

In vivo Infection and *In vitro* Stress Survival Studies of Acid Susceptible Mutant of *Mycobacterium fortuitum*

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Abstract

Background: Ubiquitous presence of *Mycobacterium fortuitum* and ability to cause infections in human beings, hints toward its integral resistance against environmental and host stress conditions. With an aim to identify genes responsible for adapting *in vitro* acidic stress of *M. fortuitum*, in the previous study, TnphoA random mutagenesis identified acid susceptible mutant MT727, with mutation in ribosomal maturation factor encoding gene *rimP*, to be mutated. The present study was conducted to explore virulent behavior as well as growth behavior under *in vitro* stress conditions. **Methods:** Acid susceptible transposon mutant MT727 was injected intravenously in female BALB/c mice and kidney tissue was analyzed for the bacillary load as well as pathological characterization. Cytokine profiling of MT727-infected mice serum was done. MT727 was also subjected to various *in vitro* stress conditions, including detergent stress, heat stress, and hypoxic stress. The viable count of bacteria under different stress conditions was determined at regular time interval. **Results:** Mutant MT727 showed slight variation in bacillary load *in vivo*; however, defective growth behavior under detergent and hypoxic stress was observed when compared to wild type strain. **Conclusion:** Results conclude probable involvement of *rimP* gene in survival of *M. fortuitum* under hypoxic stress and detergent stress conditions.

Keywords: Detergent stress, drug target, hypoxic stress, *Mycobacterium fortuitum*, *rimP*

Submission: 24-10-2019

Accepted: 30-10-2019

Published: 26-11-2019

INTRODUCTION

Mycobacterium fortuitum is an important human pathogenic nontuberculous mycobacteria, with the potential to cause multiple infections such as cutaneous, pulmonary, lymph node, bone, and eye infections.^[1,2] Variable symptoms, increasing drug resistance, long-term drug therapy, and lack of standardized drug regimen for *M. fortuitum* infections highlights its significance in medical research; however, very scarce information is available regarding the pathogenesis of *M. fortuitum*.

Employing TnphoA-based transposon mutagenesis, in the previous study, we identified ribosomal maturation factor (RimP) as one of the proteins essential for *in vitro* acidic stress survival in *M. fortuitum*.^[3] RimP protein helps in maturation of 30S ribosomal subunit, which is required for correct localization of messenger RNA (mRNA) for error-free binding of amino acid bearing transfer RNA (tRNA) with mRNA codon.^[4]

Pathogenesis of *M. fortuitum* infection in the form of bacillary load, tissue damage, and elevated immune response has been

described in murine mice infection model.^[5] During infection process in *M. fortuitum* animal infection model, *M. fortuitum* is reported to be present in necrotic granuloma in nonreplicating persistence (NRP) form.^[6] In this state, the bacilli are likely to experience unfavorable environment including low oxygen tension, i.e., hypoxic stress inside the host.^[7] Hence, mutant MT727, in which *rimP* gene is inactivated, was subjected to *in vivo* mice infection model as well as hypoxia-induced NRP model to explore the role of *rimP* gene in *in vivo* virulence and hypoxic stress survival.

M. fortuitum exploits macrophages, the phagocytic cells as its dwelling place inside the host. Ability of *M. fortuitum* to reside

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How to cite this article: Katoch P, Bisht GS, Shrivastava R. *In vivo* infection and *in vitro* stress survival studies of acid susceptible mutant of *Mycobacterium fortuitum*. Int J Mycobacteriol 2019;8:390-6.

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10.4103/ijmy.ijmy_166_19

inside macrophages affirms its level of resistance toward insulating stress conditions prevalent inside host.^[6] Hence, the importance of *rimP* gene toward cell wall stresses, including detergent stress and heat stress inside the host was also checked. The current study will help in understanding functional importance of *rimP* gene in adapting to multiple *in vitro* stress conditions as well as will help in understanding virulence potential of *rimP* gene in the pathogenesis of *M. fortuitum*.

