tuberculosis. ***Current Signal Transduction Therapy*** 2:121-28

29. Nyamath P, Alvi A, Habeeb A, Khosla S, Khan AA and Habibullah CM (2007) Characterization of hepatic progenitors from human fetal liver using CD34 as a hepatic progenitor marker. ***World Journal of Gastroenterology*** 13:2319-2323

30. Radha Rama Devi A, Reddy C K, Ranjan S and Bashyam M D (2007) Molecular genetic analysis of patients from India with hypohidrotic ectodermal dysplasia reveals novel mutations in the EDA and EDAR genes. ***British Journal of Dermatology*** 158:163-167

31. Raghavendra PB, Sreenivasan Y and Manna SK (2007) Oleandrin induces apoptosis in human, but not in murine cells: dephosphorylation of Akt, expression of FasL, and alteration of membrane fluidity. ***Molecular Immunology*** 44:2292-2302.

32. Raghavendra PB, Sreenivasan Y, Ramesh GT and Manna SK (2007) Cardiac glycoside induces cell death via FasL by activating calcineurin and NF-AT, but apoptosis initially proceeds through activation of caspases. ***Apoptosis*** 12:307-318

33. Rasheedi S, Ghosh S, Suragani M, Tuteja N, Sopory S K, Hasnain S E and Ehtesham N Z (2007) *Pisum sativum* contains a factor with strong homology to eIF5B. ***Gene*** 399: 144-151

34. Rasheedi S, Ramachandran A, Ehtesham N Z and Hasnain S E (2007) Biochemical characterization of Sf9 Sp-family-like protein factors reveals interesting features. ***Archives of Virology*** 152 :1819-1828

35. Ravi Kumar A, Sathish V, Balakrish Nair G and Nagaraju J (2007) Genetic characterization of *Vibrio cholerae* strains by inter simple sequence repeat-PCR. ***FEMS Microbiology Letters*** 272: 251-258

36. Salih B, Abasiyanik F, Ahmed N (2007) A preliminary study on the genetic profile of cag pathogenicity island and other virulent gene loci of *Helicobacter pylori* strains from Turkey. ***Infection Genetics and Evolution*** 7:509-512

37. Saxena V, Garg S, Ranjan S, Kochar D, Ranjan A and Das A (2007) Analysis of elongation factor Tu (tuf A) of apicoplast from Indian *Plasmodium vivax* isolates. ***Infection, Genetics and Evolution*** 7:618-626

38. Sechi LA, Felis GE, Ahmed N, Paccagnini D, Usai D, Ortu S, Molicotti P, Zanetti S (2007) Genome and transcriptome scale portrait of sigma factors in *Mycobacterium avium* subsp. *paratuberculosis*. ***Infection Genetics and Evolution*** 7:424-432

39. Sechi LA, Ruhel A, Ahmed N, Usai D, Paccagnini D, Felis G and Zannetti S (2007) *Mycobacterium avium* subspecies *paratuberculosis* is able to infect and persist in enteric glial cells. ***World Journal of Gastroenterology*** 13: 5731-5735

40. Shamim MTA, Anwaruddin M and Nagarajaram H A (2007) Support Vector Machine-based classification of protein folds using the structural properties of amino acid residues and amino acid residue pairs. ***Bioinformatic***s 23: 3320-3327

41. Sreenu VB, Kumar P, Nagaraju J and Nagarajaram H A (2007) Simple sequence repeats in mycobacterial genomes ***Journal of Biosciences*** 32: 1-15

42. Suresh B M and Nagarajaram H A (2007) IMEx: Imperfect Microsatellite Extractor. ***Bioinformatics*** 23:1181-1187

43. The NMITLI-BioSuite Team (…… Hasnain SE, Mande S, Nagarajaram, H A, Ranjan A….) (2007) BioSuite: A comprehensive bioinformatics software package (A unique industry academia collaboration) ***Current Science*** 92: 29-38

44. Vindal V, Ranjan S, and Ranjan A. (2007) In silico analysis and characterization of GntR family of regulators from *Mycobacterium tuberculosis.* ***Tuberculosis*** 87:242-247

