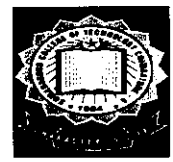


CLONING, EXPRESSION AND PURIFICATION OF THE GENE Rv0288 OF *MYCOBACTERIUM TUBERCULOSIS* IN *ESCHERICHIA COLI*

A PROJECT REPORT

Submitted by

R.SANDHIYA

In partial fulfillment for the award of the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

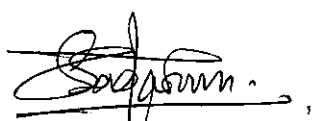
KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE

ANNA UNIVERSITY: CHENNAI 600 025

APRIL 2010

ANNA UNIVERSITY: CHENNAI 600 025
BONAFIDE CERTIFICATE

Certified that this project report entitled **"Cloning, Expression and Purification of the gene Rv0288 of *Mycobacterium tuberculosis* in *Escherichia coli*"** is the bonafide work of **"Ms. R.Sandhiya"** who carried out the project work under my supervision.



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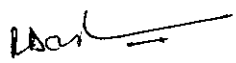

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RAJIV GANDHI CENTRE FOR BIOTECHNOLOGY
A CENTRE FOR ADVANCED RESEARCH AND TRAINING IN BIOTECHNOLOGY

26.03.2010

CERTIFICATE

This is to certify that the project report entitled "**Cloning, Expression and Purification of the gene Rv0288 of *Mycobacterium tuberculosis* in *Escherichia coli***" is an authentic record of the project work done by **Ms. Sandhiya R** at **Rajiv Gandhi Centre for Biotechnology** under my supervision, in partial fulfillment for the award for the degree of Bachelor of Technology in Biotechnology at Kumaraguru College of Technology, Coimbatore affiliated to Anna University, Chennai and that no part of this work has been submitted earlier for the award of any other degree. The tenure of her project is from 21st December 2009 to 26th March 2010.

Dr. Sathish Mundayoor

Dean & Scientist G

Mycobacteria Research Group

CERTIFICATE OF EVALUATION

COLLEGE : Kumaraguru College of Technology

BRANCH : Biotechnology

SEMESTER : Eighth Semester

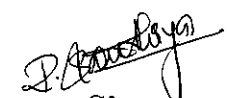
NAME OF THE STUDENT	TITLE OF THE PROJECT	NAME OF THE SUPERVISOR WITH DESIGNATION
R.SANDHIYA 71206214033	Cloning, Expression and Purification of the gene Rv0288 of *Mycobacterium tuberculosis* in *Escherichia coli*	Dr. R.BASKAR Associate Professor

The Report of the project work submitted by the above student in partial fulfillment for the award of Bachelor of Technology degree in Biotechnology of Anna University was evaluated and confirmed.

(INTERNAL EXAMINER) **(EXTERNAL EXAMINER)**

DECLARATION

I, **R.SANDHIYA,** hereby declare that the project, entitled **"Cloning, Expression and Purification of the gene Rv0288 of *Mycobacterium tuberculosis* in *Escherichia coli*"** submitted to the Anna University, in partial fulfillment of the requirements for the award of the **DEGREE OF BACHELOR OF TECHNOLOGY** is a original research work done by me under the supervision and guidance of **Dr. Sathish Mundayoor,** Scientist F, Molecular Microbiology Division and also with Co-Guidance of **Mr. D. Dhanasooraj,** SRF, RGCB, Thiruvananthapuram. This report has not formed the basis for the award of Degree/Diploma/Association /Fellowship or other similar title to any candidate in any University.



Signature

R.SANDHIYA.

AKNOWLEDGEMENT

Words are insufficient to express my whole hearted thanks and dedication to Almighty, who showered his blessings abundantly upon me. His grace and mercy has always led me through times and I will praise his name for ever.

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If words are considered as the symbol of approval and token of aknowledges, then let my words play the heralding role in expressing my gratefulness to **Mr. Dhanosooraj D.,** SRF, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, for his spontaneous and sincere help, valuble suggestions, constructive ideas and encouragement during course of my work.

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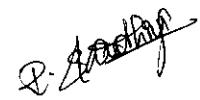
Finally, I wish to express my deep sense of appreciation to my beloved parents for their continuous encouragement and prayers, and I also thank my dear friends for their support throughout my project work.



R.SANDHIYA

Dedicated to my beloved parents

ABSTRACT

Tuberculosis caused by *Mycobacterium tuberculosis* is one of the major health problems of the world. The emergence of multidrug resistant strains and the association of the disease with human immunodeficiency virus (HIV) infection is an ominous new threat to public health. The presently available Bacillus Calmette Guerin (BCG) vaccine has been shown to have variable efficacy. A subunit vaccine consisting of a few key antigens of *Mycobacterium tuberculosis* that are capable of inducing protective immunity could have advantages over BCG. Recently, attention has been focused on the proteins that are actively secreted from the live bacilli during the early log phase of growth. These secretory proteins are released into the medium of mycobacterial culture before any significant autolysis and are collectively known as culture filtrate proteins (cfp). These proteins are reported to be major targets of T-cells in humans infected with mycobacteria. Thus, they are believed to be involved in inducing protective immunity against tuberculosis by activating the specific T-cells at an early stage. In recent years, several reports have appeared which indicate the role of culture filtrate proteins and purified secretory proteins when used alone, or in combination with other proteins in inducing protection against tuberculosis. This experiment deals with amplification, cloning, sequencing, expressing and purification of one such protein (cfp7/tb10.4/Rv0288). However, more work is needed to evaluate the protective potential of secretory protein for the development of anti- tuberculosis subunit vaccine.

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LIST OF ABBREVIATIONS:

Ab	- Antibody
AIDS	- Acquired Immuno Deficiency Syndrome
Ag85B	- Antigen 85 complex
Amp	- Ampicillin
APS	- Ammonium persulphate
ATP	- Adenosine triphosphate
BAC	- Bacterial Artificial Chromosome
BCA	- bicinchoninic acid
BCG	- Bacillus Calmette-Guérin
BCIP	- 5-Bromo-4-Chloro-3-Indolyl Phosphate
bp	- Basepair
BSA	- Bovine Serum Albumin
CBB	- Coomassie Brilliant Blue
cfp	- culture filtrate protein
CMI	- Cell Mediated Immunnity
CR	- Complement Receptors
DDA	- dimethyldioctadecylammonium
ddNTP	- Deoxy ribonucleicoside triphosphate
DNA	- Deoxy ribonucleic acid
dNTP	- Di-deoxy ribonucleicoside triphosphate
EDTA	- Ethylene diamine tetra acetic acid

ESX-1	- ESAT-6 System 1
FP	- Forward Primer
g	- Gram
G+C	- Glycine + Cytosine
His	- Histidine
HIV	- Human Immunodeficiency Virus
hr	- Hour
IgG	- Immunoglobin G
IL	- Interleukin
INF	- Interferon
IPTG	- Isopropyl β-D-1-thiogalactopyranoside
kb	- kilo Base
kDa	- kilo Dalton
LB	- Luria-Bertani
MDR	- Multi Drug Resistant
MDR-TB	- Multi Drug Resistant Tuberculosis
µg	- Micro gram
mg	- milli gram
mM	- Milli Molar
ml	- Milli litre
µl	- Micro litre
MPL	- Monophosphoryl Lipid
MOPS	- 3-(N-morpholine) propane sulfonic acid

min	- minute
M	- Molar
M.*bovis*	- *Mycobacterium bovis*
MTB	- *Mycobacterium tuberculosis*
NBT	- Nitro Blue Tetrazolium Chloride
NEB	- New England Biolabs
OD	- Optical Density
PAGE	- Poly Acrylamide Gel Electrophoresis
PBS	- Phosphate Buffer Saline
PCR	- Polymerase Chain Reaction
PGRS	- Polymeric G+C rich sequence
PMSF	- phenylmethanesulfonylfluoride
PVDF	- Poly Vinylidine Fluoride
RD	- Region of Difference
RP	- Reverse Primer
RNA	- Ribonucleic acid
rpm	- rotation per minute
SDS	- Sodium Dodecyl Sulphate
sec	- seconds
sec	- secretion pathway
TAE	- Tris Acetic acid EDTA
TB	- Tuberculosis
TBE	- Tris Borate EDTA

TBST	- Tris Buffer Saline Tween
TDB	- Trehalose 6,69-dibehenate
TFB	- Transformation buffer
TH/Th	- T-Helper cells
WHO	- World Health Organization
XDR	- Extensively Drug Resistant
XDR-TB	- Extensively Drug Resistant Tuberculosis
°C	- degree Celsius
∞	- Infinity

INTRODUCTION

INTRODUCTION

Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. The disease affects 1.8 billion people/year and is equal to one-third of the entire world population. Robert Koch, a German physician and scientist, presented his discovery of *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis (TB), on March 24, 1882 (Anderson *et al.,* 2007). Today, more than a century after Koch discovered *Mycobacterium tuberculosis*, there are still many infected individuals and around two million deaths annually resulting from the disease.

Mycobacterium tuberculosis is a fairly large non-motile rod-shaped bacterium, obligate aerobe, facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, and a physiological characteristic that may contribute to its virulence.

Kingdom	:Bacteria
Phylum	:Actinobacteria
Order	:Actinomycetales
Suborder	:Corynebacterineae
Family	:Mycobacteriaceae
Genus	:*Mycobacterium*
Species	:*tuberculosis*
Strain	: *H37Rv*

The cell wall structure of *Mycobacterium tuberculosis* deserves special attention because it is unique among prokaryotes, and it is a major

determinant of virulence for the bacterium (Anderson *et al.,* 1991 and Anderson *et al.,*1995). The cell wall complex contains peptidoglycan, but otherwise it is composed of complex lipids. Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of MTB's cell wall consists of three major components, mycolic acids, cord factor, and wax-D (Anderson *et al.,*1993).

MTB does not possess the classical bacterial virulence factors such as toxins and capsules; it has special mechanism for cell entry. The bacilli gain entry into macrophages through binding to one of several phagocyte factors. Once its entry into the cell, mycobacterium are retained in a phagocytic vacuole until the host cell dies by necrosis or apoptosis. This vacuole fails to fuse with the lysozyme. But they remain fusion competent and acquire some 'lysosomal' proteins from the synthetic pathway of the host cell. Further they fuse with other vesicles of endosomal system (Boesen *et al.,* 1995).

Bacillus Calmette-Guérin (BCG) is the present vaccine against tuberculosis (attenuated form of *Mycobacterium bovis*).The attenuation of BCG is due to the loss of *RD1* gene family (virulent genes). BCG is characterized by its capacity to induce cellular and humoral immune responses. The failure of BCG is because of the tendency of bacteria to hide in an inactive state which results in the emergence of the Multi Drug Resistant (MDR) strains. The recent emergence of multidrug-resistant and extensively drug-resistant MTB strains highlights the urgent need for extensive research unraveling the complex mechanism enabling the bacterium to be successfully parasitic in humans.

Thus, an improved vaccine is urgently needed to replace BCG and to prevent TB effectively. Several new types of TB vaccine preparations, including subunit vaccines, live attenuated vaccines, recombinant BCG (rBCG), and DNA vaccines, are currently investigated in experiments.

Recently, the genome of the MTB laboratory strain H37Rv was completely sequenced. The genomes comprise of 4,411,529 bp with 4000 genes and have a very high G+C content. The MTB H37Rv has been analyzed in order to improve the understanding of this slow growing pathogen and help in the prophylactic and therapeutic inventions. MTB differs from other bacteria in that a very large portion of its coding capacity is devoted to the production of enzymes involved in the lipogenesis and lipolysis and two families of glycine rich protein that may represent a source of antigenic variation (Fleischmann *et al.,* 2002).

The RD1 (region of difference 1) deletion region (Maharias *et al.*, 1996), is a 9,505 bp region absent in all *M.bovis* BCG strains. RD1 is commonly thought to be the primary deletion that occurred during the serial passage of *M.bovis* by Calmette and Gurein between 1908 and 1921, and is thus though possibly to be responsible for the primary attenuation of *M.bovis* to *M.bovis* BCG (Brosch *et al* 2000, Gordon *et al.,* 1999 and Behr *et al.*,1999) consequently, the genes contained in RD1 have been the object of a number of studies focusing on diagnosing of MTB infection, the search for efficient vaccine candidates and virulence (Ahmad *et al.*,1999 and Wards *et al.*,2000)

This region includes the genes that encode the secretory proteins

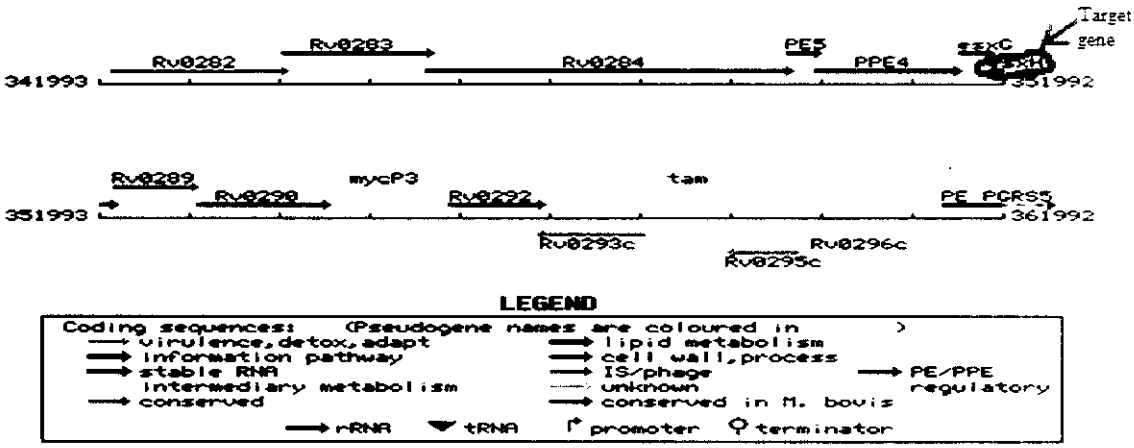
filtrate protein). The other genes encode components of a secretion system that is called ESX-1 (ESAT-6 system 1) (Takeshi Kurenuma *et al.,* 2009, Lalvani *et al.,* 2001 and de Jonge *et al.,* 2007). It is supposed that more than 14 proteins contribute to this secretion system (Abdallah *et al.,* 2007).

