Identification of an ABC transporter gene that exhibits mRNA level overexpression in fluoroquinolone-resistant *Mycobacterium smegmatis*

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Abstract **We describe here the PCR amplification of a DNA fragment (*mtp1*) from *Mycobacterium smegmatis* using primers derived from consensus sequences of the ABC family of transporters. The fragment encodes amino acid sequences that exhibited significant homology with different ABC transporters. Amino acid sequence alignment of the full length gene with other transporters identified the ABC protein as the B-subunit of the phosphate specific transporter. Strikingly, a *M. smegmatis* colony which exhibited a high level of ciprofloxacin resistance showed mRNA level overexpression of *mtp1*. Thus this is the first report in any prokaryote indicating differential expression of an ABC transporter in a fluoroquinolone resistant colony.**

Key words: ABC transporter; Active efflux; Phosphate specific transport; Drug resistance; Fluoroquinolone; Mycobacterium

1. Introduction

Selective and regulated transport of specific molecules across the cell membrane is well known in both prokaryotes and eukaryotes. This transmembrane transport is mediated through different transporters which perform a multitude of regulatory functions besides importing nutrients and exporting toxic and waste products. Involvement of these transporters in efflux mediated drug resistance in both prokaryotes and eukaryotes has already been reported [1–3]. These transporters often recognize and extrude out structurally unrelated xenobiotics thereby conferring multidrug resistance. Since drug efflux occurs against the concentration gradient, the process is energy dependent. The energy required in this event may be derived from proton motive force (PMF) or through hydrolysis of ATP.

Transporters energized by PMF are grouped in the proton antiporter family and have been extensively reported in bacteria [2–4]. They are involved in extrusion of hydrophobic cations, like ethidium bromide, acriflavin, quinolones, etc. On the other hand, ABC (ATP binding cassette) transporters, which have been reported to be involved in drug trafficking in eukaryotes [1], have been shown to be largely responsible for metabolite transport in prokaryotes [5,6]. The genes encoding these transporters are mostly located in chromosomes [3]. The efficiency of these transporters has been shown to be modulated according to need and as a consequence, induction [7], amplification [8,9] and induction followed by amplification [10] of the relevant transporter gene have been reported.

We have recently reported that the active efflux system has a major role in conferring a high level of fluoroquinolone resistance in a *Mycobacterium smegmatis* clone (CIP^r) which was serially adapted to 64 times the minimal inhibitory concentration of ciprofloxacin [11]. Surprisingly, despite mutations in the known drug target locus, DNA-gyrase A, CIP^r exhibited reversal of drug resistance when incubated with verapamil, a calcium channel blocker. Although it is quite atypical for fluoroquinolone resistance in prokaryotes [2,4], this observation was similar to findings in studies of multidrug resistance in eukaryotes [12] where ABC transporters are involved. This led us to look for the involvement of an ABC transporter in this process.

In this communication, we report the identification of an ABC transporter that distinctly shows mRNA level overexpression in the ciprofloxacin resistant clone, CIP^r, compared to the wild type. Sequencing of a 777 bp open reading frame (ORF) and homology search in the GenBank database revealed this putative gene to be the B-subunit of phosphate specific transporter (*pstB*), an ABC protein which is a part of the binding protein-dependent phosphate uptake system.

2. Materials and methods

2.1. Bacterial strains and vectors

Wild type *M. smegmatis* strain mc^2155 [13] used in this study was obtained as a gift from Dr. Anil Tyagi, Department of Biochemistry, University of Delhi South Campus, New Delhi, India. It was grown at 37°C using Middlebrook 7H9/7H10 (Himedia, Bombay, India) or Sauton's medium [14] supplemented with/without 0.01% Tween 80 (Sigma Chemical Company, St. Louis, MO, USA). Bacterial strains (*Escherichia coli* DH5α and XL1-blue) and vectors were obtained from commercial sources.

2.2. Selection of drug resistant clone

Ampicillin and tetracycline were procured from Sigma Chemical Company. Ciprofloxacin (a drug belonging to the fluoroquinolone group) was a gift from Ranbaxy (India). The procedure for selecting the clone (CIP^r) of *M. smegmatis* resistant to a high level of ciprofloxacin (32 μg/ml, at 64 times the minimal inhibitory concentration) has been described elsewhere [11].