45. Vindal V, Suma K and Ranjan A (2007) GntR family of regulators in Mycobacterium smegmatis: a sequence and structure based characterization. ***BMC Genomics*** 8: 289

46. Yellaboina S, Goyal K and Mande SC (2007) Inferring genome-wide functional linkages in E. coli by combining improved genome context methods: comparison with high-throughput experimental data. ***Genome Research*** 17:527-535

47. Yusuf A, Smanla T and Hasnain SE (2007) Novel biochemical properties of a CRP/FNR family transcription factor from *Mycobacterium tuberculosis.* ***International Journal of Medical Microbiology*** 297:451-457

Research Papers Published 2008 (until 31 March)

1 Akhter Y, Yellaboina S, Farhana A, Ranjan A, Ahmed N and Hasnain SE (2008) Genome scale portrait of cAMP-receptor protein (CRP) regulons in mycobacteria points to their role in pathogenesis. ***Gene*** 407:148-158

2 Bannantine JP, Rosu V, Zanetti S, Rocca S, Ahmed N and Sechi LA (2008) Antigenic profiles of recombinant proteins from *Mycobacterium avium* subsp. *paratuberculosis* in sheep with Johne's disease. ***Veterinary Immunology and Immunopathology*** 122:116-125

3 Bashyam MD and Ratheesh R (2008) Genetic instability in colorectal cancer. ***The ICFAI Journal of Biotechnology*** 2:13-26

4 ***Bhandari R, Juluri KR, Resnick AC, Snyder SH (2008) Gene deletion of inositol hexakisphosphate kinase 1 reveals inositol pyrophosphate regulation of insulin secretion, growth, and spermiogenesis. ***Proceedings of the National Academy of Sciences of the USA*** 105: 2349-2353

5 ***Draskovic P, Saiardi A, Bhandari R, Burton A, Ilc G, Kovacevic M, Snyder SH and Podobnik M (2008) Inositol hexakisphosphate kinase products contain diphosphate and triphosphate groups. ***Chemistry and Biology*** 15: 274-286
(***Work done elsewhere)

6 Goyal K and Mande SC (2008) Exploiting 3D structural templates for detection of metal-binding sites in protein structures. ***Proteins: Structure, Function and Bioinformatics*** 70: 1206- 1218

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Research Papers – In Press (as on 31 March, 2008)

1. Ahmed N, Dobrindt U, Hacker J and Hasnain SE (2008) Genomic fluidity and pathogenic bacteria: applications in diagnostics, epidemiology and intervention. ***Nature Reviews Microbiology***.

2. Demuth A, Aharonowitz Y, Bachmann T T, Blum-Oehler G, Buchrieser C, Covacci A, Dobrindt U, Emody L. van der Ende A, Ewbank J, Fernandez L A, Frosch M, Portillo F G, Gilmore M S, Glaser P, Goebel W, Hasnain S E, Heesemann J, Islam K, Korhonen T, Maiden M, Meyer T F, Montecucco C, Oswald E, Parkhill J, Pucciarelli M G, Ron E, Svanborg C, Uhlin B E, Wai S N, Wehland J and Hacker J (2008) Pathogenomics: An updated European Research Agenda. ***Infection, Genetics and Evolution***.

3. Farhana A, Kumar S, Rathore S S, Ghosh P C, Ehtesham N Z, Tyagi A K and Hasnain S E (2008) Mechanistic insights into a novel exporter-importer system of *Mycobacterium tuberculosis* unravel its role in trafficking of iron. ***PLoS ONE***.

4. Khan N, Alam K, Mande S C, Valluri V L, Hasnain S E and Mukhopadhyay S (2008) *Mycobacterium tuberculosis* heat shock protein 60 modulates immune response to PPD by manipulating the surface expression of TLR2 on macrophages. ***Cellular Microbiology.***

5. Khan N, Alam K, Nair S, Valluri V L, Murthy K J R and Mukhopadhyay S (2008) Strong immune responses to the PPE protein Rv1168c are associated with active tuberculosis disease. ***Clinical and Vaccine Immunology***.

6. Prabhakara K, Damien L Bruno, Priya Padman, Suma Prasad, Sudheer Kumar R, Howard R Slater and Radha Ramadevi A (2008) Prenatal detection of deletion-duplication of chromosome 3 arising from meiotic recombination of familial pericentric inversion. ***Prenatal Diagnosis*** .