The genome of MTB H37Rv has five copies of a cluster of genes known as the ESAT-6 loci. These clusters contain members of the CFP-10 (*lhp*) and ESAT-6 (*esat-6*) gene families (encoding secreted T-cell antigens that lack detectable secretion signals) as well as genes encoding secreted, cell-wall-associated subtilisin-like serine proteases, putative ABC transporters, ATP-binding proteins and other membrane-associated proteins. This membrane associated and energy-providing proteins may function to secrete members of the ESAT-6 and CFP-10 protein families, and the proteases may be involved in processing the secreted peptide (Nico Gen van Pittus *et al.,* 2001). The five ESAT-6 gene clusters present in MTB H37Rv were named region 1 (Rv3866-Rv3883c), 2 (Rv3884c-Rv38895c), 3(Rv0282-Rv0292), 4(Rv3444c-Rv3450), 5(Rv1782-Rv1798), consistent with the arbitrary numbering system used to classify five mycosin (subtilisin-like serine protease) genes identified from these regions (Brown *et.al.*,2000).

Region of difference (RD1) genes are present in virulent MTB but not the vaccine strain *M. bovis* Bacille Calmette-Guérin (BCG). The deletion of RD1 from MTB produces attenuation strikingly like that of BCG, which suggests the use of RD mutant strains for improvement of the tuberculosis (TB) vaccine. Here, we deal with the amplification, cloning, sequencing,

This gene codes for virulent proteins which can act as an effective antigen after infection which is present only in virulent strains. The encoded product of these genes can be used for the development of subunit vaccine.

Fig 1.1: Gene map



In brief, the full-length open reading frames of the genes were polymerase chain reaction (PCR) amplified using sequence specific primers followed by cloning into pET32a expression vector. The recombinant plasmids were transformed into JM109 (*Escherichia coli)* and the transformants with the correct insert and orientation were identified by DNA sequencing. For expression of the recombinant antigens, plasmids were transformed into the *E. coli* expression host BL21 (DE3). The expressed recombinant antigens were purified using His-tag protein purification resin and quantified by BCA assay.

LITERATURE REVIEW

LITERATURE REVIEW

2.1 THE DISEASE

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB), remains a major health threat. Each year, 8 million new TB cases occur and 2 million individuals die of TB (Kaufmann *et al.,* 2006). Moreover, it is estimated that one third of the population is latently infected with MTB, of which ~10% will develop active disease during lifetime. The development of active TB occurs when the balance between natural immunity and the pathogen changes (e.g. upon waning of protective immune response during adolescence and in HIV patients, (Anderson, 2007). In addition, at present ~50 million individuals are probably infected with multi drug-resistance (MDR) strains of MTB (WHO, 2006), rendering antibiotic treatment difficult. (Anat Zvi *et al.,* 2008)

A new and potentially devastating threat to TB control is the emergence of strains that cannot be cured by standard anti-tuberculosis drug regimens (Lawn *et al.,* 2006). Drug resistant tuberculosis commonly arises through the selection of mutated strains by inadequate chemotherapy. Resistance to at least the two major anti-tuberculosis drugs, isoniazid and rifampicin has been termed multidrug-resistant tuberculosis (MDR-TB). Treatment of MDR-TB requires prolonged and expensive chemotherapy using second-line drugs of heightened toxicity. Should resistance to the second line drugs also arise then the disease becomes virtually untreatable. Extensively drug resistant-tuberculosis (XDR-TB) has been reported in all

to at least rifampicin, isoniazid, a second line injectable drug (capreomycin, kanamycin or amikacin) and a fluoroquinolone. Control of drug resistant tuberculosis requires a strong health infrastructure to ensure the delivery of effective therapy coupled with surveillance and monitoring activities to enable timely intervention to limit transmission and spread of the disease. It is paradoxical that drug resistance develops and flourishes in those very settings least able to deal with it. The recent report from KwaZulu Natal Province in South Africa of an outbreak of XDR-TB where rapid progression to death was observed in 98% of patients demonstrates the vulnerability of sub-Saharan Africa to outbreaks of untreatable disease (Gandhi *et al.,* 2006).

Although studies demonstrating successful treatment outcomes for MDR-TB cases have been reported from a number of settings (Nathanson *et al.,* 2006), the allocation of resources to detect and treat MDR-TB in poor resource settings remains controversial (Epsinal *et al.,* 2005). Whereas some advocate that priority be given to the effective treatment of drug sensitive disease, thus preventing the emergence of drug resistance (Pablos-Mendez *et al.,* 2002), others argue that drug resistant cases should be detected and treated both for the good of the individual and to reduce ongoing transmission of drug resistant disease (Yong Kim *et al.*,2005). Inevitably, decisions on resource allocation are based on the perceived burden of disease. Data on the prevalence of drug resistant tuberculosis are currently presented as the proportion of cases found resistant to anti-tuberculosis drugs (Ellen Zager *et al.,* 2008).

Drug resistance surveillance data published by the WHO and the International Union against Tuberculosis and Lung Diseases Global

untreated TB cases having multi-drug resistant disease. Data was sourced from the WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance reports of 2000 and 2004 (WHO 2004 & 2000). The estimated incidence of tuberculosis for the year when the surveillance was carried out and the proportion of cases found MDR were used to calculate the incidence of MDR-TB per 100,000 of the population. Where necessary, population data was supplemented by reference to the US Census Bureau's International Data Base (US CBIDB) (Rastogi *et al.,* 1992).

The following formulae may be used to estimate incidence of MDR-TB in the population:

$$\text{Incidence of MDR-TB per 100,000 of the population} = \frac{\text{number of cases of MTB-TB in the population}}{\text{populaion surveyed}} * 100$$

2.2 PATHOGENESIS

A key characteristic of MTB infection is that this bacterium multiplies intracellular, primarily in macrophages, evading in this way many host defense mechanisms (Tiruviluamala *et al.,* 2002). Thus, internalization of MTB by macrophages is a critical step for the establishment of tuberculosis infection. Active phagocytosis seems to be the main way this bacterium gets into host cells (Schluger *et al.,* 2001). Binding and phagocytosis of MTB to macrophages can be mediated by different kinds of receptors including complement receptors (CR), mannose receptors, surfactant receptors, scavenger receptors, and GPI-anchored receptors (such as CD14) (Ernst *et al.,* 1998 and Ehlers *et al.,*1998). During MTB infection, the balance between the bacteria growth and survival and the magnitude of

effective host defense requires the production of TH1-type cytokines, such as interleukin (IL)-12 and interferon-g (IFN-g), which activate cellular immunity (Schluger *et al.,* 2001and Orme *et al.,* 1998). Under the influence of these cytokines macrophages get activated and are able then to eliminate ingested bacteria (Schluger *et al.,* 2001). In contrast to this, macrophages infected by MTB produce inhibitory cytokines, such as transforming growth factor-b1 and IL-10, which reduce macrophage activation, leading also to a decrease in the clearance of bacteria (Barnes *et al.,* 1992 & Toossi *et al.,* 1995). Despite these findings, neither the basic biology of MTB nor the host immune response against mycobacteria has been sufficiently clarified. Thus, understanding the pathogenic mechanisms of MTB and the immune response elicited by mycobacteria will aid the design of new therapies against tuberculosis (Marco Antonio Velasco-Vela´zquez *et al.,* 2003).

2.3 CURRENT VACCINE

Some of the reasons for increasing incidence of MTB are inadequate access to health care, migration, deterioration of TB control programs, low compliance with TB treatment, multidrug-resistant strains, and the acquired immunodeficiency syndrome (AIDS) epidemic.

The means used to prevent and control TB are improvement of socioeconomic conditions, case finding and treatment, chemoprophylaxis and vaccination (Rodrigues *et al.,* 1990). Improving socioeconomic conditions has proven to be slow and difficult in a world of social and political instability. Case finding and treatment, and chemoproprhylaxsis required an organized control program, which many countries do not have (Luelmo *et al.,* 1982).

Bacillus Calmette –Guerin vaccine (BCG) is an alternative preventive measure that can be achieved in new borns in a single visit. It is an attenuated strain of *Mycobacterium bovis*, applied in 1921 in France by Albert Calmette and Calmille Guerin as a vaccine against TB (Sakula *et al.*, 1983). In the first half of the 20th Century, BCG vaccines were prepared and preserved by different manufacturing laboratories. This resulted in genotypic and phenotypic differences in the daughter strains with variations in tuberculin conversion and the frequency of adverse reactions (Mauricio Castañón-Arreola *et al.*, 2004). BCG showed a progressive decrease in virulence, the most important during the first 15 passes (Tubercle 1978 and Osborn *et al.*, 1983). For BCG, a variable efficacy rate from 0% to 80% has been reported (Colditz *et al.*,1994, Brandt *et al.*,2002 & Buu *et al.*, 2002).

In regions of the world where the disease is most widespread, BCG vaccination is ineffective and therefore, the search for novel, more effective vaccines is paramount. There are a number of possible explanations for the discrepancies in BCG protector efficacy: (i) genetic host susceptibility (Buu *et al.*,2000), (ii) a wide range of virulence among MTB strains, (iii) progressive loss of BCG capacity to stimulate a durable immune response (iv) prevalence of other mycobacterial infections in the study population, (v) variations in protection against different forms of tuberculosis, and (vi) the level of exposure to environmental mycobacteria (Buddle *et al.*,2002 & Brandt *et al.*, 2002), however, none of these have strong clinical and research support (Colditz *et al.*,1994).

2.4 IMMUNOLOGICAL CONSIDERATIONS FOR NEW TUBERCULOSIS VACCINE DEVELOPMENT

The development of a new vaccine with improved protective immunity against MTB depends on the efficient recruitment of antigen-specific T-cells principally $CD4^+$ in the lungs, as well as on the cytokines that are released particularly IFN-γ, which is important for inducing the macrophage killing activation mechanism (Ellner *et al.*, 1997 and MacGuru et al., 2005).

MTB has a complex multiplicity of antigens with a diverse chemical and immune reactive nature as has been demonstrated in lipids, polysaccharides, and proteins.

Some of these induce the granuloma formation, macrophage activation, and adjuvant activity while others are immunosuppressive and enhance host toxicity (Rastogi *et al.*, 1992 and Ellner *et al.*, 1997). It should be remembered that the T-cell mediated immune response can result in protective immunity but it may also lead to pathological immunity that is detrimental to the host. The understanding of specific antigens expressed during early infection, disease, latency or reactivation and their immunological characterization are key for the development of new vaccines.

The development of new acellular vaccines composed of one or more antigens may offer a faster route for creating an alternative to BCG vaccines. However, this approach needs a distasting research to identify and defining those potential proteins recognized by human T-cells which are capable of inducing cell-mediated and long-lasting immunity. On last ten years attention has mainly been focused on the group of proteins secreted by the

growth phase. These have been recognized in the early stages of tuberculosis infection in several animal models and may be the reason that viable mycobacteria are needed for effective vaccination against MTB infection (Louise *et al.,* 2001 and Mustafa *et al.,* 1998).

Various approaches are currently being analyzed using sub-unit vaccines based on culture filtrate proteins of MTB which when are administrated with an adjuvant have been shown to induce protective immunity in mice, guinea pigs and non-human primate models (Harth *et al* .,1996). Antigen isolation has depended on the use of monoclonal antibodies (Freer *et al.,* 1998) or by direct chromatography of culture filtrates. Such isolation procedures are characterized by biochemical and immunological techniques, with the corresponding genes being sequenced and cloned for further use as a vaccine. Several antigens have been cloned and purified as recombinant proteins, due to the large doses required for immunization. The choice of an antigen or several antigens to formulate optimal immunological cocktails for vaccine use needs to be tested through *in vitro* and *in vivo* studies. In *in vitro* human studies, ESAT-6, CFP-10, MPT64, MPB70 and fusion proteins of the ESAT- 6 and antigen 85B have been tested to show their ability to induce protective T-cell response (Renshaw *et al.,* 2002). The most used criteria for tuberculosis vaccine designs are antigen-specific proliferation and IFN-γ secretion assays (Mustafa *et al.,* 1998, Harth *et al* ., 1996 and Weinrich Osborn *et al* ., 1983). ESAT-6 was a promises in this area because is an immune dominant antigens most frequently recognized by TB patients, which contains a great number of T-cell epitopes (Pollock *et al.,* 1997). In animal models, ESAT-6 has been recognized in the first phase of infection and has been demonstrated to be a strong T-cell immunogen

inducing prime memory immunity, which persisted in individuals who had recovered from disease (Wu-Hsieh *et al*., 2001 and Louise *et al*., 2001).

