GROWTH KINETICS AND CHARACTERIZATION OF *MYCOBACTERIUM FORTUITUM* SENSE AND ANTISENSE MUTANTS OF SIGMA FACTOR *SIGH*

By

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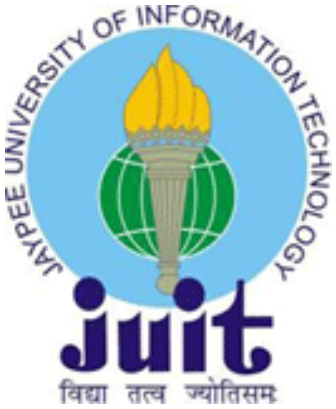

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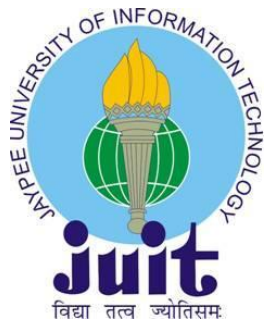

CERTIFICATE

This is to certify that the work titled **"Growth Kinetics and Characterization of *Mycobacterium fortuitum* Sense and Antisense Mutants of Sigma Factor *sigH"*** has been ***submitted*** by **Ms. Ritu Ghildiyal (142551)** in partial fulfillment for the award of degree of **M. Tech** in **Biotechnology** of Jaypee University of Information Technology, Waknaghat, Solan - 173234 has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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ABSTRACT

Mycobacterium fortuitum is an important clinical pathogen causing localized abscesses, pulmonary disease and disseminated infections in humans. It is fast growing non tuberculous mycobacteria. It has worldwide distribution and found in natural and processed water, dirt and sewage. Among all the NTM, it is responsible for 67% of infections. Severity of infection is more in immunocompromised patient. Sigma factors play vital role in different stress responses as it regulates the expression of gene. Sigma factor H (*sigH*) plays central role in a network that regulates stress responses likely to be important in *Mycobacterium tuberculosis* pathogenesis. With an aim to study role of sigma factor H in regulation of stress response and pathogenesis of *M. fortuitum*, sense and antisense knockouts were constructed using pMV261 vector. Role of *sigH* was determined under different stress conditions by subjecting the sense and antisense mutants along with wild type *M. fortuitum* as control. *sigH* was involved in hypoxia-induced, acid-induced stress response, and other in-vitro stress conditions. We have found that *sigH* plays an important role during acidic stress at pH 4.5 and nutrient starvation whereas during other stress conditions such as heat stress at 50^{o}C, oxidative stress and SDS stress, *sigH* may not have its role in *M. fortuitum.* Our study suggests that the overexpression as well as underexpression of *sigH* inhibits its own expression under invivo conditions, and the other genes responsible for the survival of *M. fortuitum* in mice model of infection.

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List of abbreviations

^{0}C	Degree Celsius
AIDS	Acquired Immuno-Deficiency Syndrome
ATCC	American Type Culture Collection
CDC	Centres for Disease Control and Prevention
CDRI	Central Drug Research Institute
CFU	Colony Forming Units
ClpB	Caseinolytic Peptidase B Protein
DnaK	70 Kilodalton Heat Shock Proteins
ECF	Extra Cytoplasmic FunctionSigma factors
HIV	Human Immunodeficiency Virus
Hrs	Hours
Hsp60	Heat shock protein 60
LAM	Lipoarabinomannan
NCBI	National Centre for Biotechnology Information
NTM	Nontuberculous Mycobacteria
O.D	Optical Density
PCR	Polymerase Chain Reaction
RGM	Rapidly Growing Mycobacteria
RNase H	Ribonuclease H
RPM	Revolutions per Minute
sigH	Sigma factor H
TB	Tuberculosis

UN	United Nation
WHO	World Health Organization

CHAPTER 1

Introduction

INTRODUCTION:

Mycobacterim fortuitum is fast growing non tuberculous mycobacteria (NTM) ubiquitously present in nature [1, 2]. NTM or environmental mycobacteria can be found in diverse environments across the world, and some species (including *M. fortuitum*) are capable of infecting humans and animals (WHO, 2004). It comes under the group of atypical mycobacteria and is capable to cause infection from normal individuals to immunocompromised patients [1, 3]. It generally causes skin, bone, and soft tissue infections following local trauma or surgical procedures [4]. *M. fortuitum* causes localized abscesses, pulmonary infections extended to disseminated infections in humans [3, 5]. Main target groups of the diseases due to *M. fortuitum* are the elderly, and the HIV-infected with compromised immunity (CDC, 1987; JIU against TB & WHO, 1982). Immunocompromised patients are more susceptible of developing an infection [6]. The route of infection was surgical site and it was deciphered that the patient had acquired the pathogen during postoperative. The pathogen does not spread from person to person [7]. NTM species does not cause tuberculosis and among all the NTM, *M. fortuitum* is responsible for 67% of infections. Mortality rate due to localized infection by *M. fortuitum* is rare but in immunocompromised patients the death rate is high due to extensive pulmonary or disseminated diseases [5]. Cases of nosocomial infection, breast infection [8], sporadic infection, cutaneous infection as well as pulmonary infection have been reported. *M. fortuitum* has been implicated in numerous outbreaks of hospital or health care associated infections including sternal wound infections [17], surgical wound infections following plastic surgery [20], post-injection abscesses [21] [22], catheter infections and respiratory disease outbreak [23]

Infection of *M. fortuitum* inside the liver and kidney creates lesions. During infection, granuloma is formed and the bacteria reside inside the macrophages. Sigma factors help the bacteria to survive inside the macrophage having a hostile environment. Macrophages have acidic environment and the bacterium have several strategies to survive inside the macrophages. Sigma factors help in the initiation of the transcription. Sigma factors are classified into four categories from group 1 to group 4. Group 4 contains extracytoplasmic sigma factors. Extracyloplasmic sigma factors (ECF) are responsible to regulate gene expression during environmental signals. Sigma factor H *(sigH*) plays a critical role during stress conditions. *sigH* regulates the expression of other sigma factors like *sigB* and *sigE*

(expressed during stress conditions). *sigH* regulates the heat and oxidative stress responses likely to be important in *Mycobacterium tuberculosis* pathogenesis. Pathogenesis of *M. fortuitum* is still not clear. *Mycobacterium tuberculosis* has 13 sigma factors and 10 of them regarded as ECF. *sigH* of *M. tuberculosis* has been well studied. Sigma factor H of *M. tuberculosis* activates genes during different stress conditions like heat stress, oxidative stress, diamide stress [57, 61] and also during macrophage infection. *sigH* induces the expression of *dnaK* and *clpB* genes during heat stress. It has been documented that the *sigH* mutant of *M. tuberculosis* showed a distinctive infection phenotype. The mutants achieved high bacterial counts in lung and spleen that persisted in tissues in a pattern identical to those of wild-type bacteria. Mutants also produced a blunted, delayed pulmonary inflammatory response, and recruited fewer CD4+ and CD8+ T cells to the lung in the early stages of infection. *sigH* of *M. tuberculosis* mediate the transcription of at least 31 genes directly and that it modulates the expression of about 150 others. *sigH* regulon also involved in the maintenance of intrabacterial reducing capacity. *sigH* gene is responsible for the bacterial growth and survival within the host as it induces thioredoxin regulon which is responsible for the reduction of reactive oxygen species and protects the cell wall [70].

sigH gene was identified in *M. fortuitum* but its role is still not known. Therefore, this study indicates the role of sigma factor H in *M. fortuitum* under different stress conditions such as oxidative stress condition, heat stress, sds stress, nutrient starvation conditions. *sigH* is involved in stress conditions so we have tried to observe its response under stress conditions. *In-vivo* analysis has also been done to elucidate the role of *sigH* in fortuitum.

CHAPTER 2

Review of Literature

REVIEW OF LITERATURE

2.1 The Bacterium: *Mycobacterium fortuitum*

Mycobacterium fortuitum is rapidly growing non-tuberculous mycobacteria (NTM)[1, 3]. The non-tuberculous mycobacteria (NTM) comprises all of those Mycobacterium species that are not members of the *Mycobacterium tuberculosis* complex [9]. Diseases caused by NTM in humans are mostly due to *M. fortuitum, M. chelonae* and *M. abscessus*. *Mycobacterium fortuitum* complex includes *Mycobacterium fortuitum* and *Mycobacterium chelonae* species [10]. On the basis of standard biochemical reactions there are three subgroups of *M. fortuitum* named as *M. fortuitum* subsp. *fortuitum and M. fortuitum* subsp. *peregrinum* and the third group unnamed third biovariant [10]. *M. fortuitum* is an acid fast gram positive bacilli. Da Costa Cruz gave the designation "*M. fortuitum*" to a strain of RGM (ATCC 6841)[2, 11]. It is ubiquitously present in nature [1]. The envelope of mycobacteria contains three structural components: plasma membrane, wall and capsule [12]. The plasma membrane is similar to other bacteria and it is surrounded by complex of carbohydrates and lipids. The whole complex is surrounded by capsule of polysaccharide, proteins and small quantity of lipids [12]. Cell wall contains mycolic acid, arabinogalactan, and peptidoglycan [13] and large amount of C_{60}-C_{90} fatty acids [14]. The fast growing *M. fortuitum* have phosphoinositol capped LAMS (lipoarabinomannan) as PILAMS while the slow growing mycobacteria like *M. tuberculosis* have the LAM capped with the mannose residue at the β-Ara residue terminal and referred as ManLAMs [14]. Due to the low permeability of envelop, the influx of small hydrophilic agents is extremely slow and is thought to be one of the major factors involved in the resistance of mycobacteria to β-lactam antibiotics [13]. *M. fortuitum* is nonpigmented rapidly growing mycobacteria (RGM) and is extremely hardy. It can survive in the most hostile of environments and also able to survive at 45°C [15].

2.2 History:

The first outbreak of a *M. fortuitum* infection in human was in 1936 from an abscess that resulted from a vitamin injection [16], nosocomial outbreaks of infection with rapidly growing mycobacteria have been documented. These outbreaks are typically associated

with surgical or clinical devices contaminated with water from a hospital or municipal water system. In the community setting, only sporadic infections have been reported, usually resulting from the contamination of a traumatic wound with soil or water [16]. *M. fortuitum* was the designation given by da Costa Cruz [2, 11] to a strain of RGM (ATCC 6841) isolated from a human post-injection abscess in 1938. Health care-associated outbreaks and pseudo-outbreaks occurred due to the exposure to tap water or water sources such as ice, ice water, and water-based solutions [17] [18] [19] . Contaminated ice machines are also an important source of *M. fortuitum*. *M. fortuitum* has been implicated in numerous outbreaks of hospital or health care associated infections including sternal wound infections [17], surgical wound infections following plastic surgery [20], post-injection abscesses [21] [22], catheter infections and respiratory disease outbreak [23] . *M. fortuitum* has also been recovered from sporadic cases of surgical wound infections and is the most common RGM species in women with surgical wound infections following augmentation mammaplasty [17, 24]. Two outbreaks of postoperative wound infections due to organisms of the *Mycobacterium fortuitum* [1, 3] complex (*Mycobacterium chelonei* and *M. fortuitum*) occurred among patients who underwent open-heart surgery were also reported. In one hospital, 19 of 80 patients who underwent cardiac surgery within an l0-week period developed sternal infection with *M. chelonei*. In the second hospital, four of nine patients who underwent cardiac surgery within a two-week period developed sternal incisional infection with *M. fortuitum* [25]. Fifteen cancer patients have developed catheter-related infections caused by the *M. fortuitum* complex (*M. fortuitum* and *Mycobacterium chelonae*) at M. D. Anderson Cancer Centre since 1978 [26]. Recent reported outbreak was an infection after tattooing in healthy individuals. This may be caused because of the unsterile instrumentation or unsterile water used for diluting tattoo ink to dilute colour [27]. Sporadic cutaneous infections were also reported in 2015 due the *M. fortuitum* [28]. Case of a 3-year-old girl with intranasal tumour-like swelling associated with cervical lymph nodes due to *M. fortuitum* infection was reported [4].