2.3. Nucleic acid extractions and manipulations

Total RNA was isolated from wild type or CIP^r cultures (in Sauton's medium, $OD_{640} \approx 1$, cell density $\approx 10^9$/ml, wet weight ≈ 10 mg/ml) following the method described elsewhere [15] with slight modifications. Briefly, cells were suspended (~100 mg/ml) in a solution (4 M guanidinium thiocyanate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol in 25 mM sodium citrate, pH 7.0), sonicated (Heat Instruments, New York, USA) with a microprobe for 3 min (30 s burst followed by 30 s cooling) and then extracted twice with acid phenol-chloroform mixture. To ensure purity of the preparation, it was subjected to RNase free RQ1 DNase (0.1 U/μl at 37°C for 2×30 min, obtained from

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Promega, USA) treatment followed by phenol-chloroform extraction and ethanol precipitation. RNA obtained in this way was dissolved in DEPC treated water, quantitated spectrophotometrically, checked qualitatively in a formamide-agarose denaturing gel and used immediately for reverse transcription (RT) reaction.

Genomic DNA from *M. smegmatis* was isolated by lysis of spheroplasts. Spheroplasts were prepared from wall deficient forms of mycobacteria following the method described by Rastogi and David [16]. Spheroplasts were lysed by incubating them (1 h at 37°C) with 3.5% SDS and proteinase K (250 μg/ml). Standard protocols [17] were followed for nucleic acids extraction with a phenol-chloroform mixture (1:1) and finally DNA was obtained by digesting RNA with RNase A, followed by ethanol precipitation. DNA obtained in this way was dissolved in TE buffer (1 mM EDTA in 10 mM Tris, pH 8.0), quantitated spectrophotometrically, checked qualitatively in an agarose gel and stored at 4°C until used.

Cosmid pHC79 [18] was used in preparing a genomic library of *M. smegmatis* mc^2155. Sub-cloning, transformation in *E. coli*, plasmid DNA isolation etc. were carried out following standard protocols [17]. Unless mentioned specifically, different modifying enzymes, restriction enzymes and molecular biological reagents etc. used in this study were obtained from either Promega or New England Biolabs (USA).

2.4. PCR and RT-PCR techniques

Two primers (synthesized from Bangalore Genei, Bangalore, India), forward P1 (5′-GGCTGCTCGGGCTGCGGCAA-3′) and reverse P2 (5′-GTCCAGAGCAGAGGTGGCTC-3′), corresponding to amino acids 429–435 and 556–562 respectively of the human multidrug resistant gene *mdr1* [19], were designed (based on conserved nucleotide binding regions of ABC transporters) using preferred codon usage for mycobacteria [20]. PCR amplification was carried out (Perkin Elmer Cetus DNA thermal cycler) with 100 ng template DNA in a final reaction volume of 25 μl containing 250 μM dNTP, 7.2 pmol of each primer, 1× buffer and 1–2 U of *Taq* DNA polymerase (Boehringer Mannheim, Germany or Amersham Life Sciences, UK). 5 min denaturation at 94°C was followed by 30 amplification cycles for: 1 min denaturation at 94°C, 1 min 30 s annealing at 50–72°C (see Fig. 1) and 1 min extension at 72°C. The final extension step was for 5 min at 72°C. The PCR product was klenowed and agarose (Sigma) gel purified and subcloned at the *Sma*I site of pUC18 following standard methods [17].

2 μg of total RNA (heated 65°C for 5 min immediately before use) was used in setting up each RT reaction (total volume of 50 μl) following standard protocol using AMV reverse transcriptase (25 U/reaction, Amersham, UK or Pharmacia, Sweden) and random primer (Pharmacia, Sweden). The reaction was carried out at 42°C for 1 h. RT products were ethanol precipitated and dissolved in autoclaved distilled water (50 μl). To carry out PCR with RT products two sets of primer (set 1: P1 and P2; set 2: forward 5′-GGTAAGTACC-ACCCGCACGGC-3′ and reverse 5′-CTCGGTGTAACGCATGG-CGGC-3′) were used. Aliquots (10 μl/25 μl reaction volume) of RT products along with necessary ingredients (primers, dNTPs and 1× buffer) except the enzyme were mixed. PCR was initiated by adding *Taq* DNA polymerase after 5 min denaturation at 94°C and carried out for 30 amplification cycles (1 min denaturation at 94°C, 1 min 30 s annealing at 50°C and 1 min of extension at 72°C), followed by a final extension step for 5 min at 72°C.

