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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 (Ms*ftsH*) 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 Ms*ftsH* locus was found to be identical to that of the *Mycobacterium tuberculosis ftsH* gene (Mt*ftsH*) and similar to that of other bacterial genera, but with divergence in the upstream region. The Ms*ftsH* gene is 2·3 kb in size and encodes the AAA (ATPases Associated with diverse cellular Activities) family Zn^{2+}-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-MtFtsH 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 Ms*ftsH* gene did not complement lethality of a Δ*ftsH*3::kan mutation in *E. coli*, but when expressed in *E. coli* cells, it efficiently degraded conventional FtsH substrates, namely σ^{32} protein and the protein translocase subunit SecY, of *E. coli* cells.

INTRODUCTION

FtsH is a membrane-bound ATP-dependent Zn^{2+}-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 Zn^{2+}-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 Zn^{2+}-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 phage 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 σ^{32} (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 σ^{W}-controlled genes

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Abbreviations: AAA, ATPases Associated with diverse cellular Activities; RRF, ribosome recycling factor.

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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 (Mt*ftsH*) 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 (Ms*ftsH*) 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 σ^{32} 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 OD_{550} 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 Ms*ftsH* gene. The Ms*ftsH* gene was cloned from two partial genomic DNA libraries of *M. smegmatis* SN2. One microgram of genomic DNA was digested with *Pst*I 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 Ms*ftsH* ORF-specific fragment in the *Pst*I digest of genomic DNA was a 364 bp PCR product, which was amplified from the *ftsH* ORF of *M. tuberculosis* H37Rv (Mt*ftsH*) present in the SCY6F7 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 Mt*ftsH* 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 *Pst*I 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 fragments were ligated to *Pst*I-digested, calf-intestinal-phosphatase-treated pBS(SK^+) vector. *E. coli* JM109 cells were electrotransformed with the ligation mixture to obtain a *Pst*I partial genomic DNA library, hereafter referred to as the *Pst*I 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 Mt*ftsH*-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 (Ms*ftsH*). Complete sequencing of the 3·2 kb insert showed that the fragment carried a partial Ms*ftsH* 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 *Pst*I library.

In order to obtain the 5′ portion of the Ms*ftsH* gene, a *Kpn*I partial genomic DNA library of *M. smegmatis* SN2 was constructed in pBS(SK^+) vector, in a manner identical to the construction of the *Pst*I library. A 548 bp *Cla*I–*Pst*I DNA fragment, which corresponded to the N-terminal transmembrane region of Mt*ftsH* gene, was used to probe the KpnI genomic DNA digest. The 548 bp fragment was obtained by *Pst*I digestion of the pBS–MtH vector (Anilkumar *et al.*, 1998). Southern hybridization of the *Kpn*I-digested, fractionated genomic DNA, with the 548 bp *Cla*I–*Pst*I 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 *Kpn*I 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 *Kpn*I fragment, was passaged through *E. coli* GM 2151 (dam^-, dcm^-), to make the *Cla*I site on the insert sensitive to cleavage by the enzyme. It was then digested with *Cla*I and *Pst*I to release a 0·576 kb fragment that represented the transmembrane region of the Ms*ftsH* gene. The *Pst*I library was digested using *Pst*I and *Xho*I enzymes to obtain the 2·3 kb DNA fragment carrying the remaining portion of the Ms*ftsH* gene, including the stop codon. The 0·576 kb *Cla*I–*Pst*I fragment and the 2·3 kb *Pst*I–*Xho*I insert were ligated to pBS(SK^+) vector, which was digested with *Cla*I and *Xho*I enzymes. The three-way ligation resulted in the cloning of a 2·876 kb DNA fragment that contained the complete ORF of the Ms*ftsH* gene as a translational fusion with the *lacZ′* gene at the N-terminus of the Ms*ftsH* gene. This construct, pBS–MsH, contained the complete Ms*ftsH* ORF of 2·31 kb.