2.3 Epidemiology and Clinical Manifestations

It widely distributed in freshwater rivers and lakes, seawater, and wastewater from hospitals [1], animal drinking troughs[9], municipal tap water [15] and in soil. Instead of this *M. fortuitum* was also isolated from raw milk and from tissue samples of feral

buffaloes [9]. Because of its ubiquity, human infections have been reported from most geographic areas in the world [15]. *M. fortuitum* is an oligotrophs as it can survive under low nutrient conditions. It is able to grow at low carbon levels [>50 µg assimilable organic carbon (AOC) l^{-1}] [9]. This property makes it an effective competitor in low nutrient, and disinfected environments (drinking water) [9]. Formation of biofilm [29] helps in its survival, persistence, and growth in drinking water distribution systems. It helps in nutrient cycling as it has ability to degrade and metabolize complex hydrocarbons present in soil [9]. *M. fortuitum* is pathogenic to humans and animals as it is widespread in the environment [30]. In most of Indian studies the infection caused by NTM is low but species like *M. fortuitum, M. avium, M. scrofulaceum* etc., have been isolated in different studies. Out of all the species of mycobacteria, nearly one third species are associated with disease in humans. NTM species associated with human disease are : *M. avium, M. intracellulare, M. kansasii, M. paratuberculosis, M. scrofulaceum, M. simiae, M. habana, M. interjectum, M. xenopi, M. heckeshornense, M. szulgai, M. fortuitum, M. immunogenum, M. chelonae , M. marinum, M. genavense, M. bohemicum, M. haemophilum, M. celatum, M. conspicuum, M. malmoense, M. ulcerans, M. smegmatis, M. wolinskyi* and *M. goodii, M. thermoresistible, M. neoaurum, M. vaccae, M. palustre, M. elephantis, and M. septicum and M. nonchromogenicum* [3].

NTM infections in humans are classified into four distinct clinical syndromes:

1. Pulmonary disease,
2. Lymphadenitis,
3. Cutaneous disease, and
4. Disseminated disease [31]

The *M. fortuitum* group causes community acquired diseases and accounts for 60% cases of localized cutaneous infections, but it rarely causes chronic mycobacterial pulmonary disease [15, 17, 24, 30]. 63% of extrapulmonary diseases were caused by the infection of *M. fortuitum* group [68]. This group also accounted for 16% of the pulmonary diseases [32], 25% infections associated with skin and soft tissue infection including cervical lymphadenitis, mastoiditis, and meningitis. It is also a common pathogen for lung infection and rarely cause disseminated diseases [15]. *M. fortuitum* in also responsible for health care-associated disease like it is responsible for 60% to 80% of cases of postsurgical wound infections and catheter infections [15].

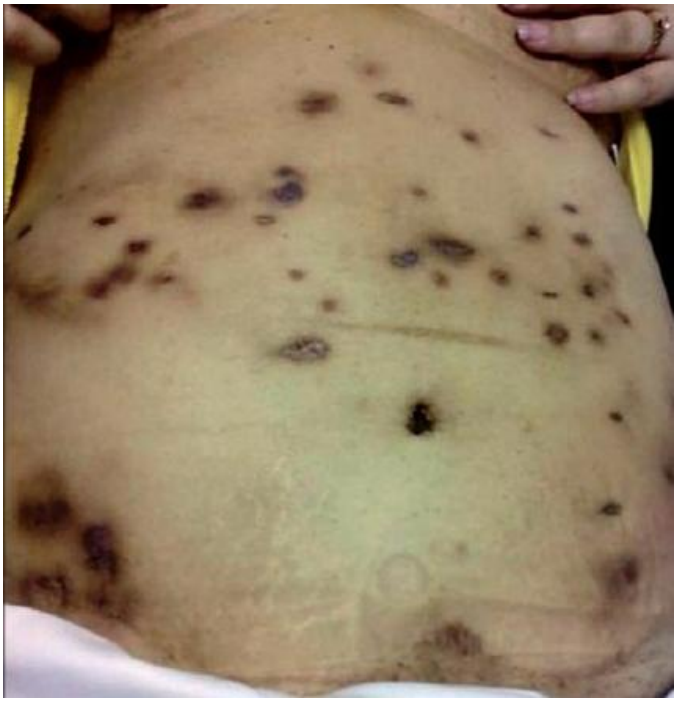

Fig 1: *Mycobacterium fortuitum* abdominal wall abscesses following liposuction [adapted from 69]

Common clinical features of infection are:

1. Local organ specific signs and symptoms to persistent high grade fever.
2. Night sweats.
3. Anaemia
4. Weight loss in addition to non-specific symptoms of malaise, anorexia, diarrhea, myalgia and occasional painful adenopathy [69].

Infection of *M. fortuitum* due to contaminated materials:

Catheter infections*:* *M. fortuitum* accounts for the infection of indwelling venous access catheters and vascular shunts. Catheter infections may occur during duration of catheter placement, immunosuppression, and prior antimicrobial therapy [33, 34]. Symptoms include local manifestations of catheter infections, such as erythema, drainage at the site, and pain. Systematic imflammatory response, fever, rigors with other mycobacterium symptoms may also occur. *M. fortuitum* is responsible for the dialysis-acquired infections in both intravascular and peritoneal mechanisms of renal replacement therapy [3, 35].

Skin and soft tissue infections: Skin and soft tissue infection is caused by using a variety of contaminated solutions such as medications not approved by the US Food and Drug

Administration, local anaesthetic agents, and steroids dispensed in multiuse vials, needles that are reused or rinsed in tap water. Persons who received adrenal cortex (during naturopathic health and weight loss programs) injections may also suffer from post injection infection with abscess formation. Cosmetic surgical infection, postsurgical wound infection has been reported after the use of contaminated solutions, instrumentation. Infection caused by *M. foruitum* usually produce painful red to violaceous nodules that can drain serosanguinous material, ulcerate, spread to deeper tissues, and form fistulous tracts [35, 36].

Pulmonary infection: pulmonary infection depends on the host conditions. Some of the predisposing factors for lung diseases are: examples Traditional lung disorders, Chronic bronchitis and emphysema (chronic obstructive pulmonary disease), bronchiectasis, fibrosis associated with ankylosing spondylitis, rheumatoid arthritis, sjogren syndrome, systemic lupus erythematosus, inflammatory bowel disease, Radiation lung injury, Malignancy (lung cancer, lymphoma, other), Histoplasmosis, Coccidioidomycosis, Sarcoidosis, Aerosol exposure/hypersensitivity pneumonitis/BOOP, Indoor hot tubs and swimming pools. For pulmonary infection there are no particular risk factors. It is associated with structural lung disease and impaired clearance of the organisms [35, 37, 38] .

Disseminated infection: disseminated infection due to RGM associated with an immunocompromising conditions such as malignancy, transplantation, HIV infection or any alternation in cytokine pathways (particular, IL-12 and IFN-g) [35, 39, 40].

2.4 Pathogenesis of *M. fortuitum*:

The pathology of *M. fortuitum* is simple as it does not spread from person to person [7]. Environmental exposure of the bacteria causes most the infections like soft tissue infections, lung infection etc**.** The mechanism underlying *M. fortuitum* persist inside the macrophage is not clearly understood. Infection of *M. fortuitum* along with other mycobacteria (*M. abscessus*, *M. fortuitum*, *M. celatum*, and *M. tuberculosis*) on THP-1 macrophages showed that the slow growing mycobacteria enters more efficiently than the fast growing bacteria (*M. fortuitum*) [41]. In animals, *M. fortuitum* has been associated with cases of cattle and sheep mastitis [42], canine pulmonary and subcutaneous abscess

[43] as well as feline cutaneous granulomas [44, 45]. Intravenous inoculation in mice causes kidney lesions and 'spinning' disease. When mice were intravenously inoculated with *M. fortuitum*, it causes spinning of head due to damage of cochlear bone, when animals are lifted up by the tail. In experimental murine models ,the bacilli localize in kidney [46], although the reason behind tropism for kidney by bacilli, as preferential niche over all other organs is not understood. The lesions produced in the kidney are attributed to mild cellular infiltrations of lymphocytes [43, 47] serous exudation in the glomeruli, and infiltration of mononuclear cells into peri-glomerular connective tissues [46]. Infection of *M. fortuitum* causes the damage in cochlea results in neurological disorder and spinning, making imbalance in body. Cochlea is the balancing organ of the body. *M. fortuitum* creates lesions in the kidney and form granulomas. Granulomas are the aggregates of immune cells surrounding the infected tissue[48]. Granulomas comprise of activated macrophages, immune cells (CD4+ T cells, CD8+ T cells), and necrotic tissues. Granulomas prevent further bacillary multiplication due to its barricade like structure and spread to other sites [48]. Most of the studies have been done for *M. tuberculosis.*

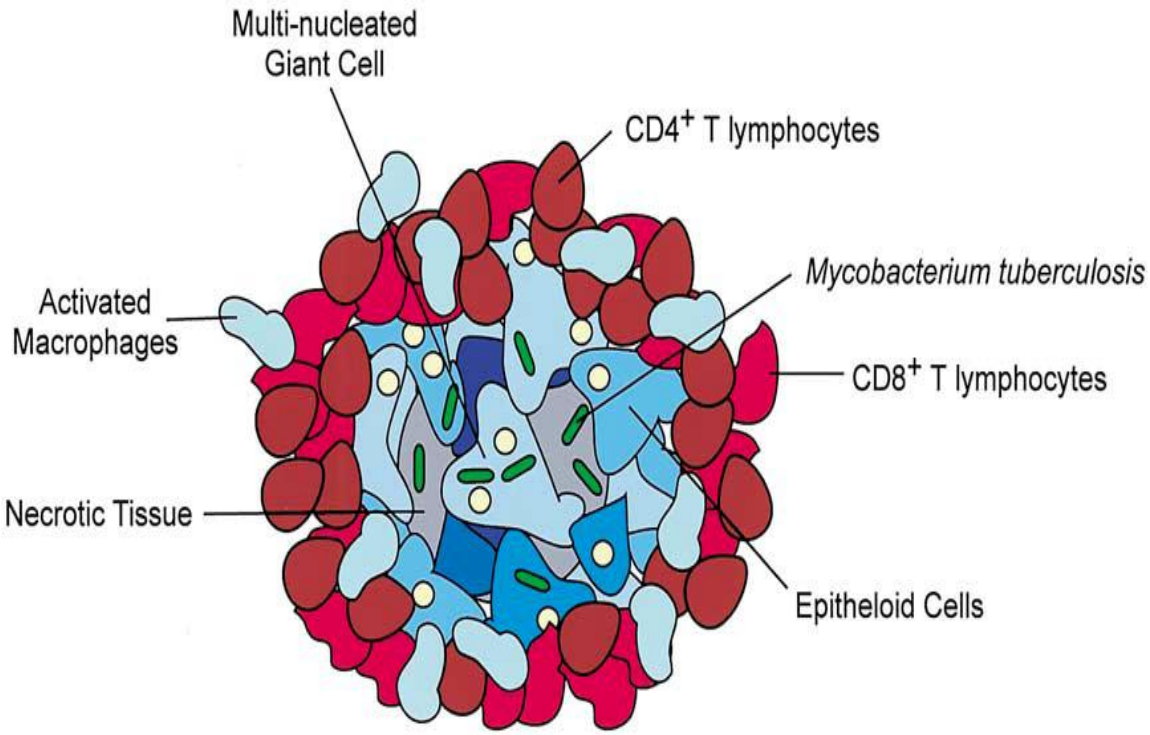

Fig 2 : Granuloma having *M. tuberculosis* infected tissue [adapted from 48]

Macrophages plays a central role in immune system as the internalise the microbe into phagosome and then kill microbes [49]. Microbes have to face certain stress inside the macrophages for its survival. Some stress conditions inside the macrophage are given below:

- Activated macrophages produces oxidizing agents like reactive oxygen intermediates, reactive nitrogen intermediates is the first stress inside macrophages.
- Second stress is the low pH of phagolysosomes
- Third stress is the damage of surface structures. Structure of mycobacteria (fatty acid rich cell envelop) could be damage by the alveolar surfactant which is having a mild detergent with antibacterial activity [50].

2.5 Sigma Factors

Sigma factors are the initiation factors that promotes the attachment of RNA polymerase to specific initiation sites and then are released [5].Sigma factors contribute a major role in the bacterial gene expression [51]. It plays a critical role in the recognition of promoters and initiates transcription (RNA synthesis) [52]. Primary check point for controlling gene expression in bacteria is transcription [53]. During initiation of transcription the RNA polymerase binds to sigma factor (σ) and then whole unit binds to the core subunits ($\beta\beta'\alpha_2\omega$) to form holoenzyme.

