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ORIGINAL ARTICLE

Ribosomal maturation factor (RimP) is essential for survival of nontuberculous mycobacteria *Mycobacterium fortuitum* under in vitro acidic stress conditions

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Abstract

Mycobacterium fortuitum is an important human pathogenic NTM, which resists stress conditions inside macrophages by exploitation of specific genes. TnphoA-based transposon mutagenesis was employed to identify membrane genes responsible for survival of *M. fortuitum* under such stress conditions. A library of about 450 mutants was constructed after electroporation of vector pRT291 into wild-type *M. fortuitum*. On the basis of blue color development and alkaline phosphatase assay, 20 mutants were shortlisted to screen for growth and survival under acidic stress at pH 6.5, 5.5, 4.5, and 3.5. Mutant MT727 showed reduced growth and survival under acidic stress. The acid susceptible mutant MT727 was subjected to other in vitro stress conditions prevalent inside macrophages including oxidative, nutrient starvation and nitrosative stress. However, the mutant showed no appreciable difference in growth behavior under oxidative, nutrient starvation and nitrosative stress conditions in comparison to the wild type. Genomic and bioinformatics analysis of MT727 led to identification of putative ribosomal maturation factor RimP of *M. fortuitum* to be affected by mutagenesis, showing closest homology to *M. abscessus* RimP. In silico functional interaction of RimP protein using STRING database showed its interaction with proteins of ribosomal assembly and maturation. Results indicate role of rimP gene in survival of *M. fortuitum* under acidic stress conditions which may be further explored for use as a potential drug target against *M. fortuitum* and other mycobacterial infections.

Keywords *Mycobacterium fortuitum* · RimP · Acidic stress · Transposon mutagenesis · Drug target · STRING

Introduction

Genus *Mycobacterium* belongs to family Mycobacteriaceae which consists of more than 180 species. The huge number of species of mycobacterium genus can be distinguished on the basis of their pathogenesis into two types, 'mycobacterium tuberculosis complex' and 'nontuberculous mycobacteria (NTM)'. The *Mycobacterium tuberculosis* complex consists of *M. tuberculosis, M. africanum, M. canettii, M. bovis, M. microti*, etc., responsible for causing tuberculosis or tuberculosis-like symptoms; whereas, nontuberculous mycobacteria, as the name indicates do not cause tuberculosis, however, are responsible for a variety of infections in immunocompromised as well as immunocompetent individuals.

NTM have gained significant attention in recent years due to their broad spectrum of virulence and ability to cause infection in different parts of the human body in immunocompromised individuals (Hermansen et al. 2017; Maurya et al. 2015). NTM can be categorized into rapid growers and slow growers on the basis of their generation time. Because of the difficulty in isolation and differentiation between NTMs, clinical and microbiologic criteria are needed for the diagnosis of NTM infections. Infections from NTM can also mislead to treatment against *M. tuberculosis*, therefore American Thoracic Society (ATS) and Infectious Disease Society of America (IDSA) recommended the identification of NTM along with *M. tuberculosis* in sputum samples. Currently recommended treatment regimens, patterns of resistance towards drugs, and treatment outcomes differ

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according to the NTM species; compounding to lengthy and complicated management of such infections with limited therapeutic options.

Mycobacterium fortuitum is one of the most prevalent rapidly growing NTMs worldwide responsible for causing skin, soft tissue, lungs, bones and blood infections (Hoefsloot et al. 2013; Raveendran et al. 2015; Umrao et al. 2016). *M. fortuitum* infections in lungs can mislead to treatment against *M. tuberculosis*, which further complicates the condition due to resistance of *M. fortuitum* to antituberculous drugs. *M. fortuitum* infections have been reported in immunocompetent individuals indicating increased virulence of the pathogen (Macente et al. 2013; Chetchotisakd et al. 2000). *M. fortuitum* infections can be fatal in immunocompromised patients, while recovery of *M. fortuitum* infections in immunocompetent individuals may also require long term drug treatment regimen (Tsai et al. 2016). Co-infection of *M. fortuitum* along with *M. tuberculosis* in ocular mycobacteriosis has been reported with no results from antituberculous drug therapy indicating requirement of multidrug treatment for *M. fortuitum* along with *M. tuberculosis* (Sharma et al. 2017).

M. fortuitum infects macrophages like *M. tuberculosis*, indicating similar pathogenesis among both pathogens. Being an intracellular pathogen, *M. fortuitum* resides inside the macrophages to evade the harmful effects of the immune system by restricting nitrogen oxide radicals and phagosome–lysosome fusion (da Silva et al. 2002). Bactericidal activity of the macrophages is majorly contributed by the acidic environment prevalent inside it, as excessive protons can damage lipids, proteins, and nucleotides of the invading mycobacteria. In addition, acidic pH inside the macrophage adversely affects the biochemical activities of the residing mycobacteria (Vandal et al. 2009). In addition to acidic stress, other stress conditions including oxidative stress, nutrient starvation stress and nitrosative stress are also prevalent inside macrophages. These stress conditions impose various bactericidal effects as defense arsenal of the host. To avoid drastic effect of stress conditions, the pathogen evolves various strategies including changing of their metabolic behavior. The change in metabolic behavior is dependent on the induction of expression of virulent genes. The aim of the present study was to get an insight into the mechanism responsible for the survival of *M. fortuitum* inside macrophage stress conditions by identification of genes involved.

Cell membrane proteins are an important part of bacteria, as it helps the bacteria to maintain its integrity, acts as a guard for entry of molecules, interacts with outside molecules and is involved in inducing signaling pathways. Membrane proteins also help the bacteria in acquisition of antibiotic resistance and combating unfavorable environment inside the host (Koebnik et al. 2000). Owing to essential functions performed by the bacterial cell membrane, the present study was undertaken for the identification of membrane proteins of *M. fortuitum* that help in survival of the bacteria under stress conditions.