2.5 SUBUNIT VACCINE

Subunit vaccine is a vaccine produced from specific protein subunits of a virus and thus having less risk of adverse reactions than whole virus vaccines. A "subunit vaccine" presents an antigen to the immune system without introducing viral particles, whole or otherwise. One method of production involves isolation of a specific protein from a virus and administering this by itself. A weakness of this technique is that isolated proteins can be denatured and will then bind to different antibodies than the proteins in the virus. A second method of subunit vaccine is the recombinant vaccine, which involves putting a protein gene from the targeted virus into another virus. The second virus will express the protein, but will not present a risk to the patient.

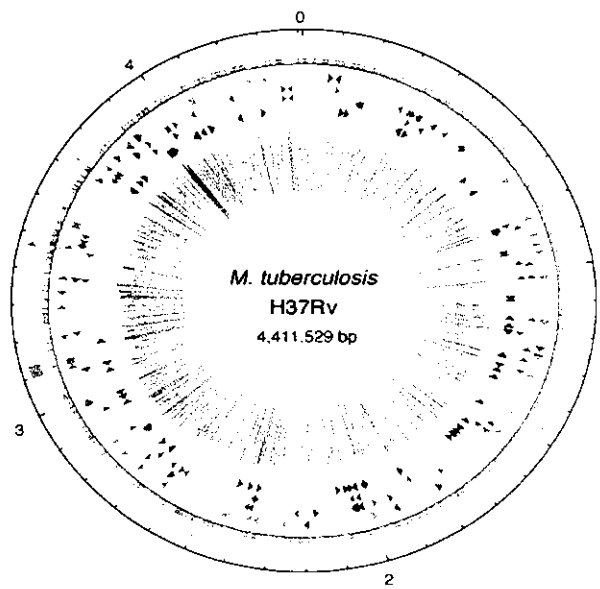
The subunit approach holds a number of advantages, such as increased safety and stability as well as the demonstrated ability to boost prior BCG immunization (Dietrich *et al.,* 2006, Brandt *et al.,* 2004). In addition, as subunit a vaccine does not appear to be influenced by environmental mycobacteria this type of vaccine may be of particular use in the developing world (Brandt *et al.,* 2002 and Talbot *et al.,* 1997). However, progress in this field has been delayed by the lack of available adjuvants that induce a strong cell-mediated immune (CMI) response. Recently Claus Aagaard *et al.,* 2000 showed that Ag85B-TB10.4 delivered in cationic dimethyldioctadecylammonium (DDA) and Monophosphoryl Lipid A (MPL) induced a strong protection against infection with MTB (Dietrich *et al.,* 2005). Furthermore, the closely related vaccine Ag85BESAT- 6

dibehenate (TDB) or in IC31H adjuvant induced a strong Th1 response that efficiently protected against infection with MTB (Christensen *et al.*, 2007 and Agger *et al* ., 2006).

2.6 GENOME

Combined approach was used that involved the systematic sequence analysis of selected large insert clones (cosmids and BACs) as well as random small-insert clones from a whole-genome shotgun library. This culminated in a composite sequence of 4,411,529 base pairs (bp), with a G + C content of 65.6%. This represents the second-largest bacterial genome sequence currently available (after that of *Escherichia coli*) (Paetzel *et al* ., 1995). The initiation codon for the *dnaA* gene, a hallmark for the origin of replication, *oriC*, was chosen as the start point for numbering. The genome is rich in repetitive DNA, particularly insertion sequences, and in new multigene families and duplicated housekeeping genes (Sassetti *et al.*, 2002).

Fig 2.1: MTB genome



The G + C content is relatively constant throughout the genome indicating that horizontally transferred pathogenicity islands of atypical base composition are probably absent. Several regions showing higher than average G + C content were detected; these correspond to sequences belonging to a large gene family that includes the polymorphic G + C-rich sequences (PGRSs) Fifty genes coding for functional RNA molecules were found. These molecules were the three species produced by the unique ribosomal RNA operon, the 10Sa RNA involved in degradation of proteins encoded by abnormal messenger RNA, the RNA component of RNase P, and 45 transfer RNAs. No 4.5S RNA could be detected (Cole *et al.,* 1998).

Drug resistance: MTB is naturally resistant to many antibiotics, making treatment difficult (Cole *et al.,* 1995). This resistance is due mainly to the highly hydrophobic cell envelope acting as a permeability barrier4, but many potential resistance determinants are also encoded in the genome. These include hydrolytic or drug-modifying enzymes such as β-lactamases and aminoglycoside acetyl transferases, and many potential drug–efflux systems, such as 14 members of the major facilitator family and numerous ABC transporters. Knowledge of these putative resistance mechanisms will promote better use of existing drugs and facilitate the conception of new therapies.

Given the scale of the global tuberculosis burden, vaccination is not only a priority but remains the only realistic public health intervention that is likely to affect both the incidence and the prevalence of the disease (Young *et al.,* 1997). Several areas of vaccine development are promising, including DNA vaccination, use of secreted or surface-exposed proteins as

(Young *et al.,* 1997). All of these avenues of research will benefit from the genome sequence as its availability will stimulate more focused approaches. Genes encoding, 90 lipoproteins were identified, some of which are enzymes or components of transport systems, and a similar number of genes encoding preproteins (with type I signal peptides) that are probably exported by the Secdependent pathway. MTB seems to have two copies of *secA*. The potent T-cell antigen Esat-6 (Sorensen *et al.,* 1995), which is probably secreted in a Sec-independent manner, is encoded by a member of a multigene family.

2.7 ESAT-6 AND CFP-10

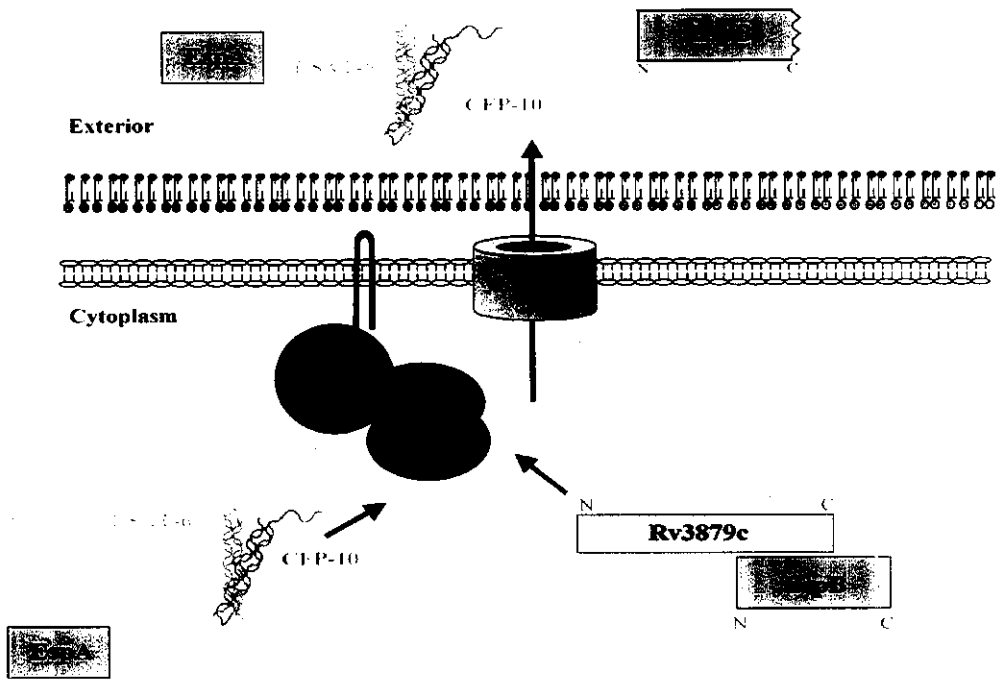
MTB are protected by an impermeable cell envelope composed of an inner cytoplasmic membrane, a peptidoglycan layer, an arabinogalactan layer, and an outer membrane. This second membrane consists of covalently linked, tightly packed long-chain mycolic acids (Hoffmann *et al.,*2008 and Zuber *et al.,* 2008) and noncovalently bound shorter lipids involved in pathogenicity (Champion *et al.,*2006, Sarah Stanley et al., 2003, Colditz *et al.,*1999 and Trivedi *et al.,*2005). To ensure protein transport across this complex cell envelope, mycobacteria use various secretion pathways, such as the SecA1-mediated general secretory pathway (Pugsley *et al.,*1993 and Wiker *et al.,*1992), an alternative SecA2-operated pathway (Braunstein *et al.,*2003), a twin-arginine translocation system (McDonough *et al.,*2008 and Saint-Joanis *et al.,*2006), and a specialized secretion pathway variously named ESAT-6-, SNM-, ESX-, or type VII secretion (Tekaia *et al.,*1999, Gey Van Pittius *et al.,*2001, Pym *et al.,*2003, Stanley *et al.,*2003, Brodin *et al.,*2004 ans Abdallah *et al.,* 2007). The latter pathway, hereafter referred to as type VII secretion (T7S), has recently become a large and competitive

of MTB (Simeone *et al.*,2009) and other pathogenic mycobacteria (Abdallah *et al.*,2007). Molecular details are just beginning to be revealed (Brodin *et al.*,2005, Champion *et al.*,2006, Smith *et al.*, 2008 and Carlsson *et al.*, 2009) showing that T7S systems are complex machineries with multiple components and multiple substrates. Despite their biological importance, there has been a lack of a clear naming policy for the components and substrates of these systems. As there are multiple paralogous T7S systems within the Mycobacteria and orthologous systems in related bacteria, we are concerned that, without a unified nomenclature system, a multitude of redundant and obscure gene names will be used that will inevitably lead to confusion and hinder future progress. In this opinion piece we will therefore propose and introduce a systematic nomenclature with guidelines for name selection of new components that will greatly facilitate communication and understanding in this developing field of research (Wilbert Bitter *et al.*, 2009).

The first T7S-associated protein to be identified was the 6-kD early secreted antigenic target ESAT-6 (Sorensen *et al.*, 1995). This small, highly immunogenic protein lacks a classical N-terminal signal sequence and is present in large amounts in the culture filtrate of MTB (Sorensen *et al.*, 1995), but is missing from the closely related attenuated live vaccine *Mycobacterium bovis* Bacille Calmette- Gue´rin (BCG) (Harboe *et al.*, 1996) due to the deletion of region of difference 1 (RD1) (Maharias *et al.*, 1996). ESAT-6 and its protein partner, the 10-kD culture filtrate protein CFP-10 (Berthet *et al.*, 1998), form a 1:1 protein complex (Renshaw *et al.*, 2005) that involves hydrophobic interaction (Renshaw *et al.*, 2005 and Brodin *et al.*, *2005*). Secretion of ESAT-6 and CFP-10 is required for the

2004). The absence of ESAT-6 secretion is responsible in part for the attenuation of the BCG and *Mycobacterium microti* vaccines (Pym *et al.*, 2003, Pym *et al.*, 2002 and Brodin *et al.*,2006), as well as for the decrease in virulence of the attenuated MTB H37Ra strain (Frigui *et al.*,2008).

Fig 2.2: Model for espB secretion:

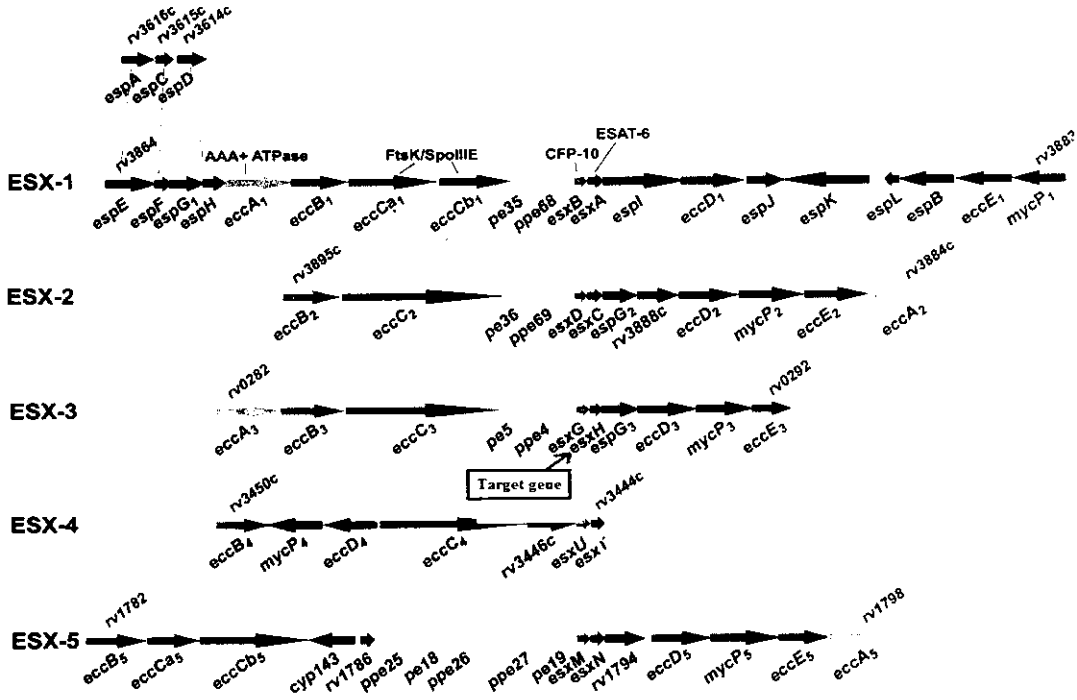


Depicted are the core ESX-1 components Rv3870, Rv3871, and Rv3877, as well as the ESAT-6/CFP-10 and EspB/Rv3879c complexes. Both cytosolic complexes require interaction with Rv3871 for substrate secretion (Bryant *et al.*, 2007). The different substrates make distinct, and potentially additive, contributions to virulence

In MTB ESAT-6 and CFP-10 belong to the WXG100 family of 23 small secreted proteins that share a size of approximately 100 amino acids, a helical structure, and a characteristic hairpin bend formed by the conserved Trp-Xaa- Gly (W-X-G) motif (Pallen *et al.*,2002). The genes encoding these proteins, many of which represent immunodominant T cell antigens (Skjot *et al.*,2000), are called esx genes in MTB (esxA-W,) and re arranged in tandem pairs at 11 genomic loci (Cole *et al.*,1998). In five of these genomic loci (ESX-1–ESX-5), the esx genes are flanked by genes coding for components of secretion machineries involved in the export of the corresponding ESX proteins. These proteins constitute the major building blocks of the T7S systems (Tekaia *et al.*,1999, Gey Van Pittus *et al.*, 2001, Brodin *et al.*, 2004, Abdallah *et al.*, 2007 and Champion *et al.*,2006). Four of these regions are also characterized by the presence of genes encoding PE and/or PPE proteins, named after their characteristic N-terminal motifs prolineglutamic acid (PE) and proline-prolineglutamic acid (PPE) (Gey Van Pittus *et al.*, 2006). Apart from genes localized in these core ESX regions, additional genes situated elsewhere on the chromosome may be required for the function of T7S systems. For example, the rv3616c-rv3614c genes are required for secretion of ESAT-6 and CFP-10 by ESX-1 (Fortune *et al.*, 2005, Mustafa *et al.*,1998 and Raghavan *et al.*, 2008).

