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***Mycobacterium tuberculosis* ftsH expression in response to stress and viability**

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SUMMARY

FtsH is an essential membrane bound protease that degrades integral membrane proteins as well as cytoplasmic proteins. We show that *Mycobacterium tuberculosis* (*Mtb*) *ftsH* expression levels are upregulated upon exposure to agents that produce reactive oxygen and nitrogen intermediates (ROI and RNI) and growth in macrophages. In partial support of this result is our observation that the *Mtb* merodiploid overexpressing *ftsH* shows increased resistance to ROI. *ftsH* transcripts levels are downregulated during stationary phase and starvation. Overexpression of *ftsH* delayed growth and reduced the viability of *Mtb* *in vitro* and *ex vivo*. Finally, we show that the intracellular levels of FtsZ, an essential cell division protein, are reduced in *ftsH* overexpressing strain. Together, our results suggest that *Mtb* FtsH is a stress response protein that promotes the pathogen's ability to deal with ROI stress and is possibly involved in the regulation of FtsZ levels.

1. Introduction

FtsH is a member of AAA-family ATPases and plays an important role in the protein quality control mechanisms. FtsH mediated degradation of misassembled membrane and cytoplasmic proteins is thought to be responsible for its role in the control of cellular functions including the heat shock response (reviewed in ^{1, 2}). FtsH is also required for the proper functioning of σ^{54} under nitrogen limitation conditions ³. FtsH expression is upregulated in response to heat shock in several bacteria, e.g., *Escherichia coli*, *Bacillus subtilis*, *Lactococcus lactis*, *Caulobacter crescentus* and *Helicobacter pylori* ^{4–7}. The FtsH protease is essential for growth in *E. coli* ^{8, 9} whereas it is dispensable in *B. subtilis* and *C. crescentus* ^{4, 10}. However, *ftsH* mutants are hypersensitive to heat, salt, and defective for sporulation and possibly cell division ^{4, 10}.

Saturated transposon mutagenesis studies indicated that *Mtb* *ftsH* (*ftsH_{TB}*) is an essential gene ¹¹ that encodes a 85 kD protein, which is 15kD larger than the 70 kD *E. coli* counterpart. The *ftsH_{TB}* can complement the *E. coli* *ftsH* phenotype and degrade heterologous substrates σ^{32} , SecY and bacteriophage λ CII protein when expressed in *E. coli* *ftsH* null mutant ¹². The expression of *M. smegmatis* *ftsH* in *E. coli* results in growth arrest and filamentation ¹³. In vitro, the FtsH_{EC} efficiently degrades FtsZ of *E. coli* (FtsZ_{EC}) and *Mtb* (FtsZ_{TB}) ¹⁴. FtsZ_{TB}, like its *E. coli* counterpart, is an essential cell division protein and initiates the process by localizing at the midcell sites in the form of Z-rings ^{15, 16}. Experiments designed to test whether FtsH_{EC} affects the stability and turnover of FtsZ_{EC} in

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#Equal contribution

vivo, however, failed to support the in vitro data¹⁷. Related experiments with *Mtb* have not been carried out. Consequently, it is unknown if *ftsH*_{TB} activity contributes to the reduction in the intracellular levels of FtsZ_{TB} in vivo. Furthermore, it is unknown if *ftsH*_{TB} expression in vivo is responsive to stress. The current study addresses these issues.

2. Materials and Methods

2.1. Bacterial growth and culture conditions

Escherichia coli Top10 was used to propagate recombinant plasmids as described^{15, 18}. *Mtb* H37Rv was grown in Middlebrook 7H9 broth supplemented with oleic acid, albumin, dextrose, and sodium chloride (OADC). Transformants were selected in the same medium supplemented with agar containing Hyg (50 µg ml⁻¹)¹⁶. Growth conditions such as hypoxia, starvation and exposure to agents that produce ROI and NO, are essentially as described earlier¹⁸. *Mtb* cultures were exposed to 500 µM DETA/NO for 16 h, 70 µM menadione or 5 mM H₂O₂ for 48 h. Growth was monitored by measuring the absorbance at 600 nm and viability by determining the CFU.

2.2. Construction of *ftsH* overexpression plasmid pACR6 and *ftsH* anti-sense plasmid pACR33

The 2.3 kb *ftsH* coding region was amplified using oligonucleotide primer pairs FtsH8 (5' – GGAATTCCATATGAACCGGAAAAACGTGACTCG – 3') and FtsH9 (5' – CTAGTCTAGACTAT-CAGCCGTGGGCCGGCTTGGTC – 3') or Fts H-A-XbaI (5'- CTAGTCTAGACTAATGAACCGGAA-AAACGTGACT -3') and Fts H-A-NdeI (5'- GGAATTCCATATGTCAGCCGTGGGCCGGTTGG -3'). The PCR products were cloned downstream of the amidase promoter in an integrating vector, pJFR19¹⁵ to create the sense plasmid, pACR6, and the antisense plasmid pACR33, respectively. Following confirmation of sequences, *Mtb* was electrotransformed with these plasmids to create *ftsH* overexpression strain Mtb-6 and antisense expression strain Mtb-33.