Sigma factors plays different roles according to the stage of initiation [52]. Sigma factors are classified into two families based on their homology to two σ factors in *Escherichia coli*:

1. The first one is σ 70 which is responsible for the bulk of transcription during growth;
2. and the structurally unrelated σ 54 (or σ N) that directs transcription in response to environmental signals, and requires the input of enhancer proteins and ATP hydrolysis to drive DNA melting [53]

Within the σ 70 family, there are four groups differ by the presence and absence of conserved regions [52, 53]. There are four structured domains σ1.1, σ2 σ3, σ4 and they interact with specific promoter and RNA polymerase. Domain σ2 is the most conserved region [52].

Lonetto et al. (1992) classified the sigma factors into primary (group 1) σ factors, a group of closely related but nonessential paralogues (group 2), and the more divergent alternative σ factors (group 3). Now group 4 having the ECF sigma factors is also added [53].

Group 1: Include all the primary sigma factors. It include sigma factor σ70 of *E. coli* and its orthologues [54]. They play an important role in the transcription of rapidly growing bacterial cells and are thus often referred to as the 'primary' factors. Size of primary sigma factors falls between 40 to 70Kda and have four conserved regions. Primary sigma factors recognize promoters of similar sequence: TTGaca near -35 and TAtaaT near -10 (where uppercase refers to more highly conserved bases) [53]

Group 2: This group comprise of all the nonessential proteins which are highly similar to the primary sigma factors but dispensible for growth. This group include *E. coli* σ^{s} (RpoS) protein. Group 2 also have three of the four Hrd (Homologue of RpoD) proteins in *Streptomyces coelicolor*. Proteins of this group also contains all the four conserved sequence regions [54]. Moreover, the regions of sigma factors for. Group 1 and group 2 have overlap in the promoter recognition regions. Most of the studied has been done on RpoS of *E. Coli* [53].

Group 3: This group comprise of the secondary sigma factors (called as alternative sigma factors) of the σ70 family. These proteins have the presence of the conserved amino acid sequences of regions 2 and 4 but in many cases conserved region 1 and often region 3 was absent. Molecular weight of the proteins of group 3 is 25 to 35Kda.

Group 4: This group include extracytoplasmic function (ECF) subfamily of sigma factors [53]. ECF plays an important role in sensing and responding to signals that are generated outside of the cell or in the cell membrane. The ECF group have at least 43 major phylogenetically distinct sub-groups [52]. ECF sigma factors recognise the promoter having 'AAC' motif in the -35 region. ECF sigma factors have functions associated with some aspect of the cell surface or transport.

M. tuberculosis has 13 sigma factors of which 10 regarded as ECF sigma factors. As the ECF sigma factors play a vital role in the pathogenecity of bacteria as well as during stress conditions such as oxidative stress [19], heat stress, cold stress etc [55].

2.5.1 Sigma Factor H (sig*H*)

Sigma factor H is another member of the ECF family of sigma factors, and is very similar to the Sigma R of Streptomyces species. Sigma R responds to certain types of oxidative stress, such as diamide treatment, that oxidize protein-SH groups, which then form intra-molecular disulfide bonds [56]. Most of the studies of sigma factor H of *M .tuberculosis* has been done which showed that the gene induced during different stress conditions like heat stress, oxidative stress, diamide stress [57-61]. *sigH* also get induced during macrophage infection. During oxidative and heat stress *sigH* induce the expression of sigma factor B, sigma factor E and the thioredoxin regulon [59].

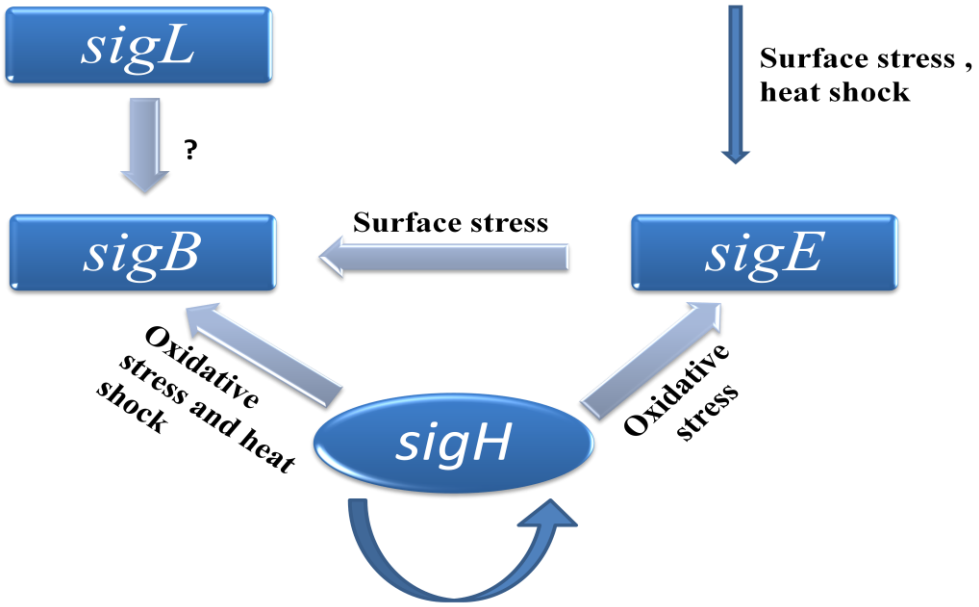

Fig 3: Transcriptional relationship among sigma factors

Sigma factor H plays a central role to regulate the expression of other sigma factors such as *sigB*, *sigE* during stress conditions [57]. During heat stress *sigH* induce the expression of *dnaK* and *clpB* genes [57, 62]. During oxidative stress the expression of *sigE* is regulated by *sigH*. *sigE* is required for the expression of *sigB* during surface stress condition however the expression of *sigB* during oxidative and heat stress depends on the *sigH*. *sigH* also promotes its own structural gene. How *sigL* is expressed upon environmental stress is still not known. [59]. Most of the studies showed that *M.*

tuberculosis sigH mutants are more susceptible to oxidative stress [51, 57]. It has been documented that the *sigH* mutant of *M. tuberculosis* showed a distinctive infection phenotype. The mutants achieved high bacterial counts in lung and spleen that persisted in tissues in a pattern identical to those of wild-type bacteria. Mutants also produced a blunted, delayed pulmonary inflammatory response, and recruited fewer CD4+ and CD8+ T cells to the lung in the early stages of infection. *sigH* of *M. tuberculosis* mediate the transcription of at least 31 genes directly and that it modulates the expression of about 150 others. *sigH* regulon also involved in the maintenance of intrabacterial reducing capacity. *sigH* gene is responsible for the bacterial growth and survival within the host. Survival inside the macrophages and the resistance to reactive oxygen and nitrogen intermediated are also related with the virulence of mycobacteria [70].

M. tuberculosis has thioredoxin system, defence mechanism against oxidative stress. Thioredoxin system composed of thioredoxin (TrxA) and thioredoxin reductase (TrxB) which requires NADH as a cofactor. The reduced form of thioredoxin is a general protein disulfide reductant, which can reactivate proteins that have been oxidized by H_2O_2. The ability of the thioredoxin system to reduce reactive oxygen species and thereby protect the cell against oxidative stress has been established in several systems. *sigH* regulates the expression of thioredoxin regulon during oxidative stress condition and helps the bacteria to survive within the macrophage[70].

2.6 Gene silencing:

Gene silencing is the epigenetic processes of gene regulation in which the gene get switched off by a mechanism other than genetic modification [65, 64]. In gene silencing the gene which would be expressed under normal circumstances is switched off. In this technique the production of protein gets reduced. In gene silencing the gene expression get intervened prior translation instead of editing DNA or inhibiting transcription process. Here the breakdown of protein occurs by designing a molecule which gets binds to the mRNA and decreases the level of protein [63].

Cellular components of gene silencing are:

- Histones
- Chromatin and heterochromatin
- MicroRNA

- siRNA
- dsRNA
- Dicer
- Transposons [67, 64]

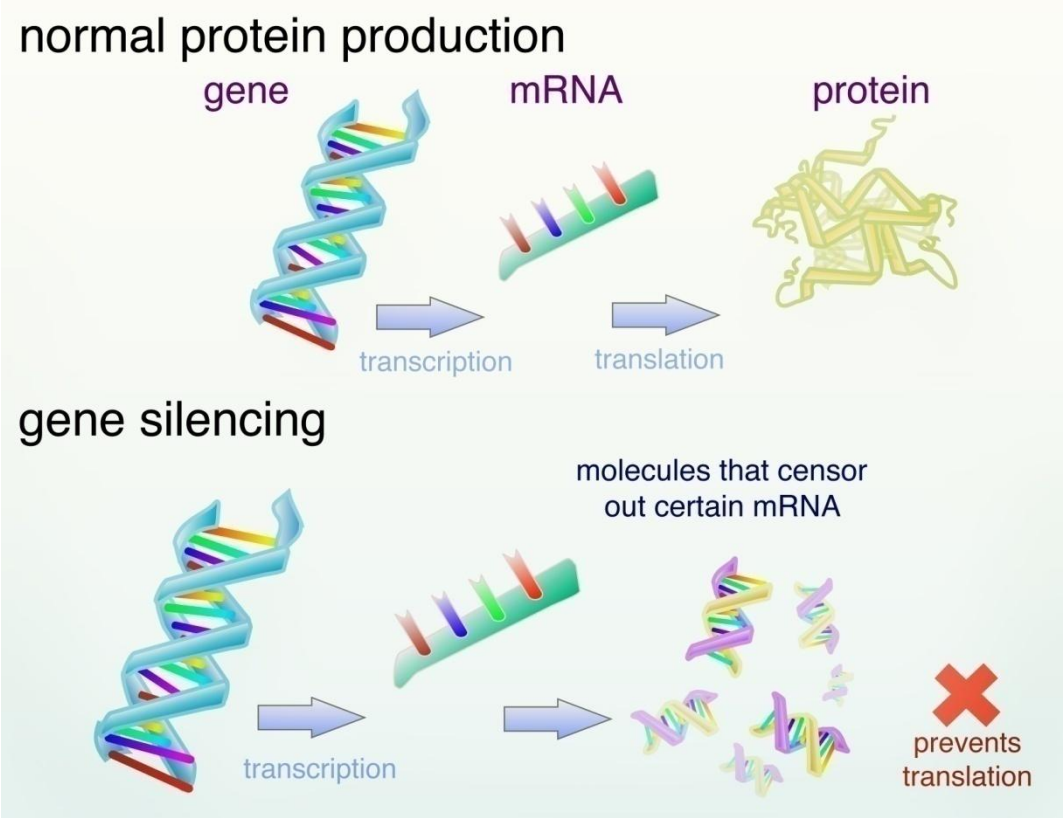

Fig 4: Antisense Method of Gene Silencing [adapted from 67]

Methods for gene silencing:

1. Transcriptional Gene Silencing: Genomic Imprinting, Paramutation, position effect, RNA-Directed DNA Methylation, Transposon silencing, Transgene silencing, Transcriptional gene silencing
2. Post-transcriptional Gene Silencing: RNA interference, Nonsense mediated decay
3. Meiotic gene silencing: Transvection

During Transcriptional Gene Silencing the gene is silenced by DNA methylation, heterochromatin formation and programmed DNA elimination. In Post transcriptional gene silencing (PTGS) the gene is silenced by the destruction of the mRNA of the gene to which the siRNA shows perfect complementarity and this is commonly known as RNA interface (RNAi) [64]. In meiotic gene silencing technique, gene is silenced by transvection. Transvection is an epigenetic phenomenon that results from an interaction between an allele on one chromosome and the corresponding allele on the homologous chromosome. Transvection can lead to either gene activation or repression. Formally it can also occur between nonallelic regions of the genome as well as regions of the genome that are not transcribed [64, 67].

RNA interference (RNAi) and Antisense oligonucleotides (ASOs) are the leading methods for gene silencing [66]

2.6.1 RNA interference (RNAi):

In RNAi interference, double stranded RNA interferes with the RNA expression of a particular gene that shares a homologous sequence with the dsRNA. Double stranded RNA can be either miRNA or small intereference RNA(siRNA) [64]. siRNA binds to the target mRNA and then degrade the target mRNA by recruiting other proteins [67] RNA interference has an important role in defending cells against parasitic genes – viruses and transposons – but also in directing development as well as gene expression in general [64,66,67]

2.6.2 Antisense oligonucleotides (ASOs):

In this technology synthetically produced complementary strand, get binds to the target mRNA and blocks its translation and finally blocks the protein production[65]. The complementary strand is called antisense strand and the target mRNA has sense strand which are involved in this technology [66]. Antisense strand can be either DNA or RNA. If DNA then it is approximately 17 base pair long and is it is RNA then it can be either catalytic or non catalytic. The catalytic antisense strands are called ribozymes which cuts at the specific position of RNA while the non catalytic antisense strands blocks the processing of proteins [67]. Antisense strand binds with the sense strand with the hydrogen bond [64, 65, 66, and 68] and the cell recognizes the double helix as foreign to

the cell. This binding recruits the cellular enzyme RNase H and degrade the target mRNA molecule thus preventing the production of protein [64, 66, 67, 68].

