The Multidrug Transporters Belonging to Major Facilitator Superfamily (MFS) in *Mycobacterium tuberculosis*

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Abstract

Background: Both intrinsic and acquired multidrug resistance play an important role in the insurgence of tuberculosis. Detailed knowledge of the molecular basis of drug recognition and transport by multidrug transport systems is required for the development of new antibiotics that are not extruded or of inhibitors that block the multidrug transporter and allow traditional antibiotics to be effective.
Materials and Methods: We have undertaken the inventory of the drug transporters subfamily, included in the major facilitator superfamily (MFS), encoded by the complete genome of *Mycobacterium tuberculosis* (MTB). These proteins were identified on the basis of their characteristic stretches of amino acids and transmembrane segments (TMS) number.
Conclusions: Genome analysis and searches of homology between the identified transporters and proteins characterized in other organisms revealed 16 open reading frames encoding putative drug efflux pumps belonging to MFS. In the case of two of them, we also have demonstrated that they function as drug efflux proteins.

Introduction

One of the major problems in combating tuberculosis disease is that *Mycobacterium tuberculosis* is resistant to most therapeutic agents because it has an unusual bacterial cell wall with intrinsically low permeability (1).

Although acquired high-level drug resistance in mycobacteria in general is due to mutational alterations of the drug target, it has become clear that low-level drug resistance to a variety of different anti-infective agents is frequently found in clinically drug-resistant isolates with no alteration of the target. Nothing is presently known about the molecular mechanisms mediating this class of resistance, although it is likely that changes in drug transport are involved (2).

Multidrug efflux systems endow on bacterial cells the ability to limit the access of antimicrobial agents to their targets. Drug efflux appears to be one of the most widespread antibiotic resistance mechanisms among microorganisms; it has been demonstrated to occur in many Gram-positive and Gram-negative bacteria including medically important species (3).

Bacterial drug efflux pumps proved to occur in five families (4). Two of these are large and ancient superfamilies known as the ATP binding cassette (ABC) superfamily and the major facilitator superfamily (MFS). The other three are smaller and more recently developing families, called the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) family, and the multidrug and toxic compounds extrusion family (MATE). The MFS, SMR, RND, and MATE families are secondary transporters, typically energized by the proton motive force. In contrast, ATP is utilized as the energy donor for members of the ABC family of multidrug efflux pumps that are often considered primary transporters. Several drug efflux pumps have been identified or characterized in mycobacteria (5–11), belonging to three of the five families just described.

First, members of the MFS have been described in several mycobacterial species. In *M. smegmatis*, the predicted LfrA protein, conferring resistance to several fluoroquinolones (5) and the Tet(V) protein, conferring resistance to tetracycline (6), appear to

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have 14 and 11 hydrophobic membrane-spanning regions, respectively. The *M. fortuitum* Tap (7) and the *M. bovis* P55 (8) proteins confer resistance to aminoglycosides and tetracycline. In *M. tuberculosis,* the putative Tap protein (7) confers resistance to tetracycline, and the EfpA protein does not seem to confer any phenotype (9).

Second, the Mmr protein of *M. tuberculosis* appears to be a multidrug efflux pump with four transmembrane regions belonging to SMR family (10).

Third, one member of the ABC transporter superfamily involved in fluoroquinolone resistance has been characterized in *M. smegmatis* (11). Recently, Braibant et al. (12), by inventory and assembly of the typical subunits of the ABC transporters encoded by the complete genome of *M. tuberculosis,* found that the genes encoding these proteins potentially implicated in the formation of the permeases account for 2.5% of the genome.

The MFS is one of the two largest families of membrane transporters. Compounds transported by MFS permeases include simple sugars, oligosaccharides, inositols, drugs, amino acids, nucleosides, organophosphate esters, Krebs cycle metabolites, and a large variety of inorganic anions and cations. They are found ubiquitously in all three domains of living organisms—bacteria, archaea, and eukaryotes (13,14). Transport proteins of the MFS contain a number of conserved amino acid sequence motifs that are either ubiquitous within the MFS or subfamily specific (15). Conservation of such motifs among proteins responsible for the transport of a wide variety of structurally disparate compounds implies that they play some vital structural or functional role rather than directly interacting with their substrate(s). In particular, Paulsen et al. (16) identified motifs conserved throughout the MFS [motifs A (GxLaDrxGrkxxxl) and B (lxxxRxxqGxgaa)]; motif C (gxxxGPxxGGxl), also known as the antiporter motif, can be found in TMS 5 of the drug/proton antiporters of either 12- or 14-TMS, but not in symporters from other MFS subfamilies, suggesting that it may be required for linking proton translocation to antiport but not to symport of a substrate (16,17); other motifs are exclusive to either the 12- or 14-TMS subfamily (motifs D to H).

