Genomic organization and in vivo characterization of proteolytic activity of FtsH of Mycobacterium smegmatis SN2

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The ftsH gene of Mycobacterium smegmatis SN2 (MsftsH) was cloned from two independent partial genomic DNA libraries and characterized, along with the identification of ephA and folE as the neighbouring upstream and downstream genes respectively. The genomic organization of the MsftsH locus was found to be identical to that of the Mycobacterium tuberculosis ftsH gene (MtftsH) and similar to that of other bacterial genera, but with divergence in the upstream region. The MsftsH gene is 2.3 kb in size and encodes the AAA (ATPases Associated with diverse cellular Activities) family Zn²⁺-metalloprotease FtsH (MsFtsH) of 85 kDa molecular mass. This was demonstrated from the expression of the full-length recombinant gene in Escherichia coli JM109 cells and from the identification of native MsFtsH in M. smegmatis SN2 cell lysates by Western blotting with anti-MsFtsH and anti-EcFtsH antibodies respectively. The recombinant and the native MsFtsH proteins were found localized to the membrane of E. coli and M. smegmatis cells respectively. Expression of MsFtsH protein in E. coli was toxic and resulted in growth arrest and filamentation of cells. The MsftsH gene did not complement lethality of a ΔftsH3::kan mutation in E. coli, but when expressed in E. coli cells, it efficiently degraded conventional FtsH substrates, namely σ²² protein and the protein translocase subunit SecY, of E. coli cells.

INTRODUCTION

FtsH is a membrane-bound ATP-dependent Zn²⁺-metalloprotease (Tomoyasu et al., 1993a, b; Akiyama et al., 1995), which is involved in the proteolytic degradation of specific integral membrane proteins (Kihara et al., 1995, 1999; Akiyama et al., 1996a, b) and cytoplasmic proteins (Tomoyasu et al., 1995; Herman et al., 1993, 1995, 1997). It contains a highly conserved 200 amino acid stretch, which has two ATP-binding motifs (Walker motifs A and B) and an AAA (ATPases Associated with diverse cellular Activities) signature motif, which are characteristic features of the members of the AAA family of ATPases (Beyer, 1997; Ogura & Wilkinson, 2001). The Zn²⁺-binding motif is present towards the C-terminal portion. These characteristics are found conserved in the FtsH molecules from eubacteria, archaea and some eukaryotes (Ogura et al., 1991; Tomoyasu et al., 1993a, b; Nilsson et al., 1994; Lysenko et al., 1997; Guelin et al., 1994; Ge & Taylor, 1996; Lindahl et al., 1996). The Zn²⁺-binding motif and one of the ATP-binding motifs are essential for the proteolytic activity (Akiyama et al., 1996a; Karata et al., 1999). FtsH protease is involved in the regulation of the levels of phase λ CII protein during phage infection (Herman et al., 1993; Shotland et al., 1997) and is the major protease involved in the degradation of the heat shock transcription factor σ²² (Herman et al., 1995; Tomoyasu et al., 1995). FtsH is also involved in the degradation of the uncomplexed forms of an essential protein translocation subunit, SecY, in Escherichia coli (Kihara et al., 1995; Akiyama et al., 1996a). FtsH has been shown to have a regulatory role not only in stress response (Deuerling et al., 1995), but also in sporulation in Bacillus subtilis and protein secretion (Deuerling et al., 1997). The FtsH of B. subtilis was found to interfere with the expression of SpoOA protein, which is a transcriptional regulator of the initiation of sporulation (Deuerling et al., 1997). Further, a 26 amino acid peptide, SpoVM, which is essential for spore formation, was found to be a substrate for FtsH (Cutting et al., 1997). The absence of FtsH protease has been found to result in the overexpression of σv²³-controlled genes.
in B. subtilis, although the reason for it is unknown (Zellmeier et al., 2003). FtsH protease has also been shown to participate in membrane biogenesis by regulating the levels of UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase [the lpxC (envA) gene product] involved at the committed step in the biosynthesis of lipid A (Ogura et al., 1999). All these diverse functions of FtsH demonstrate that it is a protease essential for the viability of bacterial cells.

These diverse, but specific, functions of stress response protease FtsH are potentially helpful for the effective adaptation to the environment involving various stress conditions, either inside host cells, as in the case of a pathogen such as Mycobacterium tuberculosis, or in the environment, as in the case of a nonpathogenic saprophyte such as Mycobacterium smegmatis. We earlier cloned and expressed the ftsH gene of M. tuberculosis H37Rv (MtftsH) in order to understand the functional role of the protease in the mycobacterial pathogen (Anilkumar et al., 1998). In order to carry out a comparative structural and functional analysis, in this communication we report: (i) structural organization, cloning and expression of the ftsH gene of M. smegmatis SN2 (MsftsH) in E. coli cells, (ii) functional complementation by the gene, and (iii) efficient proteolytic activity of MsftsH protease on the specific and typical FtsH substrates, namely heat shock transcription factor σ32 and the protein translocase subunit SecY, in vivo in E. coli cells.

METHODS

Bacterial strains, vectors, growth media and growth conditions. The bacterial strains and the plasmid vectors used in this study are given in Table 1. E. coli JM109 cells were grown in Luria–Bertani (LB) broth at 37°C, while E. coli AR 5090 cells were grown in Luria (L) broth at 30°C. M. smegmatis SN2 cells were grown in either Youman and Karlson’s (YK) medium or Middlebrook 7H9 broth supplemented with glucose and albumin. E. coli transformants were selected on LB agar or L agar with relevant antibiotics, namely ampicillin (100 µg ml⁻¹), kanamycin (25 µg ml⁻¹), tetracycline (10 µg ml⁻¹) or chloramphenicol (10 µg ml⁻¹), as appropriate.