Cloning of the Ms*ftsH* ORF for *in vivo* expression. The pBS–MsH construct was digested with *Xho*I, end-filled with T4 DNA polymerase, and then digested with *Cla*I to release the *ftsH* ORF as a *Cla*I–*Xho*I 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

Strain, plasmid or vector	Genotype	Source or reference
Strains		
E. coli JM103	[Δ(*lac–pro*) *endA sbcB15 hsdR thi rpsL supE*/F′ *lacI*^{q} *traD36 proAB*$^{+}$ *lacZ*Δ *M15*]	Messing *et al.* (1981)
E. coli JM 109	[Δ(*lac–pro*) *endA1 recA1 hsdR17 thi relA gyrA96 supE44*/F′ *lacI*^{q} *traD36 proAB*$^{+}$ *lacZ*Δ M15]	Yanisch-Perron *et al.* (1985)
E. coli GM 2151	*thr-1 araC14 leuB6*(*Am*) *fhuA31 lacY1 tsx-78 glnV44*(*AS*) *galK2*(Oc) *dcm-6 hisG1*(*Fs*) *rpsL136*(*strR*) *dam-13*::Tn*9 xylA5 mtl-1 thi-1*	M. G. Marinus, U. Mass. Med. Sch., USA
E. coli AR 423	Δ*ftsH3*::*kan* and Δ(*srl–recA*)*306*::Tn*10*/*pAR 171*; a derivative of SH392 (*met gal supE hsdR sfiC*)	T. Ogura (Akiyama *et al.*, 1994)
E. coli AR 5090	Δ*ftsH sfhC21 degP5087*/F′*lacI*^{q}; a derivative of JM103	T. Ogura (Akiyama & Ito, 2000, 2003)
E. coli C41	BL21(DE3) derivative that allows the expression of normally toxic proteins	J. E. Walker (Miroux & Walker, 1996)
M. smegmatis SN2	Wild-type isolate	Lab. collection
Plasmids/vectors		
pBS-SK+		Messing *et al.* (1981)
pKY248	Carries the Ec*secY* gene under the *lac* promoter (pACYC184 derivative)	Taura *et al.* (1993)
pSTD 113	Carries the Ec*ftsH* gene as a C-terminal myc-6×His fusion under the *lac* promoter	Y. Akiyama (Akiyama *et al.*, 1995)
pAR 171	Carries the complete Ec*ftsH* ORF including its upstream sequences *ftsH rep*^{ts} Cm^{R}	T. Ogura (Akiyama *et al.*, 1994)
pBS–*Pst*I 3·2	Carries the 3·2 kb *Pst*I fragment containing the 3′ region of the Ms*ftsH* gene and the downstream genes	This study
pBS–*Kpn*I 2·9	Carries the 2·9 *Kpn*I fragment containing the 5′ region of the Ms*ftsH* gene and the upstream genes	This study
pBS–MsH	Carries the complete Ms*ftsH* ORF under the *lac* promoter of pBS-SK^{+}	This study
pT18-zip		Karimova *et al.* (1998)
pT18	Lacks the leucine zipper region of pT18-zip	Karimova *et al.* (1998); this study
pT18–MsH	Carries the complete Ms*ftsH* ORF under the *lac* promoter of pT18	This study
pBHB1	Carries Ec*ftsH* under the arabinose promoter	Herman *et al.* (1997)
SCY6F7	Cosmid contig containing *ftsH* gene of *M. tuberculosis* H37Rv	Cole *et al.* (1998)
pBS–MtH	Contains full-length ORF of *M. tuberculosis* H37Rv	Anilkumar *et al.* (1998)
pCYB2–MsH	Contains full-length MsFtsH as C-terminal intein fusion under the *tac* promoter	This study
pGEX-4T1–MsH	Contains full-length MsFtsH as N-terminal GST translational fusion	This study
pRSET-A–MsH	Contains full-length MsFtsH as N-terminal 6×His tag under the T7 promoter	This study
pET20b–MsH	Contains full-length MsFtsH as C-terminal 6×His tag under the T7 promoter	This study