2.5. Southern hybridization

Southern/colony hybridization was carried out following standard protocols [17] using [α-^{32}P]dCTP/[γ-^{32}P]ATP (BARC, India) labeled probes (mentioned in the figure legends). PCR and RT-PCR products were separated on 1.5% agarose gels before transfer to nylon membranes (MDM membranes, Ambala, India). X-ray films were procured from Hindustan Photo Films Ltd. (India).

2.6. Nucleic acid sequencing and analysis

DNA sequencing was carried out using M13 forward and reverse or PCR primers and Sequenase kit version 2.0 (Amersham Life Sciences, UK) according to the manufacturer's recommended protocol. Nucleotide and nucleotide derived amino acid sequences were compared with both nucleotide and peptide sequence databases with the BLAST X program [21,22] using the mail server at NIH. The multiple sequence alignments of the retrieved sequences were carried out using the Clustal V program [23]. Gap introduction and extension penalties of 10 each were used during the alignments.

3. Results

3.1. A ∼600 bp band spanning the ATP binding regions of ABC transporter amplified from M. smegmatis at most stringent PCR annealing conditions

We concentrated our effort on exploring the possibility of the presence of any transporter belonging to the ABC family in *M. smegmatis*. PCR at different annealing temperatures (50, 60, 65 and 72°C) with primers P1 and P2 and *M. smegmatis* (wild type) genomic DNA revealed the amplification of several fragments, among them a ∼600 bp band was amplified consistently which could withstand the most stringent annealing conditions (72°C; calculated T_m values for P1 and P2 are 74°C and 70°C respectively) (Fig. 1). Only those reactions which contained template DNA (wild type or CIP^r), primers and enzymes showed the amplification. No apparent difference in the amplification profile was noticed between wild type CIP^r clones (data not shown). Hybridization using the primer(s) as probe(s) with the ∼600 bp amplified product confirmed the presence of both oligo sequences (data not shown). The ∼600 bp fragment (*mtp1*) was processed for further analysis and the identity of other bands obtained at low annealing temperatures remains to be determined.

3.2. RT-PCR reveals that the gene is overexpressed in drug resistant cells

In order to determine whether *mtp1* is associated with a high level of fluoroquinolone resistance in the CIP^r clone, we monitored the expression of the gene at the mRNA level using RT-PCR. The sensitivity of such a technique as opposed to a typical Northern analysis has been suggested to be a powerful alternative, particularly in situations where there is a relatively low abundance of specific mRNA [24]. Reverse transcription reactions were carried out by extracting total RNA from wild type or the CIP^r clone using random primer,

Table 1
Homology exhibited by nucleotide derived amino acid sequences of *mtp1* from the *M. smegmatis* genome with selective ABC transporters and ATP binding proteins around the nucleotide binding folds

Region	PhoT.mlep	Pstb.mtb	Hylb	Pfmdr	Lemdr	Humdr	*E. coli* ATPase α	Adenylate kinase
NB1	78	78	78	67	78	89	56	56
NB1–NB2	87	65	43	44	39	40	ND	ND
NB2	100	93	86	86	79	93	36	43

Identical as well as similar amino acid residues were considered and values are expressed in percentage rounded to the nearest integer. Amino acid stretches constituting different regions considered for calculation are 9 for NB1, 115 between NB1 and NB2 (NB1 or NB2 regions were excluded) and 14 for NB2. No appreciable homology is indicated by ND. For explanation of abbreviations see legend of Fig. 3. Sequences of *E. coli* ATPase α and adenylate kinase were taken from Chen et al. [19].

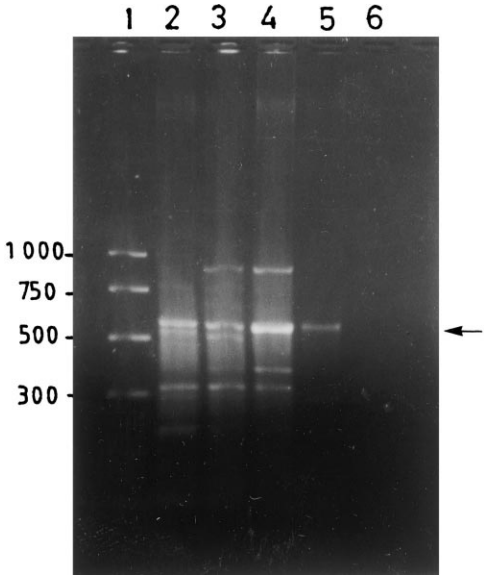


Fig. 1. PCR amplification pattern with *M. smegmatis* wild type genomic DNA using primers P1 (5′-GGCTGCTCGGGCTGCGGCAA-3′) and P2 (5′-GTCCAGAGCAGAGGTGGCTC-3′) at different annealing temperatures. The ∼600 bp band, which was consistently present at all annealing temperatures, is indicated by an arrow. Lanes: DNA molecular size markers (M); annealing at 50°C (lane 1), 60°C (lane 2), 65°C (lane 3) and 72°C (lane 4); annealing at 72°C without template DNA (lane 5).