METHODS

Bacterial cultures, plasmids, and antibiotics

M. fortuitum ATCC 6841 and *Escherichia coli* DH5 α were used in the present study.

M. fortuitum was grown in Luria Bertani (LB) broth (HiMedia) containing 0.5% glycerol and 0.2% Tween 80 (LBGT). Nutrient Agar (HiMedia) containing 0.05% Tween 80 (NAT) was used for colony-forming unit (CFU) counts. LB broth and LB agar were used to grow *E. coli* DH5 α for recombinant DNA protocols. Antibiotics, including kanamycin (30 μ g/ml) and ampicillin (100 μ g/ml), were used as required for the protocol and obtained from HiMedia. Plasmid pRT291 which contains transposon TnpA^[8] was used for transposon mutagenesis. XP (5-Bromo-4-chloro-3-indolyl phosphate) (Thermo Fisher Scientific) was used as a substrate for the screening of the mutants. Female BALB/c mice of weight 18–21 g were used for *in vivo* infection study. Mice were kept in a pathogen-free sterile environment maintained in the institute's animal house facility, and all animal infection study protocols employed in the present study were approved by the Institutional Animal Ethics Committee.

Transposon mutagenesis and the identification of *in vitro* acid susceptible phenotype of *Mycobacterium fortuitum*

M. fortuitum transposon mutant library was prepared by electroporation of TnpA based plasmid pRT291 into *M. fortuitum* ATCC 6841. The procedure for electroporation was used as described previously.^[3] After electroporation, the mutants were screened for the expression of alkaline phosphatase (reporter gene of TnpA). Further, shortlisted mutants were exposed to *in vitro* acidic stress conditions. One mutant namely, MT727, showed acid susceptible phenotype and used for the current study.^[3] Molecular and bioinformatics approaches used for identification of the mutated gene in MT727 led to the identification of ribosomal maturation factor encoding gene *rimP*. The identified gene was submitted to GenBank (accession number MH052677).

In vivo infection studies

The protocol for *in vivo* infection studies was followed as suggested previously^[6] with some modifications. Wild type *M. fortuitum* (WTMF) and mutant MT727 cells were grown till mid logarithmic phase followed by centrifugation and resuspension of pellet in 0.05% tween normal saline. Female BALB/c mice of 18–21 g weight were intravenously infected with 10⁷ cells/mice in a final volume of 200 μ l

with WTMF and MT727. Mice were observed routinely for signs and symptoms till 25 days. Three mice per group were sacrificed 10 and 25 days postinfection followed by collection of blood through cardiac puncture. The collected blood was centrifuged at 3000 rpm for 1 min for separation of serum from blood. Serum was preserved at –80°C for cytokine profiling. Kidneys were isolated from the infected mice, washed with phosphate-buffered saline (PBS), weighed and homogenized in tween normal saline for determination of CFU/g of kidney weight. One set of kidneys was preserved in 10% buffered formalin for histopathological studies.

Cytokine profiling of the serum

Cytokine profiling of the serum isolated from WTMF and mutant MT727 infected mice was done as per the manufacturer's protocol. Serum was collected after 10 and 25 days of infections and cytokine profiling was done using enzyme-linked immunosorbent assay (ELISA). Interferon gamma (IFN γ), tumor necrosis factor-alpha (TNF α), and interleukin (IL) 10 level in the serum were determined using Krishgen Biosystems ELISA kits.

Histopathological studies

For histopathological studies, kidney tissue from the infected mice was collected 10 and 25 days of intravenous infection. Histopathological studies of the infected kidney tissue were performed as described previously.^[9] The kidney tissue was fixed in 10% neutral buffered formalin followed by dehydration in ethanol, clearing in xylene and embedment in paraffin wax. The tissue section of about 4–5 μ m was then stained with hematoxylin and eosin to examine pathological changes in the infected tissue.

Growth and survival studies of MT727 under *in vitro* hypoxic stress

Exposure of *in vitro* hypoxic stress to mutant MT727 along with WTMF was performed by following the protocol described by Sood *et al.*^[10] Briefly, the cells were grown in LBGT till the logarithmic phase (0.4–0.7 optical density at 600 nm) followed by the addition of 1.5 μ g/ml oxygen depletion indicator methylene blue. 7.5 ml of culture containing methylene blue was then added into 15 ml glass vials to maintain a headspace ratio of 0.5 and closed tightly with rubber septa and vacuum grease to prevent oxygen intake into vials. The vials were incubated under anaerobic conditions at 37°C without shaking for 27 days, with determination of CFU at regular time intervals of 3 days during the incubation period to check the viability of mutant under hypoxic stress conditions.