Transposon mutagenesis used in the present study, is a valuable and useful technique to identify genes of a particular phenotype. TnphoA-based transposon mutagenesis provides a simple and efficient way to identify membrane/secreted protein encoding genes due to the presence of alkaline phosphatase (phoA) reporter gene. The fusion of phoA reporter gene with the membrane genes leads to hybrid protein expression on the surface of bacteria and can be easily identified by growing the mutants in the presence of selective media. Transposon mutagenesis has been used previously for the identification of virulence genes of *Mycobacterium* spp., *Salmonella* spp., and *Vibrio cholera* (Barquist et al. 2013; Cameron et al. 2008; Wang et al. 2011). Serine protease and Rv2136c genes have been identified in *M. tuberculosis* by transposon mutagenesis, to play a role in acidic stress (Vandal et al. 2009). Identification of virulent genes in *M. fortuitum* has also been done previously by transposon mutagenesis (Parti et al. 2008), although no genes have yet been reported to be involved in its survival under in vitro stress conditions prevalent inside macrophages. Thus, outcome of the present study will help in understanding the molecular mechanism involved in stress survival of *M. fortuitum* inside macrophages and might help in identifying new drug target(s) against the pathogen. Identification of new drug targets in *M. fortuitum* may also lead to identification of corresponding homologs as novel drug targets in other members of mycobacterium family and intracellular pathogens residing inside the macrophages.

Materials and methods

Bacterial cultures, plasmids and antibiotics

Bacterial strains used in the present study were *M. fortuitum* ATCC 6841 (received as kind gift from CDRI, Lucknow) and *Escherichia coli* DH5α. *M. fortuitum* was grown under shaking conditions at 37 °C in Luria–Bertani broth (HiMedia) supplemented with 0.5% glycerol (Fisher Scientific) and 0.2% Tween 80 (Bio Basic Inc) (LBGT), or MB7H9 broth supplemented with 0.5% glycerol and 0.2% Tween 80. Antibiotics used for the present study were purchased from HiMedia, and dissolved as per the solubility of antibiotics. Kanamycin (30 µg/mL) and ampicillin (100 µg/mL) were added to the culture medium as per the protocol requirement. Random mutagenesis of *M. fortuitum* was done using plasmid pRT291-containing transposon (Taylor et al. 1987). Solid medium used for the culture of *M. fortuitum* was nutrient agar (HiMedia) supplemented with

0.05% Tween 80 (NAT). *E. coli* DH5α strain used for cloning was grown using Luria–Bertani broth and nutrient agar. X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) (Thermo Fisher Scientific), X-P (5-bromo-4-chloro-3-indolyl phosphate) (Thermo Fisher Scientific), IPTG (isopropyl-β-D-1-thiogalactopyranoside) and glucose (Merck) were used for cloning and screening of the mutants.

Construction of transposon mutant library

Transposon mutant library of *M. fortuitum* was constructed by electroporation of TnphoA-containing plasmid pRT291 into wild-type *M. fortuitum*. The procedure for electroporation was adopted as described previously (Garbe et al. 1994; Sood et al. 2016). Briefly, 100 mL culture of *M. fortuitum* was grown in LBGT till optical density of the cells reached 0.6 at 600 nm. The cells were incubated on ice for 30 min, and then centrifuged at 5500×*g* for 10 min at 4 °C. The pellet was washed with 5% glycerol twice by centrifugation at 5000×*g* for 10 min at 4 °C. The pellet was finally suspended in 1 mL 5% glycerol. 400 µL of the cell suspension was placed in 0.2 cm electroporation cuvette (Bio-Rad) and 25 ng of plasmid DNA was added to these cells followed by incubation on ice for 5 min. Electric shock to the cells was given using gene pulser (Bio-Rad) using conditions 2500 V voltage, 25 µF capacitance and 1000 Ω resistance for 2 s. The electric shock to cells was given two times with an interval of 5 min between two electric shocks. The cells were then incubated on ice for 5 min and inoculated into 2 mL of LBGT, followed by incubation at 37 °C for 6 h. The cultures were spread on plate containing kanamycin, X-P, and 5% glucose.

Screening of transposon mutant library

Identification of mutants positive for insertion of transposon in membrane spanning gene was done on the basis of blue color of the mutants on plates containing kanamycin, X-P and 5% glucose. After primary screening, the blue mutants were further analyzed for alkaline phosphatase activity as described previously by Kaufman and Taylor (1994), with modifications. The mutants along with wild-type *M. fortuitum* were grown in MB7H9 medium till the optical density of the cells reached to 0.6 at 600 nm. 500 µL of each culture was taken and centrifuged at 15,000×*g* for 10 min. The supernatant was removed and the cells were lysed with 0.1% SDS and 2 mg/mL lysozyme. The lysed cells were vortexed properly, followed by addition of 100 µL of 1 M Tris buffer and 1 mL of 2 mM *p*-nitrophenyl phosphate. Incubation at 37 °C was done till yellow color appears (10–20 min). After appearance of yellow color, the reaction was stopped by adding 100 µL of 10 N NaOH. Optical density of the sample was measured at 420 nm, and alkaline phosphatase units were calculated as (1000 × optical density at 420 nm)/(minutes of incubation × optical density at 600 nm (initial culture) × 0.5 mL).

Survival studies under in vitro acidic stress conditions

Wild-type *M. fortuitum* and the shortlisted mutants were subjected to acidic stress conditions according to the method described by Geiman et al. with modifications (Geiman et al. 2006). Wild-type organism and the mutant strains were grown in MB7H9 medium till logarithmic growth phase and centrifuged at 5500×*g* for 10 min. Individual pellet were then washed twice with phosphate buffered saline (PBS) and resuspended into 5 mL MB7H9 medium. Each culture suspension was then used to inoculate 100 mL MB7H9 medium with different pH 6.5, 5.5, 4.5 and 3.5 and incubated at 37 °C with shaking. Samples were collected from each flask after 2, 4, 6, 12, 24 and 36 h and CFU of the collected samples was determined by serial dilution followed by plating on NAT plates.

Survival studies under in vitro oxidative stress and nutrient starvation stress

The ability of acid-susceptible mutant MT727, to survive under in vitro oxidative stress and nutrient starvation was determined by exposing the mutant and wild-type *M. fortuitum* to conditions as described previously (Kawaji et al. 2010; Geiman et al. 2006) with minor modifications. The mutant and wild-type *M. fortuitum* were grown in MB7H9 medium till mid-logarithmic phase. Centrifugation of cultures was done at 5000×*g* for 10 min, followed by washing twice with PBS. After washing, the pellet were resuspended in MB7H9 medium and, inoculated in 100 mL MB7H9 medium containing 10 mM H_2O_2 for oxidative stress and in PBS for nutrient starvation stress. The cultures were then incubated at 37 °C at 200 rpm and CFU was determined after 2, 4, 6, 12, 24 and 36 h.