followed by incubation with AMV reverse transcriptase. PCR using reverse transcription products (wild type or CIP^r or other controls) as templates with primers P1 and P2 indicated amplification of a ∼600 bp fragment in CIP^r only (Fig. 2A, lane 5). Southern analysis of the RT-PCR products using *mtp1* as a probe hybridized only with this ∼600 bp band and showed its overexpression in CIP^r compared to wild type (Fig. 2B, lanes 4 versus 5). To rule out the possibility that this differential expression of ∼600 bp fragment in CIP^r was an experimental artifact, PCR was also carried out with DNA-gyrase A primers (see Section 2) using the same reverse transcription products as templates. Southern hybridization of this 150 bp PCR product using gyrase A as a probe did not show differential expression to any significant extent between CIP^r and wild type (Fig. 2C, lanes 3 and 4). The results obtained, therefore, implied that the mRNA level overexpression of *mtp1* was specific for the CIP^r clone.

3.3. Sequencing establishes the gene as the ATP binding subunit of an ABC transporter

Comparison of the nucleotide derived predicted amino acid sequences (at +1 frame) of *mtp1* (exactly 579 bp) with the GenBank database revealed overall ∼24% similar or identical amino acids with transporters belonging to the ABC family (Fig. 3A). A pairwise analysis of the genomic sequence data of *mtp1* in comparison to selected representatives of ABC transporters as well as ATP binding proteins is listed in Table 1. *mtp1* exhibited considerable homology with ABC transporters in the entire region spanning two nucleotide binding folds [25], maximum being at NB1 or NB2 which agrees well with earlier reports [5,19]. On the other hand, this homology with ATP binding proteins is restricted only to the NB1 and NB2 regions (Table 1). Strikingly, PROSEARCH (Prosite release 12.2 of February 1995; © Amos Bairoch, University of Geneva, Switzerland) analysis also revealed that *mtp1* possesses the signature sequence (underlined amino acids identical) of the ABC transporter, namely, [LIVMFYC]-[SA]-[SAPGVYKQ]-G-[DENQMW]-[KRQAPCLW]-[KRNQSTAVM]-[KRACLVM]-[LIVMYPAN]-{PHY}-[LIVMFW]-[SAGCLIVP]-{FYWHP}-{KRHP}-[LIVMFYWSTA]. Among all ABC transporters, the *mtp1* sequence had more resemblance to mycobacterial sequences (Table 1) which highlights the presence of similar transporters in pathogenic mycobacteria as well.

We therefore screened a *M. smegmatis* mc^{2}155 genomic library using *mtp1* as probe and isolated ∼3 kbp fragment spanning *Sal*I sites followed by subcloning in pGEM-5Z(f+). Sequencing of this fragment hybridizing with *mtp1* yielded a 777 bp ORF (GenBank accession number AF045938). Since nucleotide derived amino acid sequences (Fig. 3B) of this ORF exhibited striking homology with PhoT of *M. leprae* (overall ∼85%) and PstB of *E. coli* (overall ∼66%) as well as *M. tuberculosis* (overall ∼60%), we identified this gene as *pstB*, an ABC protein which is the ATP binding subunit of the bacterial phosphate specific transporter. PstB in *E. coli* has been reported to be a part of the binding protein dependent phosphate uptake system [26].