7. Rajesh V, Singamsetti VK, Vidya S, Gowrishankar M, Elamaran M, Tripathi J, Radhika NB, Kochar D, Ranjan A, Roy SK and Das A. (2008) *Plasmodium falciparum*: genetic polymorphism in apical membrane antigen-1 gene from Indian isolates. ***Experimental Parasitoogy.***

8. Rosu V, Ahmed N, Paccagnini D, Pacifico A, Zanetti S and Sechi LA (2008) *Mycobacterium avium* subspecies *paratuberculosis* is not associated with type-2 diabetes mellitus. ***Annals of Clinical Microbiology and Antimicrobials***.

9. Victoria B, Ahmed A, Zuerner R, Ahmed N, Bulach D, Quinteiro J, and Hartskeerl R (2008) S10-spc-alpha is a conserved locus within highly plastic Leptospira genomes. ***PLoS ONE***.

10. Vindal V, Ashwantha Kumar E and Ranjan A (2008) Identification of operator sites within the upstream region of the putative mce2R gene from mycobacteria. ***FEBS Letters***.

11. Valverde MD, Ramírez JM, Oca LG, Goris MG, Ahmed N and Hartskeerl RA (2008) Arenal, a new *Leptospira serovar* of serogroup Javanica, isolated from a patient in Costa Rica. ***Infection, Genetics and Evolution.***

HUMAN RESOURCE DEVELOPMENT

PhD Program

For our PhD program we invite applications from highly motivated candidates willing to take up challenges in modern biology usually in the month of march. Keeping in view the interdisciplinary nature of modern biology, we especially encourage persons from diverse backgrounds to take up challenges in these areas. Those admitted as JRFs are considered for the PhD program of Manipal Academy of Higher Education or University of Hyderabad.

The eligibility for the program is a Masters degree in any branch of Science, Technology, Medicine or Agriculture. Candidates must have cleared the CSIR/UGC/ICMR/ICAR NET for JRF. Those who have appeared, but are awaiting results, are also eligible to apply. Those with independent SRF from CSIR can also apply. As the number of applicants outnumbers the seats available each year by a ratio of 1:10 or more, eligible candidates are invited for a written examination followed by interviews of short-listed candidates.

At present we have 59 research scholars working for their doctorates in different areas of research. 19 of our PhD students have already finished their doctoral theses and are pursuing a career in science elsewhere in India or abroad.

Postdoctoral program

In addition to our JRF program, we also carry out training at the post-doctoral level. The post-doctoral fellows are funded through the extramural grants that CDFD receives. Some post-doctoral fellows are also selected competitively by the DST fast track young scientist scheme, or the DBT post-doctoral fellowship program.

Summer training program

Every year CDFD receives more than 800 applications for carrying out a 4-6 weeks summer training project. The applications are mostly from students pursuing their M. Sc. in various Universities. Some applications are also from B. Sc. Students. In the past couple of years, CDFD has restricted admission to this program only to those students who are supported by the Indian Academy of Sciences, Bangalore or the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore or the Kishore Vigyanic Protsahan Yojana, New Delhi. On an average every year 15-20 students receive their summer training at CDFD.

Training for students from BITS Pilani and from Jamia Millia Islamia, New Delhi

CDFD has formalized an agreement with BITS Pilani to provide project training to their M. Sc. Students. Under this programme, the students spend 6 months-1 year at CDFD and work on active projects that are being carried out there. The project work helps the students in hand-on experience in modern biology. Every year 2-3 students avail of this programme.

In 2005-06, CDFD also entered into an agreement with Jamia Millia Islamia, New Delhi according to which M. Sc. Students from the University are able to visit the

CDFD for 1-week or 12-week training programs in Bioinformatics and Computational Biology.