Survival studies under in vitro nitrosative stress

The acid susceptible mutant MT727 was further studied for its ability to survive under in vitro nitrosative stress conditions, using protocol as described previously (Kawaji et al. 2010) with minor modifications. The mutant and wild-type *M. fortuitum* were grown in MB7H9 medium till mid-logarithmic phase. Centrifugation of cultures was done at 5000×*g* for 10 min, followed by washing twice with PBS. After washing, the pellet were resuspended in MB7H9 medium and, inoculated into 100 mL MB7H9 (pH 7.0) containing 1 mM sodium nitrite for nitrosative stress, 100 mL acidic MB7H9 (pH 5.0) containing 1 mM sodium nitrite for

acidonitrosative stress and 100 mL MB7H9 medium with autoclaved water as control. The cultures were then incubated at 37 °C at 200 rpm and CFU was determined at 2, 4, 6, 12, 24 and 36 h.

Genomic analysis

Genomic DNA (gDNA) of the acid-susceptible mutant MT727 was isolated using protocol as described previously by Parti et al. (2008) for *M. fortuitum*. The presence of transposon in the genomic DNA was confirmed by amplification with transposon-specific primers RS6 (5′ ACATTGCCG CCGATACCG 3′) and RS7 (5′ TGGATGGCTTTCTTTCTT GCCG 3′). After confirmation of the transposon in gDNA of the mutant, restriction digestion of the gDNA was done using EcoRV, which has no restriction site in the transposon. The digestion of gDNA with EcoRV-generated blunt ends in the digested nucleic acid. For cloning of the transposon-affected gene of *M. fortuitum* in pUC19 vector, pUC19 vector was digested with SmaI enzyme which also generates blunt ends. The digested gDNA and pUC19 vector were ligated and transformed into *E. coli* DH5α. Transformed *E. coli* cells bearing *M. fortuitum* gene with transposon insertion, were selected on LB agar plates containing ampicillin (pUC19 marker), kanamycin (TnphoA marker), X-gal and IPTG. The white transformants of *E. coli* were selected and used further for plasmid isolation. After confirmation of plasmid for the presence of transposon containing segment of DNA, the mutated gene was sequenced from both ends using universal primers M13 fwd (5′ TGTAAAACGACG GCCAGT 3′), and M13 rev (5′ CAGGAAACAGCTATGAC 3′) inwards; and transposon-specific outward primers K36 (5′ ATCGCTAAGAGAATCACGCAG 3′), and RS6 (5′ ACA TTGCCGCCGATACCG 3′) by Sanger sequencing (Xcelris genomics, Ahmedabad). Strategy used for identification of the mutated gene is described in Supplementary material Fig. 1.

Bioinformatics analysis

Gene identification by homology study

The sequence obtained was analyzed through nucleotide BLAST (BLASTn) and nucleotide translation protein BLAST (BLASTx) (https://blast.ncbi.nlm.nih.gov) of NCBI. ExPASy translate online server (https://web.expasy.org/translate) was used to translate the ORF (open reading frame) of the mutated gene, which was further subjected to multiple sequence alignment using T-Coffee (http://tcoffee.crg.cat) tool and visualized using Jalview software (http://www.jalview.org).

Protein–protein interaction studies

The identified gene was further characterized by in silico interaction study to identify potential interacting genes which may be functionally associated. This analysis would help in understanding the effect of mutation in one gene on the functions of interacting genes, which may indirectly influence the acid susceptible phenotype of the mutant. The in silico interaction studies were performed using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 10.5 (https://string-db.org). As *M. fortuitum* database was not available in STRING, in silico study was performed using RimP protein of *Mycobacterium abscessus* which showed closest homology to the identified gene. The interaction parameter was set to medium confidence level of 0.5 in the STRING database.

Statistical analysis

The data shown comprised mean of readings taken from three independent experiments with standard deviation in error bars. CFU was determined in duplicate at each time point.

Results

Short listing of blue mutants showing high alkaline phosphatase activity

TnphoA-based random mutagenesis of *M. fortuitum* resulted in construction of a library of about 450 mutants. Among these 450 mutants, 42 mutants were found to have insertion in genes coding for membrane proteins on the basis of blue color production in the presence of X-P and 5% glucose. Secondary screening by alkaline phosphatase assay of these 42 blue mutants resulted in short listing of 20 mutants having high alkaline phosphatase activity (Supplementary material Table 1). These 20 shortlisted mutants were used for acidic stress-related studies.

Mutant MT727 was unable to survive under acidic stress conditions

The shortlisted 20 mutants were subjected to acidic stress conditions to study their growth and survival. Of the 20 mutants screened, one mutant namely MT727, showed least survival under acidic stress conditions. It was observed that as the pH of the medium decreased, ability of the mutant bacilli to sustain its growth in the medium decreased. The mutant MT727 showed growth characteristics comparable to the wild type at pH 6.5 and pH 5.5. However, at pH 4.5 there was 3.8 log decrease after 36 h of acidic stress when

compared to the wild type, and at low pH of 3.5 the mutant showed 2.7 log decrease just after 2 h of exposure to acidic stress and failed to survive beyond 4 h, as no CFU could be recorded after 4 h of exposure (Fig. 1).

Mutant MT727 showed no change in growth behavior under oxidative, nutrient starvation and nitrosative stress conditions

Since the mutant MT727 showed defect in survival under acidic stress conditions, growth behavior and survival of the mutant was further studied under other macrophage-related stress conditions of oxidative, nutrient starvation and nitrosative stress. Under oxidative and nutrient starvation stress conditions, survival and growth behavior of MT727 was similar to that of wild-type *M. fortuitum* (WTMF), without any considerable difference during the observation period (Fig. 2). Growth behavior of MT727 was also found to be similar to that of WTMF when subjected to nitrosative ($NaNo_2$) and acidonitrosative (acidic $NaNo_2$) stress conditions. Under nitrosative stress, WTMF showed 3.35 log increase in CFU after 36 h of stress, which was similar to CFU increase under normal growth conditions (control). However, under acidonitrosative stress WTMF showed no increase in CFU throughout the experiment, indicating bacteriostatic effect of acidonitrosative stress on growth of *M. fortuitum*. When compared with the wild type, the mutant MT727 showed similar growth behavior as that of WTMF without any appreciable difference under nitrosative as well as acidonitrosative stress conditions (Fig. 3).