Chromosomal DNA isolation. M. smegmatis SN2 cells were grown in YK liquid medium at 37°C to an OD550 of 0.8. Glycine was added to the growing cells, to a final concentration of 0.2 M, and the incubation was continued for a further 2 h. The cells were pelleted by centrifugation at 4000 r.p.m. for 10 min, and the pellet was suspended in the lysis buffer (50 mM glucose, 25 mM Tris/HCl, pH 8-0). Lysozyme and Tween 80 were added to a final concentration of 5 mg ml⁻¹ and 0.2%, respectively, and the incubation was continued for 2 h at 37°C on a shaker. The cells were lysed by incubation in the presence of 1% SDS for 15 min at 50°C. Genomic DNA was extracted from the lysed cells, dissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), incubated in the presence of RNase A (20 µg ml⁻¹) for 12 h at 37°C, further extracted, washed with 70% ethanol, precipitated, and used directly for the construction of genomic DNA library as described by Sambrook et al. (1989).

Cloning of the MsftsH gene. The MsftsH gene was cloned from two partial genomic DNA libraries of M. smegmatis SN2. One microgram of genomic DNA was digested with PstI at an enzyme:DNA ratio of 1:10, overnight at 37°C. The digested genomic DNA was fractionated and transferred from the gel onto a nylon membrane using capillary transfer. The probe used for the detection of MsftsH ORF-specific fragment in the PstI digest of genomic DNA was a 364 bp PCR product, which was amplified from the ftsH ORF of M. tuberculosis H37Rv (MtftsH) present in the SCY67F cosmid (a kind gift from Dr Stewart Cole, Pasteur Institute, France; Cole et al., 1998) as described in our earlier work (Anilkumar et al., 1998). A non-radioactive labelling and detection system (Gene Images) was used for the labelling of the MtftsH PCR probe and for the detection of signals. Labelling of the PCR probe, hybridizations, and detection of signals were carried out as per the manufacturer’s instructions. Southern hybridization of the PstI digest revealed a single band in the 3-2 kb region, which was eluted using low-melting-point agarose gel (Sambrook et al., 1989). The eluted DNA fragment was ligated to PstI-digested, calf-intestinal-phosphatase-treated pBS(SK⁺) vector. E. coli JM109 cells were electroporated with the ligation mixture to obtain a PstI partial genomic DNA library, hereafter referred to as the PstI library. The transformants, which grew on agar containing ampicillin, were replica plated. The replica plates were used for colony hybridization (Sambrook et al., 1989) using the MsftsH-specific probe. The amino acid sequence, deduced from the partial nucleotide sequence of the inserts from a few positive clones, revealed a high percentage of identity with the sequences of FtsH proteins from other bacterial systems, confirming that the clone carried the ftsH gene of M. smegmatis SN2 (MsftsH). Complete sequencing of the 3-2 kb insert showed that the fragment carried a partial MsftsH gene containing only the highly conserved AAA domain and the protease domain. The region corresponding to the N-terminal transmembrane portion of MsftsH protein was absent from this clone obtained from the PstI library.

In order to obtain the 5’ portion of the MsftsH gene, a KpnI partial genomic DNA library of M. smegmatis SN2 was constructed in pBS(SK⁺) vector, in a manner identical to the construction of the PstI library. A 548 bp Clal–PstI DNA fragment, which corresponded to the N-terminal transmembrane region of MtftsH gene, was used to probe the KpnI genomic DNA digest. The 548 bp fragment was obtained by PstI digestion of the pBS–MtH vector (Anilkumar et al., 1998). Southern hybridization of the KpnI-digested, fractionated genomic DNA, with the 548 bp Clal–PstI DNA fragment as the probe, showed a band in the 2.9 kb region, which was used for making the second partial genomic DNA library, hereafter referred to as the KpnI library. The amino acid sequence deduced from the nucleotide sequence of the inserts from a few positive clones revealed a region corresponding to the transmembrane portion of the FtsH protein. The pBS(SK⁺) recombinant clone, containing the KpnI fragment, was passed through E. coli GM 2151 (dam⁻, cym⁻), to make the Clal site on the insert sensitive to cleavage by the enzyme. It was then digested with Clal and PstI to release a 0.576 kb fragment that represented the transmembrane region of the MsftsH gene. The PstI library was digested using PstI and Xhol enzymes to obtain the 2.3 kb DNA fragment carrying the remaining portion of the MsftsH gene, including the stop codon. The 0.576 kb Clal–PstI fragment and the 2.3 kb PstI–Xhol insert were ligated to pBS(SK⁺) vector, which was digested with Clal and Xhol enzymes. The three-way ligation resulted in the cloning of a 2.876 kb DNA fragment that contained the complete ORF of the MsftsH gene as a translational fusion with the lacZ gene at the N-terminus of the MsftsH gene. This construct, pBS–MsH, contained the complete MsftsH ORF of 2.31 kb.

Cloning of the MsftsH ORF for in vivo expression. The pBS–MsH construct was digested with Xhol, end-filled with T4 DNA polymerase, and then digested with Clal to release the ftsH ORF as a Clal–Xhol blunt-ended fragment, which was ligated to the pT18 vector. pT18-zip is a lac promoter-based vector system, derived from pBS(KS⁺), containing the T18 fragment of the adenylate cyclase (cya) gene fused to the leucine zipper at the N-terminus
**Table 1. Bacterial strains and vectors**