2.8 GENETIC ORGANIZATION

Fig 2.3: Gene organization



2.9 GENE SEQUENCE

MTB H37Rv|Rv0288|esxH: 291 bp - low molecular weight protein antigen 7 *esxh* (10 kDa ANTIGEN) (CFP-7) (PROTEIN TB10.4)

atgtcgcaaatcatgtacaactaccccgcgatgttgggtcacgccggggatatggccggatatgc
cggcacgctgcagagcttgggtgccgagatcgccgtggagcaggccgcgttgcagagtgcgtggcag
ggcgataccgggatcacgtatcaggcgtggcaggcacagtggaaccaggccatggaagatttggtgc
gggcctatcatgcgatgtccagcacccatgaagccaacaccatggcgatgatggcccgcgacacggc
cgaagccgccaaatggggcggct

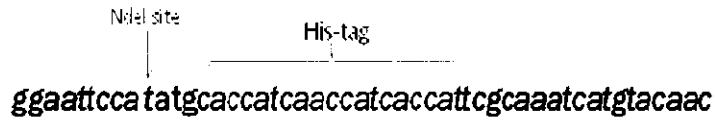
MATERIALS AND METHODS

MATERIAL AND METHODS

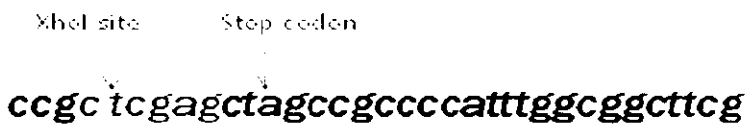
3.1 PRIMER DESIGNING

Gene specific primers were designed using primer premier software. The primers were designed such that the forward and reverse primer contained a unique restriction site at its 5' end. The *NdeI* recognition site was included in the forward primer and *XhoI* recognition site was included in the reverse primer.

3.1.1Forward primer



3.1.2Reverse primer



3.1.3. Reconstitution of Lyophilized primers

The lyophilized primers was incubated in ice for 30min and centrifuged at high speed for 1 min. Then TE buffer was added to the primer and aliquoted.

3.2 GENE AMPLIFICATION BY GRADIENT PCR

The genomic DNA of MTB strain H37Rv was amplified using the specific primers. The gradient PCR conditions are as follows:

Table 3.1: Gradient PCR conditions:

Steps	Modifications involved in DNA	No.of cycles	Temperature	Time
Step 1	Initial denaturation	1X	95°C	4min
Step 2	Denaturation	35X	98°C	30sec
	Annealing		55-65°C	30sec
	Extension		72°C	7min
Step 3	Final extension	1X	72°C	7min
Step 4	End hold	1X	4°C	∞

3.2.1 Reaction mixture

Water	:	17.85µl
10X PCR buffer	:	2.50 µl
2.5mM dNTP's	:	1.00 µl
Rv0288 FP (10pmol)	:	1.25 µl
Rv0288 RP (10pmol)	:	1.25 µl
Genomic DNA	:	1.00 µl
Taq	:	0.15 µl.
Total	:	25.00 µl

The gradient PCR was performed in Bio Rad i-cycler to find out the annealing temperature. The PCR product was then checked on 0.8% agarose gel.

3.3 PHUSION PCR AT ANNEALING TEMPERATURE

Phusion High-Fidelity DNA Polymerase provides high performance. Phusion DNA Polymerase combines the fidelity with unparalleled speed and robustness.

Table 3.2: Phusion PCR conditions:

Steps	Modifications involved in DNA	No.of cycles	Temperature	Time
Step 1	Initial denaturation	1X	95°C	4min
Step 2	Denaturation	35X	98°C	30sec
	Annealing		57°C	15min
	Extension		72°C	30sec
Step 3	Final extension	1X	72°C	7min
Step 4	End hold	1X	4°C	∞

3.3.1 Reaction mixture

Water : 23.5 µl

10X PCR buffer : 10.0 µl

2.5mM dNTP's : 4.0 µl

Rv0288 FP (10pmol) : 5.0 µl

Rv0288 RP (10pmol)	: 5.0 µl
Genomic DNA	: 2.0 µl
Phusion DNA polymerase	: 0.5 µl
Total	: 50.0 µl

The products were checked with 0.8%agarose gel.

3.3.2 Elution of the PCR product

The PCR product was eluted with GFX PCR Microspin elution kit (GE Health care) following the manufacturer's protocol and stored at -20°C.

1.4CLONING

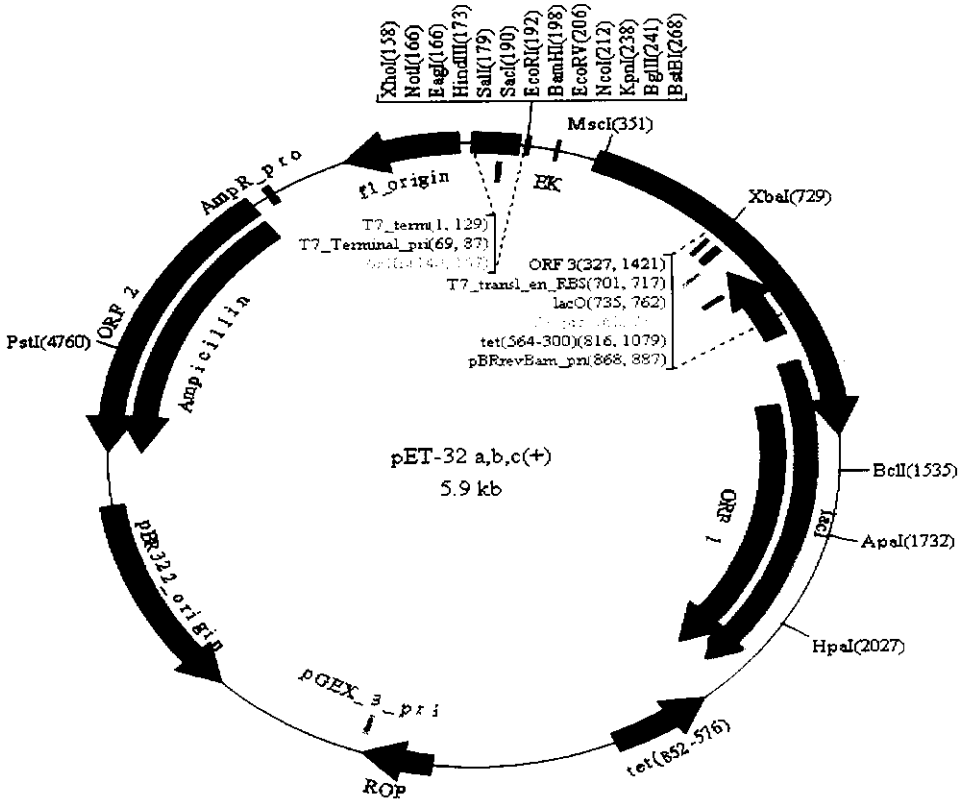
The eluted product was cloned into expression vector (pET32a) directly after the restriction digestion with enzymes. The amplified DNA insert and vector molecules were digested with two different restriction enzymes (*Nde*I and *Xho*I) to create the non-complementary sticky ends at either side of each restriction fragment. This allows the insert to be ligated to the vector (pET32a) in correct orientation and prevent vector from self ligation (direct cloning).

3.4.1 Vector

The pET System is the most powerful system developed for the cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA

are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein a few hours after induction. Although this system is extremely powerful, it is possible to attenuate the expression level simply by lowering the concentration of the inducer. Decreasing the expression level may enhance the yield of some soluble target protein. Another important benefit of this system is its ability to maintain target genes transcriptionally silent in the uninduced state. The pET32 series consists of pET32a, pET32b, pET32c. They differ from each other in the reading frames.

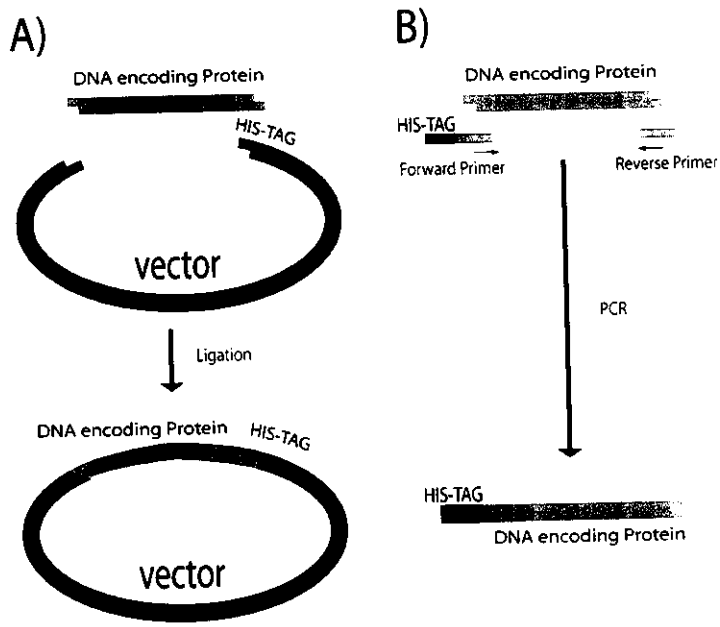
Fig 3.1: pET 32 vector



3.4.2 Vector preparation

The pET32a was used for cloning of Rv0288 in this study. The pET32a was transformed into *E.coli* JM109. The transformed cells were plated in Amp^+ Luria Bertani (LB) agar and kept at 37°C incubator for overnight. The transformed colonies were formed in the plate. One colony was selected and inoculated into 4ml LB broth containing Amp, which was incubated at 37°C shaker. After proper growth, the plasmid was isolated using plasmid preparation mini spin kit (GE health care)

Fig.3.2: Adding polyhistine tags



A) The His-tag is added by inserting the DNA coding a protein of interest in a vector that has the tag ready to fuse at the C-terminal. B) The His-tag is added using primers containing the tag, after a PCR reaction the tag gets fused to the N-terminal of the gene.

3.4.3 Restriction digestion of PCR product and expression vector-pET32a

The double digestion was performed using the enzymes, *NdeI* and *XhoI*

3.4.4 Reaction mixture

Sterile double distilled water	:	13.8µl
10XNEB Buffer	:	2.0µl
pET32a /PCR product	:	2.0µl
BSA	:	0.2µl
NdeI	:	1.0µl
XhoI	:	1.0µl
Total	:	20.0µl

After adding first enzyme, the digestion mix was immediately kept at 37°C water bath for 1hr 30min and then second enzyme was added to the digestion mixture. This was again kept at 37°C water bath for 1hr. The digested vector was checked on the 0.8% agarose gel.

3.4.5. Elution of digested vector and PCR product

Elution of the digested vector from the gel bands was carried out using GFX microspin column. The digested PCR product was eluted directly using the GFX microspin column.

3.4.6. Ligation

The eluted digest product was ligated using Fermentas ligase.

3.4.6.1 Reaction mixture

Sterile double distilled water	:	1 µl
10X ligation buffer	:	1 µl
Digested vector	:	2 µl
Digested PCR products	:	5 µl
T_4 DNA ligase	:	1 µl
Total	:	10.0 µl

The reaction mixture was mixed gently and incubated 16°C for 2hrs with Fermentas ligase.