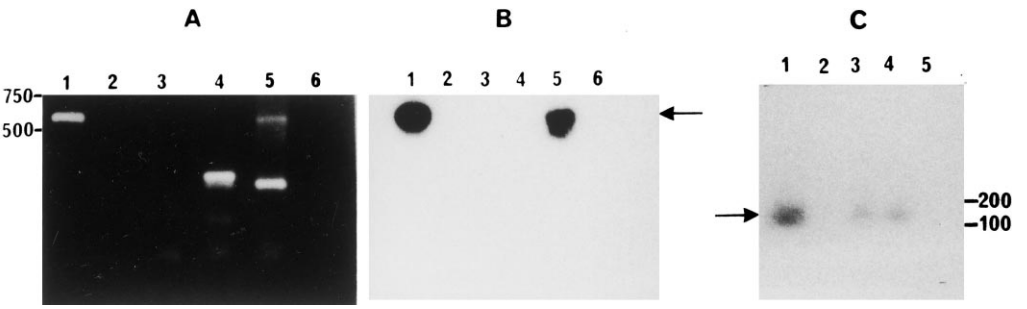
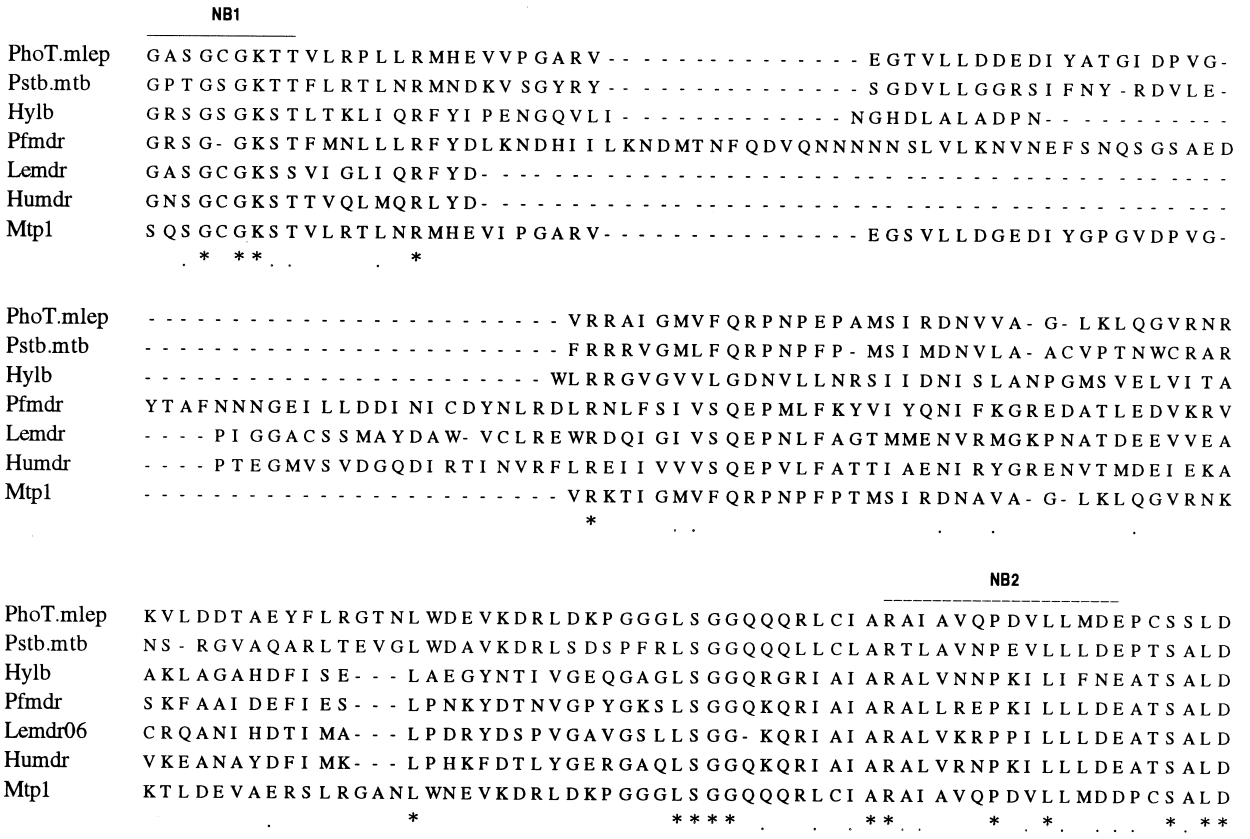


Fig. 2. RT-PCR showing overexpression of the ∼600 bp fragment in CIP^r compared to wild type. A: Gel photograph of RT-PCR products from wild type and CIP^r clones using primers P1 and P2. Details regarding the primers are mentioned in Fig. 1 and in the text. *mtp1* positive control (lane 1); PCR without template (lane 2); RT blank with wild type (lane 3); wild type (lane 4); CIP^r (lane 5); RT blank with CIP^r (lane 6). B: Southern hybridization of the same gel as in A using *mtp1* as the probe. The lanes are arranged as in A. C: Southern hybridization using the PCR amplified 150 bp *gyrA* fragment from *M. smegmatis* [11] as probe with RT-PCR products from wild type and CIP^r clones using DNA gyrase primers (see Section 2 and text for details). DNA gyrase A fragment positive control (lane 1); RT blank with wild type (lane 2); wild type (lane 3); CIP^r (lane 4); RT blank with CIP^r (lane 5). Positions of molecular size markers are indicated. The arrows denote the positions of the ∼600 bp ABC transporter and the ∼150 bp DNA gyrase A fragment.