The importance of bacterial MFS transporters in conferring drug resistance is generally accepted. In mycobacteria, the majority of drug efflux pumps characterized so far belongs to the MFS, which reinforces the importance of this family of transporters in the innate drug resistance of the tubercle bacillus. Thus, we decided to analyze the MFS transporters encoded by the *M. tuberculosis* genome (18), which may be potentially involved in antibiotic extrusion.

In this study, we applied procedures, based on the bioinformatic tools, to identify and characterize drug efflux proteins belonging to MFS in *M. tuberculosis.* To investigate the power of prediction and the substrate specificity of potential drug transporters,

we cloned 11 putative drug transporter genes of *M. tuberculosis* into different plasmids and then investigated their drug resistance phenotypes in *M. smegmatis.* In the case of two genes, we also demonstrated that they function as drug efflux proteins.

Data reported could be useful for the planning of further experimental work, necessary for the physiological characterization of these transporters and for the conception of new therapeutic agents.

Material and Methods

Computer Methods

This analysis integrates different approaches and is based on the conservation of three features between drug transporters in microorganisms: specific sequence motifs, overall sequence similarity, and structural similarities (transmembrane segments). The *M. tuberculosis* genome sequence was retrieved from the Tuberculist web server at Institut Pasteur (http://genolist.pasteur.fr/TubercuList) (18).

Searches of the stretches of amino acids containing the specific motifs conserved in and characteristic of members of the MFS among the *M. tuberculosis* proteins was done using the SEARCH PATTERN program provided at the Tuberculist web server. The MEME program (http://www.sdc.edu/MEME) (19) was also used in identifying conserved motifs within the protein families. To well characterize the specific motifs present in the *M. tuberculosis* transporters, the regions containing the motifs were aligned using the CLUSTALW program (http://www.ebi.ac.uk) (20). CLUSTALW was used also to generate the phylogenetic tree, and TreeView (21) was used to display it. Searches of sequence similarity between potential drug transporters and proteins characterized in other organisms, were done using the BLAST program (22). BLAST searches of the *M. tuberculosis* proteins were performed at the Sanger Centre web page (http://www.sanger.ac.uk/Projects/M_tuberculosis/ blast_server.shtml). This allowed for the identification of additional putative transporters encoded in the *M. tuberculosis* genome. Also, proteins encoded in the *M. smegmatis* genome were searched at the BLAST Server of The Institute for Genomic Research (http://tigrblast.tigr.org/ufmg/index.cgi?database= m_smegmatislseq).

The number and the position of the transmembrane domains have been predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0) (23).

Strains and Growth Conditions

Escherichia coli XL1-Blue, used as the cloning host, was grown in LB medium. *M. tuberculosis* H37Rv was used as the donor of chromosomal DNA. *M. smegmatis* mc^{2}155, selected to test the drug susceptibility, was grown in Middlebrook 7H9 broth and Middlebrook 7H11 agar (Difco) supplemented with 10% Middlebrook OADC enrichment (Difco) and 0.2% glycerol. All the cultures were incubated at 37 °C.

Kanamycin was added, when required, at final concentrations of 50 and 25 μg/ml for *E. coli* and *M. smegmatis*, respectively.

Cloning Procedures

Genes predicted to encode hypothetical drug efflux pumps were amplified by polymerase chain reaction (PCR) from *M. tuberculosis* H37Rv chromosomal DNA using specific primers containing the restriction enzyme sites useful for subsequent cloning. ORFs Rv0037c, Rv0849, Rv1877, and Rv2994 were cloned in pMD31 (24) and pSUM38 (25) vectors. Similarly, ORFs Rv0783c, Rv1250, Rv2333c, Rv2559, and Rv3239c were cloned in pSUM38, and ORF Rv1258c was cloned in pSUM36 (25). Rv1634 was cloned directly from cosmid Z95554 digested with *KpnI*. Each cloned ORF contained about 1000 bp upstream the start translation codon to ensure the presence of native promoter. Plasmid DNA was isolated from *E. coli* with Qiagen columns prior to transformation of *M. smegmatis* by electroporation.