CHAPTER 3

Materials and Methods

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacterial strain used: Bacterial strain used is listed in table 3.1 along with source and reference.

Table 3.1:

Strain	Reference strain	Source
Mycobacterium fortuitum	*M. fortuitum* (ATCC 6841)	CDRI Lucknow

3.1.2 Vector:

pMV261 vector used for the construction of sense and antisense construct of *sigH* of *M. fortuitum*.

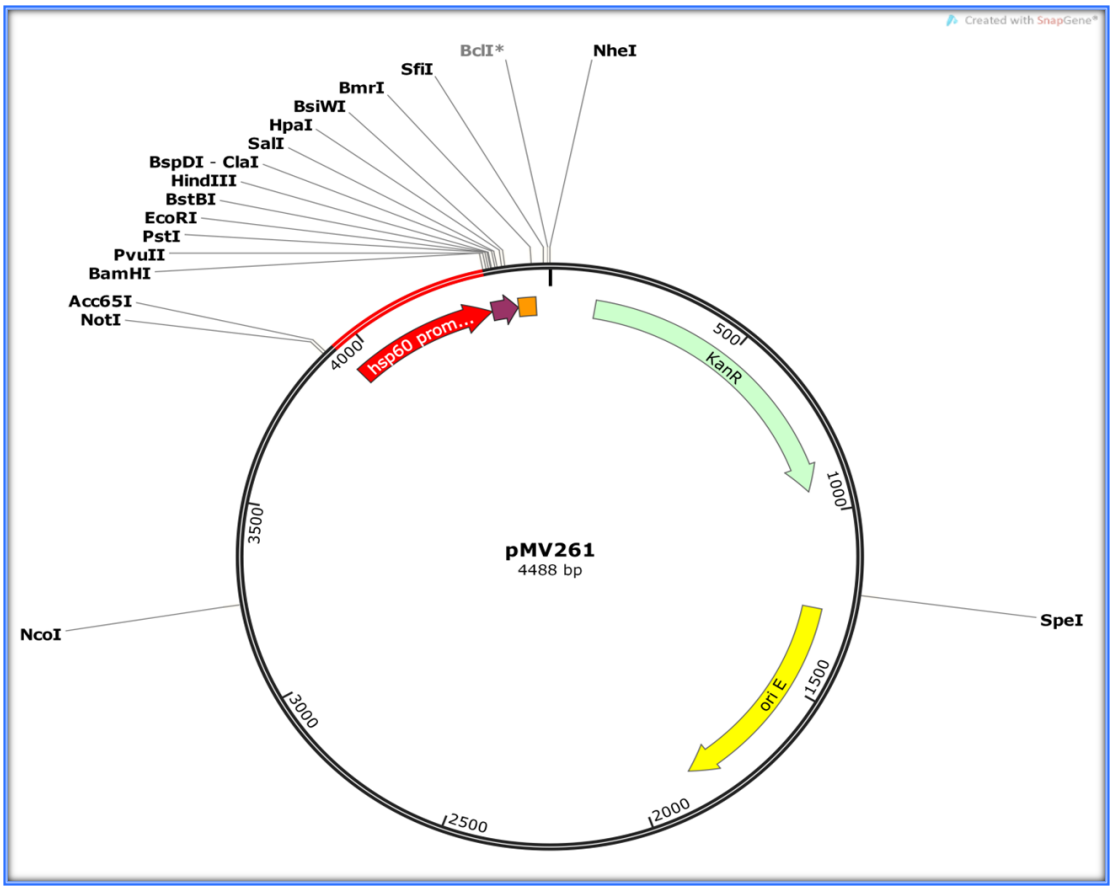

Fig 5: pMV261 Vector Map

3.2 METHODS

3.2.1 Construction of sense and antisense construct of *sigH* gene of *M. fortuitum*

Multiple sets of primers were designed from flanking region of *sigH* constant domain regions (CDS) and then PCR was done using the *M. fortuitum* genome. The PCR products were sequenced and checked for the homology with the *sigH* of *M. tuberculosis.* After doing the homology search, the longest sequence having the maximum homology was submitted to NCBI [GenBANK ID - KM282166] as *sigH* homologue. pMV261 vector was used to construct the sense and antisense mutants of *M. fortuitum sigH*. The orientation was confirmed using *BamHI* site in pMV261 vector. Presence of full length gene and its orientation was confirmed by restriction digestion and PCR using hsp60 primer (promoter). Obtained sense and antisense mutants were electroporated in *M. fortuitum.* ***(Acknowledgment: This part of the work was carried out by Ms. Shivani Sood as a part her PhD Thesis)***

3.2.2 Culturing of Microorganisms

3.2.2.1.1 Luria Bertani Broth with Glycerol and Tween-80 (LBGT): Luria broth contains tryptone, yeast extract, sodium chloride. Presence of casein enztmic hydrolysate and yeast extract makes it nutritionally rich. Osmotic equilibrium is maintained by sodium chloride. It is generally used for recombinant strains. Addition of 0.5% of glycerol helps in the growth of mycobacterium species. 0.15%-0.18% of Tween-80 in LB broth allows the diffusion of new cells. LB broth was prepared using Milli RO water and supplemented with 0.5% glycerol and 0.15%-0.18% Tween-80. The media was poured into the tubes and then autoclaved. After autoclaving antibiotics cycloheximide and kanamycin were added. LBGT with cycloheximide was used for the wild type *M. fortuitum* while LBGT with cycloheximide and kanamycin was used for the *M. fortuitum* mutants. The mycobacteria were inoculated in the LBGT tubes having antibiotics using sterile loop. The tubes were incubated at 37°C at shaking for 3-4 days.

3.2.2.2 Nutrient Agar Tween-80 (NAT): Nutrient media are basic culture media used for maintaining and cultivating microorganisms. It is also used for fastidious microorganism as the media is enriched with serum or blood and are also used for purity checking prior to biochemical or serological testing. Nutrient agar contains peptic digest of animal tissue, beef extract and yeast extract and sodium chloride. It is one of the several non-selective media useful in routine cultivation of microorganisms. Addition of different biological fluids such as horse or sheep blood, serum, egg yolk etc. makes it suitable for the cultivation of related fastidious organisms. Peptic digest of animal tissue, beef extract and yeast extract provide the necessary nitrogen compounds, carbon, vitamins and also some trace ingredients necessary for the growth of bacteria. Sodium chloride maintains the osmotic equilibrium of the medium. pH of the medium is 7.4±0.2. 0.05% of Tween-80 is added to nutrient agar. Tween-80 helps in the replication of microorganisms. Tween-80 allows diffusion of new tubercle bacilli by wetting its surface. Nutrient agar was prepared using Milli RO water and 0.05% of Tween-80 was added and then autoclaved in a flask. Antibiotics cycloheximide and kanamycin were also added and the poured into the petriplates. The working solution of cycloheximide and kanamycin is 5mg/ml and 10mg/ml respectively. The NATC petriplates were streaked with the *M. fortuitum* while NATCK plates were used for the streaking of *M. fortuitum* mutants. Streaking was done with the help of sterile loop. The plates were incubated at 37°C for 3-4 days.

3.2.2.3 Middlebrook 7H9 Broth (MB7H9)

Middlebrook 7H9 broth with Tween-80 is generally recommended for the growth of *Mycobacterium* species. The basal medium of 7H9 broth is supplemented with Middlebrook ADC Enrichment and the supplementation provides nutrients necessary for mycobacterial growth. Middlebrook ADC Enrichment conatins bovine albumin, dextrose and catalase. Albumin binds with free fatty acids which are toxic to Mycobacterium spp. and acts as a protective agent. Dextrose serves as an energy source. Catalase destroys the toxic peroxides that may be present in the medium. Glycerol, biotin and sodium citrate are also present in the basal medium. Glycerol is supplemented from outside and it acts as an abundant source of carbon and

energy for the tubercle organisms. Biotin revives the damage cells, and is involved in a variety of carboxylation and decarboxylation reactions. Sodium citrate holds inorganic cations present in the solution, when converted to citric acid. Tween-80 allows diffusion of new tubercle bacilli by wetting its surface. MB7H9 media was prepared using Milli RO water supplemented with 0.5% of glycerol and 0.15%-0.18% Tween-80 and then autoclaved. Antibiotics cycloheximide and kanamycin were also added. The working solution of cycloheximide and kanamycin is 5mg/ml and 10mg/ml respectively. *M. fortuitum* was inoculated using sterile loop or sterile tips then the culture was incubated at 37°C at shaking for 3-4 days.

3.2.2.4 Acid fast bacilli (ABF) staining: At each step purity was checked by AFB staining. A loopful of mycobacterium culture was emulsified in saline on a clean glass slide, air dried and heat fixed. The smear was stained using carbol fuchsin and heated for 5-7 min till vapours appeared. The slide was cooled and excess stain was washed with tap water and decolourised using acid alcohol mixture for 30 seconds. The slide was washed and counterstained using malachite green for 2 min. excess stain was washed off using tap water. Slides were air dried and the smear was observed under oil immersion (1000X) objective. Mycobacterial cell wall appeared pink rod shaped structure while contaminating organism stained green.

3.2.3 *In-vitro* stress analysis of *sigH* sense and antisense mutants of *M. fortuitum*

3.2.3.1 Acidic Stress:

sigH Sense and antisense constructs as well as wild type *M. fortuitum* was grown in MB7H9 media at 37^0C for 3 days. Seed cultures were passage in 100ml of middle MB7H9 media and then incubated till the O.D of culture reached 0.4. Cultures were divided into two flasks (50ml each) and then incubated at 37^0C for 2 hours. Cultures were taken in the 50ml centrifuge tubes and then centrifuged at 5000 rpm for 10 min. Supernatants were discarded and then pellets washed with MB7H9 media two times. Obtained pellets were suspended in 5ml of MB7H9 media. Cultures were inoculated in four different flasks having 50ml MB7H9 media of different pH 3.5, 4.5, 5.5 and 6.5 (pH was adjusted using dilute HCL)and then incubated at 37^oC at

shaking. The samples were collected at different time intervals 2hr, 6hr, 12hrs, 24hrs, 36hrs, 48hrs after inoculation and then diluted upto 10^6 times with tween-80 normal saline (TNS). After this, 10µl of dilution 10^2, 10^4, 10^6 of samples were spread on NATC/K plates for the determination of Colony forming unit (CFU).

3.2.3.2 Nutrient Stress:

sigH Sense and antisense constructs and wild type *M. fortuitum* was grown in MB7H9 media at 37^0C for 3 days. Seed cultures were passage in 100ml of MB7H9 media and then incubated till the O.D of culture reached 0.4. Each culture was divided into two flasks (50ml each) and then incubated at 37^0C for 2 hours. Cultures were taken in the 50ml centrifuge tubes and then centrifuged at 5000 rpm for 10 min. Supernatant was discarded and then pellet was washed with MB7H9 media. Obtained pellet was suspended in 5ml of MB7H9 media. Cultures were inoculated in 50ml phosphate buffer saline (PBS) and then incubated at 37^oC at shaking. The samples were collected at different time intervals 2hr, 6hr, 12hrs, 24hrs, 36hrs, 48hrs after inoculation and then diluted upto 10^6 times with TNS. After this, 10µl of dilution 10^2, 10^4, 10^6 of samples were spread on NATC/K plates for the determination of Colony forming unit (CFU).

3.2.3.3 Detergent stress:

sigH Sense and antisense constructs and wild type *M. fortuitum* was grown in MB7H9 media at 37^0C for 3 days. Seed cultures were passage in 100ml of MB7H9 media and then incubated till the O.D of culture reached 0.4. Each culture was divided into two flasks (50ml each) and then incubated at 37^0C for 2 hours. Cultures were taken in the 50ml centrifuge tubes and then centrifuged at 5000 rpm for 10 min. Supernatant was discarded and then pellet was washed with MB7H9 media. Obtained pellet was suspended in 5ml of MB7H9 media. Cultures were inoculated in MB7H9 media having an exposure of 0.05% SDS and then incubated at 37^oC at shaking. The samples were collected at different time intervals 2hr, 6hr, 12hrs, 24hrs, 36hrs, 48hrs after inoculation and then diluted upto 10^6 times with TNS. After this, 10µl of dilution 10^2, 10^4, 10^6 of samples were spread on NATC/K plates for the determination of Colony forming unit (CFU).

