ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/7356522

Molecular characterisation of ABC transporter type FtsE and FtsX proteins of Mycobacterium tuberculosis

Article *in* Archives of Microbiology · April 2006

Impact Factor: 1.67 · DOI: 10.1007/s00203-005-0079-z · Source: PubMed

CITATIONS
12

READS
21

4 authors, including:

Mushtaq Mir
King Khalid University
14 PUBLICATIONS **78** CITATIONS
SEE PROFILE

Usha Veeraraghavan
University of Birmingham
25 PUBLICATIONS **343** CITATIONS
SEE PROFILE

All in-text references underlined in blue are linked to publications on ResearchGate, letting you access and read them immediately.

Available from: Mushtaq Mir
Retrieved on: 07 June 2016

Arch Microbiol (2006) 185: 147–158
DOI 10.1007/s00203-005-0079-z

ORIGINAL PAPER

Mushtaq Ahmad Mir · Haryadi S. Rajeswari
Usha Veeraraghavan · Parthasarathi Ajitkumar

Molecular characterisation of ABC transporter type FtsE and FtsX proteins of *Mycobacterium tuberculosis*

Received: 25 August 2005 / Revised: 9 November 2005 / Accepted: 14 December 2005 / Published online: 14 January 2006
© Springer-Verlag 2006

Abstract Elicitation of drug resistance and various survival strategies inside host macrophages have been the hallmarks of *Mycobacterium tuberculosis* as a successful pathogen. ATP Binding Cassette (ABC) transporter type proteins are known to be involved in the efflux of drugs in bacterial and mammalian systems. FtsE, an ABC transporter type protein, in association with the integral membrane protein FtsX, is involved in the assembly of potassium ion transport proteins and probably of cell division proteins as well, both of which being relevant to tubercle bacillus. In this study, we cloned *ftsE* gene of *M. tuberculosis*, overexpressed and purified. The recombinant MtFtsE-6xHis protein and the native MtFtsE protein were found localized on the membrane of *E. coli* and *M. tuberculosis* cells, respectively. MtFtsE-6xHis protein showed ATP binding in vitro, for which the K42 residue in the Walker A motif was found essential. While MtFtsE-6xHis protein could partially complement growth defect of *E. coli ftsE* temperature-sensitive strain MFT1181, co-expression of MtFtsE and MtFtsX efficiently complemented the growth defect, indicating that the MtFtsE and MtFtsX proteins might be performing an associated function. MtFtsE and MtFtsX-6xHis proteins were found to exist as a complex on the membrane of *E. coli* cells co-expressing the two proteins.

Keywords FtsE · FtsX · ABC transporter type protein · Walker A motif · ATP binding · *Mycobacterium tuberculosis* · cell division

M. A. Mir · H. S. Rajeswari · U. Veeraraghavan
P. Ajitkumar (✉)
Department of Microbiology and Cell Biology,
Indian Institute of Science, 560012 Bangalore, India
E-mail: ajit@mcbl.iisc.ernet.in
Tel.: +91-80-2293-2344
Fax: +91-80-2360-2697

Present address: U. Veeraraghavan
Department of Biochemistry, University of Birmingham,
Birmingham, UK

Abbreviations DTSP: 3, 3′-dithio-bis(propionic acid *N*-hydroxysuccinimide ester) · ABC: ATP binding Cassette · DTT: Dithiothreitol · IPTG: Isopropylthio-*β*-D-galactopyranoside · CNBr: Cyanogen bromide · SRP: Signal Recognition Particle

Introduction

The ATP Binding Cassette (ABC) transporter type proteins (Hyde et al. 1990) in bacterial and mammalian systems are involved in the transport or translocation of diverse types of molecules namely drugs (Chen et al. 1986; Goldman and Capobianco 1990), proteins (Levy et al. 1991), ions (Riordan et al. 1989), solutes such as maltose (Davidson and Nikaido 1991; Walter et al. 1992) and polysaccharide (Reizer et al. 1992; Evans and Downie 1986) across membrane. The characteristic features of ABC transporter type proteins are the presence of two nucleotide binding (NB) domains and two transmembrane (TM) domains (Higgins 1992). The NB domain(s) and the TM domain(s) can be in the same protein or in two different proteins of a complex. The ABC transporter type proteins possess Walker A and Walker B motifs, which are involved in ATP binding and hydrolysis, and are highly conserved among these proteins (Walker et al. 1982).

Structural model proposed for ATP binding proteins associated with ion transport in cystic fibrosis, drug efflux in multidrug resistance and solute transport in bacterial systems suggest that loops 2 and 3 of the ABC transporter proteins may be responsible for coupling ATP-dependent conformational change to facilitate transport process, presumably through interaction with the hydrophobic membrane domains of the transport system (Hyde et al. 1990; Mimura et al. 1991; Panagiotidis et al. 1993). Mutations in the loops 2 and 3 of CFTR protein of human cystic fibrosis patients (Riordan et al. 1989; Cutting et al. 1990) and the functionally lethal mutations in HisP protein subunit of histidine permease (Shyamala

et al. 1991) reveal the functional importance of these domains in transport process. All the structural characteristics of ABC type transporter proteins are found conserved in *Escherichia coli* FtsE of FtsEX protein complex, where FtsE is the hydrophilic ATP-binding protein that remains associated with the inner membrane (Gill and Salmond 1987) through its interaction with the integral membrane protein FtsX (de Leeuw et al. 1999). Conditional lethal missense mutations in FtsE protein of *E. coli* were found to be similar to those found in CFTR of human patients (Gibbs et al. 1992) and one of the mutations was found to affect translocation of K^+ ion pump proteins KdpA, Kup and TrkH into inner membrane (Ukai et al. 1998). Recently FtsE and FtsX have been found to localize to septal ring in *E. coli* (Schmidt et al. 2004), with the localization requiring the cell division proteins FtsZ, FtsA, and ZipA but not FtsK, FtsQ, FtsL, and FtsI proteins (Schmidt et al. 2004), suggestive of a role for FtsEX in cell division. Thus, since FtsE of the FtsEX complex shares sequence conservation with ABC type transporter proteins, the complex could be involved in the transport or translocation processes involving drugs, ions, solutes, proteins, peptides or polysaccharides in relation to drug resistance, salt tolerance, cell division or membrane protein insertion.

Mycobacterium tuberculosis, the principal causative agent of tuberculosis (TB) in humans, is considered to be a successful pathogen owing to the elicitation of multidrug resistance, ability to survive inside macrophage phagosomes by taking nutrients from host cell cytoplasm (Ratledge 1984), and the capacity to alternate between proliferating and dormant (non-proliferating) conditions of growth (Lefford 1984; Lagrange 1984; Wayne and Hayes 1996; Lim et al. 1999). Thus, whether one looks at tubercle bacillus from the standpoint of regulation of cell division in the host system, or uptake of nutrients from the host cell cytoplasm or elicitation of drug resistance, the requirement for ABC transporter type protein complexes such as FtsEX could be of critical importance to the pathogen and therefore of clinical relevance. In fact, *M. tuberculosis* does possess *ftsE* and *ftsX* genes (Tyagi et al. 1996; Cole et al. 1998) and in this study, we analysed ATP binding characteristics of recombinant FtsE protein of *M. tuberculosis* (MtFtsE) and cellular localization of recombinant MtFtsE and MtFtsX proteins and of native MtFtsE protein in *E. coli* and *M. tuberculosis*, respectively. We further examined the possible interaction between recombinant MtFtsE and MtFtsX proteins on the membrane of *E. coli* cells and the biological relevance of the interaction in the complementation of an *E. coli ftsE* temperature-sensitive mutant.

Materials and methods

Strains, plasmids and growth conditions

E. coli strains and plasmid vectors used in this study are listed in Table 1. JM109 strain was used for the propagation of plasmid vectors. *E. coli* C41 strain (Miroux and Walker 1996) was used for expressing recombinant proteins. JM109 and C41 cells were grown in LB medium at 37°C. *E. coli ftsE* (Ts) mutant strain MFT1181 (Gibbs et al. 1992; Ukai et al. 1998) was used for complementation studies. *M. tuberculosis* H37Ra cells were always grown in Middlebrook 7H9 medium containing 0.2% Tween 80, supplemented with 0.5% BSA, 0.75% D-glucose and 0.085% sodium chloride at 37°C.