<table>
<thead>
<tr>
<th>Strain, plasmid or vector</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em> JM103</td>
<td>[Δ(lac–pro) endA sbcB15 hsdR thi rpsL supE/F’ lacI37 traD36 proAB’ lacZ ΔM15]</td>
<td>Messing et al. (1981)</td>
</tr>
<tr>
<td><em>E. coli</em> JM 109</td>
<td>[Δ(lac–pro) endA1 recA1 hsdR17 thi relA gyrA96 supE44/F’ lacI37 traD36 proAB’ lacZ ΔM15]</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td><em>E. coli</em> GM 2151</td>
<td>thr-1 araC14 leuB6(Am) fhuA31 lacY1 tsx-78 glnV44(AS) galK2(Oc) dcm-6 hisG1(Fs) rpsL136(strR) dam-13::Tn9 xylA5 nth-1 thi-1</td>
<td>M. G. Marinus, U. Mass. Med. Sch., USA</td>
</tr>
<tr>
<td><em>E. coli</em> AR 423</td>
<td>ΔftsH3::kan and Δ(srl-recA)306::Tn10 pAR 171; a derivative of SH392 (met gal supE hsdR sfiC)</td>
<td>T. Ogura (Akiyama et al., 1994)</td>
</tr>
<tr>
<td><em>E. coli</em> AR 5090</td>
<td>ΔftsH sfiC21 degP807/F’lacI37; a derivative of JM103</td>
<td>T. Ogura (Akiyama &amp; Ito, 2000, 2003)</td>
</tr>
<tr>
<td><em>E. coli</em> C41</td>
<td>BL21(DE3) derivative that allows the expression of normally toxic proteins</td>
<td>J. E. Walker (Miroux &amp; Walker, 1996)</td>
</tr>
<tr>
<td><em>M. smegmatis</em> SN2</td>
<td>Wild-type isolate</td>
<td>Lab. collection</td>
</tr>
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<td><strong>Plasmids/vectors</strong></td>
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</tr>
<tr>
<td>pBS-SK +</td>
<td>Carries the EcsecY gene under the lac promoter (pACYC184 derivative)</td>
<td>Messing et al. (1981)</td>
</tr>
<tr>
<td>pKY248</td>
<td>Carries the EcftsH gene as a C-terminal myc-6 × His fusion under the lac promoter</td>
<td>Taura et al. (1993)</td>
</tr>
<tr>
<td>pSTD 113</td>
<td>Carries the complete EcftsH ORF including its upstream sequences ftsH rep’ CmR</td>
<td>Y. Akiyama (Akiyama et al., 1995)</td>
</tr>
<tr>
<td>pAR 171</td>
<td>Carries the complete EcftsH ORF including its upstream sequences ftsH rep’ CmR</td>
<td>T. Ogura (Akiyama et al., 1994)</td>
</tr>
<tr>
<td>pBS–PstI 3-2</td>
<td>Carries the 3-2 kb PstI fragment containing the 3’ region of the MsftsH gene and the upstream genes</td>
<td>This study</td>
</tr>
<tr>
<td>pBS–KpnI 2-9</td>
<td>Carries the 2-9 KpnI fragment containing the 5’ region of the MsftsH gene and the upstream genes</td>
<td>This study</td>
</tr>
<tr>
<td>pBS–MsH</td>
<td>Carries the complete MsftsH ORF under the lac promoter of pBS-SK+</td>
<td>This study</td>
</tr>
<tr>
<td>pT18–zip</td>
<td>Lacks the leucine zipper region of pT18–zip</td>
<td>Karimova et al. (1998); this study</td>
</tr>
<tr>
<td>pT18–MsH</td>
<td>Carries the complete MsftsH ORF under the lac promoter of pT18</td>
<td>This study</td>
</tr>
<tr>
<td>pBHB1</td>
<td>Carries EcftsH under the arabinose promoter</td>
<td>Herman et al. (1997)</td>
</tr>
<tr>
<td>SCY6F7</td>
<td>Cosmid contig containing ftsH gene of <em>M. tuberculosis</em> H37Rv</td>
<td>Cole et al. (1998)</td>
</tr>
<tr>
<td>pBS–MtH</td>
<td>Contains full-length ORF of <em>M. tuberculosis</em> H37Rv</td>
<td>Anilkumar et al. (1998)</td>
</tr>
<tr>
<td>pCYB2–MsH</td>
<td>Contains full-length MsftsH as C-terminal intein fusion under the tac promoter</td>
<td>This study</td>
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<tr>
<td>pGEX–4T1–MsH</td>
<td>Contains full-length MsftsH as N-terminal GST translational fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pRSET–A–MsH</td>
<td>Contains full-length MsftsH as N-terminal 6 × His tag under the T7 promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pET20b–MsH</td>
<td>Contains full-length MsftsH as C-terminal 6 × His tag under the T7 promoter</td>
<td>This study</td>
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</table>

(Karimova et al., 1998). We removed the region encoding the leucine zipper from the vector by KpnI digestion and religation to generate the pT18 vector, which was further digested with ClaI and EcoRV. The ClaI–XhoI blunt-ended fragment containing the MsftsH ORF, which was obtained from the pBS–MsH vector, was ligated to the ClaI–EcoRV site of the pT18 vector to obtain the pT18–MsH vector, wherein the MsFtsH protein is a translational fusion with the cya gene fragment.

**Co-expression of MsFtsH and EcFtsH.** The vector pBHB1 (a kind gift from Dr. Christophe Herman, University of California San Francisco, USA; Herman et al., 1997) was used for co-expression of EcFtsH, along with MsFtsH. *E. coli* JM109 cells were co-transformed with pT18–MsH and pBHB1, and transformants were selected on L agar containing appropriate antibiotics. The cultures were grown in L medium and induced with 0.4% L-arabinose and 1 mM IPTG.

Cells were fixed onto poly-L-lysine-coated slides and photographed under a Zeiss microscope at ×100 using a CCD camera. Cell length was calculated using ImageJ software from NIH (http://rsb.info.nih.gov/ij/).

**Membrane isolation.** *E. coli* cells carrying appropriate constructs were grown to an OD600 of 0.50 and were induced with 1 mM IPTG for 150 min. The cells were pelleted at 5000 r.p.m. for 10 min and suspended in 100 mM phosphate buffer, pH 7–2, and sonicated in the presence of 1 mM PMFSF. *M. smegmatis* SN2 cells, grown in YK liquid medium to an OD550 of 0.50, were sonicated in PBS, pH 7–2, in the presence of 1 mM PMFSF. After removing debris by centrifugation at 12000 r.p.m. for 15 min at 4°C, the supernatant was used as the total-protein extract. For the protein localization studies, this supernatant was partitioned into pellet and supernatant fractions by ultracentrifugation at 40000 r.p.m. in a type 50T1 rotor.
in a Beckman ultracentrifuge for 2 h. The supernatant was used as the cytosolic fraction. The pellet was suspended in 1 M NaCl solution, incubated at 4 °C for 10 min, and separated again into pellet and supernatant by ultracentrifugation using the parameters mentioned above. The pellet fraction was suspended in 50 mM phosphate buffer, pH 7.2, and used as the membrane preparation as described by Tomoyasu et al. (1993b).