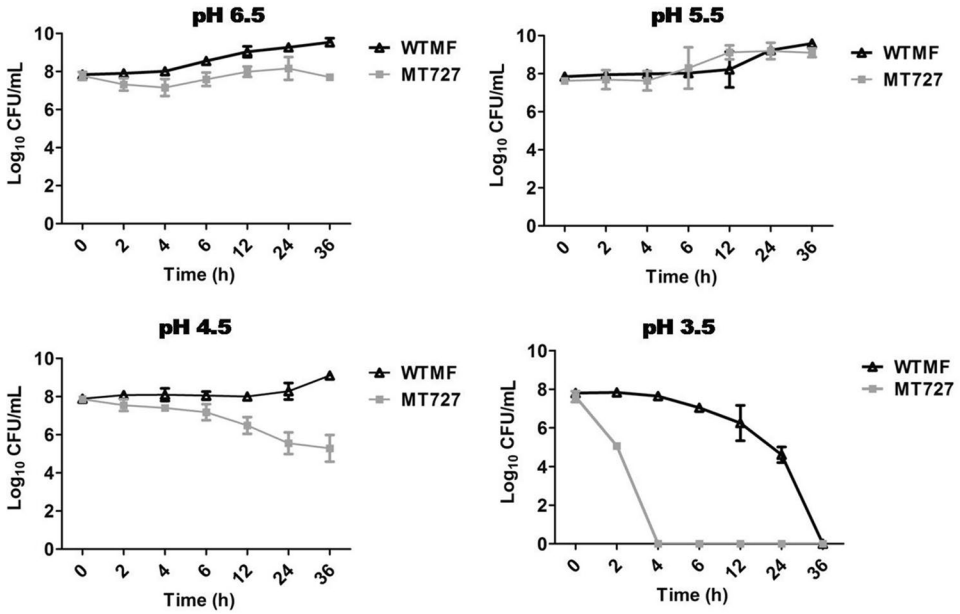

Fig. 1 Growth and survival of *M. fortuitum* wild type and mutant MT727 under acidic stress conditions. The figure shows growth behavior of mutant (MT727) at pH 6.5, pH 5.5, pH 4.5 and pH 3.5 in comparison to the wild-type strain (WTMF). The mutant shows reduced survival at pH 4.5 and no survival after 4 h at pH 3.5, in comparison to the wild type. The data represented is the mean of three independent experiments with ± standard deviation

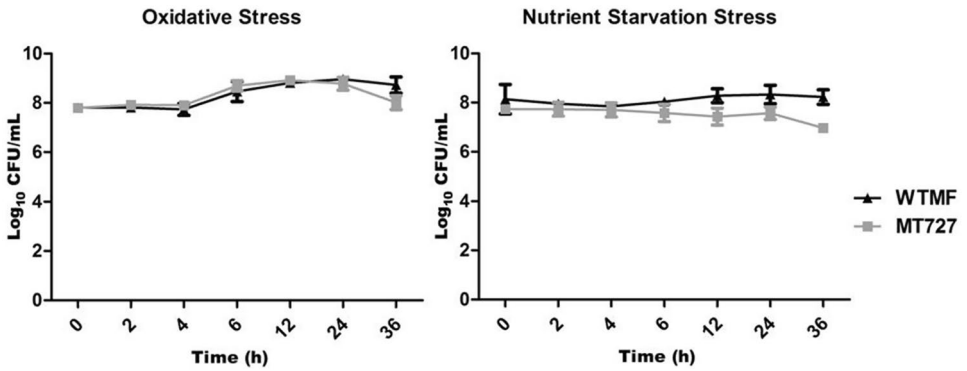

Fig. 2 Growth and survival of *M. fortuitum* wild type and mutant MT727 under oxidative and nutrient starvation stress conditions. The figure shows growth behavior of WTMF and MT727 under oxidative stress and nutrient starvation stress. The mutant and the wild-type *M. fortuitum* show similar growth curves with no considerable difference. The data represented is the mean of three independent experiments with ± standard deviation

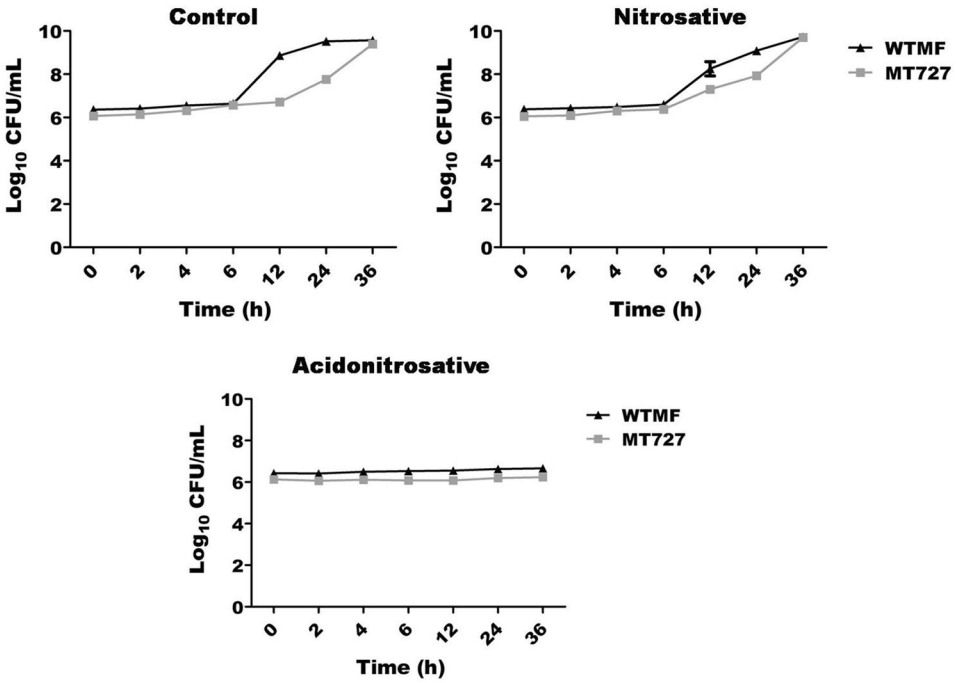

Fig. 3 Growth and survival of *M. fortuitum* wild type and MT727 under nitrosative and acidonitrosative stress conditions. The figure shows growth behavior of WTMF and MT727 under normal growth conditions (control, pH 7.0), nitrosative stress (MB7H9 with 1 mM sodium nitrite, pH 7.0), and acidonitrosative stress (acidic MB7H9 with 1 mM sodium nitrite, pH 5.0). The growth behavior of WTMF and MT727 was found to be similar with no appreciable change in CFU under both stress conditions. Acidonitrosative stress shows a bacteriostatic effect on growth of wild type and mutant *M. fortuitum* strains in comparison to the control. The data represented is the mean of three independent experiments with ± standard deviation