Chromosomal DNA isolation

M. tuberculosis H37Ra cells were grown to an OD_{550} of 0.8. Glycine was added to a final concentration of 0.2 M and incubation was continued for 2 h more. The cells were suspended in lysis buffer (50 mM glucose in 25 mM Tris-HCl, pH 8.0) and incubated with lysozyme (5 mg/ml) and Tween 80 (0.2%) for 6 h more in a bacteriological shaker. The cells were lysed by incubation at 50°C for 10 min in the presence of 1% SDS. Genomic DNA was phenol extracted, dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA), incubated in the presence of DNase-free RNase for 12 h at 37°C, extracted with phenol, washed with 70% ethanol and precipitated.

Cloning of MtftsE and MtftsE-X combined ORF of M. tuberculosis

The details of cloning are given in Table 1. Mt*ftsE* gene was PCR amplified from *M. tuberculosis* H37Ra genomic DNA, using Pfu DNA polymerase (MBI Fermentas), and MtftsE1 and MtftsE2 primers (Table 2). The PCR product was cloned in pBS (KS) to obtain pBS-Mt*ftsE*(OF). Restriction enzyme digestion and DNA sequencing confirmed Mt*ftsE* ORF in pBS-Mt*ftsE*(OF) clone. It was then subcloned under T7 promoter in pET-20b(+) expression vector to obtain pET20b-Mt*ftsE*, which provides 6xHis tag at the C-terminus. In order to express Mt*ftsE* from pBS-Mt*ftsE*(OF), the ORF was made in-frame with the ATG of *N*-terminus LacZ' to get the expression clone of pBS-Mt*ftsE*(IF). In order to clone Mt*ftsE-X* combined ORF, the *ftsE-X* ORF was amplified by PCR from genomic DNA of *M. tuberculosis* H37Ra using Pfu DNA polymerase (MBI Fermentas) and MtftsE1 and MtftsX2 primers (Table 2). The PCR product was cloned in pBS (KS) to obtain pBS-Mt*ftsEX*(OF). The Mt*ftsEX* ORF in pBS-Mt*ftsEX*(OF) was sequenced to rule out any mutation and was subsequently subcloned under T7 promoter in pET-20b(+) expression vector to obtain pET20b-Mt*ftsEX*. The protein MtFtsX expressing from this clone would have 6xHis tag at its *C*-terminus while MtFtsE would be without the tag. In order to co-express MtFtsE and MtFtsX proteins from pBS-Mt*ftsEX*(OF), the *ftsEX* ORF in pBS-Mt*ftsEX*(OF) was made in-frame with ATG of *N*-terminal *lacZ*' to obtain pBS-Mt*ftsEX*(IF). In

Table 1 Bacterial strains and plasmid vectors

Name	Genotype/Phenotype	Source or references
E. coli strains		
JM109	[*recA1 supE44 endA1 hsdR17 gyrA96 relA thiΔ*(*lac-proAB*), F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ Δ*M15)]	Yanisch-Perron et al. (1985)
C41	derivative of *BL21* (*λDE3*) [*hsdS gal* (*λcIts857 ind1 sam7 nin5 lacUV5-T7 gene1*)]	Miroux and Walker (1996)
MFT1181	*E. coli ftsE* (Ts)	Gibbs et al. (1992)
JA200	F$^+$, *ΔtrpE5, recA, thr-1, leu-6, lacY, thi, gal, xyl, ara, mtl*	A. Nishimura
Plasmid vectors		
pSTR-30-SD	Mobile plasmid, Amp^r	A. Nishimura
pBS (KS)	Cloning vector, Amp^r	Messing et al. (1981)
pQE30	Expression vector, Amp^r	Qiagen
pET-20b (+)	Expression vector with T7 promoter, Amp^r	Novagen
pBS-Mt*ftsE*(OF)	Mt*ftsE* ORF out-of-frame with *lacZ′*. Mt*fsE* PCR product cloned at BamHI/XbaI	This study
pBS-Mt*ftsE*(IF)	Mt*ftsE* ORF in-frame with *lacZ′*. pBS-Mt*ftsE*(OF) cut with BamHI, end-filled and religated	This study
pBS-Mt*ftsEX*(OF)	Mt*ftsEX* ORF out-of-frame with *lacZ′*. Mt*ftsEX* PCR product cloned at BamHI/XbaI	This study
pBS-Mt*ftsEX*(IF)	Mt*ftsEX* ORF in-frame with *lacZ′*. pBS-Mt*ftsE*(OF) cut with BamHI, end-filled and religated	This study
pET20b-Mt*ftsE*	Mt*ftsE* ORF in pET20b(+). Mt*ftsE* ORF from pBS-Mt*ftsE*(OF) subcloned at EcoRV/NotI in pET20b	This study
pET20b-Mt*ftsEX*	Mt*ftsEX* ORF in pET20b(+). Mt*ftsEX* ORF from pBS-Mt*ftsEX*(OF) subcloned at EcoRV/NotI in pET20b	This study
pET20b-Mt*ftsE*(K42R)	Mt*ftsE*(K42R) mutant ORF in pET20b(+)	This study
pQE30-Mt*ftsE*	Mt*ftsE* ORF in pQE30. MtftsE ORF from pBS-MtftsE(OF) subcloned at BamHI/SacI in pQE30	This study

order to express MtFtsE with *N*-terminus 6xHis tag, Mt*ftsE* ORF was cloned in pQE30 expression vector (see Table 1).

Site-directed mutagenesis of Mt*ftsE* gene

The K42 residue in Walker A motif of FtsE protein was mutated to R42 using protocol of QuickChange site-directed mutagenesis system (Stratagene) using Pfu DNA polymerase and the overlapping primers MtK42R1 and MtK42R2 (Table 2). The pET20b-Mt*ftsE* template DNA was denatured at 94°C for 10 min. The primers were annealed to the template DNA at 64°C for 2 min, followed by extension for 10 min at 72°C and denaturation at 94°C for 2 min. Final extension was given for 10 min at 72°C. The template DNA was removed from the PCR product by DpnI digestion for 3 h and the enzyme was precipitated with ammonium acetate at 2.5 M final concentration. The DNA, after ethanol precipitation, was used for transforming *E. coli* JM109 by calcium chloride method. The pET20b-Mt*ftsE*(K42R) clone (Mt*ftsE* gene having K42R mutation) was identified using restriction digestion and DNA sequencing.

Expression and purification of MtFtsE-6xHis protein

E. coli C41 cells were transformed with the pET20b-Mt*ftsE* recombinant clone by $CaCl_2$ method. At OD_{600} of 0.6, MtFtsE-6xHis expression was induced with 1 mM IPTG for 3 h. Cells were suspended in the lysis buffer (10 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 8 M urea), and incubated on ice for 30 min. Whole cell lysate, after removal of cell debris, was passed five times through Ni^{2+}-NTA agarose column pre-equilibrated with the lysis buffer. The column was washed with 20 bed volumes of wash buffer A (10 mM Tris/HCl, pH 8.0, containing 100 mM NaCl, 8 M urea and 20 mM imidazole) and subsequently with ten bed volumes of wash buffer B (10 mM Tris-HCl, pH 6.3,

Table 2 Oligonucleotide primers

Primers	Oligonucleotide sequence	Purpose
MtftsE1	5′gcgggatccgatatcatgatcaccctggaccatgtcaccaagcagtacaaatcg 3′	For cloning *ftsE* gene
MtftsE2	5′gcggaattctagagcgatccatcccgtagacgccacgctgttcgtc 3	For cloning *ftsE* gene
MtftsX2	5′ gcggaattctagatcgccgacgtagaggcgcagcgttaagtaagccgt 3	For cloning *ftsX* gene
MtK42R1	5′ ccgtcgggttcgggccgctcaaccttcatgcgg 3′	For K42R mutation
MtK42R2	5′ccgcatgaaggttgagcggcccgaacccgacgg 3′	For K42R mutation

Underlined sequence ggatcc and tctaga are the BamHI and XbaI sites used for cloning in pBS (KS). Underlined sequence in K42R1 and K42R2 is the mutated codon for amino acid arginine instead of lysine

containing100 mM NaCl and 8 M urea). Protein was eluted using wash buffer B containing 500 mM imidazole and refolded by dialysing against 10 mM Hepes-KOH, pH 8.0, containing 10% glycerol at 4°C. PMSF was added to a final concentration of 1 mM and the purified protein was stored at −70°C. The MtFtsE(K42R) mutant protein was also expressed, purified and refolded under identical conditions. The anti-MtFtsE antibodies, raised against purified MtFtsE-6xHis in rabbit, were affinity purified using CNBr-activated sepharose-conjugated MtFtsE (Harlow and Lane 1988), and precleared with *E. coli* whole cell lysate.