(Karimova *et al.*, 1998). We removed the region encoding the leucine zipper from the vector by *Kpn*I digestion and religation to generate the pT18 vector, which was further digested with *Cla*I and *Eco*RV. The *Cla*I–*Xho*I blunt-ended fragment containing the Ms*ftsH* ORF, which was obtained from the pBS–MsH vector, was ligated to the *Cla*I–*Eco*RV 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 OD_{600} 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 PMSF. *M. smegmatis* SN2 cells, grown in YK liquid medium to an OD_{550} of 0·50, were sonicated in PBS, pH 7·2, in the presence of 1 mM PMSF. After removing debris by centrifugation at 12 000 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 40 000 r.p.m. in a type 50Ti 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 conventional substrates of FtsH protease, namely σ^{32} (Ecσ^{32}) and SecY (EcSecY) proteins of *E. coli*. For the MsFtsH protease assay, changes in the levels of endogenous Ecσ^{32} 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 Ecσ^{32} and ectopically expressed EcSecY proteins by EcFtsH, which was induced from the pSTD113 vector, was monitored. *E. coli* AR 5090 (Δ*ftsH*::*kan*, *sfhC21*) cells were co-transformed with the plasmid vector pKY248, carrying the Ec*secY* 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 Ecσ^{32} 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 Ecσ^{32} 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, Ecσ^{32} 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 Yoshinori Akiyama, Institute for Virus Research, Kyoto University, Kyoto, Japan; Taura *et al.*, 1993), anti-σ^{32} (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 Ms*ftsH* gene locus

The nucleotide sequence of the Ms*ftsH* 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 *Pst*I and *Kpn*I partial libraries revealed the identities of the genes present in the regions downstream and upstream of Ms*ftsH* gene. The region downstream of Ms*ftsH* 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 Ms*ftsH*. Similar to the presence of the *fol* gene operon on the region downstream of Ms*ftsH*, 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 Ms*ftsH* 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* (*rrmJ*) 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 *ftsJ* 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 *ftsJ* gene in *M. smegmatis* SN2. However, from our studies, we can say that the *ftsJ* gene is not present

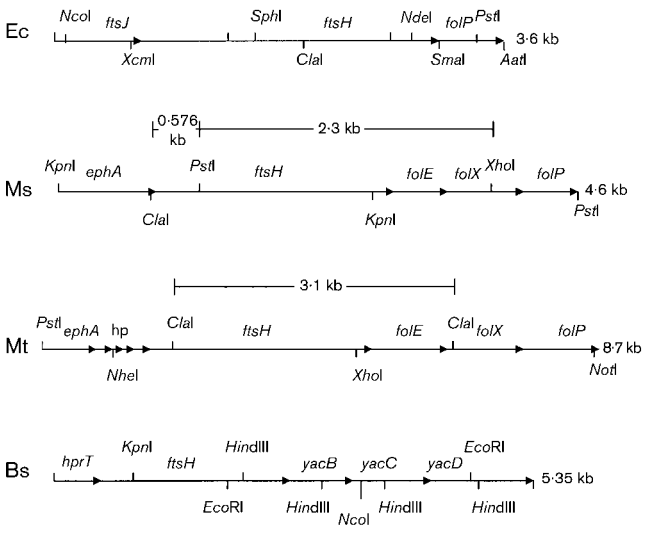

Fig. 1. Genomic organization of the *ftsH* locus of *M. smegmatis* and comparison with the *ftsH* loci of *M. tuberculosis* and *E. coli*. Arrowheads indicate the direction of the ORF. Ec, *E. coli*; Ms, *M. smegmatis*; Mt, *M. tuberculosis*; Bs, *B. subtilis*.

at least in the region immediately upstream of the *ftsH* gene in *M. smegmatis* SN2, although we cannot yet ascertain its absence from the genome. Another interesting example of divergence in the organization of the *ftsH* locus has been found in *Caulobacter crescentus* (Fischer *et al.*, 2002). In this bacterium, *yaeN* and *kinA* genes flank the *ftsH* gene on the upstream and downstream sides, respectively. In fact, *ftsH* and *folP* are interspersed by *kinA* in *C. crescentus*.