A.


NB1
PhoT.mlep GASGCGKTTVLRPLLRMHEVVPGARV----------EGTVLLDDEDIYATGIDPVG-
Pstb.mtb GPTGSGKTTFLRTLNRMNDKVSGYRY----------SGDVLLGGRSIFNY-RDVLE-
Hylb GRSGSGKSTLTKLIQRFYIPENGQVLI----------NGHDLALADPN----------
Pfmdr GRSG-GKSTFMNLLLRFYDLKNDHIILKNDMTNFQDVQNNNNNSLVLKNVNEFSNQSGSAED
Lemdr GASGCGKSSVIGLIQRFYD----------
Humdr GNSGCGKSTTVQLMQRLYD----------
Mtp1 SQSGCGKSTVLRTLNRMHEVIPGARV----------EGSVLLDGEDIYGPGVDPVG-
PhoT.mlep ----------VRRAIGMVFQRPNPEPAMSIRDNVVA-G-LKLQGVRNR
Pstb.mtb ----------FRRRVGMLFQRPNPFP-MSIMDNVLA-ACVPTNWCRAR
Hylb ----------WLRRGVGVVLGDNVLLNRSIIDNISLANPGMSVELVITA
Pfmdr YTAFNNNGEILLDDINICDYNLRDLRNLFSIVSQEPMLFKYVIYQNIFKGREDATLEDVKRV
Lemdr ----PIGGACSSMAYDAW-VCLREWRDQIGIVSQEPNLFAGTMMENVRMGKPNATDEEVVEA
Humdr ----PTEGMVSVDGQDIRTINVRFLREIIVVVSQEPVLFATTIAENIRYGRENVTMDEIEKA
Mtp1 ----------VRKTIGMVFQRPNPFPTMSIRDNAVA-G-LKLQGVRNK
NB2
PhoT.mlep KVLDDTAEYFLRGTNLWDEVKDRLDKPGGGLSGGQQQRLCIARAIAVQPDVLLMDEPCSSLD
Pstb.mtb NS-RGVAQARLTEVGLWDAVKDRLSDSPFRLSGGQQQLLCLARTLAVNPEVLLLDEPTSALD
Hylb AKLAGAHDFISE---LAEGYNTIVGEQGAGLSGGQRGRIAIARALVNNPKILIFNEATSALD
Pfmdr SKFAAIDEFIES---LPNKYDTNVGPYGKSLSGGQKQRIAIARALLREPKILLLDEATSALD
Lemdr06 CRQANIHDTIMA---LPDRYDSPVGAVGSLLSGG-KQRIAIARALVKRPPILLLDEATSALD
Humdr VKEANAYDFIMK---LPHKFDTLYGERGAQLSGGQKQRIAIARALVRNPKILLLDEATSALD
Mtp1 KTLDEVAERSLRGANLWNEVKDRLDKPGGGLSGGQQQRLCIARAIAVQPDVLLMDDPCSALD

B.