**Assay for proteolytic activity of MsFtsH in vivo.** The proteolytic activity of MsFtsH, which was expressed from pT18–MsH, was assayed in vivo using the unconventional substrates of FtsH protease, namely σ32 (Eco32) and SecY (EcSecY) proteins of *E. coli*. For the MsFtsH protease assay, changes in the levels of endogenous Ecoσ32 protein and of ectopically expressed EcSecY protein, which was induced from pKY248, were monitored independently in response to induction of MsFtsH from pT18–MsH. As a positive control for proteolytic activity, degradation of endogenous Ecoσ32 and ectopically expressed EcSecY proteins, which was induced from pKY248, were monitored independently in response to induction of MsFtsH from pT18–MsH. As a positive control for proteolytic activity, degradation of endogenous Ecoσ32 and ectopically expressed EcSecY proteins by EcFtsH, which was induced from the pSTD113 vector, was monitored. *E. coli* AR 5090 (ΔftsH::kan, sftC210) cells were co-transformed with the plasmid vector pKY248, carrying the EcSecY gene, plus pBluescript (vector control), pSTD113, pT18 (vector control) or pT18–MsH, and colonies were selected on L agar containing relevant antibiotics. Exponentially growing cultures of *E. coli* AR 5090 cells carrying two compatible plasmid vectors, pKY248 and pBluescript, pSTD113, pT18 or pT18–MsH, were induced with 2 mM IPTG for 3 h. Cells were harvested and lysed in 8 M urea in Tris/HCl, pH 8.0, containing 150 mM NaCl. The lysate was centrifuged at 12 000 r.p.m. to remove cell debris and unlysed cells. The supernatant was used for immunoblotting of Ecoσ32 and EcSecY proteins.

**Western blotting.** Equal amounts of total protein from the membrane and cytosol fractions of total lysates from transformed or untransformed *E. coli* or *M. smegmatis*, or from the uninduced or induced cultures of *E. coli* cells (25 μg and 50 μg respectively for Ecoσ32 and EcSecY Western blots), were separated on 10% SDS-polyacrylamide gel (Laemmli, 1970) or on 16-1% acrylamide–0-12% N,N'-methylene-bis-acrylamide (for SecY) and transferred onto a PVDF membrane using a semi-dry transfer apparatus, which was manufactured in the Indian Institute of Science. The membranes were blocked using 0-1% Tween 20 and 5% non-fat dried milk in phosphate-buffered saline (PBS). Primary antibodies against EcFtsH, EcSecY, Ecoσ32 and EcRRF proteins of *E. coli*, namely anti-EcFtsH (a kind gift from Dr Teru Ogura, Kumamoto University, Japan; Tomoyasu et al., 1993b), anti-EcSecY (a generous gift from Dr Yoshihiko Akiyama, Institute for Virus Research, Kyoto University, Kyoto, Japan; Taura et al., 1993), anti-σ32 (a kind gift from Dr Bernd Bukau, University of Heidelberg, Germany; Gamer et al., 1992) and anti-RRF (a kind gift from Dr Umesh Varshney, Indian Institute of Science, Bangalore, India; Rao & Varshney, 2001) were used at 1:5000, 1:5000, 1:4000 and 1:3000 dilutions respectively. The anti-MtFtsH antibody, raised against the C-terminus of FtsH protein of *M. tuberculosis* H37Rv, was used for the detection of MsFtsH at 1:5000 dilution, while protein A–HRP conjugate was used at 1:10 000 dilution. Detection was carried out using enhanced chemiluminescence detection reagents according to manufacturer’s instructions.

**RESULTS AND DISCUSSION**

**Organization of the MsftsH gene locus**

The nucleotide sequence of the MsftsH gene, which was derived from the work described in this manuscript, was deposited in 1998 in GenBank with the accession number AF037269. DNA sequence determination of the clones from the *PsI* and *KpnI* partial libraries revealed the identities of the genes present in the regions downstream and upstream of MsftsH gene. The region downstream of MsftsH contains folE, folX and folP genes, which code for proteins involved in folate biosynthesis in *M. smegmatis* SN2 (Fig. 1). However, a few potential ORFs encoding hypothetical proteins were also found interspersed between *ephA* and MsftsH. Similar to the presence of the *fol* gene operon on the region downstream of MsftsH, organization of the *ftsH* locus in *M. tuberculosis* H37Rv and *E. coli* also involves the *fol* gene operon on the region downstream of *ftsH*. The region upstream of the MsftsH gene was also conserved between *M. smegmatis* and *M. tuberculosis*. In both cases, the *ephA* gene, encoding soluble epoxide hydrolase, is immediately upstream of the *ftsH* gene. Although the gene organization in the upstream region is conserved between these mycobacterial species, it is interesting to note the divergence in the organization of the *ftsH* locus in another Gram-positive bacterium, *B. subtilis* (Kunst et al., 1997), and in the Gram-negative bacterium *E. coli* (Ogura et al., 1991). In *B. subtilis*, the gene encoding hypoxanthine guanine phosphoribosyltransferase (HprT) is placed immediately upstream of *ftsH*, while it is flanked downstream by the *yac* operon (Kunst et al., 1997). On the other hand in *E. coli*, the *ftsJ* (rmmJ) gene, encoding 23S rRNA methyltransferase (Caldas et al., 2000), is present, instead of *ephA*, immediately upstream of *ftsH* both being heat-inducible (Caldas et al., 2000; Tomoyasu et al., 1993a; Herman et al., 1995). It is interesting to mention in this context that while *fsi* is absent from the genome of *M. tuberculosis* H37Rv (Cole et al., 1998), there is no information so far regarding the presence or absence of the *fsi* gene in *M. smegmatis* SN2. However, from our studies, we can say that the *fsi* gene is not present.