Amino acid sequence and domain organization features of MsFtsH

The ORF of the Ms*ftsH* gene is 2·31 kb in size and encodes a protein of approximately 85 kDa, having 769 amino acid residues. The Ms*ftsH* ORF shares a high degree of identity (82 % at the amino acid level) with that of Mt*ftsH*, 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 GPPGTGKT and IIFVDEID, an AAA family signature motif, namely GVILIAATNRPDILDPALLRPGR, and a Zn^{2+}-binding motif, HEGGH, unlike the HEAGH motif in EcFtsH, which is characteristic of 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 *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 DVEKRPRLTMFDDFGGRVPSDKPPIKTPGELAMERGEPWPPPVPEPAFKAAI, 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 *Mycobacterium avium* (MaFtsH) and *Mycobacterium leprae* (MlFtsH) also carry a glutamine- and proline-rich stretch of residues, interestingly, MtFtsH and the FtsH protein of *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 *E. coli* (P28691), *B. subtilis* (P37476), *Arabidopsis thaliana* (O80860), *Homo sapiens* (Q9Y2Q2) and *M. tuberculosis* (P96942), using the CLUSTAL W 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 *E. coli*, *B. subtilis*, *A. thaliana* and *H. sapiens*, indicating that the 2·31 kb ORF that we obtained indeed contained the Ms*ftsH* gene. Although the extent of sequence conservation between MsFtsH and FtsH molecules of the other organisms referred

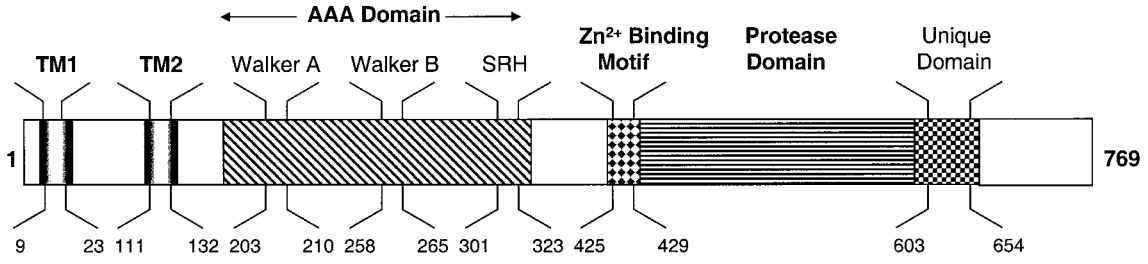

Fig. 2. Schematic representation of *M. smegmatis* FtsH protease. TM1 and TM2 represent the two transmembrane domains predicted by the Dense Alignment Surface method (DAS). SRH represents the second region of homology. The amino acid numbers encompassing the different domains are indicated.