2.3 Macrophage infection and viability determination

Monocyte derived macrophage cell line THP-1 cells were infected with various *Mtb* strains and bacterial viability was determined as described previously^{15, 19}. For some experiments, RNA was isolated from macrophage-grown Mtb¹⁹.

2.4. RNA extraction and QRT-PCR

Extraction of total RNA in RNeasy from *Mtb* cultures exposed to different conditions and synthesis of complementary strand DNA from mRNA specific to 16S rRNA and *ftsH* using reverse transcription primers and Superscript II reverse transcriptase (Invitrogen) were as described¹⁹. Quantitative real time PCR (Taqman chemistry) was carried out in a BioRad ICycler using Taq DNA polymerase (NEB). The calculated threshold cycle (Ct) value for *ftsH* was normalized to the Ct value for 16S rRNA and the fold expression was calculated using the formula: Fold change = 2^{Δ(ΔCt)}²⁰. No RT RNA samples were included as negative controls. Expression data are average from 3 independent RNA preparations, each reverse transcribed and quantitated by real time PCR in triplicate. Real-time PCR conditions: initial activation at 95°C for 3minutes; followed by 45 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds.

2.5. Immunodetection

Mtb cellular lysates were prepared by bead-beating actively growing cultures. Approximately 2 µg protein lysate per lane was loaded, resolved by SDS-PAGE and immunoblotting was carried out to detect FtsZ_{TB}, FtsH_{TB} and SigA_{TB} using the previously

described protocols^{16, 21}. Anti-FtsH_{EC} antibodies were a kind gift from Dr. Teru Ogura, Kumamoto University, Japan and were used at 1:2000 dilution, whereas anti-FtsZ_{TB} and anti-SigA_{Ecoli} were used at 1: 1000 dilution¹⁵.

3. Results and Discussion

3.1. *Mtb* ftsH expression is modulated in response to stress

Considering the roles of FtsH on the protein quality control mechanisms in other bacteria, we first evaluated by quantitative RT-PCR whether the *Mtb* ftsH transcript levels relative to 16S rRNA are modulated under the conditions that are relevant for *Mtb* survival in vivo, i.e. growth in macrophages, exposure to reactive oxygen and nitrogen intermediates, hypoxia and nutrient starvation^{22, 23}. The ftsH transcript levels were found to be sharply elevated during intramacrophage growth and when exposed to DETA-NO, a nitric oxide (NO) producer, but were downregulated during starvation and stationary phase and remained relatively unchanged during hypoxia (Fig. 1). A small, but consistent, upregulation of ftsH transcript levels was also observed in cultures exposed to menadione and hydrogen peroxide, both of which produce reactive oxygen intermediates (ROI) (Fig. 1). Presumably, altered expression levels of ftsH are necessary for the optimal survival of *Mtb* under the above growth conditions.

3.2. Altered FtsH levels and resistance to Reactive Oxygen Intermediates

The above results are consistent with a notion that elevated levels of FtsH are required for the optimal survival of *Mtb* under the conditions that produce NO and ROI. To further evaluate the correlation between altered ftsH expression and ROI and NO stress, we created and characterized merodiploid strains overexpressing ftsH mRNA (Mtb-6) and antisense RNA (Mtb-33) (see below). As can be seen, growth in the presence of acetamide for 72 hrs led to an ~3-fold increase in the FtsH protein levels in Mtb-6 and an ~ 60% decrease in Mtb-33, relative to that of H37Rv (Fig. 2A and inset). Elevated levels of FtsH slowed the growth in broth (Fig. 2B) and reduced viability by 50% (Fig. 2B inset). However, the reduction in FtsH levels did not affect the growth and viability of *Mtb* (Fig. 2B).

Next, we examined the growth and viability of *Mtb* ftsH merodiploid strains exposed to select stress conditions, i.e. hydrogen peroxide and menadione. A significant reduction in the viability of *Mtb* wild type and Mtb-33 was noted at the concentrations of the agents used (Fig. 3) indicating that the activity of FtsH protein is required for survival upon exposure to agents that produce ROI. Mtb-6 strain, producing elevated levels of FtsH (Fig. 2A), showed enhanced viability under the same growth conditions indicating that increased intracellular levels of FtsH promote the ability of *Mtb* to resist ROI (Fig. 3). Exposure to RNI intermediates and starvation had only a marginal effect on the viability of all three strains (data not shown). These results reveal that *Mtb* producing elevated levels of FtsH would weather the ROI stress better than that producing the normal or reduced levels of FtsH.

3.3. Optimal FtsH levels are needed for intramacrophage growth of *Mtb*

We next examined the consequences of altered ftsH expression on *Mtb* growth and viability upon infection. As can be seen, Mtb-6 strain expressing elevated levels of ftsH showed reduced viability in THP-1, a human macrophage cell line, as compared to the wild type parent (Fig. 4). The Mtb-33 showed delayed growth during the earlier time points of infection, but reached to the same final levels as the WT indicating a defect in replication. Macrophage growth environment is rather hostile and *Mtb* likely encounters stresses other than the ones tested in the present study, i.e. acidic pH, limiting nutrients, host secreted cytokines and antimicrobial peptides²⁴. Presumably, elevated expression of ftsH resulting in excessive levels of FtsH could trigger unregulated proteolytic activity upon infection.