3.2.3.4 Heat stress:

sigH Sense and antisense constructs and wild type *M. fortuitum* was grown in MB7H9 media at 37^0C for 3 days. Seed cultures were passage in 100ml of MB7H9 media and then incubated till the O.D of culture reached 0.4. Each culture was divided into two flasks (50ml each) and then incubated at 37^0C for 2 hours. Cultures were taken in the 50ml centrifuge tubes and then centrifuged at 5000 rpm for 10 min. Supernatant was discarded and then pellet was washed with MB7H9 media. Obtained pellet was suspended in 5ml of MB7H9 media. Cultures were inoculated in 50ml MB7H9 media and then incubated at 50^oC at shaking. The samples were collected at different time intervals 2hr, 6hr, 12hrs, 24hrs, 36hrs, 48hrs after inoculation and then diluted upto 10^6 times with TNS. After this, 10µl of dilution 10^2, 10^4, 10^6 of samples were spread on NATC/K plates for the determination of Colony forming unit (CFU).

3.2.3.5 Oxidative stress:

sigH Sense and antisense constructs and wild type *M. fortuitum* was grown in MB7H9 media at 37^0C for 3 days. Seed cultures were passage in 100ml of MB7H9 media and then incubated till the O.D of culture reached 0.4. Each culture was divided into two flasks (50ml each) and then incubated at 37^0C for 2 hours. Cultures were taken in the 50ml centrifuge tubes and then centrifuged at 5000 rpm for 10 min. Supernatant was discarded and then pellet was washed with MB7H9 media. Obtained pellet was suspended in 5ml of MB7H9 media. Cultures were inoculated in MB7H9 media having a exposure of 10mM of H_2O_2 and then incubated at 37^oC at shaking. The samples were collected at different time intervals 2hr, 6hr, 12hrs, 24hrs, 36hrs, 48hrs after inoculation and then diluted upto 10^6 times with TNS. After this, 10µl of dilution 10^2, 10^4, 10^6 of samples were spread on NATC/K plates for the determination of Colony forming unit (CFU).

3.2.4 *In-vivo* analysis of *sigH* sense, antisense constructs of *M. fortuitum*

3.2.4.1 Preparation of inoculum:

sigH sense, antisense constructs and wild type *M. fortuitum* were grown in MB7H9 media and incubated at 37^0C till the O.D reached 0.4. 1ml. Culture was taken in micro centrifuge tubes and then centrifuged at 4000 rpm for 3

min. Supernatant was removed and the weight of pellet was taken. Then the pellet was resuspended in 0.5% tween normal saline so that the final concentration of cells would be 6mg/ml and then vortexed properly and diluted upto 4 times. For the determination of initial CFU of the inoculum. 500µl culture was taken and spread on the NATC plates.

3.2.4.2 Intravenous infection to mice:

Mice (BALB/c) were heated for 5-10 minutes for the proper visualization of veins in the tail then 200µl of inoculum was taken in 1ml insulin syringe and injected in the tail veins of the mice. Proper labelling of the mice were done using different colour codes.

3.2.4.3 Analysis of symptoms of infected mice:

Symptoms such as neck paralysis, spinning, and tilting of infected mice were observed daily till 35 days after infection.

3.2.4.4 Determination of CFU in kidney of mice:

Dissection of mice was done on 10^{th} and 35^{th} day of infection. One kidney was isolated and homogenized in the homogenizer with tween normal saline and another kidney was preserved for the histopathology in tween normal saline. CFU was determined by diluting the homogenized kidney upto 10000 times in tween normal saline. 100µl of 100 times and 10000 times dilution were taken for spreading and then spread on NATC/K plates.

CHAPTER 4

Results

RESULTS

4.1 Construction of sense and antisense construct of *sigH* gene of *M. fortuitum*

Multiple primer sets were designed from conserved region of *M. tuberculosis sigH* to amplify full length gene sequence of its homologue in *M. fortuitum*. Longest sequence showing highest similarity 78% with *Mycobacterium tuberculosis sigH* was submitted to NCBI [GenBANK ID - KM282166] as *M. fortuitum* ATCC 6841 homologue of sigma factor H. *M. fortuitum sigH* homologue was cloned in Sense and antisense *orientation at BamHI* site in *pMV261* vector. Presence of full length gene and its orientation was confirmed by restriction digestion and PCR using *hsp60* primer (promoter). Sense and antisense knockouts were electroporated in *M. fortuitum*. ***(Acknowledgment: This part of the work was carried out by Ms. Shivani Sood as a part her PhD Thesis)***

4.2 *In-vitro* stress analysis of *sigH* sense and antisense mutants of *M. fortuitum*

4.2.1 CFU Under Acidic Stress: CFU has been taken under different pH 6.5, 5.5, 4.5, 3.5**.**

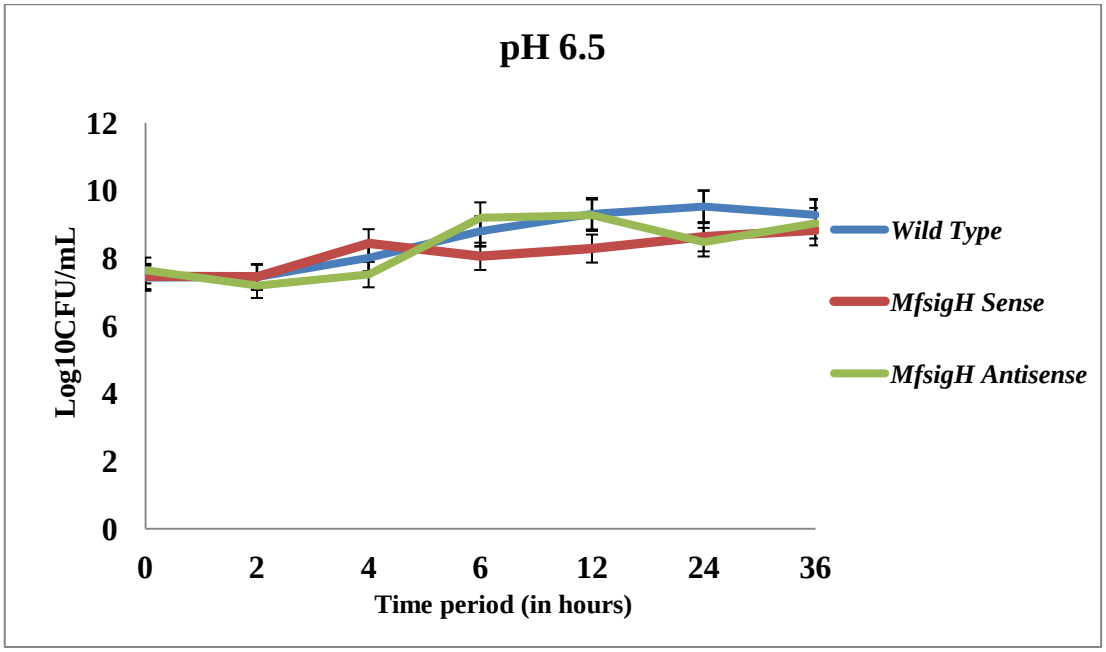

Fig 6: CFU at pH 6.5: Wild type *M. fortuitum*, *MfsigH* senses construct and *MfsigH* antisense construct survived under normal pH (6.5).

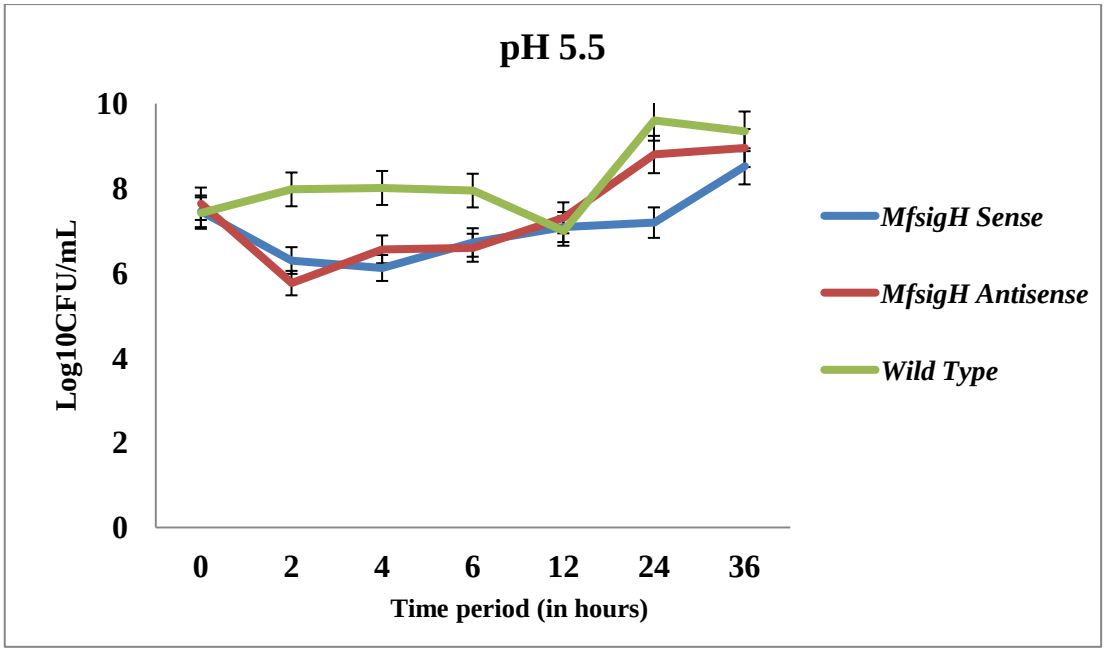

Fig7: CFU at pH 5.5: Wild type *M. fortuitum* survived at pH 5.5 but initially the growth of *MfsigH* sense construct and *MfsigH* antisense construct declined but after 12 hours, constructs showed revival in cell count.

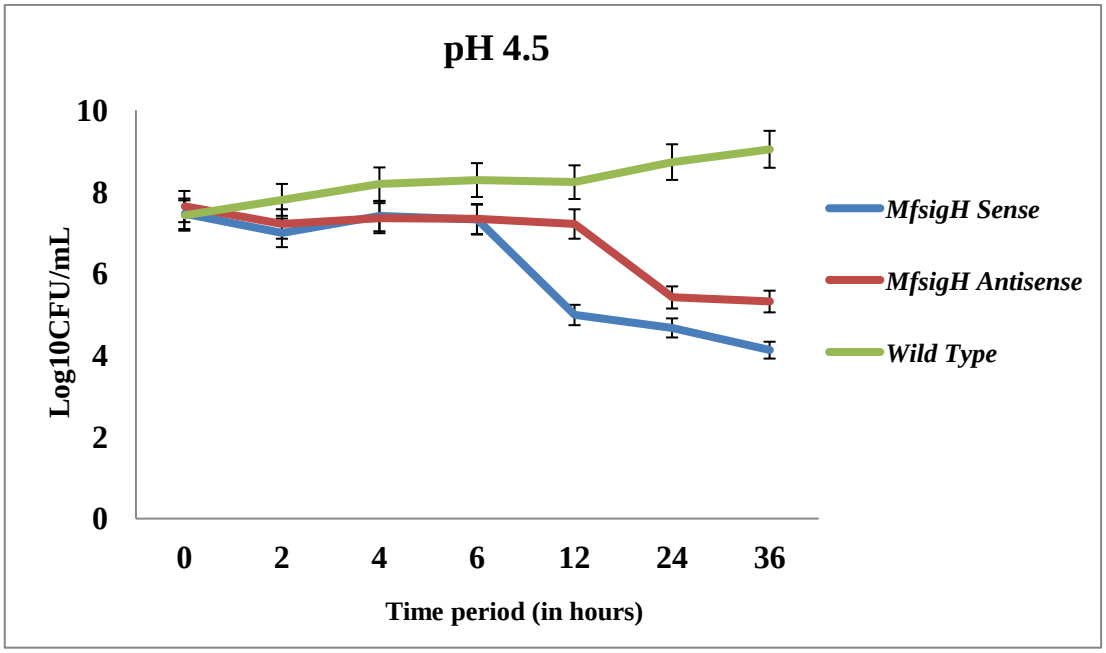

Fig 8: CFU at pH4.5: *MfsigH* sense and antisense constructs showed a decrease in growth as compared to wild type