Preparation of membrane and cytosol fractions

M. tuberculosis H37Ra cells were grown to an OD_{600} of 0.8. The cells were resuspended in 1X PBS, containing protease inhibitor PMSF (1 mM) and lysed by sonication. The supernatant was subjected to ultracentrifugation at 32,000 rpm for 2 h. The pellet (membrane fraction), which was solubilized in 1X PBS containing 1% NP-40, and the supernatant (cytosol) fractions were stored at −70°C until further use. Similarly, the whole cell lysate of *E. coli* C41 cells, wherein MtFtsE-6xHis or MtFtsX-6xHis or both were expressed, were also partitioned into membrane and cytosol fractions. For the protein–protein interaction study, membrane solubilisation was carried out in 50 mM Hepes-KOH, pH 7.4, containing 50 mM KCl and 1 mM PMSF.

ATP binding assay

Filter binding assay was used to study ATP binding of MtFtsE-6xHis protein, wherein 50 µl reaction mixture of MtFtsE-6xHis protein (0.8 µM) was used in the presence of varying concentrations of [α^{32}P] ATP (specific activity 3000 Ci/mmole, 10 μCi/µl) for 1 h at 25°C in the binding buffer (50 mM Hepes-KOH, pH 7.7, containing 50 mM KCl and 6 mM DTT). For competition experiments, 70-fold molar excess of unlabelled individual NTPs were added to the reaction mixture. The entire reaction volume was spotted on 0.22 µm diameter cellulose nitrate filters pre-wetted with the binding buffer. Unbound ATP was removed by rapid filtration with five volumes of 300 µl of binding buffer. The filters were air-dried and exposed to X-ray film for 2 min. Quantitation was carried out using image gauge V2.54.

Western blotting

Proteins in the membrane and cytosol fractions were fractionated using 12% SDS-PAGE. After fractionation, the proteins were transferred to PVDF membrane and the membrane was blocked for 3 h with 1X PBS buffer containing 0.2% Tween 20 and 5% skimmed milk. The PVDF membrane was incubated for 3 h with anti-MtFtsE antibodies or anti-polyhistidine antibodies at a dilution of 1:4,000 or 1:6,000, respectively. The membrane was washed thrice with 1X PBS containing 0.2% Tween 20, incubated with protein A-HRP for 3 h, washed, and developed using ECL western blotting detection kit (Amersham Biosciences).

Ni^{2+}-NTA agarose pulldown assay

The membrane fractions from *E. coli* C41 cells transformed with pET20b-Mt*ftsE* or pET20b-Mt*ftsEX* were incubated with 1 mM DTSP (3,3′-dithio-bis(propionic acid N-hydroxysuccinimide ester) in 50 mM Hepes/KOH and 50 mM KCl for 1 h on ice. The reaction was stopped with one volume of 0.1 M Tris-HCl, pH 8.0. Urea was added to a final concentration of 8 M and incubated with Ni^{2+}-NTA agarose beads at room temperature for 30 min. The beads were washed with five bed volumes of 50 mM Hepes-KOH, pH 7.4, containing 50 mM NaCl and 8 M urea. The beads were boiled in 1X SDS-PAGE loading buffer and fractionated on 12% SDS-PAGE. MtFtsE and MtFtsX protein bands were identified by western blotting with anti-MtFtsE or anti-polyhistidine antibodies, respectively.

Results

Complementation of *E. coli ftsE* (Ts) strain MFT1181 with Mt*ftsE* gene

The primary structure of MtFtsE protein is 50% identical and 62% similar to *E. coli* FtsE protein (EcFtsE) sequence (NCBI BLASTP) and is highly homologous to FtsE proteins of other bacterial genera (Fig. 1). Like in the case of EcFtsE, MtFtsE protein does not possess transmembrane domain as revealed by "DAS" transmembrane prediction server. The predicted amino acid sequence of MtFtsE contains Walker A motif consisting of a glycine-rich sequence followed by a conserved stretch of lysine, serine and threonine residues (GPSGSGKST). Multiple sequence alignment of primary structure of FtsE from different bacterial species showed that Walker A motif of MtFtsE is conserved among them (Fig. 1). Owing to the sequence homology with EcFtsE, we wanted to verify whether MtFtsE protein could complement the function of *E. coli* FtsE protein in the MFT1181 (Ts) strain at non-permissive temperature. The MFT1181 (Ts) strain harbouring pBS-Mt*ftsE*(IF) plasmid could grow on LB agar at non-permissive temperature (Fig. 2a), but the cells were filamentous to an extent of 1.5-fold in length (Fig. 2b). However, when MFT1181 (Ts) strain was transformed with pBS-Mt*ftsEX*(IF) construct, which co-expresses MtFtsE and MtFtsX, it could efficiently complement growth defect of the Ts strain at non-permissive temperature by almost abolishing the filamentous phenotype. These observations indicate that although MtFtsE by itself could partially substitute for the loss of EcFtsE