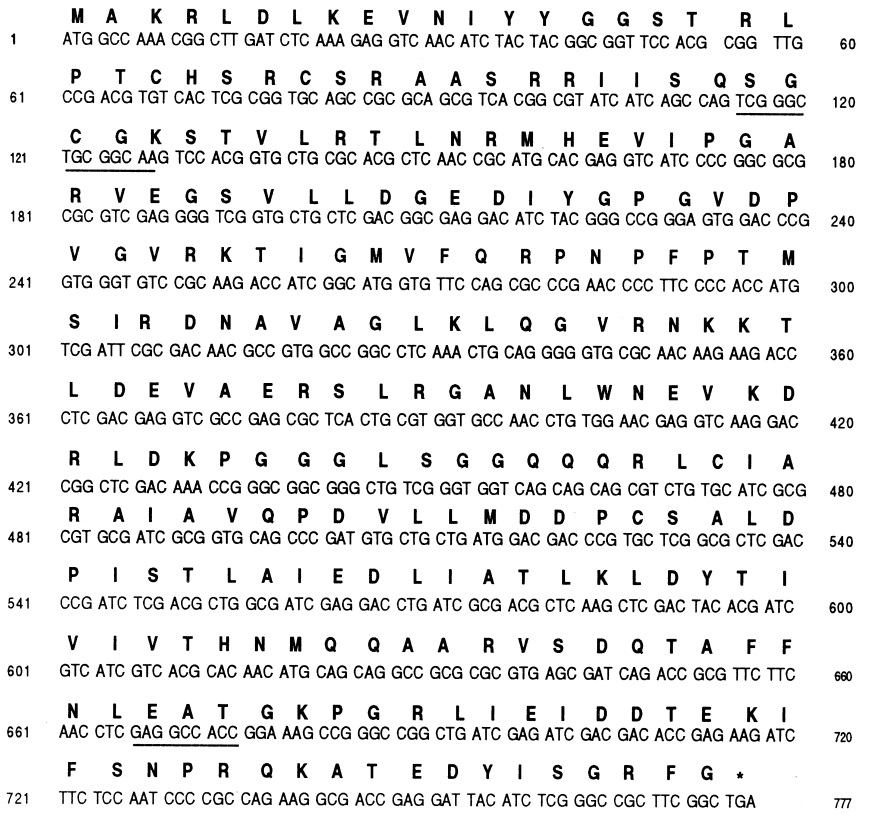

M A K R L D L K E V N I Y Y G G S T R L
1 ATG GCC AAA CGG CTT GAT CTC AAA GAG GTC AAC ATC TAC TAC GGC GGT TCC ACG CGG TTG 60
P T C H S R C S R A A S R R I I S Q S G
61 CCG ACG TGT CAC TCG CGG TGC AGC CGC GCA GCG TCA CGG CGT ATC ATC AGC CAG TCG GGC 120
C G K S T V L R T L N R M H E V I P G A
121 TGC GGC AAG TCC ACG GTG CTG CGC ACG CTC AAC CGC ATG CAC GAG GTC ATC CCC GGC GCG 180
R V E G S V L L D G E D I Y G P G V D P
181 CGC GTC GAG GGG TCG GTG CTG CTC GAC GGC GAG GAC ATC TAC GGG CCG GGA GTG GAC CCG 240
V G V R K T I G M V F Q R P N P F P T M
241 GTG GGT GTC CGC AAG ACC ATC GGC ATG GTG TTC CAG CGC CCG AAC CCC TTC CCC ACC ATG 300
S I R D N A V A G L K L Q G V R N K K T
301 TCG ATT CGC GAC AAC GCC GTG GCC GGC CTC AAA CTG CAG GGG GTG CGC AAC AAG AAG ACC 360
L D E V A E R S L R G A N L W N E V K D
361 CTC GAC GAG GTC GCC GAG CGC TCA CTG CGT GGT GCC AAC CTG TGG AAC GAG GTC AAG GAC 420
R L D K P G G G L S G G Q Q Q R L C I A
421 CGG CTC GAC AAA CCG GGC GGC GGG CTG TCG GGT GGT CAG CAG CAG CGT CTG TGC ATC GCG 480
R A I A V Q P D V L L M D D P C S A L D
481 CGT GCG ATC GCG GTG CAG CCC GAT GTG CTG CTG ATG GAC GAC CCG TGC TCG GCG CTC GAC 540
P I S T L A I E D L I A T L K L D Y T I
541 CCG ATC TCG ACG CTG GCG ATC GAG GAC CTG ATC GCG ACG CTC AAG CTC GAC TAC ACG ATC 600
V I V T H N M Q Q A A R V S D Q T A F F
601 GTC ATC GTC ACG CAC AAC ATG CAG CAG GCC GCG CGC GTG AGC GAT CAG ACC GCG TTC TTC 660
N L E A T G K P G R L I E I D D T E K I
661 AAC CTC GAG GCC ACC GGA AAG CCG GGC CGG CTG ATC GAG ATC GAC GAC ACC GAG AAG ATC 720
F S N P R Q K A T E D Y I S G R F G *
721 TTC TCC AAT CCC CGC CAG AAG GCG ACC GAG GAT TAC ATC TCG GGC CGC TTC GGC TGA 777