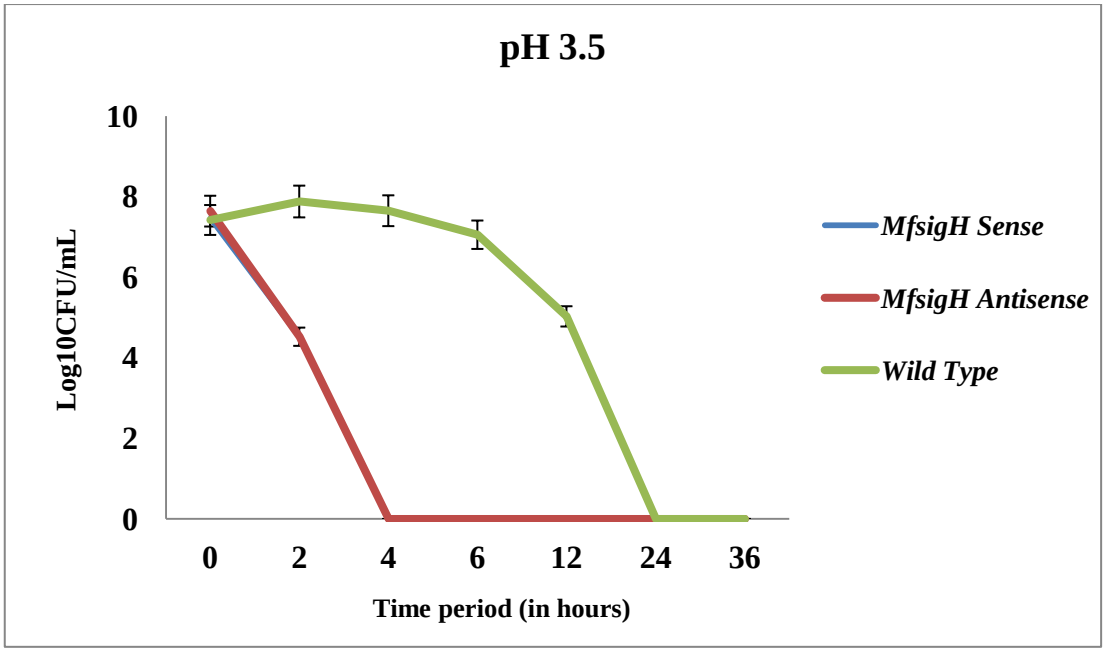

Fig 9: CFU at pH 3.5: *MfsigH* sense and antisense construct showed negligible growth at pH 3.5 as compared to wild type.

4.2.2 CFU Under Heat Stress

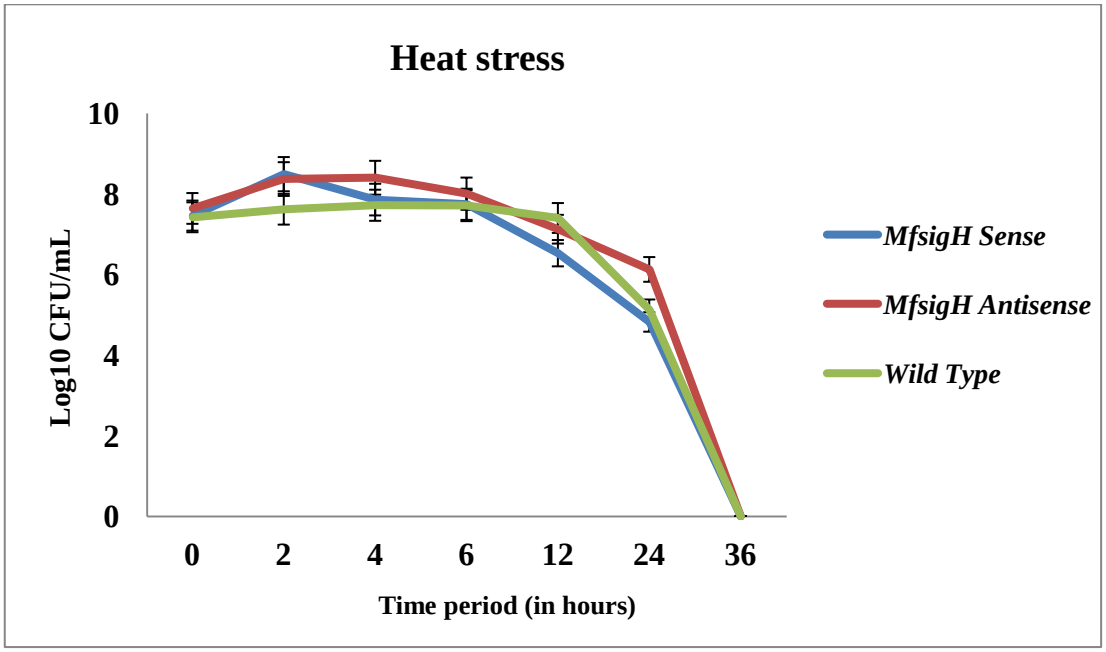

Fig 10: CFU when subjected to heat stress: The graph pattern of *MfsigH* sense and antisense along with wild type was observed to be similar during heat stress at 50^0C.

4.2.3 CFU Under Oxidative Stress

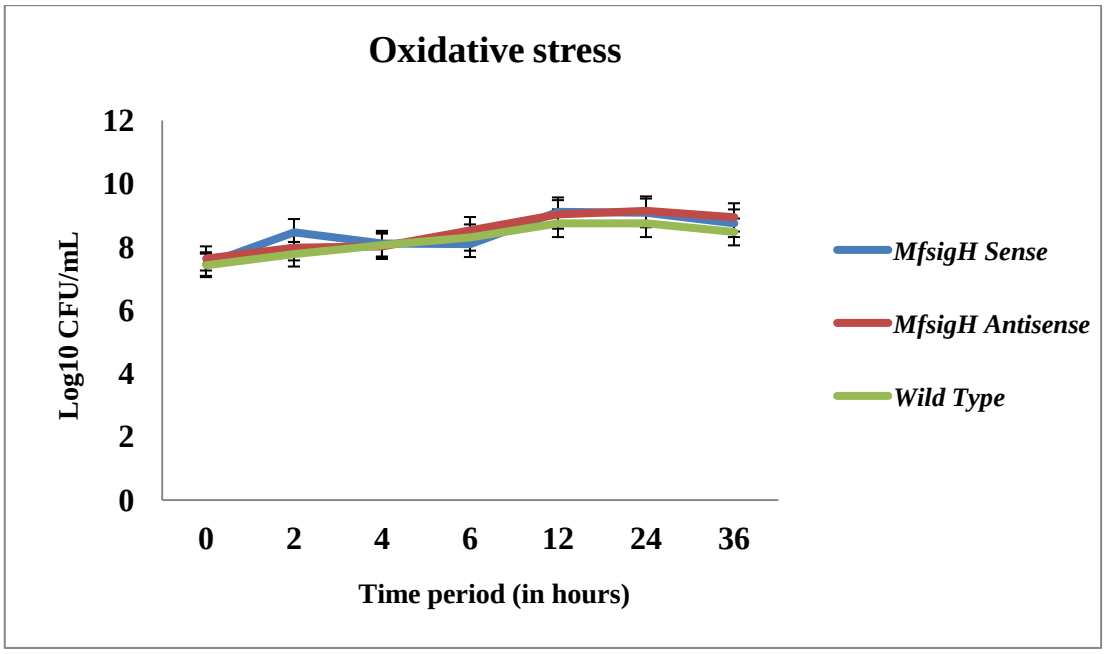

Fig 11: CFU under oxidative stress: Wild type *M. fortuitum* along with *MfsigH* sense and antisense construct survived under oxidative stress condition.

4.2.4 CFU Under Nutrient Starvation

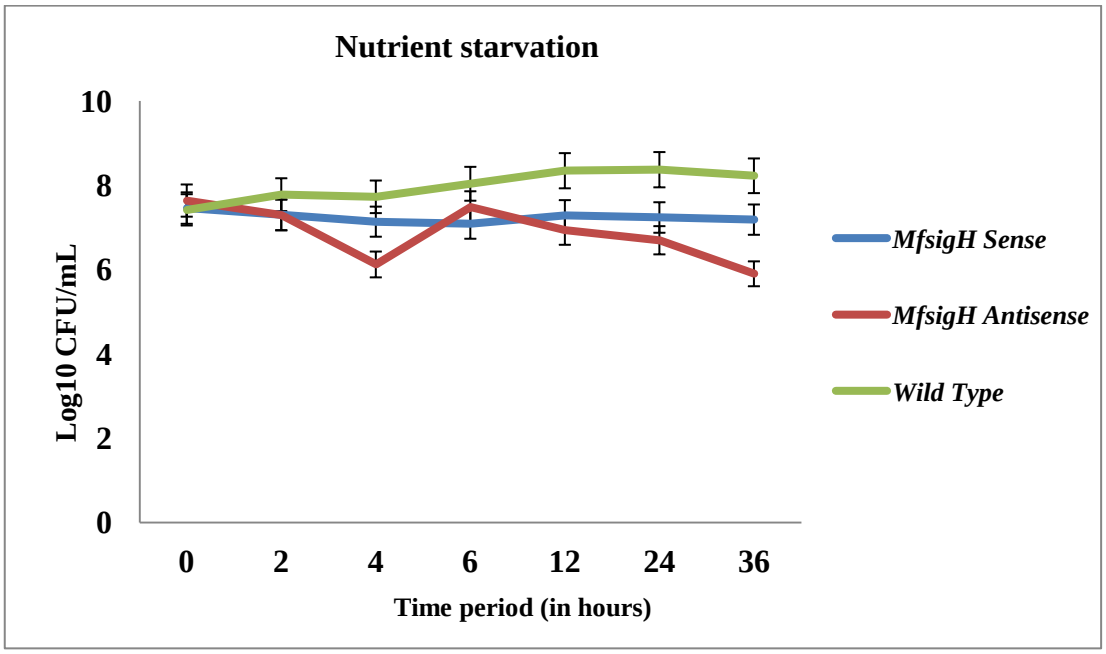

Fig12: CFU under nutrient starvation: *MfsigH* antisense construct showed decrease in growth as compared to *MfsigH* sense construct and wild type*M. fortuitum*.

4.2.5 CFU Under SDS Stress

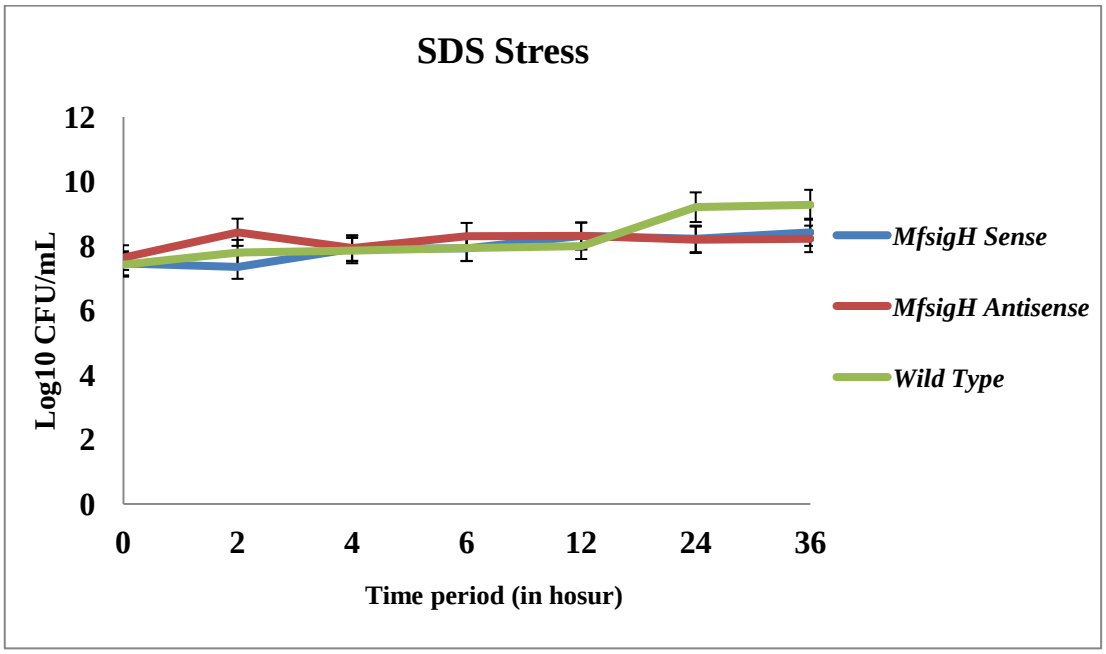

Fig 13: CFU under SDS stress: *MfsigH* sense and antisense construct showed constant CFU whereas wild type *M. fortuitum* showed increase in cell count after 12 hours during detergent stress condition.