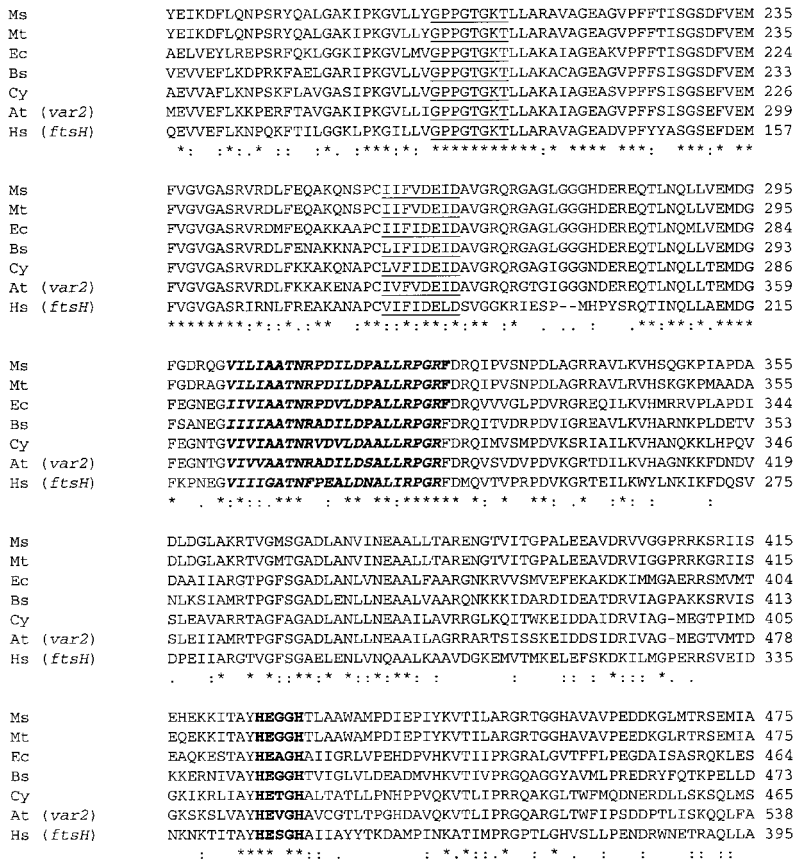

Fig. 3. Comparision of the conserved sequence of *M. smegmatis* FtsH with other FtsH homologues (bacterial and eukaryotic counterparts). Amino acid sequences were aligned using the program CLUSTAL W. Ms, *M. smegmatis* SN2 (O52395); Mt, *M. tuberculosis* H37Rv (P96942); Ec, *E. coli* (P28691); Bs, *B. subtilis* (P37476); Cy, *Cyanidioschyzon merolae* (Q9TJ83); At, *Arabidopsis thaliana* (O80860); Hs, *Homo sapiens* (Q9Y2Q2). ATP-binding motifs are underlined. AAA signature motifs are shown in italics and bold. The Zn^{2+}-binding motif is shown in bold. Asterisks represent identical amino acids; colons represent conserved amino acids. The SwissProt or TrEMBL accession numbers are given in parentheses.

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*ftsH* ORF shares 82 % identity at the amino acid level with that of Mt*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 *M. bovis*, *M. leprae* and *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 *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 *M. smegmatis* SN2 cell lysates. Western blot analysis of the membrane fraction, prepared from *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 Ms*ftsH* gene was cloned into several prokaryotic expression vectors to obtain pBS–MsH, pGEX-4T1–MsH, pRSET-A–MsH, pCYB2–MsH and pET20b–MsH. *E. coli* C41 (Miroux & Walker, 1996) was used for transformation with pRSET-A–MsH and pET20b–MsH, while *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 Ms*ftsH* was not be expressed from any of the constructs, namely pBS–MsH, pGEX-4T1–MsH, pRSET-A–MsH, pCYB2–MsH and pET20b–MsH, we had no choice but to use pT18–MsH for the expression of Ms*ftsH in vivo* to demonstrate proteolytic activity. Even using this vector, the protein was not detected by Coomassie blue staining, but it was detected as an 85 kDa band in Western blotting (Fig. 4b). The possible reason for the expression of Ms*ftsH* could be that the C-terminal translational fusion of the T18 fragment of the *cya* gene might have contributed to the stability of the protein, although the *cya* fragment is cleaved off *in vivo*, as inferred from the molecular mass of the recombinant protein observed upon immunoblotting with anti-EcFtsH and anti-MtFtsH antibodies.