SENIOR STAFF AND OFFICERS OF CDFD

Scientific Staff

Dr J Gowrishankar, Director

Dr E A Siddiq, Adjunct Faculty

Prof T Ramasarma, Adjunct Faculty

Dr J Nagaraju, Staff Scientist

Dr Shekhar C Mande, Staff Scientist

Dr S Mahalingam, Adjunct Faculty

Dr A Radha Rama Devi, MD (Ped), Consultant

Dr Murali D Bashyam, Staff Scientist

Dr Sunil Kumar Manna, Staff Scientist

Dr H A Nagarajaram, Staff Scientist

Dr Akash Ranjan, Staff Scientist

Dr Sangita Mukhopadhyay, Staff Scientist

Dr Gayatri Ramakrishna, Staff Scientist

Dr G V Rao, Staff Scientist (under suspension)

Dr Sanjeev Khosla, Staff Scientist

Dr Ranjan Sen, Staff Scientist

Dr Rupinder Kaur, Staff Scientist

Dr Madhusudan R Nandineni, Staff Scientist

Dr Niyaz Ahmed, Staff Scientist

Dr Ashwin Dalal, Staff Scientist

Dr Abhijit A Sardesai, Staff Scientist

Dr Rashna Bhandari, Staff Scientist

Dr P Janila, Staff Scientist (Science Communication)

Administrative Staff

Mr K Ananda Rao, Staff Scientist (Engg.)

Mr Raghavendrachar J, Staff Scientist (Instrumentation)

Mr J Sanjeev Rao, Head - Administration

Mr A K Prakash, Head - Finance & Accounts

COMMITTEES OF THE INSTITUTE

MEMBERS OF SOCIETY

Shri Kapil Sibal Minister for S&T and of Earth Sciences	President
Prof M K Bhan Secretary, DBT, New Delhi	Member
Prof P Balaram Director, IISc, Bangalore	Member
Director General, CSIR, New Delhi	Member
Prof V S Chauhan Director, ICGEB, New Delhi	Member
Dr Siddhartha Roy Director, IICB	Member
Joint Secretary (PM), MHA, New Delhi	Member
Joint Secretary, Ministry of Law, New Delhi	Member
Joint Secretary & Financial Advisor, DBT, New Delhi	Member
Director General Bureau of Police Research Development	Member
Dr Alka Sharma Jt.Director, DBT, New Delhi	Member
Dr J Gowrishankar Director, CDFD	Member - Secretary

MEMBERS OF GOVERNING COUNCIL

Prof M K Bhan Secretary, DBT, New Delhi	Chairperson
Director General, CSIR, New Delhi	Member
Prof V S Chauhan Director, ICGEB, New Delhi	Member
Prof P Balaram Director, IISc, Bangalore	Member
Dr Siddhartha Roy Director, IICB, New Delhi	Member
Joint Secretary, Ministry of Law, New Delhi	Member
Joint Secretary (PM), MHA, New Delhi	Member
Joint Secretary & Financial Advisor, DBT, New Delhi	Member
Director General Bureau of Police Research & Development, New Delhi	Member
Dr Alka Sharma Jt.Director, DBT, New Delhi	Member
Dr J Gowrishankar, Director, CDFD	Member-Secretary

MEMBERS OF CDFD FINANCE COMMITTEE

Dr V S Chauhan Director, ICGEB, New Delhi	Chairman
Dr Siddhartha Roy Director, IICB, New Delhi	Member
Dr J Gowrishankar, Director, CDFD	Member
Joint Secretary & Financial Advisor, DBT, New Delhi	Member
Shri Virendra Kapoor Deputy Secretary, DBT, New Delhi	Member
Joint Secretary, MHA, New Delhi	Member
Shri A K Prakash Head Finance & Accounts, CDFD	Member Secretary

MEMBERS OF BUILDING COMMITTEE

1) Prof V S Chauhan - Chairman
   Director, ICGEB, New Delhi

2) Dr. J. Gowrishankar - Member
   Director, CDFD

3) Shri Samant - Member
   Joint Secretary, DBT, New Delhi

4) Shri Virendra Kapoor - Member
   Deputy Secretary, DBT, New Delhi

5) Shri B Bose - Member
   Management Consultant, NII, New Delhi

6) Shri J Sanjeev Rao - Member
   Head Administration, CDFD

7) Shri A K Prakash - Member
   Head Finance & Accounts, CDFD

8) Shri K Ananda Rao - Member & Convenor
   Staff Scientist (Engg), CDFD

LECTURES, MEETINGS, WORKSHOPS AND IMPORTANT EVENTS

Dr Soumen Basak, University of California, San Diego delivered a lecture on "Module to System: The NF-kB Signaling and Beyond" (23.05.2007)