Growth and survival studies of mutant under *in vitro* detergent and heat stress conditions

Surviving ability of MT727 under *in vitro* detergent and heat stress conditions was followed as mentioned previously^[11] with some modifications. Briefly, MT727 and WTMF were grown in MB7H9 medium till mid logarithmic phase. Afterward, pellet of cells was collected by centrifugation at 5500 \times g for 10 min, followed by twice washing with PBS. Then, the pellet was resuspended in MB7H9 medium and inoculated in 0.05%

sodium dodecyl sulfate containing MB7H9 for detergent stress and normal MB7H9 medium for heat stress. The cultures were then kept at 37°C (detergent stress) and 45°C (for heat stress) with shaking at 200 rpm followed by calculation of CFU by plating on NAT plates after regular time interval.

Statistical analysis

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

RESULTS

Mutant MT727 showed a slight difference in bacillary load during later stages of infection

Infection kinetics of mutant MT727 as a function of bacillary load in infected kidney tissue of mice was determined at different time intervals. Mutant MT727 bacillary load in the kidney during the early stages of infection, i.e., after 10 days of inoculation in mice did not show appreciable difference relative to WTMF, however, at later stages of infection, i.e., 25-day postinfection, there was a slight decrease of 1.8 log relative to WTMF in kidney of mice infected with mutant MT727 [Figure 1]. The mice infected with mutant MT727 also showed characteristic symptoms of *M. fortuitum* infection within 8 days of postinfection similar to WTMF. Characteristic symptoms, including spinning neck, restlessness, and neck tilting, were observed in mice infected with both WTMF and MT727. No mortality was found in mice with either WTMF and MT727. The observation of detrimental effect of MT727 infection in tissues of mice showed white patches on the surface of the kidneys. Histopathological analysis of the kidney showed the presence of granuloma in MT727 and WTMF [Figure 2]. There was no observable difference in tissue pathology among both WTMF and MT727.

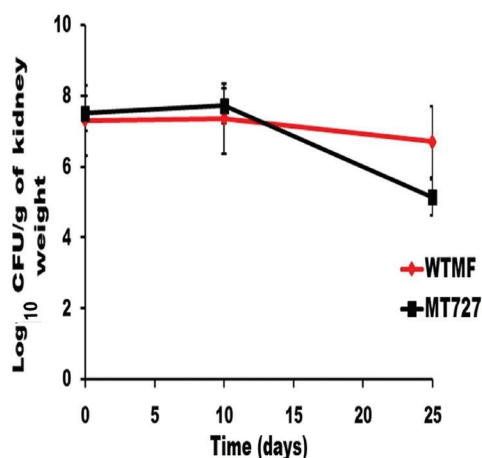


Figure 1: Bacillary load in kidney of mice infected with mutant MT727. Bacillary load of mice infected with mutant MT727 after 10 days and 25 days of infection in comparison to wild type *Mycobacterium fortuitum*. The data represented is the mean of three independent experiments with \pm standard deviation

MT727-infected mice showed deviation in immune response relative to wild type *Mycobacterium fortuitum*

Cytokine profiling of two groups of mice which are infected with WTMF and MT727 showed decreased concentration of pro-inflammatory cytokines in the case of MT727; however, the concentration of anti-inflammatory cytokine was similar between both groups [Figure 3]. After 10 days of infection, the level of IFN γ decreased by 1.8 fold in mice serum infected with MT727 when compared to WTMF. However, there was 1.7-fold increase in cytokine concentration after 25 days of infection in mice infected with MT727 relative to WTMF infected mice serum. Serum of MT727 infected mice also showed 3.89 fold and 1.57-fold decrease in TNF α after 10 days and 25 days of infection, respectively, compared to WTMF-infected mice serum. The concentration of anti-inflammatory cytokine, i.e., IL 10 did not show any observable difference between both groups.