Homology study revealed rimP gene to be inactivated by mutagenesis

Presence of a transposon-specific 300-bp fragment (Supplementary material Fig. 2) confirmed the insertion of transposon in the mutant MT727. Sequencing of the DNA fragment mutated by insertion lead to identification of a 312 bp ORF translating to 103 amino acids (Supplementary material Fig. 3). The nucleotide sequence obtained was submitted to GenBank as 'Putative ribosomal maturation factor RimP of *Mycobacterium fortuitum'* with accession number MH052677. Homology study of the gene sequence obtained using BLASTx showed 61% homology with ribosomal maturation factor rimP of *M. abscessus* subspecies *abscessus*, 32% identity with *Mycobacterium marinum* and 36% identity with *M. tuberculosis*. Multiple sequence alignment of translated product obtained by ExPASy translate tool showed 60.8% identity with RimP protein sequence of *M. abscessus* subspecies *abscessus*, 33.6% identity with *M. marinum* and 27.9% identity with *M. tuberculosis* (Supplementary material Fig. 4). As the percentage identity of the gene was most significant with *M. abscessus* subspecies *abscessus*, hence, the sequence obtained was identified as a putative homologue of *M. abscessus* subspecies *abscessus* RimP in *M. fortuitum* and designated as MfRimP.

Interaction of RimP with ribosomal assembly factors

Functional interaction of RimP protein with a confidence score of more than 0.5 on STRING was observed with translation termination and antitermination protein (NusA), 30S ribosomal protein s12 (RpsL), translation initiation factor IF-2 (InfB), probable 5′-methylthioadenosine phosphorylase (Pnp), probable ribosomal binding factor (RbfA) and five other hypothetical proteins (Supplementary material Fig. 5). Reported function(s) of the interacting proteins was deduced from Mycobrowser database (https://mycobrowser.epfl.ch) (Supplementary material Table 2). All these genes are involved in ribosomal assembly and translation, thus functional interdependency of these genes is not uncommon.

Discussion

Nontuberculous mycobacteria have broad range of virulence, and are responsible for a wide variety of infections. Identification and diagnosis of NTM is a difficult and complicated process. Variation in the treatment regimen among NTM further raises the importance of this group of mycobacteria. *M. fortuitum* is an important member of this group causing a range of infections including skin and soft tissue infections, bone infections and pulmonary infections (Umrao et al. 2016). Disseminated infections due to *M. fortuitum* have also been reported in immunocompromised (Unni et al. 2005) as well as immunocompetent patients (Chetchotisakd et al. 2000), requiring long-term drug therapy for treatment. Emergence of antibiotic resistance and complicated drug treatment regimen for immunocompromised patients warrant development of standard drug therapy against *M. fortuitum* infections. Hence, deeper understanding of pathogenesis and

survival mechanism of the bacteria, and subsequent identification of novel drug targets would aid in the effective treatment of complications due to *M. fortuitum* infections.

M. fortuitum, like other pathogenic mycobacteria resides inside macrophage and resists its obliterating effects (Parti et al. 2008). Activation of macrophages by IFN-γ and bacterial lipopolysaccharides induces the vacuolar proton ATPase inside phagolysosome, which results in greater efflux of protons inside the vacuole, thus leading to prevalence of acidic conditions (Ehrt and Schnappinger 2009). The pH of intracellular phagolysosome compartment ranges from 6.5 to 4.5 depending upon the activation stage of the macrophage, however, pH inside macrophage phagolysosome has been demonstrated to be as low as 3.5 (Vandal et al. 2009). Thus, the present study was undertaken with acidic stress conditions ranging from pH 6.5 to 3.5 mimicking pH conditions inside macrophages to identify mutant defective in survival under low pH conditions.

Cell membrane proteins are involved in a variety of cellular processes and are essential for survival of bacteria. Membrane proteins help in interaction, transport, signaling and resistance of pathogens to various adverse conditions (Koebnik et al. 2000), functioning as critical virulence factors, and thus, act as potential drug targets. The role of cell membrane proteins as drug target has also been reviewed by Schwegmann and Brombacher (2008) that can be further explored in discovering new and effective treatment strategies against intracellular pathogens.

TnphoA-based transposon mutagenesis is an effective tool for the identification of membrane genes, as it contains alkaline phosphatase reporter for identification of transposon insertion in genes encoding for membrane proteins (Taylor et al. 1987). TnphoA is a derivative of Tn5 that contains alkaline phosphatase (phoA) gene lacking signal sequence. Genes encoding for membrane protein contain a signal sequence for protein exportation to the cell surface. Thus, the active insertion of TnphoA in membrane protein encoding gene leads to formation and exportation of the hybrid protein to the cell surface, which can be easily visualized by blue color formation on selection plates.

A library of about 450 mutants was constructed by transposon mutagenesis, of which 42 mutants were selected on the basis of their blue color in presence of X-P and 5% glucose on NAT plates. Blue color of colonies after electroporation of the TnphoA-containing plasmid pRT291 into wild type indicates expression of alkaline phosphatase reporter gene (phoA) fused with a membrane protein encoding gene of the host genome. Further, alkaline phosphatase assay of the 42 blue mutants resulted in shortlisting of 20 mutants, which were then screened for their ability to survive under in vitro acidic stress conditions.

Of the 20 mutants screened, mutant MT727 showed reduced growth and survival under acidic stress conditions as compared to the wild-type strain. Genomic analysis of the mutant was done by cloning the transposon-containing fragment into pUC19 followed by sequencing. Bioinformatics analysis of the obtained nucleotide sequence using BLASTn did not show significant homology with other mycobacteria, hence, BLASTx was used to analyze the sequence. As protein sequence is more conserved in comparison to nucleotide sequence on the basis of evolutionary relationship, BLASTx translates nucleotide sequence into protein to identify homologous proteins (Khandelwal et al. 2017). Homology study carried out with the translated protein sequence led to identification of putative ribosomal maturation factor 'RimP' of *M. fortuitum* (MfRimP) as the mutated gene, on the basis of closest homology to the RimP protein of *M. abscessus* subspecies *abscessus*. The novel identified ORF was submitted to GenBank (ID: MH052677).