**Fig. 1.** Genomic organization of the *ftsH* locus of *M. smegmatis* and comparison with the *ftsH* loci of *M. tuberculosis* and *E. coli*. Arrowheads indicate the direction of the ORF. Ec, *E. coli*; Ms, *M. smegmatis*; Mt, *M. tuberculosis*; Bs, *B. subtilis.*
at least in the region immediately upstream of the \textit{ftsH} gene in \textit{M. smegmatis} SN2, although we cannot yet ascertain its absence from the genome. Another interesting example of divergence in the organization of the \textit{ftsH} locus has been found in \textit{Caulobacter crescentus} (Fischer et al., 2002). In this bacterium, \textit{yaeN} and \textit{kinA} genes flank the \textit{ftsH} gene on the upstream and downstream sides, respectively. In fact, \textit{ftsH} and \textit{folP} are interspersed by \textit{kinA} in \textit{C. crescentus}.

\textbf{Amino acid sequence and domain organization features of MsFtsH}

The ORF of the \textit{MsftsH} gene is 2-31 kb in size and encodes a protein of approximately 85 kDa, having 769 amino acid residues. The \textit{MsftsH} ORF shares a high degree of identity (82\% at the amino acid level) with that of \textit{MtftsH}, which has 760 amino acid residues encoding a protein of 84 kDa (Anilkumar et al., 1998). Analysis of the deduced amino acid sequence of the protein showed the presence of two transmembrane regions, two ATP-binding motifs (Walker A and B motifs), namely GPPGTGTK and IIFVDEID, an AAA family signature motif, namely GVILAVIRDPDA-LLRPGR, and a \(\text{Zn}^{2+}\)-binding motif, HEGGHH, unlike the HEAGH motif in EcFtsH, which is characteristic of \(\text{Zn}^{2+}\)-metalloprotease (Fig. 2). Prediction of transmembrane regions using the Dense Alignment Surface (DAS) method (Cserzo et al., 1997) (http://www.sbc.su.se/~miklos/DAS/maindas.html) showed the presence of two transmembrane helices at the N-terminus, one spanning from T9 to F23, and another spanning from 111V to 132F (Fig. 2). These features are characteristic of FtsH proteases from all bacterial genera (Beyer, 1997; Ogura & Wilkinson, 2001). These motifs exhibited 100\% identity at the amino acid level with the FtsH protease of \textit{M. tuberculosis} (MtFtsH) protein.

Examination of domain organization using Pfam analysis, available at the Sanger Centre, UK and at Washington University at St Louis, USA (http://pfam.wustl.edu/hmmsearch.shtml), demonstrated the presence of a unique domain adjacent to the protease domain. This domain, having the primary structure DVEKRPRLLMFDFDGFGRVPSPDKPPKTPGELAMERGEWPPPPVPEPAFKAI, was found to be unique to all the FtsH proteases known so far from mycobacterial species, but is absent from the FtsH proteases of all other organisms (Fig. 2). Its placement towards the proteolytic domain at the C-terminus raises the possibility that this domain might be involved in the interaction with specific substrates unique to mycobacteria. This possibility can be verified only after determination of the profile of FtsH substrates in mycobacteria. Further, from the nature of unique substrates alone, one could elucidate the biological relevance of this unique domain.

While the unique domain containing 51 amino acid residues is common to all mycobacterial FtsH proteins, MsFtsH possesses, in addition, a unique stretch of sequence, which is rich in glutamine and proline residues, at the C-terminal portion of the protein (Fig. 2). While FtsH proteases of \textit{Mycobacterium avium} (MaFtsH) and \textit{Mycobacterium leprae} (MlFtsH) also carry a glutamine- and proline-rich stretch of residues, interestingly, MtFtsH and the FtsH protein of \textit{Mycobacterium bovis} (MbFtsH) conspicuously lack the stretch of glutamine and proline residues (Anilkumar et al., 1998). It may be noted here that glutamine-rich sequences, but not proline-rich sequences, are known to be one of the characteristic features of transcriptional activators (Wykoff et al., 1999). However, the biological role of the glutamine- and proline-rich sequence in MsFtsH, MaFtsH and MlFtsH, and the consequent biological differences due to its absence in MtFtsH and MbFtsH, need further investigation. One potential use of this sequence might be for the construction of a phylogenetic tree among mycobacterial species.

The amino acid sequence of conserved regions of MsFtsH was aligned with the corresponding sequences of the FtsH proteases of \textit{E. coli} (P28691), \textit{B. subtilis} (P37476), \textit{Arabidopsis thaliana} (O80860), \textit{Homo sapiens} (Q9Y2Q2) and \textit{M. tuberculosis} (P96942), using the \textsc{clustalw} program at the European Bioinformatics Institute (Thompson et al., 1994) (http://www2.ebi.ac.uk/clustalw) (Fig. 3). The MsFtsH protein shares 44.8\%, 47.2\%, 40.2\% and 53\% identity, respectively, at the amino acid sequence level with the amino acid sequences of the FtsH proteases of \textit{E. coli}, \textit{B. subtilis}, \textit{A. thaliana} and \textit{H. sapiens}, indicating that the 2-31 kb ORF that we obtained indeed contained the MsftsH gene. Although the extent of sequence conservation between MsFtsH and FtsH molecules of the other organisms referred

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Schematic representation of \textit{M. smegmatis} FtsH protease. TM1 and TM2 represent the two transmembrane domains predicted by the Dense Alignment Surface method (DAS). SRH represents the second region of homology. The amino acid numbers encompassing the different domains are indicated.}
\end{figure}
to above is only to the extent of about 40–50 %, there is a high level of sequence conservation among mycobacterial FtsH proteases. The Ms\textit{ftsH} ORF shares 82 % identity at the amino acid level with that of Mt\textit{ftsH}, which has 760 amino acid residues encoding a protein of 84 kDa (Anilkumar et al., 1998). Similarly, MsFtsH shares sequence identity of 80–2%, 79–7 % and 79–3 %, respectively, with the FtsH proteases of \textit{M. bovis}, \textit{M. leprae} and \textit{M. avium}.