Fig. 3. Alignment of nucleotide derived amino acid sequences of *mtp1* from the *M. smegmatis* genome with other ABC transporters spanning the nucleotide binding regions (A) and nucleotide sequences of full length *mtp1* (B). A: NB1 and NB2 indicate two ATP binding folds. Gaps in the sequences have been introduced for optimum alignment. Asterisks and dots are used to denote identical and similar amino acids respectively. Abbreviations: PhoT.mlep = PhoT of *M. leprae* (accession number U15182); PstB.mtb = PstB of *M. tuberculosis* (accession number Z47981); Hylb = bacterial hemolysin transport protein [33]; Pfmdr = *Plasmodium falciparum* multidrug resistant transporter [8]; Lemdr = *Leishmania donovani* multidrug resistant transporter [9]; Humdr = Human multidrug resistant transporter, Mdr1 [19]. B: Nucleotide derived single letter amino acid codes are shown in bold. Numbers indicate base numbers. Identical bases of primers P1 and P2 in the *mtp1* sequence are underlined.
←

4. Discussion

We generated a *M. smegmatis* clone (CIP^r) exhibiting a high level of fluoroquinolone resistance, by serially passaging it from lower to higher concentrations of ciprofloxacin. Although it has been reported that mutations in the quinolone resistance determining region of DNA gyrase A subunit are the prime cause for a high level of fluoroquinolone resistance in mycobacteria ([27] and references therein), we have convincingly demonstrated that active and accelerated drug efflux has a predominant role in conferring such resistance [11]. This notion has further been strengthened by the fact that a transporter (LfrA) belonging to the proton antiporter family has recently been described for mycobacteria which seems to be responsible only for low level (but not for high level) fluoroquinolone resistance [28,29]. Thus at the high level of such resistance [11], involvement of other transporter(s) besides LfrA seems to be a possibility. The association of an ABC transporter does not seem to be remote since the CIP^r clone exhibited reversal of drug resistance by verapamil, a situation reported in eukaryotic multidrug resistance where involvement of such transporters is well known [1,12]. We therefore concentrated our efforts on investigating whether any transporter belonging to the ABC family is present in *M. smegmatis* and if so whether it is associated with the drug resistance.

We have exploited the conservation of sequences [5] of the nucleotide binding domain of the ABC transporters, especially between the NB1 and NB2 regions [25], to design PCR primers. To ensure full representation of the sequences between the NB1 and NB2 regions in the PCR amplified fragment, the forward primer in our study was designed choosing only the sequences from the NB1 region. The reverse primer, on the other hand, was designed from the conserved sequences carboxy-terminal to the NB2 region (see Fig. 3A,B). Amplification of a band at most stringent annealing conditions in PCR (Fig. 1) and subsequent hybridization of the band with the mycobacterial genomic DNA (data not shown) suggested that the amplified product (*mtp1*) was not an artifact. The most striking observation in our study is the mRNA level overexpression of the *mtp1* in the CIP^r clone (Fig. 2). Such an event strongly argues the involvement of transporters in conferring drug resistance [3,7]. In this connection, our results also imply the association of this ABC transporter with fluoroquinolone resistance in the CIP^r clone, which is the first report of its kind in any prokaryote.

Nucleotide derived amino acid (at +1 frame) sequence (Fig. 3B) comparison with the GenBank database of the full length gene with various ABC proteins revealed that it is *pstB*. In *E. coli* PstB is a traffic ATPase [30] and is a part of the binding protein dependent phosphate uptake system. Available reports on *E. coli* indicate that the phosphate specific transport (Pst) system is operative to scavenge phosphate and is active only in a situation when the surrounding phosphate concentration is less than 1 mM [26]. The medium phosphate concentration in our studies was ~3.7 mM. Therefore the differential expression of this gene in the CIP^r clone (Fig. 2) does not highlight its apparent involvement in phosphate scavenging. The role of mycobacterial Pst in coping with adverse situations has already been postulated [31,32]. Therefore our work is the first experimental evidence in favor of these reports. So it remains to be seen whether Pst acts directly as a drug transporter or regulates other exporter(s) in effluxing out drug. At this juncture it would be appropriate to mention that among prokaryotes, the presence of multiple copies of the Pst system has recently been reported only in mycobacteria [31,32]. In such a situation, genetic redundancy is apparent if all the Pst perform the same function. To ensure economy of organization, mycobacterial Pst may have versatile functions besides phosphate transport. Further studies will help to resolve these issues.

Finally, we have carried out our experiments utilizing a saprophyte, *M. smegmatis* which has often been used as a model system in genetic studies for *M. tuberculosis* [28]. Hybridization (data not shown) and sequence homology of this transporter gene with the *M. tuberculosis* genome suggest that the novelty in our findings is not restricted to the laboratory generated fluoroquinolone resistant mycobacteria only.

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