Dr Krishna Murari Sinha, Memorial-Sloan Kettering Cancer Center, New York delivered a lecture on “DNA replication and repair in pathogenic parasite and bacteria” (12.06.2007)

Dr Sudha Rajagopalan, Indian Institute of Science, Bangalore delivered a lecture on “Applications of mass spectrometry for proteomics studies and structural analysis” (12.07.2007)

Dr Neeraj Dhar, Collaborator Scientifique, Global Health Institue, Switzerland delivered a lecture on “Persisters in *Mycobacteria* : Novel insights into a persisting problem using genetic & single-cell studies” (7.12.2007)

Dr Ramakrishna Vadrevu, University of Massachusetts Medical School, Worcester, USA delivered a lecture on "Structural Analysis of Partially-Folded States in a 29 kDa TIM Barrel Protein: Back to *BASiCs"* (06.08.2007)

Dr Dibyendu Bhattacharyya, University of Chicago, Chicago, USA delivered a lecture on “Deciphering ER Exit Sites” (22.11. 2007)

Dr Rammohan V. Rao, Buck Institute for Age Research, Novato, USA delivered a lecture on “Coping with cellular stress: ER to the rescue” (06.12.2007)

Dr Vivek Ramakrishnan, M/s Intellectual Ventures, USA delivered a lecture on "Intellectual Ventures: Idea focused Innovation" (18.12.2007)

Dr A A Jeyaprakash, MPI International Research Fellow, Department of Structural Cell Biology, Max-planck Institute of Biochemistry, Germany delivered a lecture on “Structure of a Survivin-Borealin-INCENP core complex reveals how chromosomal passengers travel together” (03.01.2008)

Dr Raju Chaganti, William E. Snee Chair, Medicine Memorial Sloan-Kettering Cancer Center, New York delivered a lecture on "Array CGH in lymphomas: clinical correlations and target genes" (08.01.2008)

Dr Sudip Mandal, University of California, Los Angeles, USA delivered a lecture on "Regulation of cell cycle by mitochondrial signaling" (17.01.2008)

Prof Gerald R Smith, Fred Hutchinson Cancer Research Centre, Seattle, USA delivered a lecture on "Homologous recombination in E.coli: pathways, enzymes, and hot spots" 04.02.08)

Dr Takaaki Daimon, Assistant Professor, University of Tokyo, Japan delivered a lecture on "Silkworm research in the post-genomic era" (05.02.2008)

Dr V Sriram Fellow, NCBS, Bangalore delivered a lecture on "Molecular mechanisms of mitochondrial remodeling in Drosophila" (06.02.2008)

Prof Guy Dodson, FRS, University of York, Heslington, York, UK & National Institute of Medical Research, London delivered a lecture on "The Structural and Chemical Factors that Govern Insulin Biosynthesis and Activity" (19.02.2008)

CDFD celebrated its 11th Foundation Day on 28.1.08. On the occasion **Prof. Stewart Cole**, FRS Director, Global Health Institute, Ecole Polytechnique Federale de Lausanne, Switzerland delivered a lecture on "A World Without Tuberculosis?"

Dr Sumeena Bhatia, National Cancer Institute, NIH, USA delivered a lecture on "Organization of co stimulatory molecules on the cell surface : Implications for T cell activation" (13.03.08)

Dr Shweta Tyagi, University of Lausanne, Switzerland delivered lecture on "Epigenetic Changes that Coordinate Cell Cycle Response: Linking the E2F1 Transactivator and MLL Family of Histone Methyltransferases via HCF-1" (20.03.2008)

Dr Sharon Master, Dept of Molecular Biology and Microbiology, University of New Mexico, USA delivered lecture on "Inhibition of Host Defence Pathways by Mycobacteria " (26.03.08)

CDFD Retreat-2008 was held on 26th and 27th March, 2008 at Gandipet Campus.