MT727 showed reduced survival under *in vitro* hypoxic stress

Mutant MT727 exposure to *in vitro* hypoxic stress conditions resulted in the inability of the mutant to survive for extended period of time relative to WTMF. The faded color of methylene blue was observed after 5 days, which indicated the presence of hypoxic stress inside culture containing vials. Mutant MT727 showed 2.2 log reduction in the growth after 6 days; which continues to decrease and reduced to 4.5 log after 12 days when compared to WTMF. A sharp decline was observed after 15 days of hypoxic stress in mutant due to complete loss of cell viability, whereas WTMF sustained its growth until 27 days [Figure 4].

Mutant MT727 showed defective growth under detergent stress conditions

Exposure of MT727 to *in vitro* detergent stress resulted in defective growth of MT727 comparable to WTMF. MT727

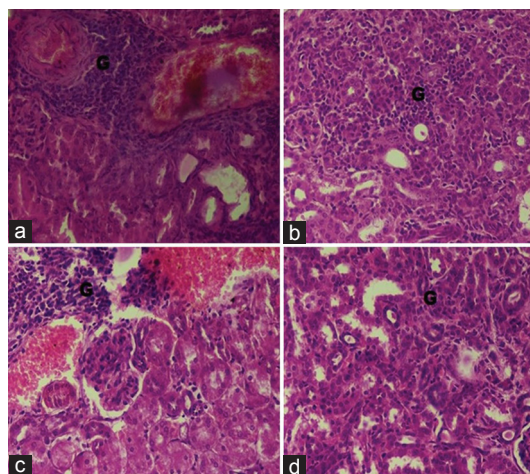


Figure 2: Pathological characteristics of kidney tissue infected with MT727. Histopathological analysis of kidney tissue section which shows pathological characteristics of wild type *Mycobacterium fortuitum* 10-day postinfection (a) and 25 days postinfection (b). (c and d) Pathological characteristics after 10 days and 25 days of infection of mice kidney tissue infected with mutant MT727. “G” indicates leukocyte infiltration at the site of infection which leads to “granuloma” formation