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

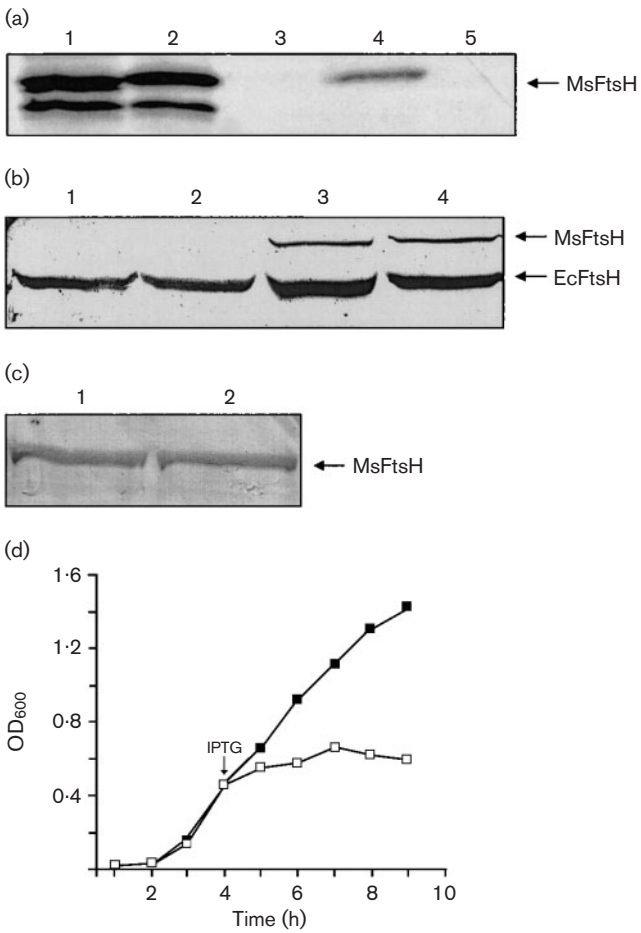

Fig. 4. (a) Membrane localization of MsFtsH protein in *M. smegmatis* SN2. Membrane fractions were prepared from *M. smegmatis* cells as described in Methods. Membrane proteins were resolved by 12% SDS-PAGE. FtsH was detected with anti-EcFtsH antibodies. Lanes: 1, whole-cell lysate from *M. smegmatis*; 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 blotting was 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-MtFtsH antibodies. (d) Growth arrest of *E. coli* JM109 cells upon induction of Ms*ftsH*. □, Cells carrying the Ms*ftsH* gene induced with 1 mM IPTG; ■, cells carrying the pT18 plasmid alone. The arrow indicates the time at which IPTG was added.

case of MtFtsH (Anilkumar *et al.*, 1998). The expression of Ms*ftsH* 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).

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.*, 1995). 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 (Δ*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

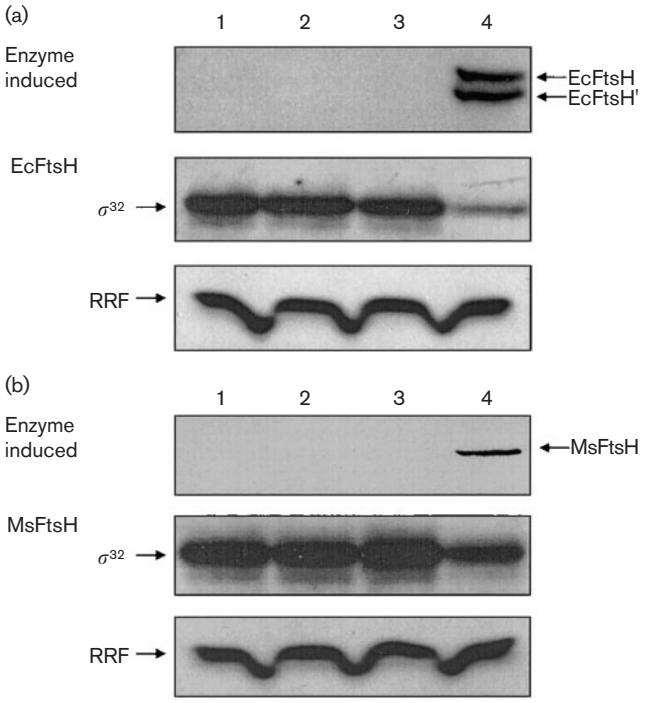

Fig. 5. (a) *In vivo* degradation of Ecσ^{32} by EcFtsH protease. Upper panel, the expression of Ec*ftsH* from pSTD113 in *E. coli* AR 5090 cells (Δ*ftsH*::*kan sfhC21*). FtsH′ indicates the C-terminally processed FtsH. Middle panel, the degradation of Ecσ^{32} by EcFtsH. Lower panel, the amount of RRF 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 (Ec*ftsH*), uninduced and induced with 2 mM IPTG respectively. Lane 4 shows the degradation of Ecσ^{32} by EcFtsH, while levels of RRF remain unchanged. (b) *In vivo* degradation of Ecσ^{32} by MsFtsH protease. Upper panel, the expression of Ms*ftsH* in *E. coli* AR 5090 cells (Δ*ftsH*::*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 Ecσ^{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 (Ms*ftsH*), uninduced and induced with 2 mM IPTG respectively. Lane 4 shows the degradation of Ecσ^{32} by MsFtsH, while the levels of RRF remain the same.

pSTD113 (Ec*ftsH* 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^{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^{32} could be either the differences in the expression levels of the two proteases or the possibility that σ^{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^{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^{32} protein is a substrate (Goff & Goldberg, 1985; Ito *et al.*, 1986; Kanemori *et al.*, 1997). In this case, degradation of σ^{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^{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^{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^{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 Ec*FtsH*-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

unassembled SecY (Kihara *et al.*, 1995). Therefore, the protease activity of FtsH on the membrane-bound substrates can be assessed by its ability to degrade overexpressed and uncomplexed SecY.