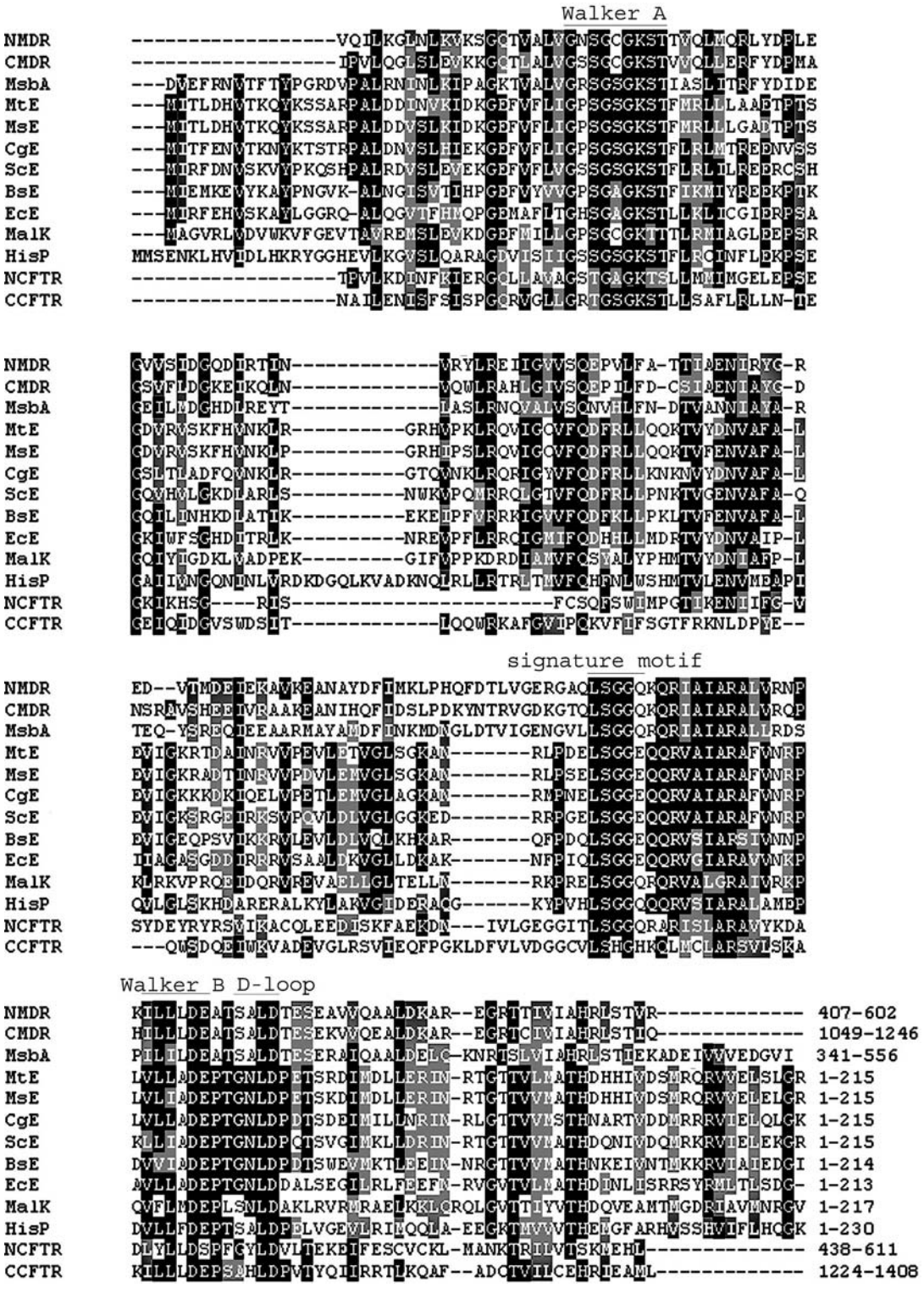

Fig. 1 Sequence alignment of FtsE protein from various bacterial genera in comparison with other ABC transporters. Multiple alignments were carried out using clustalW (http://www.ebi.ac.uk/clustalw). *NMDR and CMDR N*- and *C*-terminal domains of mouse *MDR1, MsbA C*-terminal domain of *E. coli* lipid A flippase, *MtE* FtsE of *M. tuberculosis*, *MsE* FtsE of *M. smegmatis*, *CgE* FtsE of *C. glutamicum*, *ScE* FtsE of *S. coelicolor*, *BsE* FtsE of *B. subtilis*, *EcE* FtsE of *E. coli*, *MalK N*-terminal domain of ATP binding subunit of *T. litoralis* maltose transporter, *HisP* the ATP binding subunit of histidine transporter of *S. typhimurium*, *NCFTR and CCFTR N*- and *C*-terminal domains of human CFTR. Amino acids marked with *black* or *gray boxes* indicate sequence identity or similarity, respectively. The *dashes* indicate the gaps introduced to maximize sequence alignment

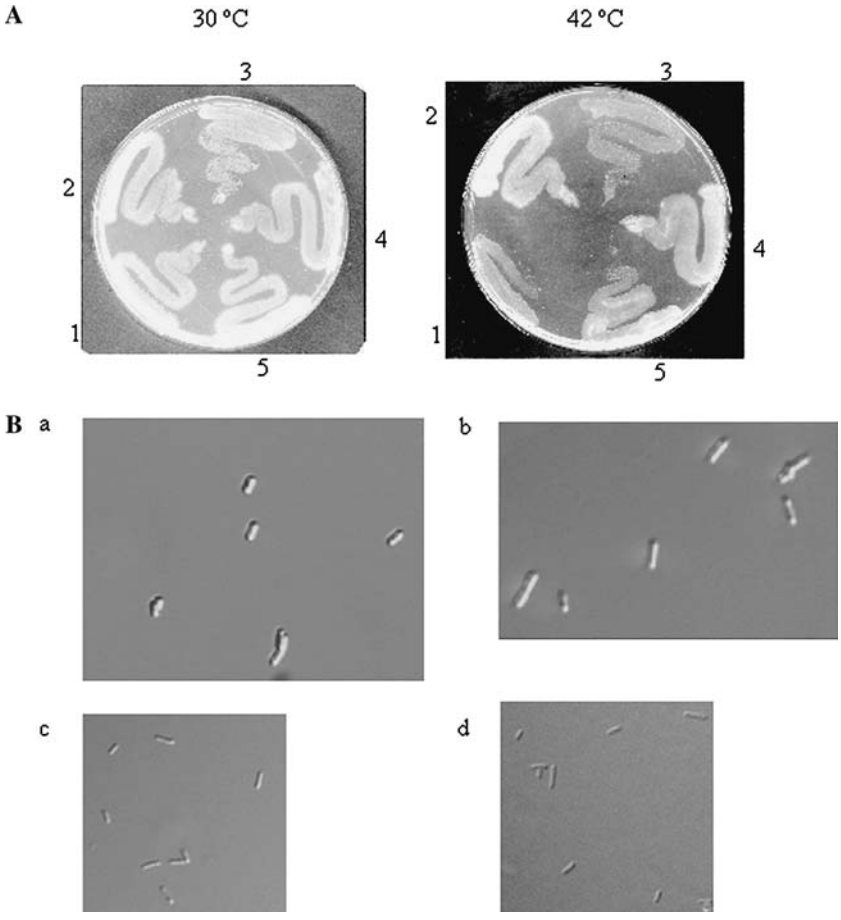

Fig. 2 Complementation of growth defective phenotype of *E. coli ftsE* (Ts) MFT1181 strain by Mt*ftsE* and Mt*ftsX*. **a** MFT1181 cells transformed with: pBS II KS (1), *E. coli* FtsE-expressing plasmid pSTR-30-SD (2), pBS-Mt*ftsEX*(IF) (4) and pBS-Mt*ftsE*(IF) (5); and wild-type JA200 cells transformed with pBS (KS) (3) were streaked identically on two LB agar plates containing ampicillin. One plate was incubated at 30°C and other was incubated on 42°C. **b** Morphology of *E. coli* MFT1181 cells complemented with pSTR-30-SD (a), pBS-Mt*ftsE*(IF) (b), pBS-Mt*ftsEX*(IF) (c) and of wild-type strain JA200 transformed with pBS II KS (d). Cells were photographed at 25,000 magnification, brightness and contrast have been adjusted using Adobe Photoshop 6.0

function to some extent, MtFtsE and MtFtsX, when co-expressed, could much more efficiently substitute for the loss of function of EcFtsE.

Generation of MtFtsE(K42R) mutant gene

The lysine residue in Walker A (GXXGXGKS/T) motif has been found conserved among ABC transporter type proteins (Fry et al. 1986). X-ray crystallographic and NMR data on these proteins have shown that the conserved lysine residue is in contact with the β- and γ-phosphates of the bound ATP (Pai et al. 1977; Hung et al. 1998; Diederichs et al. 2000). Further, site-directed mutagenesis experiments have shown that the lysine residue is critical for ATP binding (Reinstein et al. 1990). Similar to that in all the ABC transporter type proteins, the conserved lysine at position 41 in *E. coli* FtsE, has been demonstrated to be essential for ATP binding (de Leeuw et al. 1999). MtFtsE also harbours a lysine residue at position 42 in the ATP binding motif, also called the P-loop or Walker A motif. We wanted to verify whether the K42 residue in MtFtsE is critical for ATP binding. For this purpose, we created a site-directed mutation wherein the K42 residue was changed to arginine (K42R).

Overexpression and purification of MtFtsE-6xHis and MtFtsE(K42R)-6xHis mutant proteins

For the biochemical characterization of MtFtsE protein, Mt*ftsE* gene was expressed from pET20b-Mt*ftsE* construct by induction with 1 mM IPTG for 3 h at 37°C in *E. coli* C41 cells. The overexpressed recombinant MtFtsE-6xHis protein migrated as a prominent 32 kDa species on SDS-PAGE, which is 7 kDa above its calculated molecular weight of 25 kDa, being the contribution of pelB leader sequence from pET-20b (+) vector backbone and 6xHis tag (Fig. 3a, lane 2). The MtFtsE(K42R)-6xHis mutant protein was overexpressed under identical conditions and it migrated as a 32 kDa species (Fig. 3b, lane 2). Subsequently, recombinant MtFtsE-6xHis and MtFtsE(K42R)-6xHis proteins were purified to homogeneity under denaturing conditions by lysing C41 cells in 8 M urea, followed by affinity chromatography using Ni^{2+}-NTA agarose resin and refolding (Lane 3 of Fig. 3a and b, respectively). The respective total cell lysate containing 8 M urea, prior to affinity purification, and also purified MtFtsE-6xHis and MtFtsE(K42R)-6xHis proteins revealed small proportion of a 60 kDa protein species in the absence of 2–mercaptoethanol (Lane 4 of Fig. 3a and b, respectively). The 32 kDa single species MtFtsE-6xHis protein,