3.4.7 Transformation into propagation host (*E.coli* JM109)

- Preparation of competent cells
- Transformation

3.4.7.1 Preparation of competent cells

a) Requirements

1. Luria Bertani broth.
2. TFB-I(transformation buffer I)

Potassium acetate (30mM)	: 0.294g
Calcium chloride (10mM)	: 0.147g
Manganese chloride (50mM)	: 0.9895g
Rubidium chloride (1000mM	: 1.209g

Adjusted to pH 5.8 with 1M acetic acid and made up to 100ml with water. Filter sterilized (0.2μm filter) and stored at 4°C.

3. TFB-II(transformation buffer-II):

MOPS* (10mM)	: 0.2082g
Calcium chloride	: 1.1025g
Rubidium chloride (10mM)	: 0.1209g
Glycerol (15%)	: 15ml

Adjusted to pH 6.5 with 1M KOH and made up to 100ml. Filter sterilized (0.2 μm filter) and stored at 4°C.

*MOPS- 3-(N-morphiline) propane sulfonic acid.

b) Method

- Inoculated *E.coli* JM109 and *E.coli* BL21 (DE3) competent cells to 4ml broth and kept for 3-4hrs in shaker at 180rpm, 37°C.
- Once 0.6OD was reached, 3ml was inoculated into 100ml broth and kept in shaker for 0.6OD at 180rpm, 37°C.
- Chilled the pre-autoclaved oakridge tubes and transformation buffers.
- Poured the 100ml culture into four oakridge tubes in equal amount and centrifuged at 4500rpm for 5min, 4°C.
- Removed the supernatant, dissolved the pellet in 1ml TFB-I initially. Slightly tapped for dissolving. All the pellets were collected in one tube and made up to 25ml with TFB-I(ice cold)
- Incubated in ice for 5min.
- Centrifuged at 4500rpm for 5min, 4°C.
- Resuspended the pellet first in 1ml TFB-II and then add 3ml TFB-II.

- Labeled 1.7ml eppendrof tubes for aliquots.
- Aliquoted 100µl of competent cells into each eppendrof and stored immediately at -70°C for further use.

3.4.7.2. Transformation

After incubation of ligation mix, this mix was initially transformed into host, *E.coli* JM109 competent cell.

a) Requirements

1. *E.coli* JM109 competent cell

   This strain is convenient for initial cloning of target DNA into pET32a vector and for maintain plasmids because they give high transformation efficiencies and good plasmid yields.

2. Ampicillin

b) Method

- Competent cells from -70°C was kept in ice for thawing for 5min.
- Added 2µl of ligation solution to competent cells and mixed slightly with the pipette tip.
- Incubated in ice for 30min.
- Heat shock was given at 42°C and immediately incubated in ice for 5min.
- Added 400 µl LB into the mixture.
- Kept in shaker at 160rpm, 37°C for 45min.
- 100 µl was plated on LB agar Amp^{+}.
- The plate was incubated overnight in 37°C incubator

3.4.8 Isolation of plasmid from transformed colony

After overnight incubation, the transformed colonies were formed. Single colony was inoculated into 4ml LB broth Amp^{+} and incubated in 37°C shaker for 0.6OD. Plasmid isolation was done from this culture using plasmid preparation mini spin kit (GE health care)

3.5 SEQUENCING PCR

Sequencing was based on Sanger's Dideoxy Chain Termination method. It was carried out in ABI 310 Automated Sequencer (Applied Biosystems, Perkin Elmer) using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 3.2 (PE Biosystems).

3.5.1 Thermocycling conditions

The thermo cycling conditions were as follows,

Cycle1(1X)	step 1 :	95°C for 4min
Cycle2(25X)	step 1 :	95°C for 30sec
	Ramp to 96°C at 1°C/sec	
	Step 2 :	55°C for 30sec
	Ramp to 55°C at 1°C/sec	
	Step 3 :	66°C for 4min
	Ramp to 66°C at 1°C/sec	
Cycle3(1X)	Step 1 :	4°C for ∞

3.5.2 Reaction mixture

Sterile double distilled water	: 5.15µl
Sequencing buffer (5X)	: 1.50 µl
Magnesium chloride	: 0.50 µl
Forward/reverse primer	: 0.35 µl
Sequencing mix	: 0.50 µl
DNA	: 2.00 µl
	Total: 10.00µl

3.5.3 Post PCR clean-up

a)Requirements (for single tube)

1. Master mix I:
   i. Sterile double distilled water : 11.5 µl
   ii. 0.5M EDTA : 0.5 µl
2. Master mix II:
   i. Absolute alcohol : 50 µl
   ii. 3M sodium acetate : 2 µl
3. 70% ethanol :100 µl

b)Method

- Transferred the PCR products into 1.7ml tube each.
- Added 12 µl of master mix I.

- Then added 52 µl of master mix II.
- And the mixture was incubated at room temperature for 15 min.
- Centrifuged at 13000rpm for 15min, 20°C.
- Discarded the supernatant and added 100 µl of 70% ethanol (pure grade).
- Centrifuged at 13000rpm for 10min, 25°C.
- Aspirated the liquid using pipette tip.
- After aspiration, the tubes were placed on a tissue paper (inverted for 10min) for air dry.
- Tubes were labeled and gave for sequencing.

3.6 EXPRESSION

Transformation of Rv0288/pET32a (ligated) into expression host BL2 (DE3)

BL21 carries the gene for T7 RNA polymerase under lacUV5 control. It is therefore suited for expression from T7 promoters. This strain contains a pET32a compatible plasmid that produces T7lysozyme, thereby reducing basal expression of target gene.

3.6.1 Gene over expression by induction with IPTG

The over expression of gene was achieved by inducing with isopropyl β-D-thiogalactosidase (IPTG). Induction was performed at different IPTG concentration (0.1mm, 0.5mm, 1mM) and induction time (0hr, 2hr, 4hr, 6hr, 8hr).

a)Method:

- The isolated plasmid was transformed into expression host BL21 (DE3). The transformed culture was plated in LB agar Amp^+ and kept in 37°C incubator for overnight.
- Selected one colony and inoculated it into 4ml LB broth containing ampicillin.
- Kept in 37°C shaker with 180rpm for 0.6 OD.
- Once the required growth is reached, 1ml of culture were inoculated into three 7ml LB broth containing ampicillin.
- This was then kept at 37°C shaker with 180rpm for 0.6 OD.
- As proper growth is obtained, IPTG induction was given at different concentration in each tube say 0.1mM, 0.5mM, 1mM.
- The tubes were then kept at 28°C shaker.
- Starting from 0^{th} hour, 1ml samples were collected for every 2hrs up to 8^{th} hr.
- These samples were centrifuged for 4min at 4000rpm.
- The pellets were stored at -20°C.
- The optimized induction time and IPTG concentration was obtained by SDS-PAGE.

3.6.2 Analysis of gene over expression by SDS-PAGE

a)Requirements (Refer appendix 1)

1. 30% acrylamide
2. 4X Tris-Cl(pH6.8)

b)Method

1. Separating gel (15%,for 10ml):

Sterile double distilled water	: 2.30ml
Acrylamide mix	: 5.00ml
Tris(pH 8.8)	: 2.50ml
SDS	: 0.10ml
APS 10%	: 0.10ml
TEMED	: 0.004ml

Mixed the ingredients gently in the order shown above, ensuring no air bubble formation. And the mix was poured into glass plate assembly. The Bio-Rad mini PAGE gel apparatus was used for performing the experiment. Overlaid gel with water to ensure a flat surface and to exclude air.

2. Stacking gel (4ml):

Sterile double distilled water	: 2.700ml
Acrylamide mix	: 0.670ml
Tris(pH 6.8)	: 0.500ml
SDS	: 0.040ml
APS 10%	: 0.040ml
TEMED	: 0.004ml

Mixed as before, poured on top of set resolving gel, after removing water layer. Inserted the comb, allowed to settle, removed comb and filled with electrophoresis buffer. Top tank onto glass plate assembly was assembled. Then the electrophoresis tank was filled with tris-glycine buffer.

3.6.3 Sample loading

a) Requirements (Refer appendix 1)

1. Phosphate buffer saline
2. 6X-SDS- PAGE sample loading buffer
3. Staining solution
4. De-staining solution
5. Tris-glycine buffer(running buffer)

b) Method

- Cell pellet was taken on every 2hr interval and dissolved in 200µl PBS. From that 17µl sample was taken and mixed with 3µl 6Xloading dye. Then the samples were boiled for 5min at 100°C before loading into gel.
- About 80-100V current was applied for gel running. As soon as the sample reaches the bottom of the gel, it is kept in Coomassie Brilliant Blue (CBB) for staining for 10 min. Then the gel was distained with 7%acetic acid for overnight.

3.6.4 Western blot

a) Requirements (Refer appendix 1)

1. Towbin's buffer(pH 8.3)
2. TBST
3. Developing solution(pH 9.5)

b) Sandwich assembly

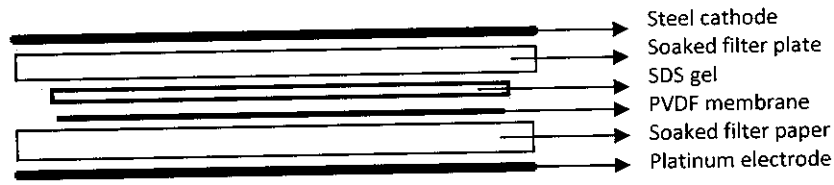


Fig 3.3: Sandwich assembly

c) Method

- Protein was separated by SDS-PAGE.
- Polyvinylidene difluride(PVDF) membrane was cut to the size of gel.
- PVDF membrane was activated with 10ml methanol for 5min (kept in shaker) and kept at room temperature.
- The gel was equilibrated by dipping in 10ml Towbins Buffer (pre-chilled) for 15min.
- The sandwich was assembled. The air bubbles (blocked) removed were by rolling a glass pipette over the sandwich.
- Poured few ml of 1X transfer buffer on the top of the sandwich to avoid drying out during membrane transfer.
- Transfer was done at 20V for 30min in Semi Dry Transfer Cell (Bio-Rad).
- Removed the membrane and washed in 10ml TBST for 2min.
- 10% non-fat milk powder (sagar) was added to the membrane and kept at room temperature in shaker for 1hr followed by TBST wash-3times.
- 1°Ab was added (anti HBC Ab raised in mouse).
- Overnight incubation in cold room (in shaker).
- Washed with TBST for 2min – 3times.

- 2°Ab was added (antimouse IgG fat specific) prepared in 3% BSA. And then kept in shaker for 1hr.
- Washed with TBST for 10 min – 4times.
- Added 10ml of developing solution in absence of light.
- After color change, immediately the membrane was washed with TBST and air dried.

3.7 PURIFICATION

3.7.1 Native lysis

a) Requirements

1. Lysozyme 1mg/ml
2. Lysis buffer:

(i) Sodium dihydrogen phosphate : 50mM
(ii) Sodium chloride : 300mM
(iii) Imidazole : 10mM
(iv) Triton-X-100 : 0.25%

The solution was made up to 100ml.

b) Method

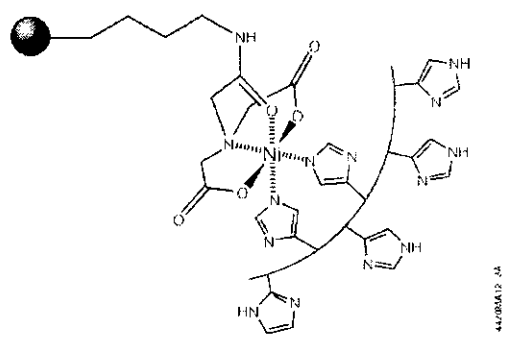
- The bacterial culture at optimized IPTG concentration and induction time was pelleted at 4000rpm for 4min,4°C
- Added 1ml lysis buffer.
- Added 1mg/ml lysozyme and incubated in ice for 30min.
- Cells were lysed by sonicator, 10 pulses for 6times.

- Centrifuged at 10000g for 20min, 4°C.collected the supernatant and pellet separately.
- These supernatant and pellet was loaded in the SDS-PAGE to find whether the protein is solubilized or not. As the protein was not solubilized in supernatant, urea lysis was performed followed by purification through His Link Purification System.

3.7.2 Urea lysis & Purification using His Link Purification System (Promega)

Protein purification of polyhistidine- or HQ-tagged, expressed proteins from a crude *E. coli* cell lysate is done by His Link Protein Purification resin.

Fig 3.4: Chemical structure of His Link Purification Resin



a) Requirements (Refer appendix 1)

1. Urea buffer at pH 6.8,5.9&4.5 : 20ml each
2. PMSF : 2 µl
3. His Link Purification resin :100µl

RESULTS

b) Method

- 100ml of bacterial culture induced with optimized IPTG concentration & induction time was pelleted in two 50ml tubes.
- To the pellet added 2ml of urea buffer (pH6.8) and 2µl of PMSF. This is then transferred to 2ml tube and kept at vortex for 2hr.
- Centrifuged at 10000g for 20min, 25°C.
- The pellet and supernatant was kept separately and was loaded in SDS-PAGE to know where the protein is present i.e., in supernatant or pellet.
- As the protein was present in supernatant, the supernatant was transferred into the 200µl resin. Initially the ethanol in the resin was removed by washing it in water.
- Incubated the sample and resin for about 45min, on a rotating platform to optimize binding.
- The tube was allowed to spin to obtain clear liquid solution.
- The resin was washed 5times with 1ml urea buffer (pH 5.9) –a washing buffer.
- Then the protein was eluted 5times from the resin using 200µl (each time) urea buffer (pH 5.9)-a elution buffer.
- And finally the resin was washed 5times with urea buffer (pH 4.5).
- All the samples were loaded in the SDS-PAGE to know the eluting pH.
- Thus the proteins elution pH was optimized.