4.3 In-vivo analysis of *sigH* sense and antisense mutants of *M. fortuitum*

4.3.1 Symptoms of infected mice:

Mice infected with *MfsigH* sense construct, *MfsigH* antisense constructs and wild type *M. fortuitum* showed spinning movement of head, weight loss.

Symptoms of mice after 35 days of infection:

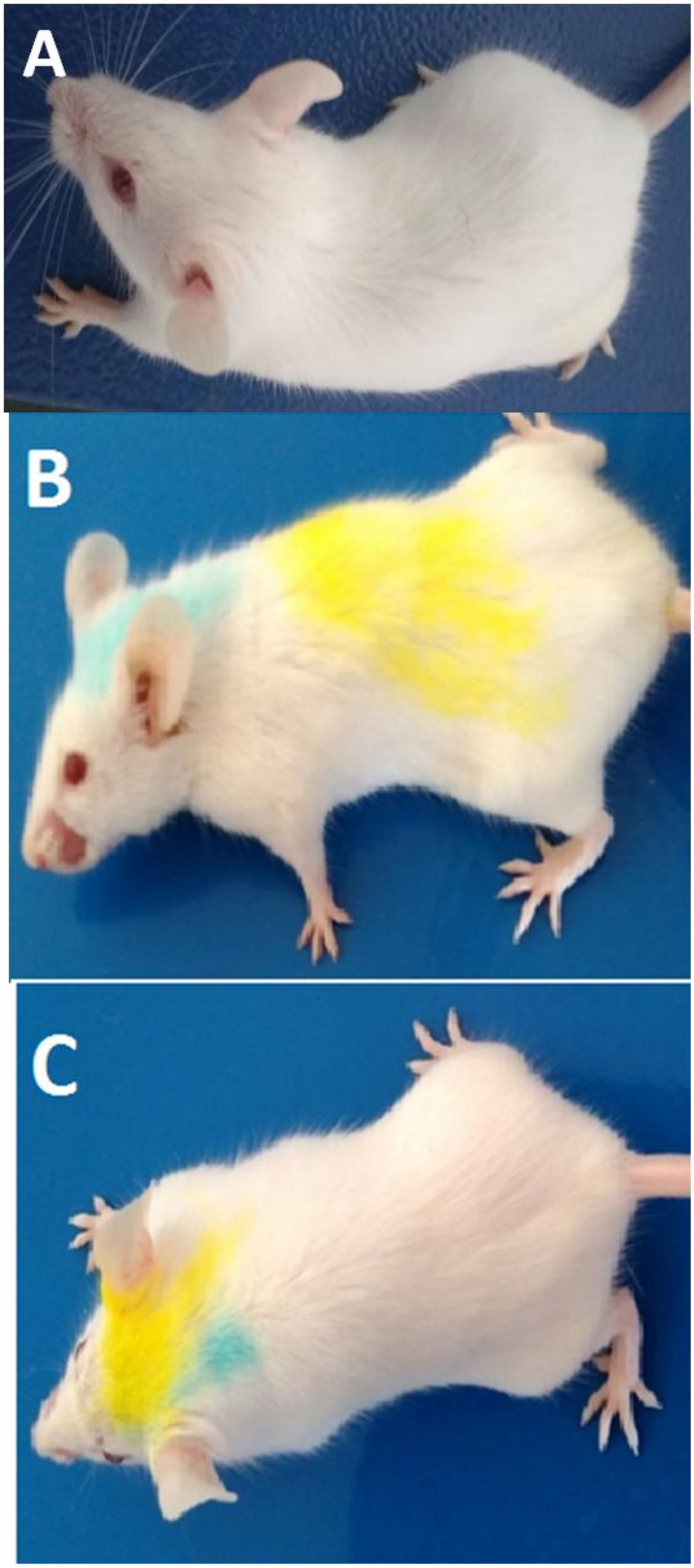

Fig 14: Mice infected with Wild Type *M. fortuitum* along with their mutants

In figure A, the mice infected with wild type *M. fortuitum* showed paralysed neck and weight loss. In figure B, the mice infected with *MfsigH* antisense construct -weight loss, unstable neck and spinning movement were observed. In figure C, mice infected with *MfsigH* sense construct showed weight loss and other symptoms like unstable neck and spinning movement but less in comparison to antisense construct infection and wild type

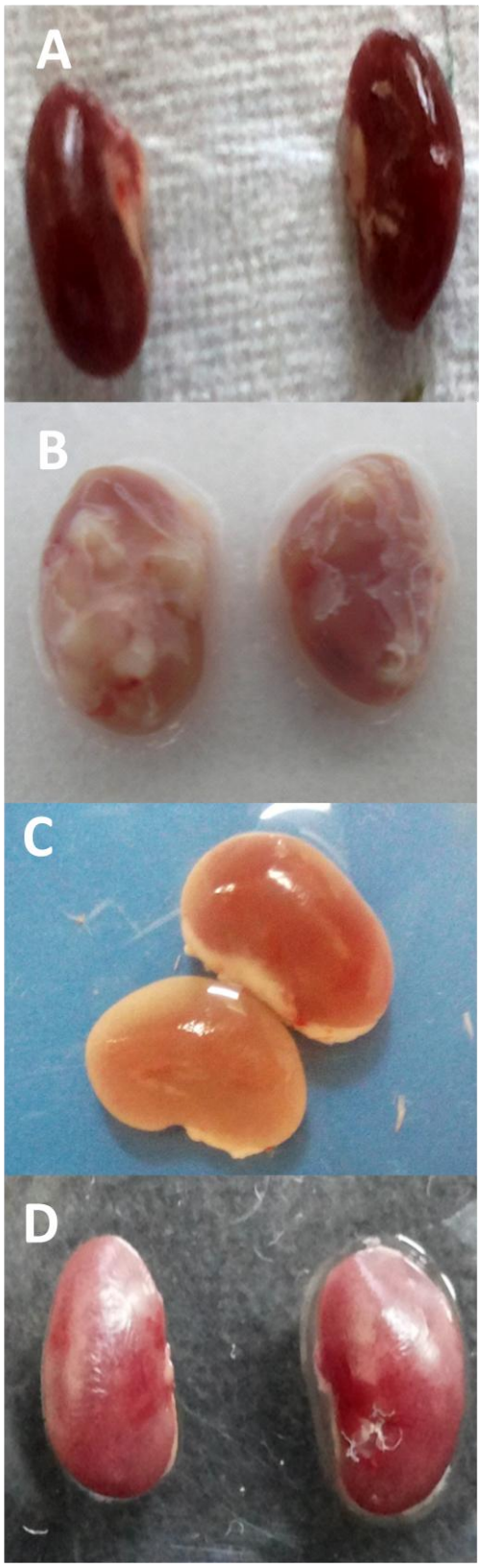

Fig 15: Kidneys having infection of wild type *M. fortuitum* and *MfsigH* mutants

Figure A showed the kidney of normal healthy mice.

Figure B showed the kidney of mice infected with wild type *M. fortuitum*. Figure C showed the kidney of mice infected with *MfsigH* antisense construct

Figure D showed the kidney of mice infected with *MfsigH* sense construct.

It is observed that normal kidneys have red colour while infected kidneys show white patches (due to the formation of granulomas). And the kidneys infected by *sigH* sense construct have less white patches than the kidneys infected with *sigH* antisense construct and wild type.

4.3.2 Bacillary load in kidney

Bacillary load in kidney after infection

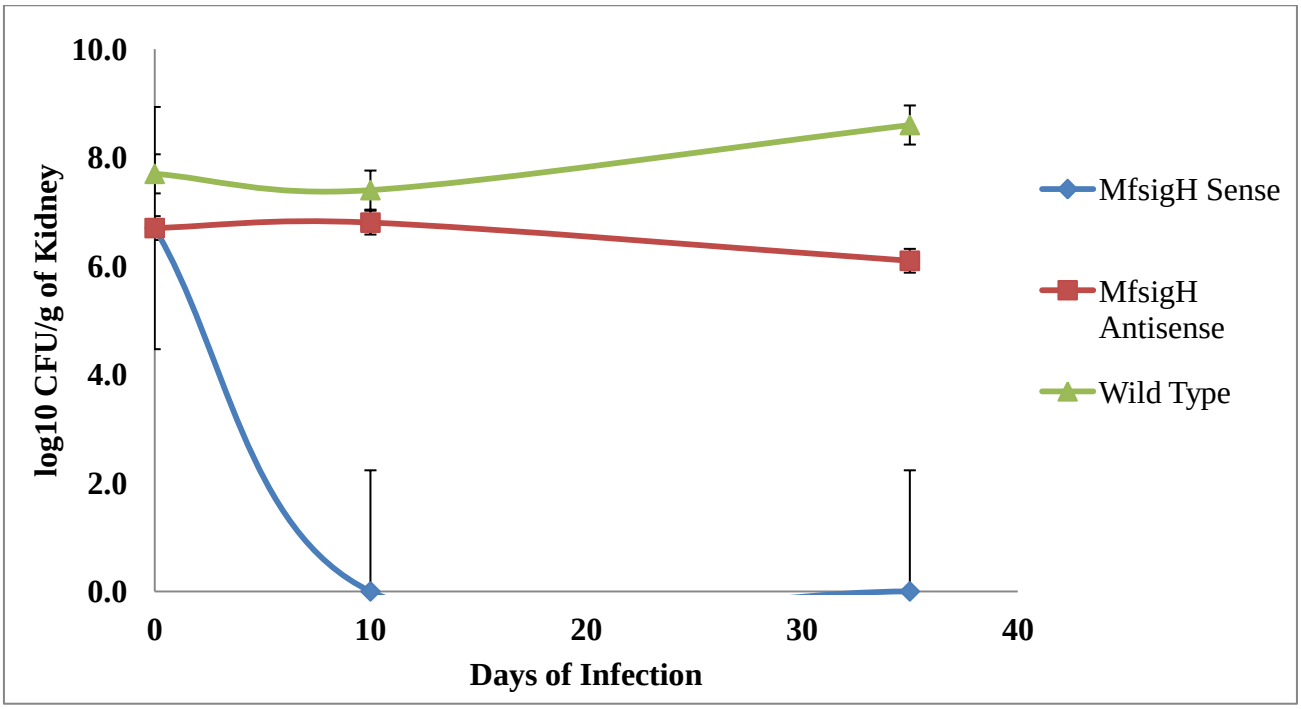

Fig 16: Figure shows bacillary load in kidneys after infection: *In-vivo* analysis showed that the *MfsigH* sense construct do not able to survive after a certain time but antisense construct survived 40 days of observation period.

CHAPTER 5

Discussion and Conclusion

DISCUSSION AND CONCLUSION

Mycobacterium fortuitum is a versatile fast growing pathogen causing infection in human and animals [1]. It is present in the environment. *M. fortuitum* causes pulmonary, skin and soft, catheter-related, tissues, joint, bone, wound infections, lymphadenitis as well as disseminated infections [57, 67]. The mortality rate is high in immunocompromised patients particularly HIV [1, 3]. The exact pathogenesis of *M. fortuitum* is still not clear but it has been documented that kidney forms lesions during the infection. Granuloma formation takes place upon infection of *M. fortuitum*. Immune system recruits the macrophages during infection and acts as a first line of defence. Macrophages engulf the bacteria but bacteria have several evasion strategies. The bacteria survive under the hostile environment of macrophages. Extracytoplasmic sigma factors are the one which helps the bacteria to survive inside macrophages. Among all the sigma factors, sigma factor H has a critical role as it regulates the expression of other sigma factors which helps the bacteria to survive under heat and oxidative stress. Sigma factor H responses during environmental stimuli and helps the mycobacteria to survive inside the macrophages [70].