Similarly, decreased expression of *ftsH* resulting in the reduced in vivo pools of FtsH could lead to the accumulation of undesirable proteins. Both scenarios could compromise the ability of *Mtb* to replicate in macrophages optimally. These results are consistent with a notion that optimal FtsH levels are essential for survival of *Mtb* in vivo (Fig. 4).

3.4. Intracellular FtsZ protein levels are altered in *ftsH* merodiploids

Optimal intracellular levels of the essential cell division protein FtsZ are critical for cell division and viability¹⁶. FtsZ levels are growth-phase dependent^{16, 25}. To evaluate the correlation, if any, between the decreased growth of *ftsH* merodiploids and FtsZ levels, we determined the intracellular FtsZ levels in Mtb-6 and Mtb-33 strains by immunoblotting. Since SigA levels are known to remain unchanged under several conditions that have been tested^{15, 26}, we normalized the data to SigA (Fig. 5). These analyses revealed that FtsZ levels were reduced by approximately 34% in Mtb-6, but remained unaffected in Mtb-33 (Fig. 5). These results, however, do not provide direct evidence that FtsH_{TB} protease degrades FtsZ_{TB} protein. Our earlier data showed that the FtsZ levels are similar in broth and macrophage grown bacteria¹⁵, although the *ftsH* expression is shown to be upregulated during intramacrophage growth (Fig. 1). Thus, it is possible that FtsH could directly regulate the FtsZ levels by its proteolytic activity or indirectly influence its levels by degrading hitherto unidentified proteins that stabilize FtsZ_{TB}, thereby facilitating the action of other proteases on FtsZ. Nonetheless, reduction in the FtsZ levels under FtsH overproduction conditions leads to a speculation that proliferation of *Mtb* depends, in part, on the optimal ratio of FtsH to FtsZ proteins. Tipping that balance by elevating the FtsH levels could influence the intracellular FtsZ pools and compromise growth and viability.

4. Conclusions

1. Upregulation of *ftsH* transcription in response to ROI induced stress and increased survival of *Mtb* merodiploids producing elevated levels of FtsH suggests a potential role for FtsH protease in dealing with ROI stress.
2. Reduced intracellular levels of FtsZ in *Mtb* merodiploids producing elevated levels of FtsH suggest that an optimal ratio of FtsH to FtsZ is one of the contributing factors necessary for *Mtb* proliferation.

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Figure 1.

ftsH expression levels under different growth conditions. *ftsH* expression levels in *Mtb* grown under various indicated conditions were quantitated by QRT-PCR. cDNA was synthesized for *ftsH* along with 16S rRNA and QRT-PCR was carried out as described under material and methods. The expression levels of *ftsH* mRNA were normalized to 16S mRNA levels. Mean $\pm$ SD from three independent experiments are shown.

Figure 2.

FtsH protein levels and growth of *ftsH* strains. **A.** FtsH protein levels in *Mtb* strains were quantitated by immunoblotting. Protein lysates from *ftsH* strains were resolved on SDS-PAGE, transferred to nitrocellulose membrane and probed with α -FtsH and α -sigma³² antibodies. Bands were visualized on a BioRad molecular imager and quantitated using the QuantityOne software. FtsH levels were normalized to SigA. Symbols: WT – wild type *Mtb*, Mtb-6 – *ftsH* overexpressing strain and Mtb-33 – *ftsH* antisense strain. Inset: Immunoblot showing FtsH and SigA bands. **B.** Growth of *ftsH* strains was monitored by measuring absorbance at 600 nm and plotted. Actively growing cultures were also spread on agar plates for determining CFU (inset).

Figure 3.

FtsH protein levels and resistance to ROI. Exponential cultures of Mtb-6, Mtb-33 and wild type strains were grown in the presence of menadione (70 μ M) or H₂O₂ (5mM) for 48 hrs and plated on 7H10-OADC plates. CFU were counted and percent survival was calculated. Data presented are normalized to no-treatment control for each strain.

Figure 4.

Growth of *ftsH* strains in THP-1 macrophages. Monolayers of human macrophage cell line, THP-1, were infected with wild type (H37Rv, squares) or merodiploids overexpressing *ftsH* (Mtb-6, diamonds) or *ftsH* antisense (Mtb-33, triangles) at a moi of 1:1. At indicated periods of time, macrophages were lysed and viable bacterial count was determined by plating for CFU.

Figure 5.

FtsZ levels in *ftsH* strains. Exponential cultures of *ftsH* strains were grown in the presence of 0.2% acetamide for 3 days and examined for FtsZ levels by immunoblotting as described above in Fig. 2. Symbols: WT – wild type *Mtb*, Mtb-6 – *ftsH* overexpressing strain and Mtb-33 – *ftsH* antisense strain. Inset: Immunoblot showing FtsZ protein in the above strains.