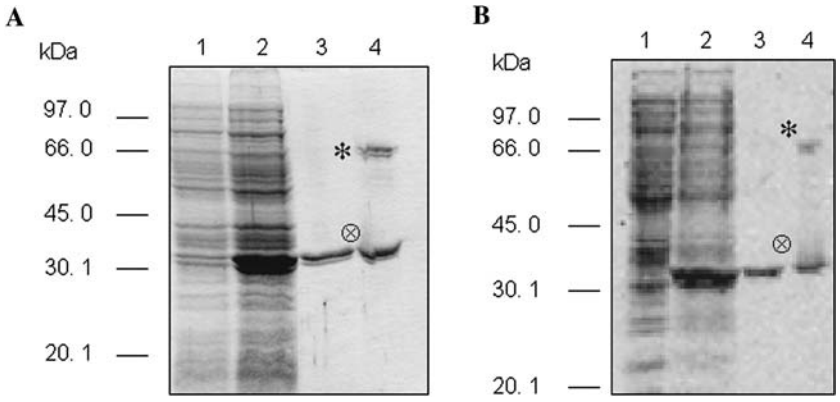

Fig. 3 Profile of overexpressed and purified MtFtsE and MtFtsE(K42R) mutant proteins. **a** Coomassie Blue-stained SDS-PAGE profile of lysate from *E. coli* C41 cells harbouring pET20b-M*tftsE* and of purified MtFtsE protein. Lysates from uninduced (*lane 1*) and induced (*lane 2*) C41 cells; affinity purified MtFtsE-6xHis protein: loaded in the presence of β-mercaptoethanol indicating only the monomeric form of MtFtsE-6xHis (*lane 3*); loaded in the absence of β-mercaptoethanol indicating the monomeric and dimeric forms of MtFtsE-6xHis (*lane 4*). **b** Coomassie Blue-stained SDS-PAGE profile of lysate from *E. coli* C41 cells harbouring pET20b-M*ftsE*(K42R) and of purified MtFtsE(K42R)-6xHis protein. Samples of MtFtsE(K42R)-6xHis protein, which are identical to those of MtFtsE-6xHis were loaded in the respective lanes as in **a**. *Hatched circle* indicates monomer and *asterisk* indicates dimer

which was purified under denaturing conditions and refolded by dialysis, was used to generate anti-MtFtsE antibody in rabbit. Expression of MtFtsX protein was toxic to the host cells and the level of expression of recombinant MtFtsX was very low.

MtFtsE-6xHis protein binds ATP in vitro

Incubation of MtFtsE with increasing concentrations of [α^{32}P] ATP for a definite period showed that at saturating concentrations of ATP, 19 micromoles of ATP was found to bind per mole of MtFtsE-6xHis protein (Fig. 4a). Maximum ATP binding was found to occur at 1.3 nM concentration of ATP. At 1.3 nM concentration of [α^{32}P] ATP, 70 molar excess of ATP, ADP, AMP, and GTP competed out respectively 97%, 87%, 73% and 57% of the [α^{32}P] ATP bound to MtFtsE-6xHis (Fig. 4b). Filter-binding assay with MtFtsE(K42R)-6xHis mutant protein under identical conditions showed that the K42R mutation in the Walker A motif abolished ATP binding (Fig. 4a). It indicated that the lysine residue at position 42 in the Walker A motif is critical for ATP binding. Use of oxidized [α^{32}P] ATP for ATP binding studies (Clertant and Cuzin 1982) did not yield conclusive results as the MtFtsE protein having 11 lysine

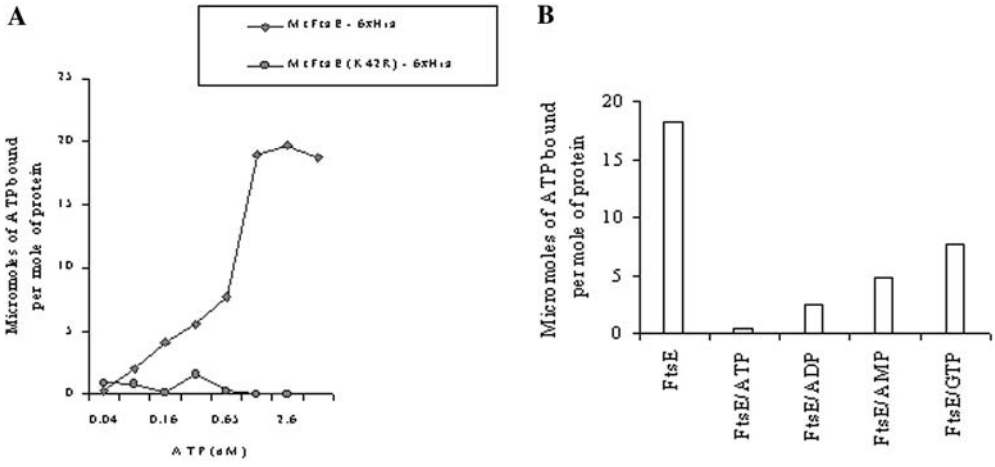

Fig. 4 ATP binding and specificity of binding of MtFtsE-6xHis and MtFtsE(K42R)-6xHis proteins using filter binding method. **a** Purified MtFtsE-6xHis or MtFtsE(K42R)-6xHis protein was incubated with varying concentrations of [α^{32}P] ATP. The whole reaction volume was subjected to rapid filtration through 0.22 micron cellulose nitrate filter. The arbitrary values obtained by using image gauge V2.54 program were converted into number of micromoles of ATP bound per mole of protein from the arbitrary value of known amount of ATP (in nanomolar concentration) used. The number of micromoles of ATP bound per mole of protein was plotted against the nanomolar concentration of [α^{32}P] ATP used in the respective reaction. **b** Purified MtFtsE-6xHis or MtFtsE (K42R)-6xHis protein was incubated with 1.3 nM [α^{32}P] ATP in the presence of 70-fold molar excess of ATP, ADP, AMP, or GTP. The whole reaction volume was subjected to rapid filtration through 0.22 micron cellulose nitrate filter. The number of micromoles of [α^{32}P] ATP bound per mole of protein in the presence of the respective unlabelled nucleotide was plotted