In addition to acidic stress, pathogenic mycobacteria encounter different stress conditions inside the macrophage. As the mutant MT727 exhibited an acid-sensitive phenotype, with the mutant gene having a potential role during macrophage infection, growth behavior of the mutant was studied under other stress conditions prevalent inside macrophage including oxidative, nutrient starvation and nitrosative stress.

Presence of reactive oxygen species (ROS) acts as one of the major defense strategies inside macrophages by causing oxidation of proteins and DNA of infectious organism (Shi et al. 2008; Cumming et al. 2014). *M. fortuitum* has been reported to survive in vitro oxidative stress (Núñez et al. 2008) indicating presence of mechanism for combating such stress condition. However, when exposed to in vitro oxidative stress conditions, no appreciable difference in survival and growth behavior of the mutant MT727 was observed in comparison to WTMF, indicating that mutation in rimP does not influence oxidative stress survival of *M. fortuitum*. Similar results were reported by Weng (2016), where rimP function was not associated with oxidative stress resistance.

The ability to inhibit active growth rate by suppressing the transcription and translation of genes required for rapid growth describes the 'cleverness' or 'expertise' of pathogenic mycobacteria to survive under stress conditions. Under nutrient stress, binding of guanosine pentaphosphate (p) ppGpp with RNA polymerase results in scarcity of GTP inside the cell leading to inhibition of transcription of genes required for active growth (Gaca et al. 2015). Under nutrient starvation, MT727 showed growth behavior similar to WTMF pointing towards no association of rimP gene with growth or survival under nutrient starvation stress. It might be due to the regulation of nutrient starvation stress at transcription level only, while inactivation of rimP gene might lead to defective translation of proteins.

The presence of reactive nitrogen intermediates (RNI) in activated macrophages is an important antimicrobial

weapon of the host immune system. Nitric oxide and its physiological intermediates play a major role in defending the host from pathogen. However, pathogenic mycobacteria have evolved survival strategies to overcome the bactericidal effect of RNI. When exposed to in vitro nitrosative and acidonitrosative stress conditions, no appreciable difference in growth and survival pattern of the mutant MT727 was observed in comparison to the WTMF, indicating no direct role of rimP gene in combating nitrosative stress. Although a previous study by Weng (2016) indicated role of rimP gene under nitrosative stress in non pathogenic mycobacteria *M. smegmatis*; a different mechanism not involving rimP gene might be operational in a pathogenic mycobacterial species like *M. fortuitum*. Results obtained in our experiment can further be correlated to previous reports where down regulation of ribosomal proteins and translation factors was observed under acidonitrosative stress in *M. tuberculosis* and *M. smegmatis* (Cossu et al. 2013). This is first study demonstrating bacteriostatic effect of in vitro acidonitrosative stress on the growth of *M. fortuitum*, corresponding to previous studies where *Mycobacterium bovis* showed constant growth under nitrosative stress conditions (Gallant et al. 2016).

The gene rimP is involved in maturation of 30S ribosomal subunit (Rene and Alix 2011). Ribosomal maturation factors RimM and RimP are required during early and late stage of ribosomal maturation, respectively (Bunner et al. 2010). The work done by Bylund et al. (2001) showed reduced growth of RimM deletion mutant under normal growth conditions, which signifies the essentiality of ribosomal maturation factors for the growth of bacteria. RimP has been reported to increase the affinity of 30S ribosomal protein s12 (RpsL) with 16SRNA in *M. smegmatis*, which is a major step in 30S ribosomal subunit biogenesis. Defect in 30S ribosomal subunit can modulate the mRNA decoding efficiency of ribosome and result in defective translation. Significance of RimP has been known in other bacterial genera as well, as less number of polysomes were observed in rimP deletion mutant of *E. coli* as compared to the wild type leading to translation deficient phenotype (Nord et al. 2009).

Under stress conditions including acidic stress, translational variations occur in most pathogenic bacteria that are required for survival of the organism. RimP deletion mutant of *M. smegmatis* was unable to survive under nitrosative stress suggesting the requirement of rimP under stress condition for protein synthesis (Weng 2016). Significance of ribosomal maturation factors under stress conditions has also been established in *E. coli* where delay in ribosomal assembly or defective ribosomal maturation leads to heat sensitive (Rene and Alix 2011), and cold-sensitive phenotype of *E. coli* (Bryant and Sypherd 1974), respectively. As rimP is involved in the maturation of 30S ribosomal subunit (Rene and Alix 2011), mutation in rimP gene of *M. fortuitum* may lead to less polysomes formation under acidic stress conditions. Thus, reduction in translation of proteins essential for survival under acidic stress might be the reason of acid susceptible phenotype of MT727 due to rimP inactivation. However, the mutant did not show any appreciable deviation in its growth behavior under oxidative, nutrient starvation and nitrosative stress conditions indicating lack of essentiality of rimP gene for survival under these stress conditions.

To have an insight into mechanism of functioning of RimP, as well as to determine the probable effect of mutation in RimP encoding gene on other genes, in silico analysis was done using STRING database. STRING database is an important online tool to understand cellular processes in the form of functional interactions between proteins (Szklarczyk et al. 2011). STRING uses experimentally validated information and computational prediction approaches to predict functional association between proteins in the form of network. As *M. fortuitum* database was not available in STRING, in silico study was performed using RimP protein of *M. abscessus* showing closest homology (60.8%) to the identified sequence.

Functional interaction of RimP protein using STRING demonstrated its association with translation termination and antitermination protein (NusA), 30S ribosomal protein s12 (RpsL) translation initiation factor IF-2 (InfB), probable 5′-methylthioadenosine phosphorylase (Pnp) and probable ribosomal binding factor (RbfA) which are involved in ribosomal assembly and subsequent translation process. Functional association of RimP with RpsL has been established previously in *M. smegmatis* where RimP helps in increasing the affinity of RpsL with 16SRNA (Weng 2016; Chu et al. 2019). RbfA and InfB have been reported to be upregulated under in vitro lysosomal stress conditions (Lin et al. 2016). Interaction of RimP with RbfA and InfB proteins has been observed using online STRING database in our study, further emphasizing a prospective role of rimP gene in acidic stress survival of *M. fortuitum*. It may be hypothesized that rimP is required for survival of *M. fortuitum* under acidic stress conditions of phagolysosome, confirmed by reduced survival of the mutant MT727 having inactivation of rimP.

RimP protein also showed predicted interaction with putative flavoprotein MAB_3133 and four hypothetical proteins MAB_3138, MAB_3134, MAB_3132 and MAB_3137. The functions of these proteins are unknown, however, MAB_3132, MAB_3133 and MAB_3134 were found to be present in a single operon. MAB_3138 was found to be associated with nusA, both genes sharing a single operon (http://www.lgcm.icb.ufmg.br/cmregnet).