Expression and localization of native MsFtsH protein

The antibody raised in our laboratory against the C-terminus of MtFtsH did not detect native MsFtsH from \textit{M. smegmatis} SN2 cell lysate, probably due to low titre, although the antibody did detect both the proteins when overexpressed in their recombinant form. Therefore, anti-EcFtsH antibody was used to detect native MsFtsH in the membrane fraction from \textit{M. smegmatis} SN2 cell lysates.

Western blot analysis of the membrane fraction, prepared from \textit{M. smegmatis} SN2 cells, with anti-EcFtsH antibody showed that MsFtsH protein was found only in the membrane fraction and not in the cytoplasmic fraction (Fig. 4a).

Expression and localization of recombinant MsFtsH protein

The complete ORF of the MsftsH gene was cloned into several prokaryotic expression vectors to obtain pBS–MsH, pGEX-4T1–MsH, pRSET-A–MsH, pCYB2–MsH and pET20b–MsH. \textit{E. coli} C41 (Miroux & Walker, 1996) was used for transformation with pRSET-A–MsH and pET20b–MsH, while \textit{E. coli} JM109 cells were used for transformation with pBS–MsH, pGEX-4T1–MsH and pCYB2–MsH. Although clones were obtained, expression of MsFtsH was not achieved even within the detection limits of Western blotting. In all these cases, host cells showed filamentation, with further growth severely hampered. Since MsftsH was not be expressed from any of the constructs, namely pBS–MsH, pGEX-4T1–MsH, pRSET-A–MsH, pCYB2–MsH and pET20b–MsH, \textit{E. coli} C41 (Miroux & Walker, 1996) was used for transformation with pRSET-A–MsH and pET20b–MsH, while \textit{E. coli} JM109 cells were used for transformation with pBS–MsH, pGEX-4T1–MsH and pCYB2–MsH. Although clones were obtained, expression of MsFtsH was not achieved even within the detection limits of Western blotting. In all these cases, host cells showed filamentation, with further growth severely hampered.

The recombinant MsFtsH protein was recovered from the pellet fraction obtained after incubation with 1 M NaCl (Fig. 4c), suggesting that it was localized to the inner cell membrane (Tomoyasu et al., 1993a, b), as reported in the.
alone. The arrow indicates the time at which IPTG was added.

Fig. 4. (a) Membrane localization of MsFtsH protein in *M. smegmatis* SN2. Membrane fractions were prepared from *M. smegmatis* cells as described in Methods. Membrane proteins were resolved by 12% SDS-PAGE. FtsH was detected with anti-EcFtsH antibodies. Lanes: 1, whole-cell lysate from *M. smegmatis*; 2, proteins from membrane fraction; 3, proteins from the cytoplasmic fraction; 4, whole-cell lysate from AR5090/pT18–MsH induced with 1 mM IPTG; and 5, whole-cell lysate from AR5090/pT18 induced with 1 mM IPTG (negative control for the protein band in lane 4). The arrow indicates the 85 kDa MsFtsH protein. (b) Expression of MsFtsH in *E. coli* JM109 cells. Cultures were induced with 1 mM IPTG for 3 h and equal amounts of protein were separated using 10% SDS-PAGE. Western blots were carried out with anti-EcFtsH antibodies. Lanes: 1, JM109/pT18 uninduced; 2, JM109/pT18 induced; 3, JM109/pT18–MsH uninduced; and 4, JM109/pT18–MsH induced. The 85 kDa MsFtsH protein expressed from the pT18–MsH vector is present in lanes 3 and 4. (c) Membrane localization of MsFtsH protein in *E. coli* JM109 cells. Cultures were induced with 1 mM IPTG for 3 h and membranes were prepared as described in Methods. Lanes: 1, total membrane fraction; 2, pellet fraction obtained after incubation with 1 M NaCl. Western blotting was carried out with anti-MsFtsH antibodies. (d) Growth arrest of *E. coli* JM109 cells upon induction of MsFtsH. □, Cells carrying the MsFtsH gene induced with 1 mM IPTG; ■, cells carrying the pT18 plasmid alone. The arrow indicates the time at which IPTG was added.

Protease activity of recombinant MsFtsH in *E. coli* cells

The protease activity of MsFtsH, induced from the pT18–MsH construct, was tested in vivo in *E. coli* cells on σ^32 protein and SecY protein, both of which are specific substrates for EcFtsH (Tomoyasu et al., 1995; Kihara et al., 1999). These heterologous substrates from *E. coli* had to be used for the demonstration of protease activity of MsFtsH in vivo for the following reasons. A homologue for σ^32 protein is absent in mycobacteria, although a heat-shock-inducible sigma factor, sigH, exists (Cole et al., 1998; Fernandes et al., 1999). Recombinant SecY proteins of *M. smegmatis* or *M. tuberculosis* were not obtained, since expression of secY genes of both these mycobacteria in *E. coli* cells resulted in severe toxicity to host cells, with resultant growth arrest.