3.7.3 BCA assay

The BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 570 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 µg/ml). The BCA method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.

a) Requirements

BCA Protein Assay Kit (Pierce).

b)Method

The protein assay was performed as per the instructions of Pierce BCA Protein Assay Kit manual.

RESULTS

4.1 GRADIENT PCR

Initially, gradient PCR was done to find out the annealing temperature of individual primer pairs.

4.2 AGAROSE GEL ELECTROPHORESIS

The amplified product (Rv0288) was visualized in a 0.8% agarose gel (stained with EtBr at 5µg/ml) along with a 100bp ladder. Bands of size 331bp were observed respectively for Rv0288 (**Fig 4.1**). The band size is 331bp which includes gene sequence, His Tag sequence, restriction enzyme sequence, additional nucleotide sequence.

4.3 PHUSION PCR

The genes were amplified again at the respective annealing temperature of the gene by Phusion PCR for its high fidelity. The PCR products were then loaded in 0.8% agarose gel (**Fig 4.2**) and the gel were eluted in 20µl of sterile double distilled water.

4.4 PREPARATION OF COMPETENT CELL

Competent cells *E.coli* JM109 and *E.coli* BL21 (DE3) were prepared by using transformation buffers and stored at -70°C.

4.5 CLONING AND TRANSFORMATION

4.5.1 Digestion

The pET32a vector and the amplified genes were double digested with the NdeI and XhoI restriction enzymes. The digested product (pET32a) was run in the 0.8% agarose gel along with undigested vector. The digested vector was then gel eluted. The insert was eluted directly.

4.5.2 Ligation

The digested insert and vector was ligated using Fermentas ligase.

4.5.3 Transformation

Competent cell *E.coli* JM109 were transformed with the ligation mixture and selected on LB agar with ampicillin. Colonies were obtained after overnight incubation at 37°C. The colonies were then inoculated into LB broth with ampicillin and plasmids were isolated. The isolated plasmids were subjected to sequence PCR with T7 promoter and T7 terminator primer of the vector to confirm the presence of gene in the vector. A 340bp expected band size was respectively observed for Rv0288.

4.6 SEQUENCING

Sequencing of pET32a+Rv0288 was done and sequenced in an automated sequencer. The sequence were analyzed and checked. The sequence showed maximum homology (**Refer appendix 2**) to the genome sequence of MTB H37Rv available at tuberculist website. To confirm the presence of gene of interest in vector, PCR was performed with the vector specific primers at annealing temperature. The amplified products was run in agarose gel and the presence of gene was confirmed (**Fig 4.3**)

4.7 EXPRESSION

The plasmids were then transformed into *E.coli* BL21 (DE3) for expression studies.

4.7.1 Analysis of expression using SDS-PAGE

E.coli BL21 (DE3) containing recombinant plasmid were grown until mid log phase. They were then induced with different concentration of IPTG and incubated for different time period to find out the optimum condition of expression of maximum protein. Following the induction, the bacterial

cultures were centrifuged at maximum rpm for 2min and the pellet was dissolved in 1X PBS for protein analysis.

E.coli BL21 (DE3) with recombinant vector was subjected to 15% SDS-PAGE gel. The maximum expression was found in **lane 6 of Fig 4.4.,** which was induced with 0.1mM IPTG and incubated for 6hr. The protein size was also compared with the protein marker

4.7.2 Western blotting

Western blot was done to confirm the presence of expressed proteins. As the protein contains six histidine residues in terminal their N-terminal end, a rabbit anti-his antibody was used to detect the protein of interest which was then detected using mouse anti-rabbit Ab conjugated with alkaline phosphatase. After developing, a band was obtained which was then compared with the pre-stained protein marker and the size of protein was found to be 10kDa (**Fig 4.5**).

4.8 PURIFICATION

4.8.1 Native lysis

Native lysis was done with the lysozyme followed by the centrifugation. After centrifugation the pellet and supernatant was analyzed to fine whether the protein is solubilized. By SDS (**Fig 4.6**) it was confirmed that protein was not solubilized. To solubilize the protein urea lysis was performed. Solubilization is important because only solubilized protein can be easily isolated and purified.

4.8.2 Urea lysis

In addition of urea, the protein was solubilized. The solubilized protein was then confirmed by SDS (**Fig 4.7**).

4.8.3 His Link Purification Resin

The resin was initially bound with protein. The proteins were bound to the Ni in the resin. During elution, the bound proteins start to elute at different rate according to the pH of the buffer.

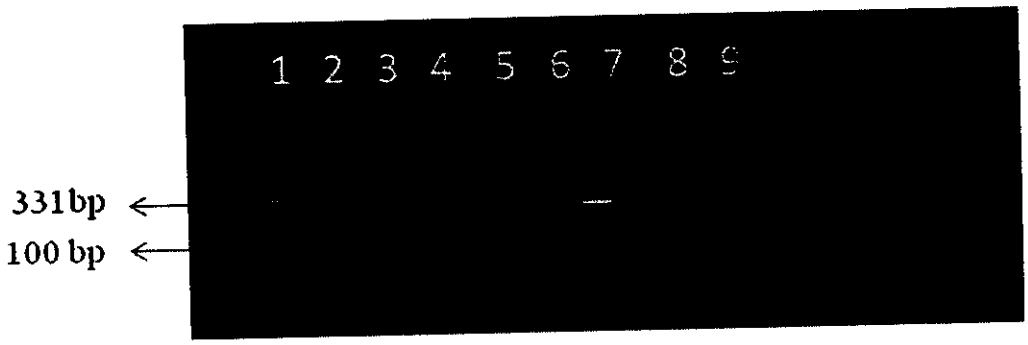
While washing with wash buffer (pH 6.9), all the unbound proteins was washed away and while using buffer at pH 5.8 (**Fig 4.8**), the protein started to elute. Again when pH 4.5 buffer was used the rate elution decreased (**Fig 4.9**). At pH 5.8 the protein was eluted in maximum amount.

4.8.4 BCA assay

The protein concentration was found to be 50μg/ml by BCA assay.

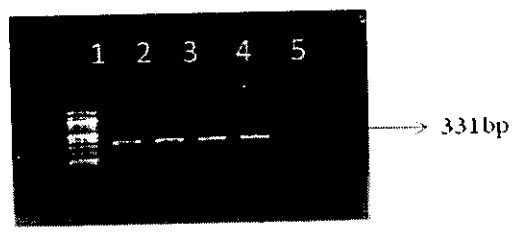
4.9 Figures

Fig 4.1: Gradient PCR of cfp7



Lane1 :100bp marker
Lane2 : Amplification at 65.0°C
Lane3 : Amplification at 64.3°C
Lane4 : Amplification at 63.1°C
Lane5 : Amplification at 61.2°C
Lane6 : Amplification at 58.7°C
Lane7 : Amplification at 57.0°C
Lane8 : Amplification at 55.8°C
Lane9 : Amplification at 55.0°C

Fig 4.2: Phusion PCR of cfp7

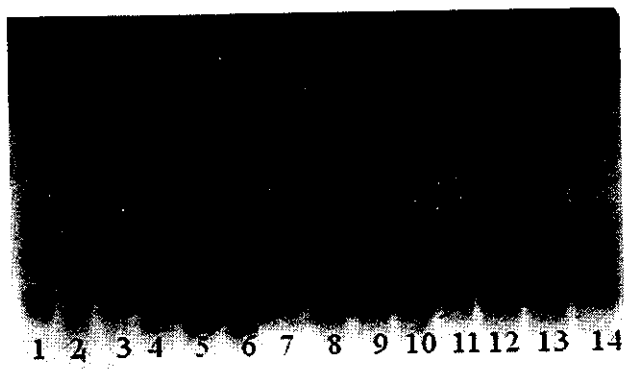


Lane1 :100bp marker
Lane2-5 : Amplification at 57.0°C

Fig 4.3: Amplification of cloned gene

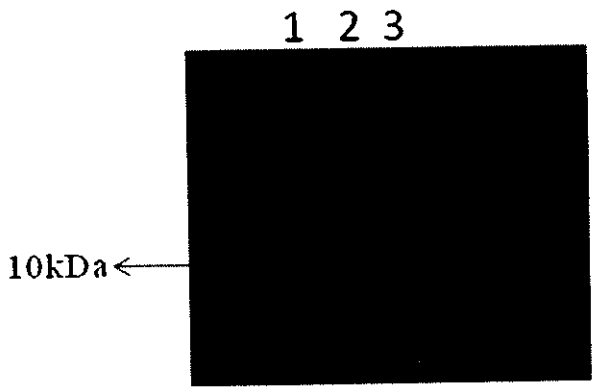
Lane 1 : 100kb marker
Lane 2-4 : cloned plasmid (from colony 1)amplified at 57°C
Lane 5-7 : cloned plasmid (from colony 2)amplified at 57°C

Fig 4.4: Expression at different induction time and IPTG concentration



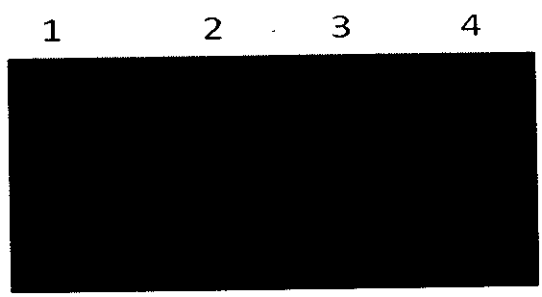
Lane1 : protein marker
Lane 2 : protein without lysis
Lane 3 : 0.1mM IPTG at 0hr
Lane 4 : 0.1mM IPTG at 2hr
Lane 5 : 0.1mM IPTG at 4hr
Lane 6 : ***0.1mM IPTG at 6 hr (optimized conditions)***
Lane 7 : 0.5mM IPTG at 0hr
Lane 8 : 0.5mM IPTG at 2hr
Lane 9 : 0.5mM IPTG at 4hr
Lane 10 : 0.5mM IPTG at 6hr
Lane 11 : 1mM IPTG at 0hr
Lane 12 : 1 mM IPTG at 2hr
Lane 13 : 1 mM IPTG at 4hr
Lane 14 : 1 mM IPTG at 6hr

Fig 4.5: Western blot



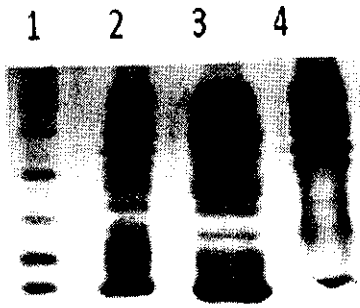
Lane 1, 2 : Eluted protein of cfp7 gene
Lane 3 : Pre-stained protein marker (10kDa)

Fig 4.6: Native lysis



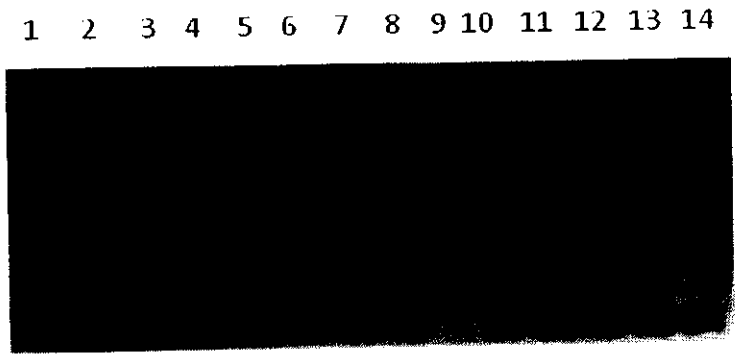
Lane 1 : protein marker
Lane 2 : protein without lysis
Lane 3 : supernatant of native lysis
Lane 4 : pellet of native lysis

Fig4.7: Urea lysis



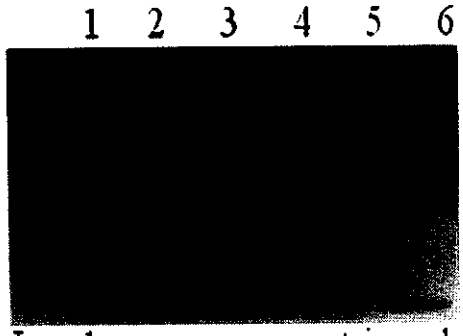
Lane 1 :protein marker
Lane 2 :protein without lysis
Lane 3 :supernatant sample after lysis
Lane 4 :pellet sample after lysis

Fig 4.8: Protein purification (gel A)



Lane 1 :protein marker
Lane 2 :protein after washing the resin
Lane 3 to 9 :represents binding of protein to resin at buffer pH 6.5
Lane 10 to 14 :binded protein starts to elute as the pH of buffer changes(i.e.,pH5.9)

Fig 4.9: Protein purification (gel B)



Lane 1 :protein marker

Lane 2to6 :represents elution was decreased at buffer pH 4.5

DISCUSSION

DISCUSSION

Striking advances were made in our understanding of the basic biology and immunology of tuberculosis in the past decade, capped by sequencing of the genome of MTB (Cole *et al.*, 1998). These advances will lead to identification of mycobacterial proteins that may be a useful vaccine components or diagnostic reagents. In the present study cloning, expression, purification of Rv0288, a MTB gene was carried out. This gene belongs to RD3 and codes for low molecular weight protein of unknown function. The genes contained in RD have been the object of a number of studies focusing on diagnosing of MTB infection, the search for efficient vaccine candidates and virulence (Kearns *et al.*, 1999 and Kaufmann et al., 2006)

The gene was amplified by gradient PCR and the annealing temperatures were found. The amplified gene was separately cloned into NdeI and XhoI site of pET32a vector. Both the T7 promoter and the lac operon are located at 5' to the genes of interest. When T7 RNA polymerase is present, the lac operon is not repressed and the transcription of gene of interest proceeds rapidly. Because T7 is a viral promoter, it transcribes rapidly and profusely for as long as the T7 RNA polymerase is present (Studier *et al.*, 1986). The expression of the gene of interest increases rapidly as the amount of mRNA transcribed from gene of interest increases. Within a few hours the inserted gene is one of the most prevalent components of the cell (Unger *et al.*, 1997).