Exponentially growing cultures of *E. coli* AR 5090 (Ec*ftsH* 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 FtsH 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 σ^{32} 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.

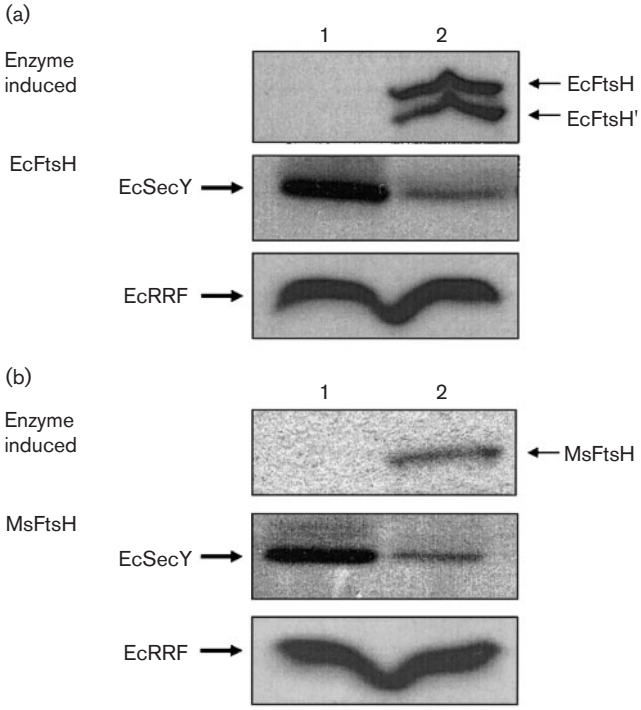

Fig. 6. (a) *In vivo* degradation of EcSecY by EcFtsH protease. Upper panel, the expression of Ec*ftsH* from pSTD113 in *E. coli* AR 5090/pKY248 cells. FtsH′ indicates the C-terminally processed FtsH. Middle panel, the degradation of EcSecY by EcFtsH. Lower panel, the amount of RRF present in these samples. Lane 1: AR 5090/pKY248 cells carrying vector alone (pBluescript), induced with 2 mM IPTG. Lane 2: AR 5090/pKY248 cells carrying pSTD113 (Ec*ftsH*), 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 Ms*ftsH* 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 (Ms*ftsH*), induced with 2 mM IPTG. Levels of RRF do not vary.

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 (Δ*ftsH*::*kan*/pAR171) with Ms*ftsH*

E. coli AR 423 (Δ*ftsH*::*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 esssential Ec*ftsH* gene under its own promoter and a chloramphenicol resistance (Cm^R) marker. Complementation assays were carried out exactly as described by Nilsson *et al.* (1994), using several constructs, namely pBS–MsH, pGEX-4T1–MsH, pCYB2–MsH and pT18–MsH, all of which carried an ampicillin resistance (Ap^R) marker. *E. coli* AR 423 (Δ*ftsH*::*kan*/pAR171) was transformed with the constructs, and the transformants were selected for Cm^R, Km^R and Ap^R 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 Km^R and Ap^R, indicating that they had retained the Ms*ftsH*-containing constructs and the mutation Δ*ftsH3*::*kan*. All the colonies obtained were also Cm^R, showing that there was no loss of pAR171. However, unlike the case of complementation by *Lactococcus lactis ftsH* (Ll*ftsH*), Cm^S colonies were not obtained. Thus, none of these constructs were able to complement *E. coli* AR 423 (Δ*ftsH*::*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

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 Ecσ^{32} and EcSecY in *E. coli* cells, determination of proteolytic activity of MsFtsH against LpxC would ascertain the reason for the inability of Ms*ftsH* to complement the Ec*ftsH* mutant.

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