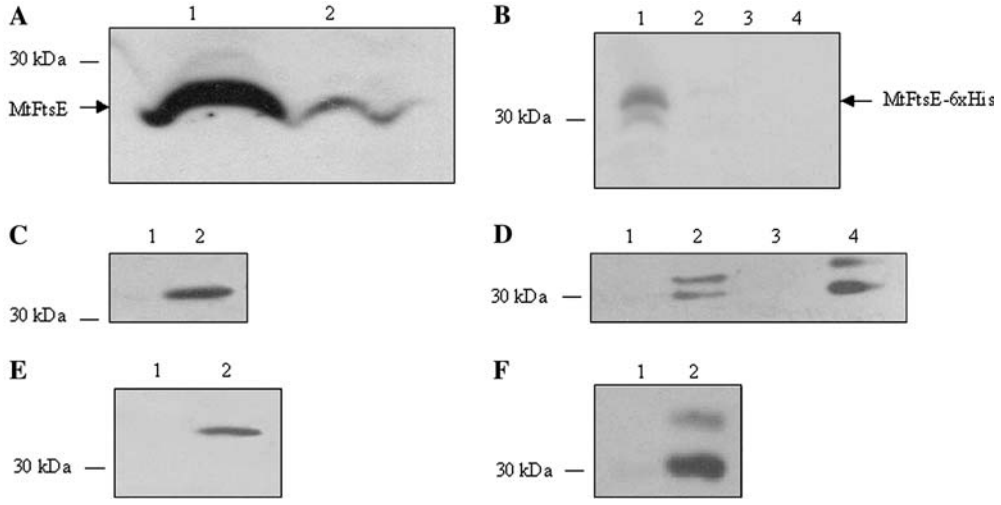

Fig. 5 (**a**, **b**) Western blot analysis of native MtFtsE protein in *M. tuberculosis* cells and of recombinant MtFtsE-6xHis protein in *E. coli* cells. (**a**) Native MtFtsE protein in the membrane (*lane 1*) and cytosol (*lane 2*) fractions of *M. tuberculosis* H37Ra cells, detected using anti-MtFtsE-6xHis antibody. (**b**) Recombinant MtFtsE-6xHis protein in the membrane (*lane 1*) and cytosol (*lane 2*) fractions of *E. coli* C41 cells induced for the overexpression of Mt*ftsE*-6xHis gene from pET20b-Mt*ftsE* vector, detected with anti-polyhistidine antibody; *lanes 3* and *4* contain the fractionated membrane and cytosol proteins of induced *E. coli* C41 cells harbouring pET-20b(+) vector control. (**c** – **f**) Existence of MtFtsE and MtFtsX as a complex in the membrane. (**c**, **d**) Western blot of membrane fraction from *E. coli* C41 cells co-expressing MtFtsE and MtFtsX proteins: MtFtsX-6xHis from pET20b-Mt*ftsEX* (**c**, *lane 2*) and pET20b(+) (**c**, *lane 1*) (probed with anti-polyhistidine antibodies), MtFtsE from pBS-Mt*ftsEX* (IF) (**d**, *lane 2*) and pET20b-Mt*ftsEX* (**d**, *lane 4*) (both probed using anti-MtFtsE antibodies). *Lanes 1* and *3* contain membrane fractions from cells harbouring pBS(KS) (**d**, *lane 1*) and pET20b(+) (**d**, *lane 3*). (**e, f**) Western blot of DTSP-cross-linked and Ni^{2+}- NTA agarose pulled down proteins from the membrane fractions of *E. coli* C41 cells co-expressing MtFtsE and MtFtsX proteins: from pET20b(+) (**e**, *lane 1*) and pET20b-Mt*ftsEX* (**e**, *lane 2*, binding of MtFtsX-6xHis to the beads) (both probed using anti-polyhistidine antibodies), pBS-Mt*ftsEX*(IF) (**f**, *lane 1*, lack of direct binding of MtFtsE to the beads per se) and pET20b-Mt*ftsEX* (**f**, *lane 2*, binding of MtFtsE to the beads through MtFtsX-6xHis) (both probed using anti-MtFtsE-6xHis antibodies)

residues and the MtFtsE (K42R) mutant with ten lysine residues did not show significant difference in the respective competition experiments and also in the individual reactivity between the two proteins in the extent of ATP binding (data not shown). Our attempts to UV cross-link [α^{32}P] ATP to purified MtFtsE protein, according to published protocols (Berrios et al. 1983; Guhan and Muniyappa 2003), were also unsuccessful, like in the case of UV cross-linking of ATP to FtsH (Karata et al. 1999).

Subcellular localization of native and recombinant MtFtsE proteins

In order to determine subcellular localisation of native MtFtsE in *M. tuberculosis* H37Ra cells and recombinant MtFtsE-6xHis in *E. coli* C41 cells, the membrane and cytoplasmic fractions of *M. tuberculosis* H37Ra cells and of *E. coli* C41 cells harbouring MtFtsE-6xHis (induced from pET20b-Mt*ftsE*) were analysed by immunoblotting with affinity-purified anti-MtFtsE-6xHis antibodies and anti-polyhistidine antibodies, respectively. Native MtFtsE protein, with the expected molecular size of 25 kDa, and recombinant MtFtsE-6xHis protein of 32 kDa size were found in the membrane fraction (lane 1 of Fig. 5a and b, respectively).

MtFtsE and MtFtsX exist as a complex in the membrane

Partial complementation of the growth-defective phenotype of *E. coli ftsE* (Ts) cells with recombinant MtFtsE alone, but more efficient complementation of the defective phenotype of the cells with the co-expression of MtFtsE and MtFtsX proteins implied that the two proteins might be interacting with each other and functionally participating inside *E. coli* cells. In order to examine whether the MtFtsE and MtFtsX proteins of *M. tuberculosis* interact on the membrane, the proteins were co-expressed in *E. coli* C41 cells from pET20b-Mt*ftsEX* construct, where MtFtsX protein would have 6xHis tag at its *C*-terminus but MtFtsE would be without the tag. The expression of MtFtsX-6xHis (Fig. 5c, lane 2) and of MtFtsE (Fig. 5d, lane 4) in the membrane fraction was confirmed using anti-polyhistidine antibody and anti-MtFtsE antibody, respectively. The membrane fraction was then treated with DTSP in order to crosslink MtFtsE with MtFtsX-6xHis, if the two proteins were interacting, and MtFtsX-6xHis was pulled down with Ni^{2+}-NTA agarose. The Ni^{2+}-NTA agarose beads were washed several times with the buffer (50 mM Hepes-KOH, pH 7.4, containing 50 mM NaCl and 8 M urea), and boiled. Immunoblotting of the supernatant, obtained by boiling the beads in 1X

SDS-PAGE loading buffer containing β-mercaptoethanol in order to reverse the crosslink and to release the proteins into the supernatant, independently with anti-polyhistidine (for MtFtsX-6xHis) and anti-MtFtsE antibodies detected MtFtsX-6xHis (Fig. 5e, lane 2) and MtFtsE (Fig. 5f, lane 2). Western blotting of even 1.5-fold more membrane protein from *E. coli* C41 transformants containing the vector controls pBS (KS) or pET20b(+) under identical conditions of induction did not detect either MtFtsE (Fig. 5d, lanes 1 and 3, respectively) or MtFtsX (Fig. 5e, lane 1). These control experiments confirmed that the FtsE protein, which was detected in the sample where MtFtsE and MtFtsX-6xHis were co-expressed, was not the endogenous EcFtsE protein. These experiments showed that MtFtsE and MtFtsX, when co-expressed from pET20b-Mt*ftsEX*, existed probably as a complex in the membrane since they could be crosslinked.

However, it is possible that the presence of MtFtsE in the Ni^{2+}-NTA agarose pull-down experiments could have been due to the non-specific interaction of the protein with agarose beads and not due to crosslinking of MtFtsE with MtFtsX-6xHis. However, Ni^{2+}-NTA agarose pull-down and DTSP crosslinking experiments, and immunoblotting with anti-MtFtsE antibody, under identical conditions, using membrane fraction from *E. coli* C41 transformants containing pBS-Mt*ftsEX*(IF), wherein both MtFtsX and MtFtsE proteins were co-expressed, did not detect MtFtsE (Fig. 5f, lane 1). MtFtsE protein could not be detected in an identical experiment even with 1.5-fold more of the membrane protein, although MtFtsE was expressed from pBS-Mt*ftsEX*(IF) and present in the membrane fraction (Fig. 5d, lane 2). This control experiment showed that MtFtsE did not interact with Ni^{2+}-NTA agarose and that the MtFtsE protein detected in the Ni^{2+}-NTA agarose pulldown of the membrane fractions from C41 cells containing co-expressed MtFtsE and MtFtsX-6xHis proteins was indeed the MtFtsE that interacted with the MtFtsX-6xHis. Thus, these results showed that the MtFtsE and MtFtsX-6xHis proteins, which were co-expressed from pET20b-Mt*ftsEX* in *E. coli* C41 cells, indeed existed in the membrane as a complex.