The present study revealed probable role of rimP gene under in vitro acidic stress survival of *M. fortuitum*. Although the role of rimP has been reported for survival under stress conditions in *M. smegmatis*, its requirement under acidic stress conditions has not been reported in

pathogenic mycobacteria, to the best of our knowledge. Thus, the present study is a novel finding indicating probable role of rimP gene under in vitro acidic pH, which can be correlated to similar acidic stress conditions prevalent inside macrophages, although no association of the gene was observed with other stress conditions prevalent inside macrophage. Complementation of the disrupted gene and/or independent knock out studies in the same gene will provide comprehensive information about the role of rimP under acidic stress conditions. The gene can serve as a potential candidate for drug designing, albeit, in vivo studies of the mutant are required to confirm its role as virulent protein for infection and in vivo pathogenesis. The present study provides basis for the future development of therapeutic approaches using RimP as a druggable target against *M. fortuitum* in particular and other mycobacterial infections in general.

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Author contributions Poonam performed all experiments and wrote the manuscript. RMY supervised computational studies for the paper. GSB edited the manuscript and helped in idea development of the project. RS designed and supervised the project. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

Barquist L, Langridge GC, Turner DJ, Phan MD, Turner AK, Bateman A, Parkhill J, Wain J, Gardner PP (2013) A comparison of dense transposon insertion libraries in the *Salmonella* serovars Typhi and Typhimurium. Nucleic Acids Res 41(8):4549–4564. https://doi.org/10.1093/nar/gkt148

Bryant RE, Sypherd PS (1974) Genetic analysis of cold-sensitive ribosome maturation mutants of *Escherichia coli*. J Bacteriol 117(3):1082–1092

Bunner AE, Nord S, Wikström PM, Williamson JR (2010) The effect of ribosome assembly cofactors on in vitro 30S subunit reconstitution. J Mol Biol 398(1):1–7. https://doi.org/10.1016/j.jmb.2010.02.036

Bylund GO, Lövgren JM, Wikström PM (2001) Characterization of mutations in themetY-nusA-infB operon that suppress the slow growth of a ΔrimM mutant. J Bacteriol 183(20):6095–6106. https://doi.org/10.1128/JB.183.20.6095-6106.2001

Cameron DE, Urbach JM, Mekalanos JJ (2008) A defined transposon mutant library and its use in identifying motility genes in *Vibrio cholerae*. Proc Natl Acad Sci 105(25):8736–8741. https://doi.org/10.1073/pnas.0803281105

Chetchotisakd P, Mootsikapun P, Anunnatsiri S, Jirarattanapochai K, Choonhakarn C, Chaiprasert A, Ubol PN, Wheat LJ, Davis TE (2000) Disseminated infection due to rapidly growing mycobacteria in immunocompetent hosts presenting with chronic lymphadenopathy: a previously unrecognized clinical entity. Clin Infect Dis 30(1):29–34. https://doi.org/10.1086/313589

Chu T, Weng X, Law CO, Kong HK, Lau J, Li S, Pham HQ, Wang R, Zhang L, Kao RY, Lau KF (2019) The ribosomal maturation factor P from *Mycobacterium smegmatis* facilitates the ribosomal biogenesis by binding to the small ribosomal protein S12. J Biol Chem 294(1):372–378. https://doi.org/10.1074/jbc.RA118.002298

Cossu A, Sechi LA, Bandino E, Zanetti S, Rosu V (2013) Expression profiling of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium smegmatis* in acid-nitrosative multi-stress displays defined regulatory networks. Microb Pathog 65:89–96. https://doi.org/10.1016/j.micpath.2013.10.004

Cumming BM, Lamprecht DA, Wells RM, Saini V, Mazorodze JH, Steyn AJ (2014) The physiology and genetics of oxidative stress in mycobacteria. Microbiol Spectr. https://doi.org/10.1128/microbiolspec.MGM2-0019-2013

da Silva TRM, de Freitas JR, Silva QC, Figueira CP, Roxo E, Leao SC, de Freitas LAR, Veras PST (2002) Virulent *Mycobacterium fortuitum* restricts NO production by a gamma interferon-activated J774 cell line and phagosome-lysosome fusion. Infect Immun 70(10):5628–5634. https://doi.org/10.1128/IAI.70.10.5628-5634.2002

Ehrt S, Schnappinger D (2009) Mycobacterial survival strategies in the phagosome: defence against host stresses. Cell Microbiol 11(8):1170–1178. https://doi.org/10.1111/j.1462-5822.2009.01335.x

Gaca AO, Colomer-Winter C, Lemos JA (2015) Many means to a common end: the intricacies of (p) ppGpp metabolism and its control of bacterial homeostasis. J Bacteriol 20:JB–J02577. https://doi.org/10.1128/JB.02577-14

Gallant JL, Viljoen AJ, Van Helden PD, Wiid IJ (2016) Glutamate dehydrogenase is required by *Mycobacterium bovis* BCG for resistance to cellular stress. PLoS One 11(1):e0147706. https://doi.org/10.1371/journal.pone.0147706

Garbe TR, Barathi J, Barnini S, Zhang Y, Abou-Zeid C, Tang D, Mukherjee R, Young DB (1994) Transformation of mycobacterial species using hygromycin resistance as selectable marker. Microbiology 140(1):133–138. https://doi.org/10.1099/13500872-140-1-133

Geiman DE, Raghunand TR, Agarwal N, Bishai WR (2006) Differential gene expression in response to exposure to antimycobacterial agents and other stress conditions among seven *Mycobacterium tuberculosis* whiB-like genes. Antimicrob Agents Chemother 50(8):2836–2841. https://doi.org/10.1128/AAC.00295-06

Hermansen TS, Ravn P, Svensson E, Lillebaek T (2017) Nontuberculous mycobacteria in Denmark, incidence and clinical importance during the last quarter-century. Sci Rep 7(1):6696. https://doi.org/10.1038/s41598-017-06931-4

Hoefsloot W, Van Ingen J, Andrejak C et al (2013) The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples: an NTM-NET collaborative study. Eur Respir J 42(6):1604–1613. https://doi.org/10.1183/09031936.00149212

Kaufman MR, Taylor RK (1994) Identification of bacterial cell-surface virulence determinants with TnphoA. In: Methods in enzymology, vol 235. Elsevier, pp 426–448. https://doi.org/10.1016/0076-6879(94)35159-7