One of the most important parts of the pET32a expression system involves the fact that gene of interest is not transcribed unless T7 RNA

polymerase is present. Prokaryotic cells do not produce this type of RNA and therefore the T7 RNA polymerase must be added. Usually, the host cell for this expression system is the bacterium which has been genetically engineered to incorporate the gene for T7 RNA polymerase, the lac promoter and the lac operator into genome. When lactose or molecule similar to lactose is present inside the cell, transcription T7 RNA polymerase is activated (Novagen, 2003).

Control of pET expression system is accomplished through the lac promoter and operator. Before gene of interest can be transcribed, T7 polymerase must be present. The gene on the host cell chromosome usually has an inducible promoter which is activated by IPTG. This molecule, IPTG, displaces the repressor from the lac operon. Since there are lac operators both the gene encoding T7 polymerase and also the gene of interest, IPTG activates both the genes. Therefore, when IPTG is added to the cell, T7 polymerase is expressed, and quickly begins to transcribe gene of interest which is translated as protein of interest (Stratagene, 2006).

cfp7 was expressed when cloned into pET32a and introduced into *E.coli* BL21 (DE3) strain. This was analyzed by SDS-PAGE and confirmed by western blotting. As the protein was insolublized in lysozyme, urea lysis method was used for solubilization. A protein that is expressed as an inclusion body and has been solubilized with urea was purified using His Link Protein Purification System.

Protein purification of polyhistidine- or HQ-tagged, expressed proteins from a crude *E. coli* cell lysate is done by His Link Protein

silica resin modified to contain a high level of tetradentate chelated nickel (>20mmol Ni/ml settled resin). The high level of nickel available on the particle surface means that larger quantities of polyhistidine- or HQ-tagged protein can be purified per volume of resin used. The resin is designed to efficiently capture and purify bacterially expressed polyhistidine- or HQ-tagged proteins. Polyhistidine binding to immobilized nickel is most efficient at a pH well above the pKa of polyhistidine (~ 6.0). Above pH 7, more than 90% of the imidazole moieties of histidine will be deprotonated and available to bind to nickel. Lowering the pH of a binding reaction below the pKa of histidine leads to protonation of the histidine tag and release from the resin. Thus the proteins were eluted from the resins (Promega, 2009).

Finally, concentration of the protein in the eluted samples was found by BCA (bi-cinchonic acid) protein assay kit.

MTB interferes directly with the macrophages, inhibiting the acidification and maturation of phagasome. The above proteins may be expressed only when it infects host cells, as previous studied in the MRG laboratory, RGCB Thiruvananthapuram, have shown that their promoters get activated upon infection. The cloning, expression and purification of individual mycobacterial antigen are essential for understanding the pathogenic mechanism of mycobacteria and immune response against them. Therefore, this gene may play an important role in survival of mycobacterium in host macrophages.

The purified gene products may be further studied to develop the subunit vaccine. These subunit vaccines will be an effective and cheapest agent against MTB (Anderson *et al.*, 1992, Brandt *et al.*, 2002 and Talbot *et al.*, 1997).

SUMMARY

SUMMARY

MTB genome contains about 4000 genes, of which approximately one-third codes for unknown functions and are classified as hypothetical proteins and a few of them were recently found to have certain functions. The present work is on cloning, expression and purification of cfp7 gene that encode for low molecular weight proteins (unknown functions) which provokes cell mediated immunity. This may be promising subunit vaccine candidates in future (Kearns *et al.*, 1999 and Kaufmann et al., 2006).

The steps involved in cloning, expression and purification includes,

- Amplification cfp7 from H37Rv (virulent strain) using gradient PCR and found the optimized annealing temperature.
- Restriction digestion and ligation of the genes in to pET32a vector.
- Transforming *E. coli* JM109 strain for increasing plasmid efficiency.
- Sequencing the plasmid clones for checking the ORF.
- Transforming the plasmid (insert+vector) into *E. coli* BL21 (DE3) for expression.
- Expression studies after IPTG induction.
- The proteins were analyzed by SDS-PAGE and confirmed by performing Western Blotting.
- Purification of expressed proteins using His Link Protein Purification System.
- Protein assay by BCA method.

Still many procedures are there for complete purification of the protein. The purified protein can be studied and analyzed for its therapeutic uses

APPENDICES

APPENDICES

APPENDIX 1

REAGENTS AND CHEMICALS

REAGENTS AND CHEMICALS

AGAROSE GEL ELECTROPHORESIS

10X TBE buffer

Tris-base - 108g

Boric acid - 55g

0.5M EDTA - 20ml

This is made up to 1000ml with double distilled water.

50X TAE buffer

0.5M EDTA - 200µl

Tris-base - 242g

Glacial acetic acid - 57.1 ml

This is made up to 1000ml with double distilled water.

TRANSFORMATION

Ampicillin - 1mM

SDS-PAGE

4X Tris-Cl (pH 6.8)

Tris-base - 6.05g

Water - 100ml

The pH was adjusted with 1N HCl and 0.4g of SDS was added.

4X Tris-Cl (pH 8.8)

Tris-base - 18.28g

Water - 60ml

The pH was adjusted with 1N HCl and 0.4g of SDS was added.

Electrophoresis buffer (pH 8.3)

Tris -3.02g

Glycine -14.4g

SDS - 1g

The pH was adjusted with 1N HCl and made up to 1000ml with double distilled water.

Acrylamide

Acrylamide - 30g

Bis- Acrylamide - 0.8g

Water - 80ml

The above reagents was mixed thoroughly in magnetic stirrer and made up to 500ml with double distilled water.

6X-PAGE loading dye

4X- Tris stacking buffer - 3.5ml

Glycerol - 1.5ml

Bromophenol blue - 0.6mg

The mixture was made up to 5ml with double distilled water and stored at -20°C.

15% separating gel (for 10ml)

Sterile double distilled water - 2.3ml

Acrylamide mix - 5ml

Tris (pH8.8) - 2.5ml

SDS - 0.1ml

Ammonium persulphate (10%) -0.1ml

TEMED -0.0004ml

15% stacking gel (for 4ml)

Sterile double distilled water - 2.7ml

Acrylamide mix - 0.670ml

Tris (pH8.8) - 0.5ml

SDS - 0.04ml

Ammonium persulphate (10%) -0.04ml

TEMED -0.0004ml

Staining solution

Coomassie Brilliant Blue - 0.1ml

Methanol - 20%

Acetic acid - 0.5%

Destaining solution

Methanol - 30%

Acetic acid - 7%

1X PBS (pH 7.4)

NaCl - 8g

KCl -0.2g

Na_2HPO_4 - 1.44g

KH_2PO_4 - 0.24g

This was made up to 1000mlwith sterile double distilled water.

WESTERN BLOT

Towbin's buffer (pH-8.3)

Tris - 3g

Glycine -14.4g

Methanol - 200ml

This was made up to 1000ml with sterile double distilled water and stored at 4°C.

TBST

Tris - 12.14g

NaCl - 9g

Tween20 -1ml

This was made up to 1000ml with sterile double distilled water.

Developing solution

1M tris (pH-9.5) - 1ml

0.5M NaCl - 0.2ml

300mM $MgCl_2$ -0.1ml

This was made up to 1oml with sterile double distilled water.

NBT : 5% in 70%DMF

BCIP : 5mg of BCIP in 0.1ml sterile double distilled water

Primary antibody

Anti-HBC antibody raised in mouse.

Secondary antibody

Mouse monoclonal anti IgG. (sigma, AP conjugate)

Milk powder

Dissolved 1g of milk powder (sagara) in 10ml TBST.

NATIVE LYSIS

Lysis buffer

NaH_2PO_4	-50mM
NaCl	- 300mM
Imidazole	- 10mM
Triton-X-100	-0.25%

The pH was adjusted to 8 and made up to 100ml with sterile double distilled water.

APPENDIX 2

MULTIPLE ALIGNMENT SEQUENCES

FORWARD PRIMER AND CLONED VECTOR SEQUENCE

DSD1-G08-026.seq - Forward primer sequence
DSD2-H08-025.seq - Forward primer sequence
Pet wt insert Insilico – Cloned vector sequence

APPENDIX 3

CLONED VECTOR SEQUENCE

PeT 32a – Rev Compl sequence and gene sequence

TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCG
TGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCC
ACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGC
TTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCT
GATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAA
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GGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAA
CGTTTACAATTTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTC
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GCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGT
GCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGA
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CATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCG
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ATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATA
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GCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAG
AGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTG
TAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAG
TCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAAC
GGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGC
GTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGC
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ATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGA
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TGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATATGGTGCACTCTCA
GTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGG
TCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCG
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ATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGATGCTCACGATACGGGTTACTG
ATGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAAACAACTGGCGGTATGGATGCGGCGG
GACCAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATGTAGGTGTTCCACA
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TTTCCAGACTTTACGAAACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTCGCAGACGTT
TTGCAGCAGCAGTCGCTTCACGTTCGCTCGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCA
ACCCCGCCAGCCTAGCCGGGTCCTCAACGACAGGAGCACGATCATGCGCACCCGTGGGGCCGCCA
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- T7 terminator

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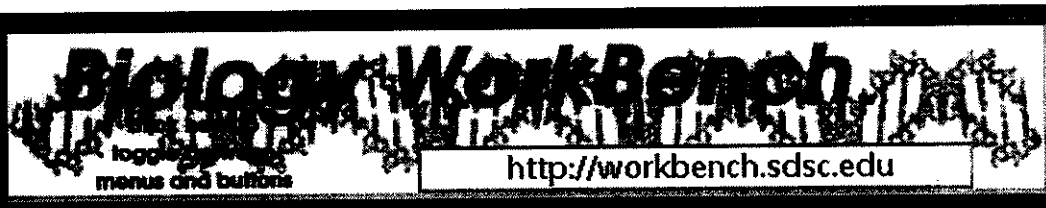

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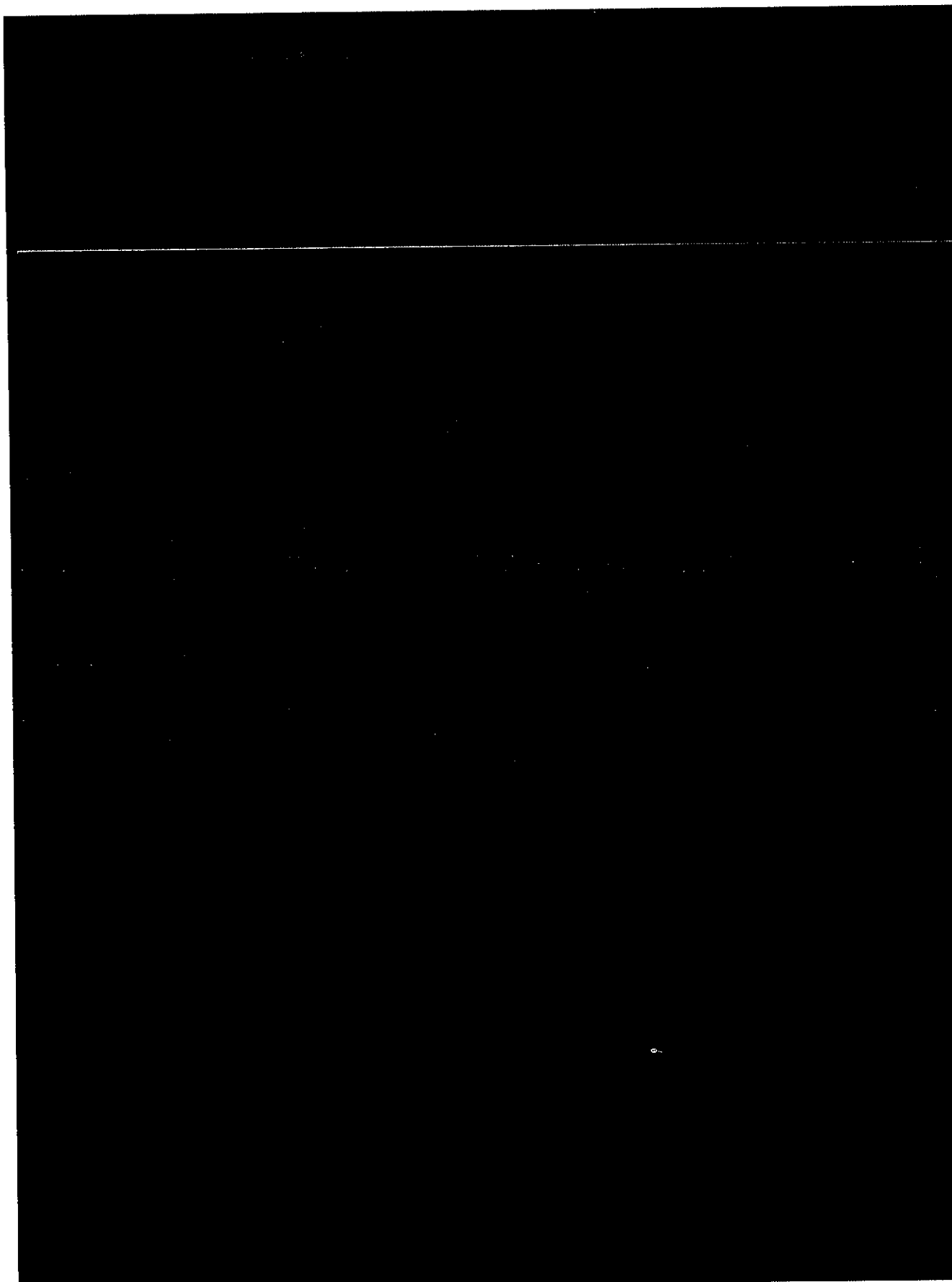