Discussion

The present study was aimed at the biochemical characterization of FtsE and FtsX proteins of *M. tuberculosis* with respect to complementation of EcFtsE (Ts) phenotype, ATP binding, subcellular localisation and mutual interaction. As native MtFtsE and MtFtsX exist at very low levels in the cell, purification of the respective native proteins for biochemical characterization was not possible. Therefore, respective recombinant proteins were used for biochemical characterization. We could not get the overexpressed protein in soluble fraction for purification even when the cells were grown at lower temperature of 18 and 25°C and/or induced with lower concentration of IPTG (100 μM) or for shorter durations. The fusion of 6xHis tag at the *N*-terminus of MtFtsE protein, by cloning into pQE30 expression vector, as compared with the *C*-terminus 6xHis tag when expressed from pET20b vector, also could not get the overexpressed protein into soluble fraction for purification. Therefore, we had no choice but to purify MtFtsE-6xHis protein under denaturing conditions followed by refolding using dialysis in the presence or absence of DTT. Refolding in the absence of DTT resulted in the formation of the dimeric form of MtFtsE-6xHis, probably through disulphide linkage involving the sole cysteine residue, whereas the presence of DTT during refolding prevented formation of the dimeric form of MtFtsE-6xHis protein. Overexpression and purification of MtFtsE(K42R)-6xHis mutant protein was also carried out under identical conditions. Neither the mobility nor the stability of MtFtsE-6xHis protein was affected by the point mutation. Contrary to the expression of MtFtsE-6xHis and MtFtsE(K42R)-6xHis proteins, expression of MtFtsX protein was toxic even to *E. coli* C41 cells, which tolerate expression of toxic proteins (Miroux and Walker 1996). Accordingly, the level of expression of recombinant MtFtsX was very low and could be detected only with western blotting using anti-polyhistidine antibodies when expressed from the pET20b-Mt*ftsEX* construct in *E. coli* C41 cells.

Based on the high conservation of Walker A motif among FtsE protein sequences from different bacterial genera, FtsE of *M. tuberculosis* is predicted to be an ATP binding molecule. Binding of ATP to MtFtsE-6xHis protein confirmed the functionality of the Walker A motif present in MtFtsE protein and conferred the characteristics of an ABC-type protein on the molecule. We suspect that the low level of binding of ATP by MtFtsE-6xHis (19 micromoles of ATP per mole of the protein) might be due to the denatured-refolded status of the protein and not due to any contamination since the K42R MtFtsE mutant, which was purified under identical conditions, did not show ATP binding. Competition by GTP for the Walker A motif is consistent with the conservation of Walker A motif in GTPase proteins (Saraste et al. 1990). Loss of binding of ATP by the MtFtsE(K42R)-6xHis mutant protein indicates that K42 in the Walker A motif is critical for ATP binding. Also, specificity of nucleotide binding as revealed by competition experiment and site-directed mutagenesis of Walker A motif shows that the MtFtsE-6xHis protein, which was overexpressed and purified under denaturing conditions, had refolded to yield a biochemically active molecule.

Detection of MtFtsE in the membrane fraction of *M. tuberculosis* by immunoblotting with anti-FtsE antibodies indicated that the native MtFtsE is a membrane-associated protein, like in the case of *E. coli* FtsE (Gill and Salmond 1987). In the case of the recombinant MtFtsE-6xHis as well, we found that it remained associated with the membrane in *E. coli* cells. Since "DAS" transmembrane prediction server predicted lack of

transmembrane domain in MtFtsE, the recombinant MtFtsE-6xHis protein might probably be remain associated with the *E. coli* inner membrane on the cytoplasmic side. The membrane localization of the native and of the recombinant MtFtsE-6xHis indicated that the protein might be having a function similar to that of FtsE protein of *E. coli*. This premise prompted us to look for complementation of *E. coli ftsE* (Ts) strain MFT1181 with MtFtsE at non-permissive temperature. Our study showed that Mt*ftsE* gene could partially complement the growth defect due to the loss of function of FtsE in *E. coli ftsE* (Ts) MFT1181 strain at non-permissive temperature. MFT1181 cells expressing MtFtsE were mildly filamentous at non-permissive temperature, indicating that the complementation with MtFtsE per se was not efficient. However, co-expression of MtFtsE and MtFtsX complemented the growth defect much more efficiently than by MtFtsE alone, as could be inferred from the abolition of filamentous phenotype of MFT1181 to a significant extent. It has been reported that FtsE and FtsX might function as a complex in *E. coli*, as indicated by the complementation of the growth defect of *E. coli ftsE* null mutant RG60 by the co-expression of Ec*ftsE* and Ec*ftsX* genes (Schmidt et al. 2004). Therefore the lack of efficient complementation by MtFtsE per se might be indicative of the possibility that MtFtsE might not be functionally compatible with EcFtsX as its partner. The efficient complementation of Ec*ftsE* (Ts) phenotype with MtFtsE-MtFtsX complex, when co-expressed from pBS-Mt*ftsEX* (IF), underscores the possibility that this complex is functionally more competent to substitute for the loss of function of EcFtsE. It implies that MtFtsE and MtFtsX might be functioning as a single functional complex inside the cells, like in the case of the complex formed by EcFtsE and EcFtsX (de Leeuw et al. 1999). In *M. tuberculosis*, *ftsE* and *ftsX* are placed next to one another without sequence gap, although *ftsX* has been found to have an independent promoter of its own located inside *ftsE* ORF (Tyagi et al. 1996). Lack of complementation reported for *E. coli ftsX* (Ts) strain JS10 by Mt*ftsX* gene alone (Tyagi et al. 1996), also probably could be due to the absence of co-expression of Mt*ftsE* and MtFtsX. Thus, it is quite likely that Mt*ftsE* and Mt*ftsX* would be co-transcribed and translated, and hence might be forming a functional complex. Pulldown assay of MtFtsE by MtFtsX-6xHis using Ni^{2+}-NTA agarose, indicating interaction of the two proteins on the membrane, is also suggestive of this possibility.

If MtFtsE is the ATP binding component of an ABC transporter type complex, then there should be an integral membrane protein with which MtFtsE could interact and form a complex. In that respect as well, MtFtsX, being predicted to be a membrane protein (DAS — transmembrane prediction server), might be the candidate protein that would interact with MtFtsE on the membrane to constitute an ABC transporter type protein complex. Localisation of FtsE and FtsX to the septal ring in *E. coli* has been found to require the cell division proteins FtsZ, FtsA and ZipA but not FtsK, FtsQ, FtsL, and FtsI proteins (Schmidt et al. 2004). This observation is suggestive of a direct or indirect role for FtsEX complex in cell division, probably as the transporter of other mycobacterial cell division proteins downstream of the hierarchy of recruitment of FtsZ, FtsA and ZipA. The protein FtsQ, which is not required for FtsZ ring formation (Addinall et al. 1996), is cross-linked to the SRP subunit Ffh (Valent et al. 1997) and localized to mid-cell site downstream of FtsE/FtsX (Schmidt et al. 2004). It is likely that FtsEX complex might be involved in the targeting of FtsQ into the divisome on the membrane. Further, MtFtsE shares significant homology with lipid flippase MsbA from *E. coli*, which transports lipid A — a major component of the bacterial outer cell membrane (see Fig. 1). Therefore, it is also possible that MtFtsE in association with MtFtsX might be acting as a lipid flippase at the mid-cell site to properly flip the lipids in the outer leaflet of the membrane during septum formation. It would not be surprising if MtFtsEX complex would have a role in the transport of specific ion pump proteins under conditions of ion requirement for the pathogen inside macrophages, like in the case of the involvement of EcFtsE for the translocation of K+-ion pump proteins KdpA, Kup and TrkH into inner membrane (Ukai et al. 1998). Thus from any standpoint, in terms of requirement for transport ions or cell division proteins or drugs, the FtsE-FtsX complex would be of critical importance to tubercle bacillus.

Acknowledgments The authors are immensely thankful to Dr. A. Nishimura, National Institute of Genetics, Japan, for providing us *E. coli* MFT1181 *ftsE* (Ts) mutant, pSTR-30–SD vector and the wild-type strain *E. coli* JA200 for the complementation experiments. We thank Ramanujam Srinivasan for critical comments on the work reported here. The work was supported in part by the Genomics Initiative on Microbial Pathogens — Structural Genomics Initiative, funded by the Department of Biotechnology, Government of India, in the Division of Biological Sciences, at Indian Institute of Science. Authors acknowledge the infrastructure and equipment facilities in the Department of Microbiology and Cell Biology supported by the FIST grant from Department of Science and Technology, Government of India, Departmental Special Assistance Programme from University Grants Commission, Government of India, and ICMR Centre for Advanced Study in Molecular Medical Microbiology, at the Department of Microbiology and Cell Biology, Indian Institute of Science. Use of DBT-supported phosphorimager facility is acknowledged. MAM is recipient of the scholarship from Indian Institute of Science and UV acknowledges financial assistance from the Structural Genomics Programme.