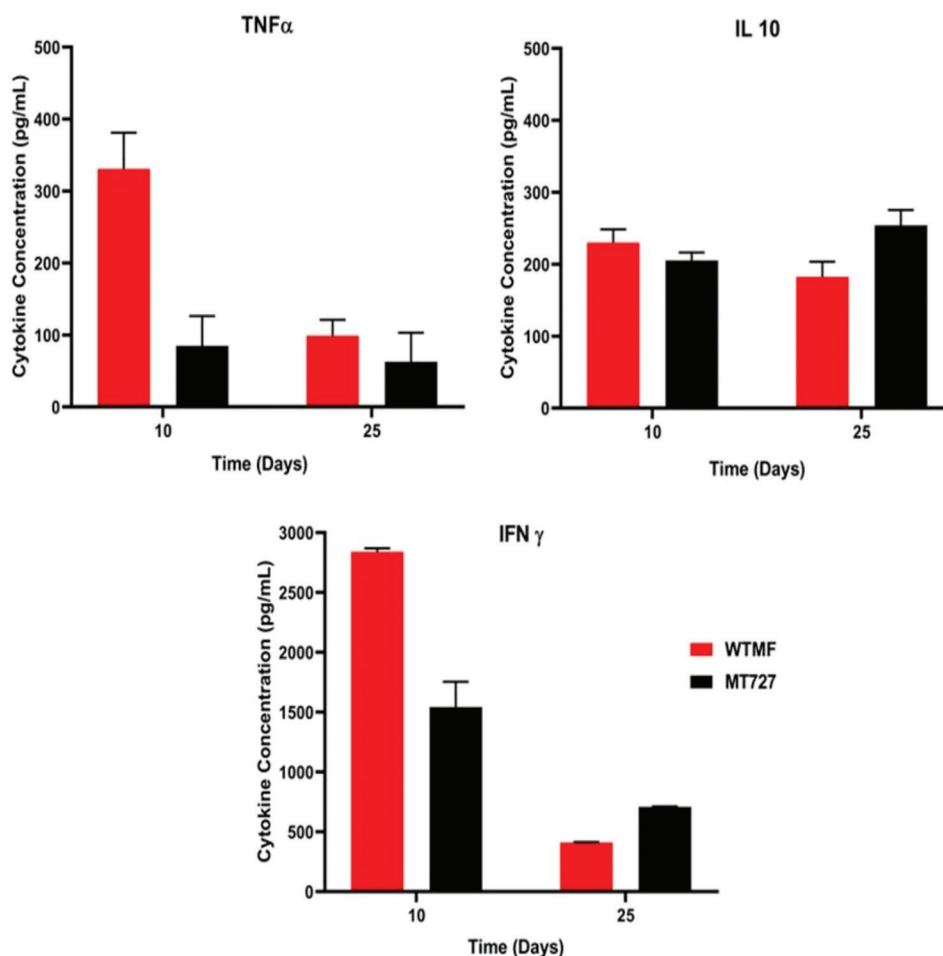


Figure 3: Cytokine profiling of serum of mice infected with mutant MT727. The figure shows proinflammatory (interferon gamma and tumor necrosis factor alpha) and antiinflammatory cytokine (interleukin 10) concentration in mice serum infected with MT727 and wild type *Mycobacterium fortuitum*. Appreciable difference in pro inflammatory cytokines was observed between both groups after 10 days and 25 days of infection, whereas, no difference was observed in case of anti inflammatory cytokine concentration

showed a decrease of 2.3 log in CFU after 12 h of stress. The growth of mutant continued to decrease afterward and showed 3.6 log reduction in CFU relative to WTMF after 36 h of observation period [Figure 5]. In case of heat stress, decline in CFU was observed in both mutant MT727 and WTMF during the observation period. Mutant MT727 and WTMF showed complete loss of viability within 36 h of stress. Thus, under heat stress, no appreciable change in the kinetics of growth of MT727 was detected when compared to WTMF [Figure 6].

DISCUSSION

Apart from being an environmental bacterium, *M. fortuitum* can cause range of infections, including skin and soft-tissue infections, bone infections, and pulmonary infections.^[12] Disseminated infections due to *M. fortuitum* have also been reported in immunocompromised^[13] as well as immunocompetent individuals.^[14] *M. fortuitum* is an intracellular pathogen, invade macrophages and resist phagocytosis. Regardless of the presence of an active immune response against *M. fortuitum* inside the host, it can

establish itself as a successful pathogen.^[6] In the presence of immune response, the bacilli must come across various adverse conditions, including acidic stress, nitrosative stress, oxidative stress, heat stress, and other cell surface stresses. Although this response effectively controls the replication of *M. fortuitum*, the bacteria can resist eradication by transforming itself into a slow-growing persistent form, for extended periods inside granuloma. Hypoxic stress is present inside these granulomatous structures, suggesting the potential of *M. fortuitum* to survive under such conditions.

Membrane proteins help the pathogen to establish inside host by adhesion, invasion, and host-pathogen interaction. Owing to the significance of membrane proteins as a virulent factor, in the previous study, we applied TnphoA-based transposon mutagenesis, for the identification of membrane, transmembrane, and secretory protein (s) as potential virulent factor in *M. fortuitum* pathogenesis. We identified, ribosomal maturation factor *rimP* (probable membrane protein) to be mutated due to TnphoA mutation and suggested functional importance of *rimP* gene in surviving under *in vitro* acidic