Consultant Physician in HIV/AIDS & TB, Chelsea & West Minister Hospital, London who is advisor to Blue Peter Research Centre (BPRC), **Dr Anton Poznaik**, was visiting India for the purpose of "improving the preparedness of BPRC for clinical trail in the area of HIV and STI" visited CDFD on 7.3.08

A five members delegation from 'Centre of Excellence in Genomics Medicines Research, Kind Abdulaziz University, Jeddah including Dr Mohammed Al Qahtan visited CDFD on 05.03.08 and an MoU was signed for collaboration in research and training.

Directors and Senior scientists of Indian Council of Forest Research and Education (ICFRE), Ministry of Environment and Forests, attending training at Administrative Staff College of India visited the Centre on 13.03.08.

DEPUTATIONS ABROAD OF CDFD PERSONNEL

SCIENTISTS & STAFF

Dr Gowrishankar (Singapore) To visit the Institute of Molecular and Cell Biology and the Bioinformatics Institute, 02-05 May 2007

Dr Gowrishankar (Brisbane, Australia) To participate in the 4th Indo-Australian Conference on Biotechnology at the Queen land Institute of Medical Research, 06-10 May 2007

Dr Shekhar C Mande (Brisbane, Australia) To attend the 4th Indo-Australian Biotechnology Conference at QIMR, 07 – 09 May 2007

Dr N Madhusudan Reddy (Germany) To conduct research in human genetic diversity in Prof. Mark Stoneking's Lab at the Department of Evolutionary Genetics, Max Planck Institute for Evolutionary Anthropology, Leipzig, 7 May to 11 June, 2007

Dr Gowrishankar (Melbourne, Australia) To visit the University of Melbourne for discussions and attend Seminar, 11-12 May 2007

Dr Gowrishankar (Marseille, France) To attend the Scientific Council meeting of the Indo-French Council for Promotion of Advanced Research (IFCPAR), 28 May to 5 June, 2007

Dr Gowrishankar (Paris, France) To visit the Laboratory of Prof. Miroslav Radman for a seminar and discussions, 06-07 June 2007

Dr Shekhar C Mande (Tokyo, Japan) To present a talk on his work at the Asia-Pacific Conference organized by Nature, 6 June 2007

Dr J Nagaraju (Villeurbanne, France) To pursue the accomplished work with his collaborator Prof. Pierre Couble on their Indo-French Collaborative project entitled "Baculovirus-resistance in transgenic silkworms", 07-22 June 2007

Dr J Nagaraju (Naples, Italy) To visit the Institute of Genetics and Biophysics, Naples and Department of Genetics at University of Naples and for interactions with two research groups actively working on "Isolation of Sex Determining Genes in Insects", 16-20 June 2007

Dr Shekhar C Mande (Plymouth, NH, USA) To visit the Broad Institute in Massachusetts and to attend the Gordon Research Conference on Proteins held at Holderness School, Plymouth 16 – 27 June 2007

Dr Niyaz Ahmed (Germany) To visit the laboratory of Prof. Jorg Hacker, Director of Institute for Molecular Infections Biologie at the University of Wuerzburg for a collaborative framework and explore collaboration in the area of evolution of virulence in pathogenic bacteria, 21 June - 03 July, 2007

Dr Ranjan Sen (Vermont, USA) To attend the FASEB Summer Conference on "Mechanism & Regulation of Prokaryotic Transcription", 23 – 28 June 2007

Dr Gowrishankar (Belgium) As a Member of Science & Technology delegation, led by the Secretary, DST, 25-27 June 2007

Dr K Anupama (Paris) To undertake collaborative research in the Laboratory of Prof. Miroslav Radman, 15 September to 02 October, 2007

Dr Niyaz Ahmed (Turkey) To attend the XXth Conference and International Workshop of the European Helicobacter Study group, 20-24 September 2007

Dr Gowrishankar (Toronto, Canada) To attend the International Cancer Genomics Consortium Strategy Meeting, 30 September to 02 October, 2007

Dr Sanjeev Khosla (Kobe, Japan) To visit the Centre for Developmental Biology under the "JSPS-DST Exploratory Exchange Programe", 21 – 25 October 2007