References

Addinall SG, Bi E, Lutkenhaus J (1996) FtsZ ring formation in fts mutants. J Bacteriol 178:3877–3884

Berrios M, Blobel G, Fisher PA (1983) Characterization of an ATPase/dATPase activity associated with the Drosophila nuclear matrix-pore complex-lamina fraction. Identification of the putative enzyme polypeptide by direct ultraviolet photoaffinity labeling. J Biol Chem 258:4548–4555

Chen C-J, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, Robinson IB (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell 47:381–389

Clertant P, Cuzin F (1982) Covalent affinity labeling by periodate-oxidized [(-^{32}P] ATP of the large-T proteins of polyoma and SV40 viruses. J Biol Chem 257:6300–6305

Cutting GR, Kasch LM, Rosenstein BJ, Zielenski J, Tsui LC, Antonarakis SE, Kazazian HH Jr (1990) A cluster of cystic fibrosis mutations in the first nucleotide-binding fold of the cystic fibrosis conductance regulator protein. Nature 346:366–369

Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE 3rd, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393:537–344

Davidson AL, Nikaido H (1991) Purification and characterization of the membrane-associated components of the maltose transport system from *Escherichia coli*. J Biol Chem 266:8946–8951

de Leeuw E, Graham B, Phillips GJ, Ten Hagen-Jongman CM, Oudega B, Luirink J (1999) Molecular characterization of Escherichia coli FtsE and FtsX. Mol Microbiol 31:983–993

Diederichs K, Diez J, Greller G, Muller C, Breed J, Schnell C, Vonrhein C, Boos W, Welte W (2000) Crystal structure of MalK, the ATPase subunit of the trehalose/maltose ABC transporter of the archaeon Thermococcus litoralis. EMBO J 19:5951–5961

Evans IJ, Downie JA (1986) The nodI gene product of *Rhizobium leguminosarum* is closely related to ATP-binding bacterial transport proteins; nucleotide sequence analysis of the nodI and nodJ genes. Gene 43:95–101

Fry DC, Kuby SA, Mildvan AS (1986) ATP-binding site of adenylate kinase: mechanistic implications of its homology with ras-encoded p21, F1-ATPase, and other nucleotide-binding proteins. Proc Natl Acad Sci USA 83:907–911

Guhan N, Muniyappa K (2003) *Mycobacterium tuberculosis* RecA intein, a LAGLIDADG homing endonuclease, displays Mn2+ and DNA-dependent ATPase activity. Nucleic Acids Res 31:4184–4191

Gill DR, Salmond GP (1987) The *Escherichia coli* cell division proteins FtsY, FtsE and FtsX are inner membrane-associated. Mol Gen Genet 210:504–508

Gibbs TW, Gill DR, Salmond GP (1992) Localised mutagenesis of the fts YEX operon: conditionally lethal missense substitutions in the FtsE cell division protein of *Escherichia coli* are similar to those found in the cystic fibrosis transmembrane conductance regulator protein (CFTR) of human patients. Mol Gen Genet 234:121–128

Goldman RC, Capobianco JO (1990) Role of an energy-dependent efflux pump in plasmid pNE24-mediated resistance to 14- and 15-membered macrolides in Staphylococcus epidermidis. Antimicrob Agents Chemother 34:1973–1980

Higgins CF (1992) ABC transporters from microorganisms to man. Ann Rev Cell Biol 8:67–113

Harlow E, Lane D (1988) In Antibodies — A Laboratory Manual. Cold Spring Harbour Laboratory, New York, pp313–315

Hyde SC, Emsley P, Hatshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE, Higgins CF (1990) Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature 346:362–365

Hung LW, Wang IX, Nikaido K, Liu PQ, Ames GF, Kim SH (1998) Crystal structure of the ATP-binding subunit of an ABC transporter. Nature 396:703–707

Karata K, Inagawa T, Wilkinson AJ, Tatsuta T, Ogura T (1999) Dissecting the role of a conserved motif (the second region of homology) in the AAA family of ATPases. Site-directed mutagenesis of the ATP-dependent protease FtsH. J Biol Chem 274:26225–26232

Lagrange PH (1984) Cell-mediated immunity and delayed-type hypersensitivity. In: Kubica GP, Wayne LG (eds) The mycobacteria: a sourcebook Part B. Marcel Dekker Inc., New York, pp681–720

Lefford MJ (1984) Disease in mice and rats. In: Kubica GP, Wayne LG (eds) The mycobacteria: a sourcebook, Part B. Marcel Dekker Inc., New York, pp947–977

Levy F, Gabathuler R, Larsson R, Kvist S (1991) ATP is required for in vitro assembly of MHC class I antigens but not for transfer of peptides across the ER membrane. Cell 67:265–274

Lim A, Eleuterio M, Hutter B, Murugasu-Oei B, Dick T (1999) Oxygen depletion-induced dormancy in Mycobacterium bovis BCG. J Bacteriol 181:2252–2256

Miroux D, Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol 260:289–298

Messing J, Crea R, Seeburg PH (1981) A system for shotgun DNA sequencing. Nucleic Acids Res 9:309–321

Mimura CS, Holbrook SR., Ames GF (1991) Structural model of the nucleotide binding conserved component of periplasmic permeases. Proc Natl Acad Sci USA 88:84–88

Nishino K, Yamaguchi A (2001) Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. J Bacteriol 183:5803–5812

Pai EF, Sachsenheimer W, Schirmer RH, Schulz GE (1977) Substrate positions and induced-fit in crystalline adenylate kinase. J Mol Biol 114:37–45

Panagiotidis CH, Reyes M, Sievertsen A, Boos W, Shuman HA (1993) Characterization of the structural requirements for assembly and nucleotide binding of an ATP-binding cassette transporter. The maltose transport system of *Escherichia coli*. J Biol Chem 268:23685–23696

Ratledge C (1984) Metabolism of iron and other metals by mycobacteria. In: Kubica GP, Wayne LG (eds) The mycobacteria: a sourcebook, Part A. Marcel Dekker Inc., New York, pp603–627

Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, et al (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245:1066–1073

Reinstein J, Schlichting I, Wittinghofer A (1990) Structurally and catalytically important residues in the phosphate binding loop of adenylate kinase of *Escherichia coli*. Biochemistry 29:7451–7459

Reizer J, Reizer A, Saier MH Jr (1992) A new subfamily of bacterial ABC-type transport systems catalyzing export of drugs and carbohydrates. Protein Sci 1:1326–1332

Saraste M, Sibbald PR, Wittinghofer A (1990) The P-loop—a common motif in ATP- and GTP-binding proteins. Trends Biochem Sci 15:430–434

Shyamala V, Baichwal V, Beall E, Ames GF (1991) Structure-function analysis of the histidine permease and comparison with cystic fibrosis mutations. J Biol Chem 266:18714–18719

Schmidt KL, Peterson ND, Kustusch RJ, Wissel MC, Graham B, Phillips GJ, Weiss DS (2004) A predicted ABC transporter, FtsEX, is needed for cell division in Escherichia coli. J Bacteriol 186:785–793

Tyagi JS, Das TK, Kinger AK (1996) An *M. tuberculosis* DNA fragment contains genes encoding cell division proteins ftsX and ftsE, a basic protein and homologues of PemK and small protein B. Gene 177:59–67

Ukai H, Matsuzawa H, Ito K, Yamada M, Nishimura A (1998) *ftsE*(Ts) affects translocation of K+-pump proteins into the cytoplasmic membrane of *Escherichia coli*. J Bacteriol 180:3663–3670

Valent QA, de Gier JW, von Heijne G, Kendall DA, ten Hagen-Jongman CM, Oudega B, Luirink J (1997) Nascent membrane and presecretory proteins synthesized in *Escherichia coli* associate with signal recognition particle and trigger factor. Mol Microbiol 25:53–64

Walter C, Honer-zu-Bentrup K, Schneider E (1992) Large scale purification, nucleotide binding properties, and ATPase activity of the MalK subunit of *Salmonella typhimurium* maltose transport complex. J Biol Chem 267:8863–8869

Wayne LG, Hayes LG. (1996) An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. Infect Immun 64:2062–2069

Walker JE, Saraste M, Runswick MJ, Gay NJ (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J 1:945–951

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13 mp18 and pUC19 vectors. Gene 33:103–119