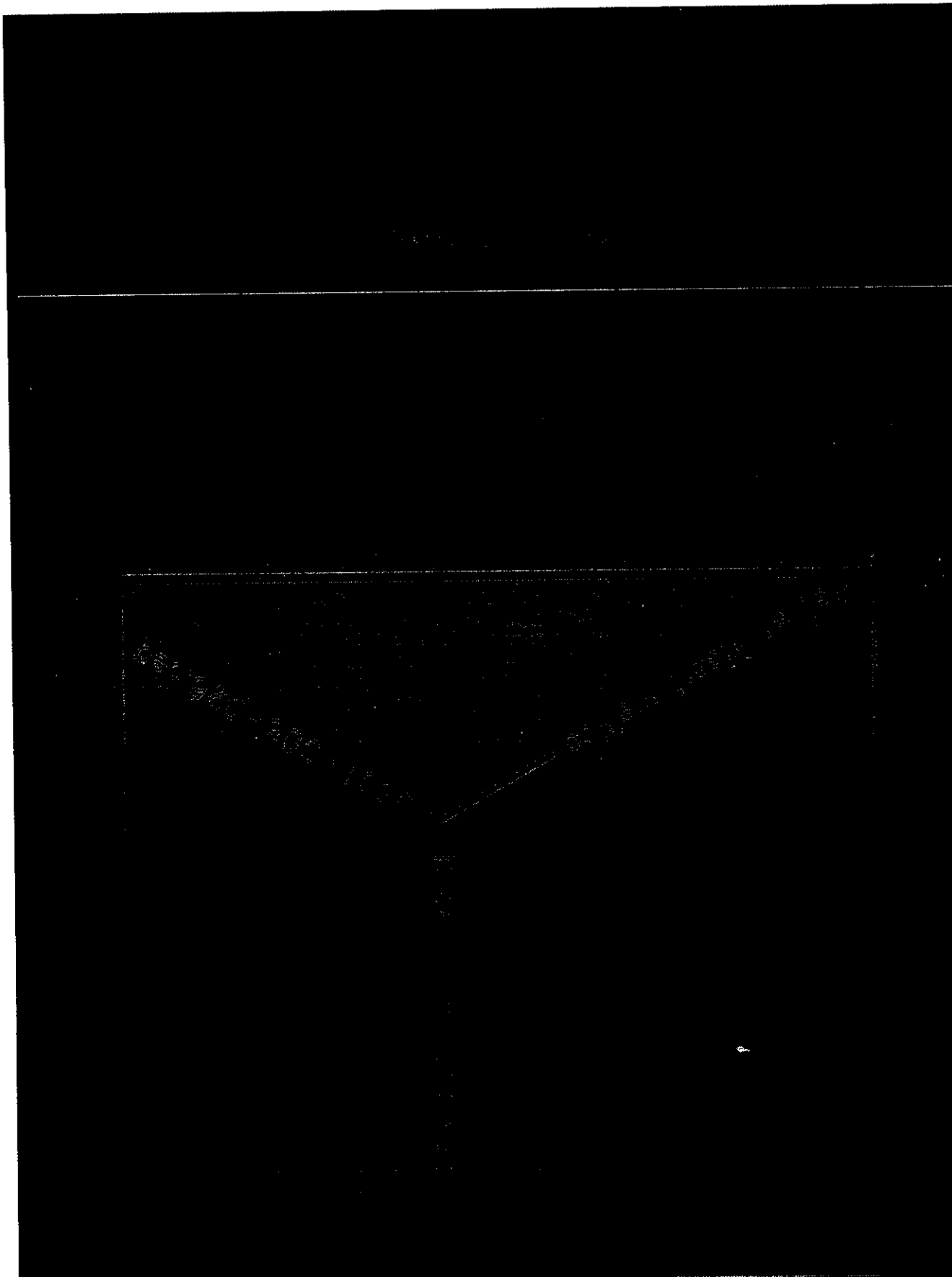

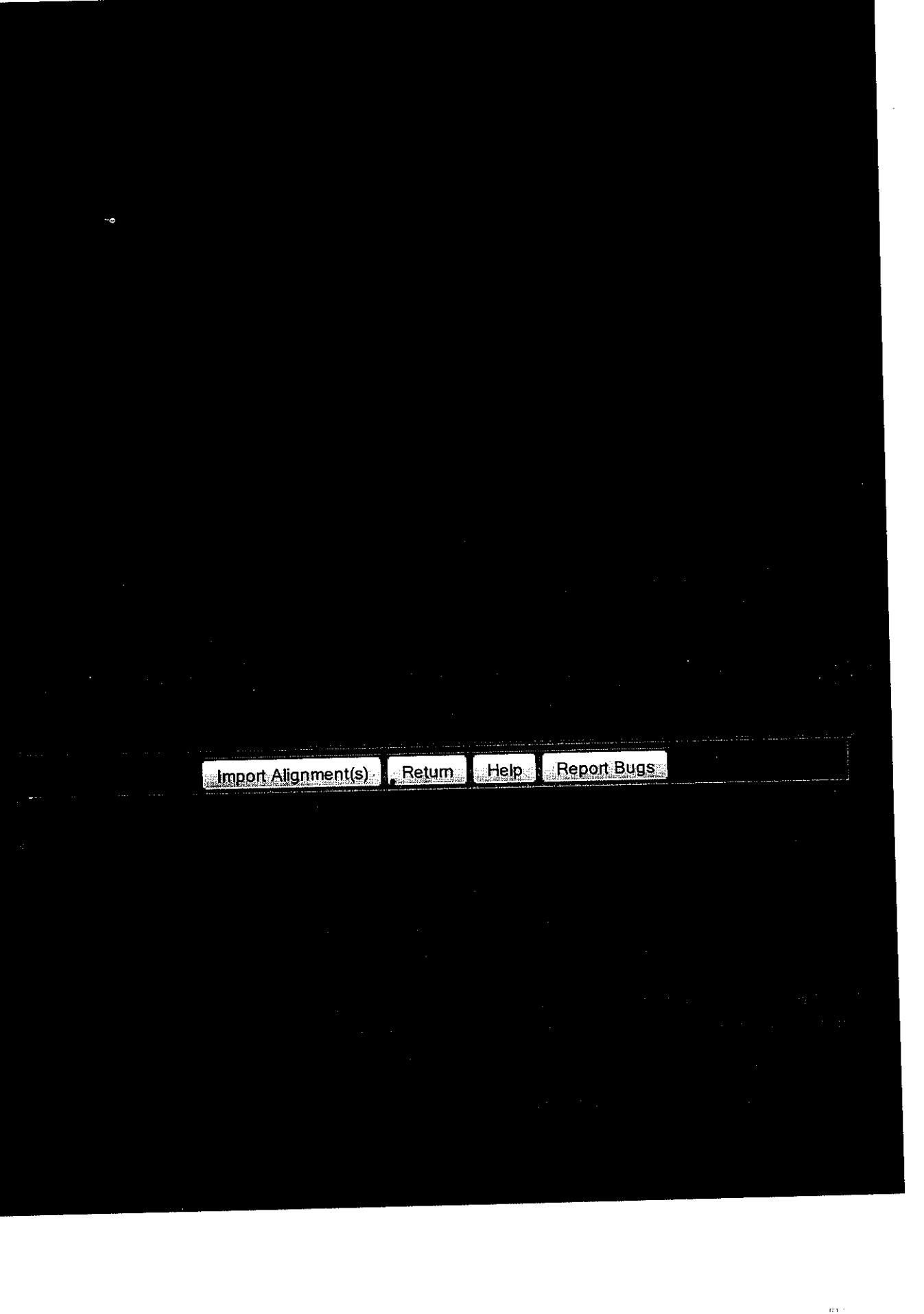
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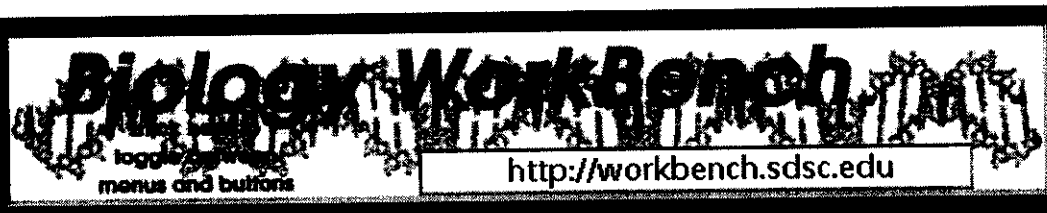

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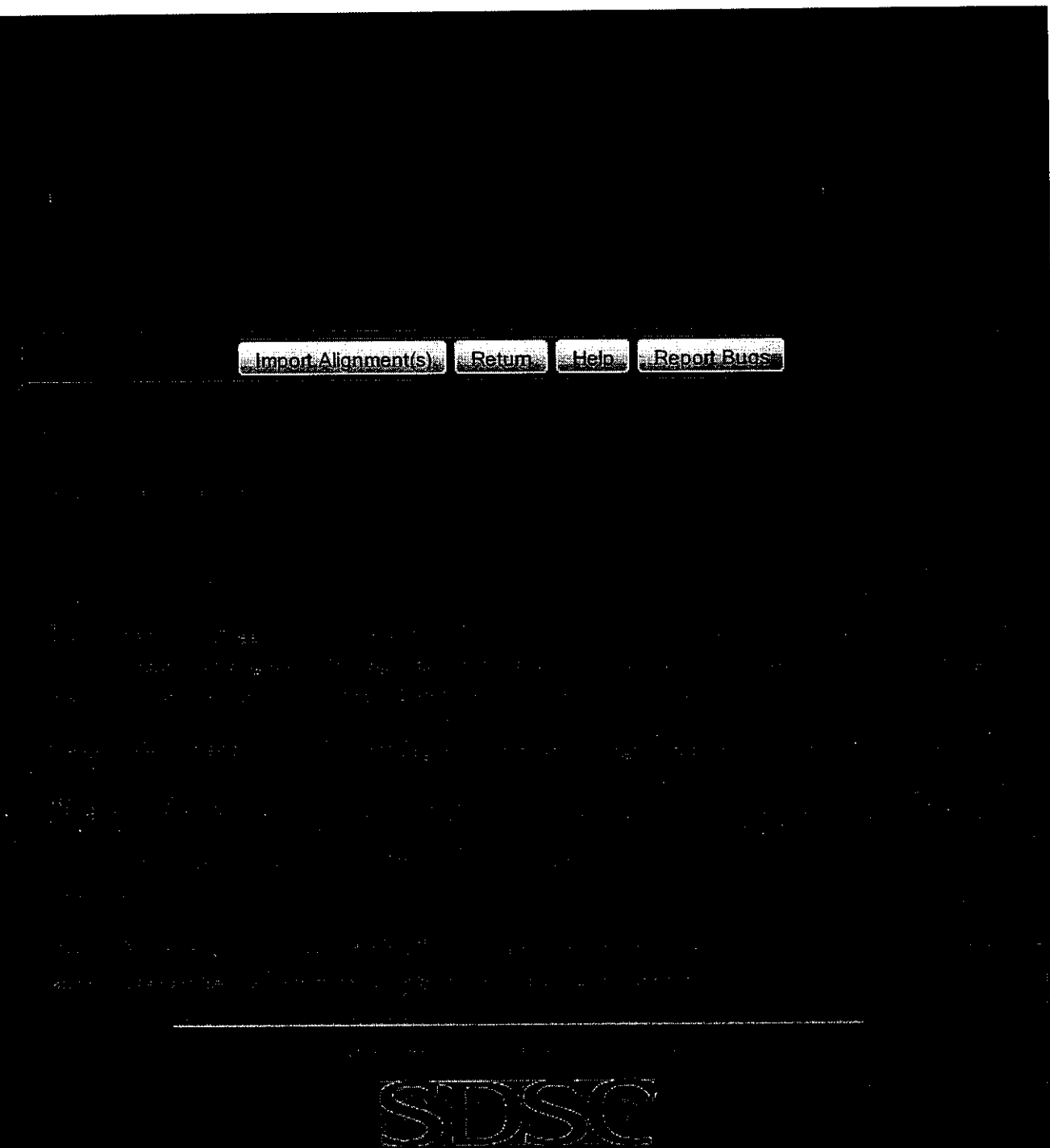
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