Kawaji S, Zhong L, Whittington RJ (2010) Partial proteome of *Mycobacterium avium* subsp. *paratuberculosis* under oxidative and

nitrosative stress. Vet Microbiol 145(3–4):252–264. https://doi.org/10.1016/j.vetmic.2010.03.025

Khandelwal I, Sharma A, Agrawal PK, Shrivastava R (2017) Bioinformatics database resources. In: Ram S (ed) Library and information services for bioinformatics education and research. IGI Global, Hershey, pp 45–90. https://doi.org/10.4018/978-1-5225-1871-6.ch004

Koebnik R, Locher KP, Van Gelder P (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol Microbiol 37(2):239–253. https://doi.org/10.1046/j.1365-2958.2000.01983.x

Lin W, de Sessions PF, Teoh GHK, Mohamed ANN, Zhu YO, Koh VHQ, Ang MLT, Dedon PC, Hibberd ML, Alonso S (2016) Transcriptional profiling of *Mycobacterium tuberculosis* exposed to in vitro lysosomal stress. Infect Immun 84(9):2505–2523. https://doi.org/10.1128/IAI.00072-16

Macente S, Helbel C, Souza SFR, Siqueira VLD, Padua RAF, Cardoso RF (2013) Disseminated folliculitis by *Mycobacterium fortuitum* in an immunocompetent woman. An Bras Dermatol 88(1):102–104. https://doi.org/10.1590/S0365-05962013000100014

Maurya A, Nag V, Kant S, Kushwaha R, Kumar M, Singh A, Dhole T (2015) Prevalence of nontuberculous mycobacteria among extrapulmonary tuberculosis cases in tertiary care centers in Northern India. Biomed Res Int. https://doi.org/10.1155/2015/465403

Nord S, Bylund GO, Lövgren JM, Wikström PM (2009) The RimP protein is important for maturation of the 30S ribosomal subunit. J Mol Biol 386(3):742–753. https://doi.org/10.1016/j.jmb.2008.12.076

Núñez MC, Menéndez MC, Rebollo MJ, García MJ (2008) Transcriptional analysis of *Mycobacterium fortuitum* cultures upon hydrogen peroxide treatment using the novel standard rrn A-P1. BMC Microbiol 8(1):100. https://doi.org/10.1186/1471-2180-8-100

Parti R, Shrivastava R, Srivastava S, Subramanian A, Roy R, Srivastava BS, Srivastava R (2008) A transposon insertion mutant of *Mycobacterium fortuitum* attenuated in virulence and persistence in a murine infection model that is complemented by Rv3291c of *Mycobacterium tuberculosis*. Microb Pathog 45(5–6):370–376. https://doi.org/10.1016/j.micpath.2008.08.008

Raveendran R, Oberoi JK, Wattal C (2015) Multidrug-resistant pulmonary and extrapulmonary tuberculosis: a 13 years retrospective hospital-based analysis. Indian J Med Res 142(5):575. https://doi.org/10.4103/0971-5916.171285

Rene O, Alix J-H (2011) Late steps of ribosome assembly in *E. coli* are sensitive to a severe heat stress but are assisted by the HSP70 chaperone machine. Nucleic Acids Res 39(5):1855–1867. https://doi.org/10.1093/nar/gkq1049

Schwegmann A, Brombacher F (2008) Host-directed drug targeting of factors hijacked by pathogens. Sci Signal 1(29):re8. https://doi.org/10.1126/scisignal.129re8

Sharma K, Gautam N, Sharma M, Dogra M, Bajgai P, Tigari B, Sharma A, Gupta V, Sharma SP, Singh R (2017) Ocular mycobacteriosis—dual infection of *M. tuberculosis* complex with *M. fortuitum* and *M. bovis*. J Ophthalmic Inflamm Infect 7(1):2. https://doi.org/10.1186/s12348-016-0121-0

Shi L, Sohaskey CD, North RJ, Gennaro ML (2008) Transcriptional characterization of the antioxidant response of *Mycobacterium tuberculosis* in vivo and during adaptation to hypoxia in vitro. Tuberculosis 88(1):1–6. https://doi.org/10.1016/j.tube.2007.08.007

Sood S, Kaur S, Shrivastava R (2016) A lacZ reporter-based strategy for rapid expression analysis and target validation of *Mycobacterium tuberculosis* latent infection genes. Curr Microbiol 72(2):213–219. https://doi.org/10.1007/s00284-015-0942-3

Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, Doerks T, Stark M, Muller J, Bork P (2011) The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. Nucleic Acids Res 39(suppl_1):D561–D568. https://doi.org/10.1093/nar/gkq973

Taylor RK, Miller VL, Furlong DB, Mekalanos JJ (1987) Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc Natl Acad Sci 84(9):2833–2837. https://doi.org/10.1073/pnas.84.9.2833

Tsai SC, Chen LH, Liao HH, Chiang CY, Lin WL, Chen SC, Tsao SM, Hung HC, Lee YT (2016) Complicated skin and soft tissue infection with *Mycobacterium fortuitum* following excision of a sebaceous cyst in Taiwan. J Infect Dev Ctries 10(12):1357–1361. https://doi.org/10.3855/jidc.7356

Umrao J, Singh D, Zia A, Saxena S, Sarsaiya S, Singh S, Khatoon J, Dhole TN (2016) Prevalence and species spectrum of both pulmonary and extrapulmonary nontuberculous mycobacteria isolates at a tertiary care center. Int J Mycobacteriol 5(3):288–293. https://doi.org/10.1016/j.ijmyco.2016.06.008

Unni M, Jesudason MV, Rao S, George B (2005) *Mycobacterium fortuitum* bacteraemia in an immunocompromised patient. Indian J Med Microbiol 23(2):137. https://doi.org/10.4103/0255-0857.16058

Vandal OH, Nathan CF, Ehrt S (2009) Acid resistance in *Mycobacterium tuberculosis*. J Bacteriol 191(15):4714–4721. https://doi.org/10.1128/JB.00305-09

Wang X, Wang H, Xie J (2011) Genes and regulatory networks involved in persistence of *Mycobacterium tuberculosis*. Sci China Life Sci 54(4):300–310. https://doi.org/10.1007/s11427-011-4134-5

Weng X (2016) Functional characterization of ribosome maturation factor RimP in *Mycobacterium smegmatis*. Dissertation, City University of Hong Kong