Degradation of σ^32 protein by MsFtsH in vivo

Cellular levels of *E. coli* heat shock transcription factor σ^32 are controlled by the ATP-dependent proteases such as Lon, ClpXP, HslVU and FtsH (Kanemori et al., 1997). The σ^32 protein is stabilized in *ftsH*-null strains of *E. coli*, and its levels are at least 20-fold higher than those in wild-type cells (Ogura et al., 1999). One such strain, AR 5090 (*ftsH::kan, sfhC21*), offers an excellent in vivo assay system for monitoring the protease activity of FtsH (Akiyama & Ito, 2000; Karata et al., 2001; Akiyama & Ito, 2003). MsFtsH was expressed from the pT18–MsH recombinant vector in *E. coli* AR 5090 cells. Synthesis of 85 kDa MsFtsH protein, upon induction with 2 mM IPTG for 3 h, was confirmed with Western blotting using anti-EcFtsH antibodies (Fig. 5b). Use of anti-EcFtsH antibodies also confirmed the absence of the 74 kDa EcFtsH protein from the AR 5090 lysates. Degradation of endogenous σ^32 protein of *E. coli* was monitored with Western blotting using anti-σ^32 antibody (a kind gift from Dr Bernd Bukau, University of Heidelberg, Germany). Recombinant MsFtsH degraded σ^32 protein in vivo (Fig. 5b). Degradation of σ^32 by MsFtsH was not as efficient as that by EcFtsH expressed from the plasmid case of MrFtsH (Anilkumar et al., 1998). The expression of MsftsH in *E. coli* JM109 cells was lethal and growth stopped within 1 h of induction with 1 mM IPTG (Fig. 4d). The cells were filamentous as compared with the control cells that carried the pT18 vector alone, but they did not show any defect in nucleoid segregation (data not shown). The cells were filamentous even in the absence of IPTG. Expression of an algal FtsH has been reported to result in a similar phenotype (Itoh et al., 1999). It is possible that sequestration of EcFtsH by MsFtsH in oligomerization, thereby making EcFtsH unavailable for biological function, could be the cause of the filamentation. In such case, overexpression of EcFtsH might rescue the cells from filamentation or toxicity caused by the expression of MsFtsH. However, co-expression of EcFtsH from pBHB1 did not suppress the toxicity to or filamentation of *E. coli* JM109 cells caused by the expression of MsFtsH (data not shown).
Fig. 5. (a) In vivo degradation of Eco\textsuperscript{32} by EcFtsH protease. Upper panel, the expression of Ecfsh from pSTD113 in E. coli AR 5090 cells (ΔftsH::kan sfhC21). FtsH\textsuperscript{H} indicates the C-terminally processed FtsH. Middle panel, the degradation of Eco\textsuperscript{32} by EcFtsH. Lower panel, the amount of RRF present in these samples. Lanes 1 and 2: AR 5090 cells carrying vector alone (pBluescript), uninduced and induced with 2 mM IPTG respectively. Lanes 3 and 4: AR 5090 cells carrying pSTD113 (EcfshH), uninduced and induced with 2 mM IPTG respectively. Lane 4 shows the degradation of Eco\textsuperscript{32} by EcFtsH, while levels of RRF remain unchanged. (b) In vivo degradation of Eco\textsuperscript{32} by MsFtsH protease. Upper panel, the expression of Msftsh in E. coli AR 5090 cells (ΔftsH::kan sfhC21). Cultures were induced with 2 mM IPTG and expression of MsFtsH protein was detected by immunoblotting with anti-EcFtsH antibodies. Middle panel, the degradation of Eco\textsuperscript{32} by MsFtsH. Lower panel, the amount of RRF present in these samples. Lanes 1 and 2: AR 5090 cells carrying vector alone (pT18), uninduced and induced with 2 mM IPTG respectively. Lanes 3 and 4: AR 5090 cells carrying pT18-MsH (Msftsh), uninduced and induced with 2 mM IPTG respectively. Lane 4 shows the degradation of Eco\textsuperscript{32} by MsFtsH, while the levels of RRF remain the same.

pSTD113 (EcfshH expressed from lac promoter; a kind gift from Dr Yoshinori Akiyama, Institute of Virus Research, Kyoto University, Japan; Akiyama et al., 1995). About a twofold decrease in ς\textsuperscript{32} protein levels was observed when MsFtsH was expressed, as compared to the fivefold decrease found in the case of degradation by EcFtsH. Reasons for the inefficient degradation of ς\textsuperscript{32} could be either the differences in the expression levels of the two proteases or the possibility that ς\textsuperscript{32} is a relatively poor substrate for MsFtsH. Expression levels of EcFtsH and MsFtsH could not be compared by Western blot because of possible differences in the cross-reactivity of the anti-EcFtsH antibodies to MsFtsH and EcFtsH. As an additional internal control, the lysates were immunoblotted with antibodies against ribosome recycling factor (RRF) (a kind gift from Dr Umesh Varshney, Indian Institute of Science, Bangalore, India). RRF did not show any changes in its level upon overexpression of either EcFtsH or MsFtsH (Fig. 5a, b).

One possible objection to the specificity of degradation of ς\textsuperscript{32} protein by MsFtsH could be that the protein was degraded by some protease other than MsFtsH. This would be possible if the induced expression of heterologous MsFtsH protein had induced a classical stress response in E. coli host cells, resulting in the induction of cellular proteases such as Lon and/or ClpXP for which ς\textsuperscript{32} protein is a substrate (Goff & Goldberg, 1985; Ito et al., 1986; Kanemori et al., 1997). In this case, degradation of ς\textsuperscript{32} protein could be effected by Lon and/or ClpXP, and not by MsFtsH itself. Although expression of heterologous proteins is known to result in the induction of heat shock response involving ς\textsuperscript{32} protein, such a heat shock response has been found to be associated with an increase rather than a decrease in the levels of ς\textsuperscript{32} protein in the absence of FtsH protease (Kanemori et al., 1997). Moreover, expression of heterologous FtsH has not been reported to result in a classic stress response. Therefore, it is quite unlikely that degradation of ς\textsuperscript{32} protein, concomitant with the induction of MsFtsH, might have been caused by proteases such as Lon and/or ClpXP, and not by MsFtsH.

Nevertheless, in order to further substantiate the protease activity of recombinant MsFtsH in E. coli cells, EcSecY, the degradation of which is not dependent upon a classical stress response, was used for the in vivo assay. Moreover, FtsH is the only protease hitherto known to be involved in the degradation of SecY. Therefore, degradation of EcSecY was monitored in response to induction of MsFtsH. Thus, any reduction in the levels of SecY protein, upon induction of MsFtsH in the EcFtsH-null AR 5090 strain, would be due solely to MsFtsH protease expressed from the plasmid.