Dr Shekhar C Mande (Montpellier, France) To participate in the Indo-French Workshop on Bioinformatics, 17 – 24 November 2007

Dr H A Nagarajaram (Montpellier, Germany) To participate in Indo-French Bioinformatics Meeting, 19 – 21 November 2007

Dr Gowrishankar (Seville, Spain) To make two scientific presentations in the Second International Conference on Environmental, Industrial and Applied Microbiology BioMicroWorld 2007, 28 November to 01 December, 2007

Dr Niyaz Ahmed (Italy) To visit the Laboratory of Prof. Leonardo A Sechi, Professor of Microbiology at University of Sassari, 12-31 January 2008

Dr J Nagaraju (Narita, Tokyo, Japan) To promote collaboration between India and Japan (Advanced Industrial Science and Technology (AIST)) researchers, 21-25 January 2008

Dr Shekhar C Mande (USA) to participate in an International Workshop on "How do I find job in my home country" on 25th February 2008 ; to give an invited talk at the Centre for Structural Biology, University of Birmingham, Alabama on 26th February 2008; to organize a session on Biomedical Engineering in the Frontiers of Engineering Symposium at Irvine, California during 28th February 2008 to 1st March 2008 ; and for academic discussions at the University of California at San Diego on 3rd March 2008, 24 February to 05 March, 2008

STUDENTS

Mr Smanla Tundup, (Australia) to attend the Indo-Australian Conference on Biotechnology to be held at Brisbane, Australia from May 5 – 11, 2007

Mr Smanla Tundup, (Germany) to visit the laboratory of Prof. Jorg Hacker, Wurzburg, Germany to carry out part of his Ph.D. work for a maximum period of 6 weeks from

20.06.2007 without any financial commitment on CDFD for this trip 6 weeks from 20.06.2007

Mr Laishram Rakesh Singh, (USA and Germany) To attend "The Molecular Genetics of Bacteria and Phages Meeting" for the year 2007 held in Madison, Wisconsin, USA from 07.08.07 to 12.08.07 and also to visit Fox Chase Cancer Center, Philadelphia and University of Cologne, Germany from 13.08.07 to 30.08.07

Ms Aisha Farhana, (UK) To attend the Gorden Research Conference (GRC) meeting on "Tuberculosis Drug Development" organized by the GRC organizing committee at Magdalene College, Oxford, United Kingdom 26 - 31 August, 2007

Ms Aisha Farhana, (Australia) To attend the Fourth Indo-Australian Conference on Biotechnology held at Brisbane, Australia from 05 - 11 May, 2007

Mr Tabrez Anwar Shamim, (Austria) To attend the "15th Annual International Conference on Intelligent Systems for Molecular Biology (ISMB) & 6th European Conference on Computational Biology (ECCB) held at Vienna, Austria from 21 - 25 July, 2007

Mr MRK Subba Rao, (UK) To attend "The Mechanisms of Cell Signalling" conference held at Magdalene College, Oxford, United Kingdom from 16 - 21 September, 2007

Mr VLN Reddy, (Japan) For participating in 5th International Symposium of Rice Functional Genomics (ISRFG) held at Tsukuba, Japan from 15 - 17 October, 2007

Mr KP Arun Kumar, (Japan) to visit Dr. Toru Shimada's lab in University of Tokyo and Dr. Kazui Mita's Lab in NIAS, Tsukuba, Japan to carry out collaborative research work in the project on "Comparative genomics of wild silkmoths" from 24.10.2007 to 23.11.2007

Mr KP Arun Kumar, (CSHL, USA) to attend the CSHL hosted conference entitled "The Biology of Genomes" and present an abstract entitled "Construction, characterization and analysis of expressed sequences from an Indian Wild Silkmoth, Antheraea Assama" at CSHL, New York, USA from 04.05.07 to 16.05.07

Mr Jayendranath Shukla, (USA) SRF To attend CSHL hosted conference entitled "Systems Biology: Global Regulation of Gene Expression" held at New York, USA from 27.03.08 to 30.03.08

Mr Sandeep Kumar, (USA) To attend the "Keystone symposia held at Santa Fe, New Mexico, USA for the session titled "DNA Replication and Recombination (B5)" from 10.02.08 to 15.02.08