The role of *sigH* in *M. tuberculosis* has been already document as it helps the *M. tuberculosis* to survive during stress conditions such as heat stress, detergent stress, diamide stress and during hypoxia [57, 61]. In *M. tuberculosis, sigH* get induced inside the macrophages. *M. tuberculosis sigH* mutants were more susceptible to heat stress and oxidative stress. As *M. fortuitum* also resist the stress conditions inside macrophages like *M. tuberculosis*, in this study we have tried to find out the role of *sigH* in *M. fortuitum*.

In this study we have observed that the wild type *M. fortuitum* survives during oxidative stress conditions concluding that *M. fortuitum* is residing inside the macrophages during oxidative stress condition whereas there is no difference between the growth pattern of *MfsigH* sense and *MfsigH* antisense construct indicating that *sigH* may not play a role in oxidative stress. The results of our study are not comparable to the study done by S. Raman., *et al.*,(2001) which showed the *sigH* mutant of *M. tuberculosis* were more suseptible during oxidative stress condition [57].

M. fortuitum wild type was not able to survive during heat stress after 24hours indicating that *M. fortuitum* cannot resist heat stress at 50^0C. Studies has been done for *sigH* of *M. tuberculosis* at 45^0C to 52^0C.S.Raman., *et al*.,(2001) observed that the *sigH* mutant of *M. tuberculosis* are more suseptible to heat stress at 52^0C [57]. The result of our study are not similar to thet study done by Fernandes et al., which showed that the *M. smegmatis sigH* mutant also showed decrease in their survival at 50^0C [58].

During nutrient starvation wild type *M. fortuitum* was able to survive and have constant cell count whereas the growth of *MfsigH* antisense construct decreased. We can say that the sigma factor play a role in the survival of *M. fortuitum* during nutrient starvation. Our results are comparable to the study done by Singh, et al., (2009) in which *M. smegmatis* was able to survive for long during nutrient starvation [51].

Wild type *M. fortuitum* as well as *MfsigH* sense and antisense constructs showed their survival during sds stress. It means that the bacteria was able to survive inside macrophages but there was no cell count difference between the mutants and the wild type *M. fortuitum* indicating that may be *sigH* do not have its role during sds stress condition.

Wild type *M. fortuitum* and *MfsigH* mutants were survived under normal pH i.e at 6.5 pH. But at pH 5.5 there was a slight difference in the cell count. Cell count of *MfsigH* sense and antisense construct get lowered as compared to wild type. At 4.5 pH the *MfsigH* sense and antisense constructs showed decline in there growth but wild type was able to survive at pH4.5. At pH 3.5 wild type along with constructs were not able to survive but wild type survived for certain period of time as compared to constructs. This study showed that the role of *sigH* under acidic condition. The overexpression as well as the underexpression of *sigH* gene inhibits the growth of bacteria. The overexpression may inhibiting the other genes which have role in the survival during stress conditions. Previsoly it has been studied that the *M. smegmatis sigH* mutant was found to be similar in logarithmic growth, stationary phase survival, and survival following acidic and detergent stress condition [58].

In-vivo studies showed that the *MfsigH* mutants were not survived as compared to wild type. This study may conclude that the overexpression of *sigH* inhibits its

own expression through feedback inhibition and it may also inhibit the expression of other genes responsible for survival in stress conditions.

CHAPTER 6

References

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CHAPTER 7

Appendix

APPENDIX

7.1 Bacteriological Media

All the media were prepared in Milli RO grade water and autoclaved at 15 pounds per square inch.

7.1.1 LB Broth (Luria Bertani Broth)

Tryptone	10g
Yeast Extract	5g
NaCl	10g

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.5 with 5N NaOH and the volume adjusted to 1000ml with Milli RO water.

7.1.2 LB Broth with Glycerol and Tween80 (LBGT)

0.5% 0f Glycerol and 0.15% to 0.18% of Tween80 were added to 1L of LB broth and then autoclaved.

7.1.3 Nutrient Broth tween80 (NAT)

Peptone	13g
Yeast Extract	1.5g
Beef Extract	1.5g
NaCl	5g
Agar (1.5%)	15g
Tween 80(0.05%)	0.5ml

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.4 with 5N NaOH and the volume adjusted to 1000ml with Milli RO water.

7.1.4 Nutrient Agar tween80 cycloheximide/ kanamycin(NATC/K)

Working concentration of cycloheximide and kanamycin was 50µl/ml and 30µl/ml respectively. Cycloheximide and kanamycin were added in sterilized NAT.

Cycloheximide

The stock solution of cycloheximide was 5mg/ml. 520.8mg of cycloheximide (potency=96units/mg) was dissolved in 100ml of Milli RO water. The solution was mixed and then filtered through syringe filter (0.22µ).

Kanamycin:

The stock solution of kanamycin was 10mg/ml. 13.33mg/ml of knamycin (potency= 750µg/mg) was dissolved in 100ml of 100ml of Milli RO water. The solution was mixed and then filtered through syringe filter (0.22µ).

7.1.5 Middlebrook 7H9 Broth (MB7H9)

Disodium Phosphate	2.5gm
Monopotassium Phosphate	1.0gm
L-Glutamic Acid	0.5gm
Ammonium Sulfate	0.5gm
Sodium Citrate	0.1gm
Magnesium Sulfate	50.0mg
Ferric Ammonium Citrate	40.0mg
Zinc Sulfate	1.0mg
Copper Sulfate	1.0mg
Pyridoxine	1.0mg
Calcium Chloride	0.5mg
Biotin	0.5mg
Glycerol	2.0ml
Bovine Albumin	5.0gm
Dextrose	2.0gm
Beef Catalase	3.0mg
Tween80 (0.15%-0.18%)	1.5ml
Glycerol (0.5%)	5ml
Milli RO water	900ml

All the components were mixed using Milli RO water and the pH should be around 7.2. The final volume adjusted to 1000 ml and then sterilised.

7.2 Reagents for Acid Fast Staining

7.2.1 Carbol Fuchsin (primary stain)

Basic Fuchsin	3g
Phenol	5%
Ethanol (96%)	10ml

Mixed 10ml of Basic Fuchsin to 90ml of phenol and the solution was filtered through Whatman filter paper.

7.2.2 Acid alcohol (Decolourizer)

HCL (conc.)	3ml
Ethanol (96%)	97ml

7.2.3 Malachite Green solution (Counter stain)

Malachite green 0.25g in Milli RO water

7.3 Reagents and Buffers

7.3.1 Pohsphate Buffer Saline (PBS)

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g

All the components were dissolved in 800ml Milli RO water and then pH adjusted to 7.4 with HCL. Final volume adjusted to 1000ml using Milli RO water. Finally the PBS was sterilized.

7.3.2 Sodium Dodecyl sulphate (SDS 10%)

10g SDS was mixed in 100ml Milli RO water. 0.05% SDS was prepared using prepared stock solution (10%). For detergent stress, 250µl of 10% SDS was mixed in 50ml MB7H9 media.

7.3.3 Hydrogen peroxide 10mM

56.6 µl of H_2O_2 were mixed in the 50ml of MB7H9 media for oxidative stress condition.

7.3.4 Tween normal saline

Tween 80(0.1%)	1ml
NaCl (0.89%)	8.9g

Components were mixed using 1000ml of Milli RO water and the autoclaved.

7.3.5 MB7H9 Media for acidic stress

For acidic stress, pH of 50ml MB7H9 media was adjusted to 3.5, 4.5, 5.5, and 6.5 using dilute HCL

Publications

Mycobacterium fortuitum sigH **antisense knock-out mutant shows reduced Survival under in vitro stress conditions**

Ritu Ghildiyal, Shivani Sood, Jitendraa Vashistt, Rahul Shrivastava

Poster presentation at - 56th Annual Conference of Association of Microbiologists of India, organized by JNU, New Delhi,

December 7- 10, 2015

Poster:

Mycobacterium fortuitum sigH antisense knock-out mutant shows reduced survival under in vitro stress conditions

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ABSTRACT

Mycobacterium fortuitum is an important clinical pathogen causing localized abscesses, pulmonary disease and disseminated infections in humans. Sigma factors play vital role in different stress responses. *Sigma factor H* (*sigH*) plays central role in a network, that regulates stress responses likely to be important in *M. tuberculosis* pathogenesis. With an aim to study role of *sigma factor H* in regulation of stress response and pathogenesis of *M. fortuitum*, multiple primer sets were designed from conserved region of *M. tuberculosis sigH* to amplify full length gene sequence of its homologue in *M. fortuitum*. Longest sequence showing highest similarity *with M. tuberculosis sigH* was submitted to NCBI as *M. fortuitum* ATCC 6841 homologue of *sigma factor H*. To determine the role of *sigH* in survival under different stress conditions, sense and antisense knockouts were constructed using pMV261 vector. Role of *sigH* was determined in various stress conditions by subjecting the sense and antisense mutants along with wild type *M. fortuitum* as control to different stress conditons. *sigH* was involved in hypoxia-induced, acid-induced stress response, and other in-vitro stress conditions.

INTRODUCTION

- *Mycobacterium fortuitum* is an ubiquitous non-tuberculous fast growing mycobacterium
- Causes pulmonary, skin and soft, catheter-related, tissues, joint, bone, wound infections, lymphadenitis as well as disseminated infections
- Responsible for high mortality rates in immunocompromised patients particularly HIV
- Sigma factors are involved in heat shock, cold shock, hypoxic , nitric oxide shock, carbon, iron and zinc starvation
- *sigH* plays central role in a network, that regulates stress responses likely to be important in *M. tuberculosis* pathogenesis
- *sigH* regulates the expression of *sigB* and *sigE*
- *sigH* is also responsible for *trx operon* regulation

MATERIALS AND METHODS

Multiple sets of primers were designed from flanking region of *sigH* CDS and PCR amplified on *M. fortuitum* genome

↓

Amplified product were sequenced and checked for homology with sigH of *M. tuberculosis*

↓

Longest sequence having maximum homology was submitted to NCBI [GenBANK ID - KM282166].

↓

Presence of full length gene and its orientation was confirmed by restriction digestion and PCR

↓

Sense and antisense knockouts were electroporated in *M. fortuitum*

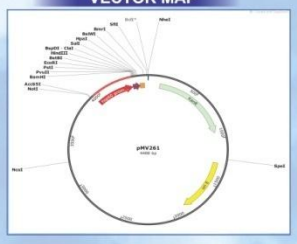
↓

Role of these knockout constructs was determined under *in vitro* different stress conditions

↓

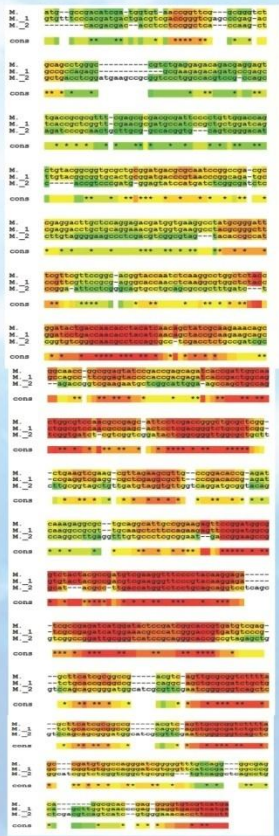
In-vivo analysis of knockout construct was done

VECTOR MAP

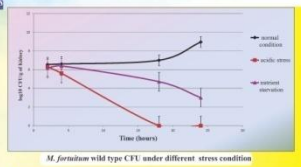


RESULTS

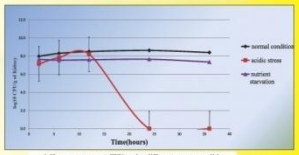
M. fortuitum sigH showed 51.15% homology with *sigH* of *M. tuberculosis*



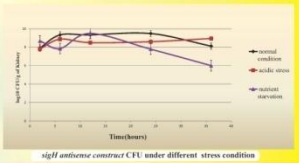
IN VITRO



M. fortuitum wild type CFU under different stress condition

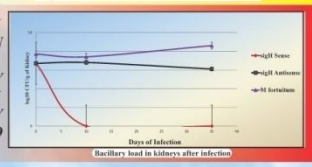


sigH sense construct CFU under different stress condition



sigH antisense construct CFU under different stress condition

IN VIVO



Bacillary load in kidneys after infection

SIGNIFICANCE

-*sigH* homologue of *M. fortuiutm* was identified and submitted

-Study shows the importance of *sigma factor H* in the pathogenesis of *M. fortuitum as M. fortuitum* survives under stress conditions

-Overexpression of *sigH* factor in *sigH* sense construct shows inhibition under acidic conditions as wells in *in-vivo* studies, indicates the inhibiton of *sigH* (feedback inhibition) or other genes responsible for survival in stress conditions

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