Degradation of EcSecY by MsFtsH in vivo. SecY encodes an integral transmembrane protein, which spans the cytoplasmic membrane ten times (Akiyama & Ito, 1987) and plays an essential role, in conjunction with SecE and SecG, in forming the peptide translocase complex (Brundage et al., 1990; Ito, 1992). In E. coli, moderately overexpressed and unassembled SecY is rapidly degraded with a half-life of 2 min (Taura et al., 1993). The degradation of SecY that failed to associate with its partner SecE was dependent upon FtsH (Akiyama et al., 1996a), and mutations that resulted in the loss of function or underexpression of the ATP-dependent FtsH protease stabilized the overexpressed SecY (Kihara et al., 1995). Overexpression of FtsH accelerated the degradation of
Exponentially growing cultures of expressed and uncomplexed SecY. Therefore, the protease activity of FtsH on the membrane-bound substrates can be assessed by its ability to degrade over-expressed and uncomplexed SecY.

Exponentially growing cultures of E. coli AR 5090 (EcftsH null) carrying two compatible plasmids, pKY248 with pBluescript, pSTD113, pT18 or pT18–MsH, were induced with 2 mM IPTG for 3 h. Western blotting with anti-SecY antibodies (a generous gift from Dr Akiyama, Institute of Virus Research, Kyoto University, Japan) was used to monitor SecY levels in the induced cultures. Co-expression of MsFtsH resulted in the degradation of SecY molecules that were produced in excess (Fig. 6b). The level of expression achieved for SecY, when MsFtsH was co-expressed, was 10-fold less than that achieved in an ftsH-null strain carrying the vector alone. EcFtsH, expressed from pSTD113, was used as the positive control for the degradation of SecY (Fig. 6a). As an internal control, immunoblotting with anti-RRF antibodies showed no changes in the levels of RRF, indicating that the degradation of SecY by EcFtsH was specific. Densitometric comparison of the levels of SecY in the absence of FtsH, and in the induced presence of FtsH, showed that the degradation of EcSecY by MsFtsH was twofold less efficient than that by EcFtsH. The degradation of EcSecY by MsFtsH demonstrated that the recombinant MsFtsH protein, expressed in E. coli cells, was proteolytically active. This observation alludes to the possibility of the existence of conserved mechanisms of protein translocation and their control across bacterial genera. It indirectly supports the contention that degradation of σ32 protein, concomitant with the induction of MsFtsH, might also have been due to MsFtsH protease itself, and not to some other classical stress-response-specific protease.

Thus, these experiments demonstrate that the MsFtsH protease, induced from the pT18–MsH construct, is capable of degrading heterologous substrates, which are cytoplasmic or membrane bound, in E. coli cells.

**Complementation of E. coli strain AR 423 (ΔftsH::kan/pAR171) with MsftsH**

E. coli AR 423 (ΔftsH::kan/pAR171; Akiyama et al., 1994; a kind gift from Dr Teru Ogura, Kumamoto University, Japan) was used for the complementation studies. The pAR171 plasmid, which has a ts ori, is defective for replication at 42 °C, and carries the essential EcftsH gene under its own promoter and a chloramphenicol resistance (CmR) marker. Complementation assays were carried out exactly as described by Nilsson et al. (1994), using several constructs, namely pBS–MsH, pGEX–4T1–MsH, pCYP2–MsH and pT18–MsH, all of which carried an ampicillin resistance (ApR) marker. E. coli AR 423 (ΔftsH::kan/pAR171) was transformed with the constructs, and the transformants were selected for CmR, KmR and ApR at 30 °C. These transformants were grown in LB at 42 °C for 6 h and then plated on LB agar at 30 °C. The colonies that were obtained were all KmR and ApR, indicating that they had retained the MsftsH-containing constructs and the mutation ΔftsH3::kan. All the colonies obtained were also CmR, showing that there was no loss of pAR171. However, unlike the case of complementation by Lactococcus lactis ftsH (LjftsH), CmR colonies were not obtained. Thus, none of these constructs were able to complement E. coli AR 423 (ΔftsH::kan/pAR171).

A possible reason for lack of complementation could be that none of the constructs could give an optimum level of MsFtsH comparable to that of the endogenous EcFtsH protein. However, this could not be ascertained since expression from most of these constructs was not detected, even with Western blotting. Another reason for the inability of MsFtsH to complement could be a probable deficiency in the protease activity of the recombinant protein, as it is

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**Fig. 6.** (a) In vivo degradation of EcSecY by EcFtsH protease. Upper panel, the expression of EcFtsH from pSTD113 in E. coli AR 5090/pKY248 cells. FtsH' indicates the C-terminally processed FtsH. Middle panel, the degradation of EcSecY by EcFtsH. Lower panel, the amount of RRF present in these samples. Lane 1: AR 5090/pKY248 cells carrying vector alone (pBluescript), induced with 2 mM IPTG. Lane 2: AR 5090/pKY248 cells carrying pSTD113 (EcftsH), induced with 2 mM IPTG. Levels of RRF do not vary. (b) In vivo degradation of EcSecY by MsFtsH protease. Upper panel, the expression of MsFtsH in E. coli AR 5090/pKY248 cells. Middle panel, the degradation of EcSecY by MsFtsH. Lower panel, the amount of RRF present in these samples. Lane 1: AR 5090/pKY248 cells carrying vector alone (pT18), induced with 2 mM IPTG. Lane 2: AR 5090/pKY248 cells carrying pT18–MsH (MsftsH), induced with 2 mM IPTG. Levels of RRF do not vary.
known that the proteolysis of LpxC by FtsH is essential for viability (Ogura et al., 1999). It has also been shown that a mutant EcFtsH that retains ATPase activity, but lacks protease activity, does not complement the growth defect of an FtsH-depleted strain (Jayasekera et al., 2000). Although the MsFtsH protein, expressed from pT18–MsH, showed protease activity in vivo against two known substrates of EcFtsH, namely Eσ32 and ESecY in *E. coli* cells, determination of proteolytic activity of MsFtsH against LpxC would ascertain the reason for the inability of MsftsH to complement the EcftsH mutant.

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FtsH protease of Mycobacterium smegmatis