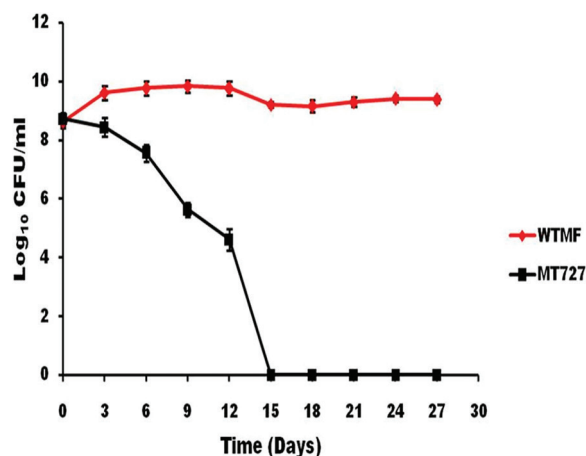


Figure 4: Growth and survival of MT727 under *in vitro* hypoxic stress. The figure shows growth and survival of mutant MT727 under *in vitro* hypoxic stress in comparison to wild type *Mycobacterium fortuitum*. MT727 showed reduced survival as compared to wild type *Mycobacterium fortuitum* and was unable to survive beyond 15 days of exposure to *in vitro* hypoxic stress conditions. The data represented is the mean of three independent experiments with \pm standard deviation

stress. In a recent study, requirement of *rimP* for survival under nitrosative stress was reported in *M. smegmatis*,^[4] which further emphasizes on importance of *rimP* in adapting to stress conditions. Thus, in the present study, *rimP* gene affected mutant MT727, was checked for its ability to cause infection as well as for its ability to survive under detergent, heat, and hypoxic stress, to explore functional significance of *rimP* gene.

Ribosomal maturation factor RimP is involved in basic cellular processes for the growth of bacteria, including its involvement in maturation of 30S ribosomal subunit.^[15] Under stress condition cellular modification in terms of transcription as well as translation takes place that might be correlated with modification in the ribosomal maturation and assembly factors during such conditions.^[16]

Ribosomal maturation factor RimP plays an important role in central metabolism, it can function as an important drug target against bacterial pathogens. In the present study, *M. fortuitum* *rimP* mutant, i.e., mutant MT727 virulence study in mice infection model showed no appreciable difference during early stages of infection, whereas, a slight decrease was observed in viable count during later stages of infection. Based on the tissue pathology, no appreciable difference in pathology between WTMF and mutant observed, highlighting, no major effect of mutation of *rimP* gene on the virulence of *M. fortuitum*, however, in *Salmonella enteridis*, the involvement of *rimP* has been reported in virulence.^[15] Although *rimP* gene has not been explored for virulence-related studies, Chu *et al.* hypothesized *rimP* as drug target in *M. tuberculosis* by studying *rimP* gene behavior in *M. smegmatis*,^[17] which is a model organism to study tuberculosis virulence. Kolker *et al.* also suggested *rimP* gene involvement in other molecular processes including DNA unbinding in *Haemophilus influenzae*.^[18]

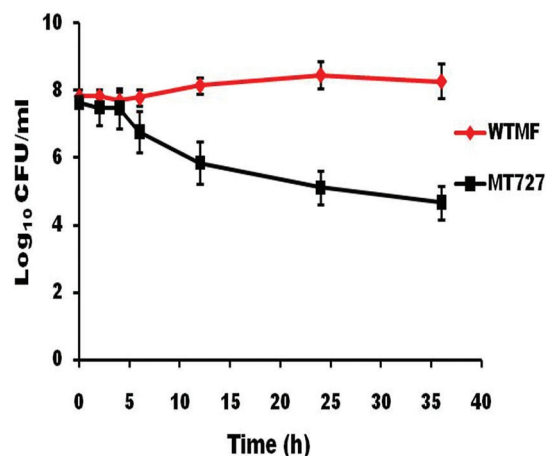


Figure 5: Growth kinetics of MT727 under *in vitro* detergent stress. The figure shows growth behavior of mutant MT727 under *in vitro* detergent stress, where mutant shows 3.6 log decrease after 36 h of the observation period in comparison to wild type *Mycobacterium fortuitum*. The data represented is the mean of three independent experiments with \pm standard deviation

Mice infected with mutant MT727 showed low levels of proinflammatory cytokines, TNF α and IFN γ than the mice infected with WTMF; however, no attenuation in virulence of MT727 was observed. As *rimP* is involved in protein translation of bacteria, *rimP* gene mutation in MT727, may affect translation of protein required for active immune response leading to low levels of cytokine production.

In pathogenic mycobacteria, the stability of ribosomes is an essential phenomenon for transformation of pathogen into slow-growing persistent form. The stability of 30S ribosome is regulated by DosR and DevR regulon.^[19] Slow growth of bacteria under hypoxic stress conditions highlights limited translation of proteins. In the present study, mutant MT727 showed reduced survival for extended period of time under hypoxic stress, proposing *rimP* gene role under hypoxic stress survival. The translation is a process of coordinated function of various factors related to ribosomes, tRNA as well as mRNA, the results might be due to involvement of RimP protein in the maintenance of 30S ribosomal subunit stability, which is essential for slow growth rate of bacilli under such harsh conditions.

Antimicrobial peptide cathelicidin is produced inside macrophages through toll-like receptor 2 signaling pathway, during infection. In addition to the production of antimicrobial peptides in macrophages itself, other antimicrobial peptides are also delivered inside macrophages through T lymphocytes as well as through neutrophils.^[20] Antimicrobial peptide behaves in a detergent-like manner by binding to lipids present on the surface of pathogens and permeabilizes the membrane.^[21] In addition to the presence of antimicrobial peptides, pulmonary surfactants on alveoli also bear detergent like behavior as their bactericidal mechanism. *M. fortuitum* is known to reside inside the macrophages, and

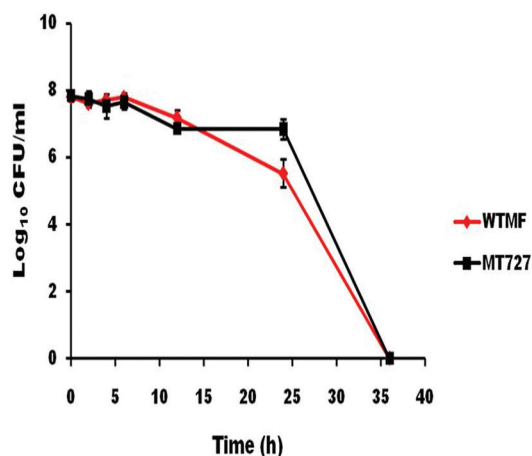


Figure 6: Growth and survival of MT727 under *in vitro* heat stress. The figure shows the growth and survival of mutant MT727 under *in vitro* heat stress in comparison to wild type *Mycobacterium fortuitum*, where both mutant and wild type showed constant decline in colony-forming unit, followed by complete loss of growth within 36 h of the observation period. The data represented is the mean of three independent experiments with \pm standard deviation

it also causes pulmonary infections even in the presence of pulmonary surfactants, signifying its resistance ability to cell surface stresses. In the present study, exposure of mutant MT727 to *in vitro* detergent stress showed an appreciable reduction in growth during observation period. The defective growth of mutant MT727, recommends functional significance of *rimP* in detergent stress survival. To the best of our knowledge, *rimP* gene has not been explored to determine *in vitro* detergent stress survival, and thus, the present finding is a novel report which can be further explored in other intracellular microorganisms.

Our study concludes no association of *rimP* gene in virulence of *M. fortuitum*, however, *rimP* gene may be considered to be important for immune response stimulation. The importance of *rimP* in survival under detergent stress and hypoxic stress in *M. fortuitum* has been reported in our study. However, the role of *rimP* gene in combating detergent stress and hypoxic stress conditions is only putative as further studies, including gene complementation is required to confirm its role. As, *rimP* mutation did not affect virulence capability of *M. fortuitum*, *rimP* gene may-not be used as drug target to treat human infection; however, it can be used as drug target to reduce the growth of *M. fortuitum* on hospital equipment's and instruments which are major source of *M. fortuitum* infection.

Acknowledgments

We would like to acknowledge Jaypee University of Information Technology, Waknaghat, Solan (Himachal Pradesh) administration for providing research facility, and fellowship to Ms. Poonam. Authors are grateful to CDRI, Lucknow, for providing bacterial strains and plasmids.

Financial support and sponsorship

Financial support in the form of research grant was provided to RS by the Department of Science and Technology, Government of India-Science and Engineering Research Board with sanction number SR/FT/LS-117/2012.

Conflicts of interest

There are no conflicts of interest.

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