Drug Susceptibility Test

Minimal inhibitory concentrations (MIC) of drugs were determined on 7H11 medium supplemented with OADC and containing various drugs (chloramphenicol, tetracycline, ciprofloxacin, lomefloxacin, isoniazid, ethambutol, rifampicin, erythromycin, ethidium bromide, rhodamine 123, doxorubicin, gentamicin, streptomycin, and 2'-N- and 6'-N-ethylnetilmicin) at various concentrations as indicated. Plates were inoculated with a total of 10^4 cells and incubated at 37 °C for 4–6 days and then growth was evaluated.

Measurement of Norfloxacin Accumulation

The modified fluorimetric method of Mortimer and Piddock (26) was altered slightly to accommodate the growth characteristics of the mycobacteria, as described by Williams et al. (27).

Tetracycline Accumulation Assays

The accumulation of titriated tetracycline in *M. smegmatis* cells was monitored as described previously (7,8).

Results and Discussion
Analysis of MFS Drug Transporters ORFs

To identify the *M. tuberculosis* drug efflux proteins belonging to MFS, we first scanned the *M. tuberculosis* predicted proteins for the presence of the transporter family signature motifs A and B, using the program SEARCH PATTERN at the TubercuList web server. In motifs A (GXL[AG]D[RI]XGX[KR]XX[LF][LI]) and B (L[VL]XXRXX[QLA]GXG[AG][AG]), X represents any amino acid, and residues in brackets indicate those most frequently observed in a single position in more than 75% of transport proteins. Allowing none or one mismatch in the A and B transporter family signatures, no proteins were identified. By increasing progressively the number of mismatches to four in motifs A and B, 540 potential motifs in 290 different proteins were identified. Because, in this superfamily, motifs A and B are separated by 18–30 amino acids, we checked if the identified proteins contained each of these two motifs at a correct relative position. If not, they were discarded.

The proteins in which motifs A and B were properly located were subjected to TMHMM to analyze the transmembrane profile; because MFS proteins are characterized by more than 12-TMS, we considered initially the following 19 ORFs: Rv0102 with 16-TMS, Rv3239c and Rv3728 with 15-TMS, Rv2846c, Rv1877, Rv2333c, Rv2459, Rv0783c, Rv1634, Rv1250, Rv1410c with 14-TMS, and Rv1258c, Rv0849, Rv0842, Rv1217c, Rv1510, DinF, NarK1, NarK2, and SugI with 12-TMS.

BLASTP analysis of these proteins confirmed they all belong to the MFS. However, this analysis also revealed that only 12 of them (Rv3239c, Rv3728, Rv2846c, Rv1877, Rv2333c, Rv2459, Rv1410c, Rv1250, Rv1258c, Rv0783c, Rv1634, and Rv0849) showed sequence similarity to drug efflux pumps, previously characterized in other bacteria. As shown in Figure 1, motifs A and B, typical of MFS transporters, are present. Further, as expected, motif C, typical of drug antiporters, could be found in these twelve proteins, as revealed by ClustalW alignment (Fig. 1).

Proteins Rv3728 and Rv3239c are unusually long for being drug transporters; they have 1065 and 1048 amino acids, respectively. The sequence similarity with multidrug efflux transporters and the position of the transmembrane segments is restricted to the first 500–600 residues. However, they were also considered because they showed the MFS motifs A, B, and C clearly conserved and properly located (Fig. 1).

By ClustalW alignment of the ORFs with 14 and 15 TMS, motifs D1, H, E, and F were identified (Fig. 2A); in the 12 TMS ORFs, motif G has been identified (Fig. 2B).

To identify possible additional drug efflux proteins not selected by the strategy mentioned, a search of sequence similarity (BLASTP) within all proteins of *M. tuberculosis* H37Rv was done using each of the 12 proteins previously identified as a query. This search allowed the identification of four additional hypothetical efflux pumps: Rv0191, Rv0037c, Rv2456c, and Rv2994. ClustalW alignment of these hypothetical efflux pumps revealed the presence of motifs A and B (data not shown), indicating their belonging to MFS superfamily; concerning motif C, typical of the drug efflux transporters, a possible *consensus* sequence was identified, although not well conserved (data not shown). Interestingly, Rv0037c, Rv2456c, and Rv2994 were retrieved among the first set of 290 proteins containing motifs A and B that were
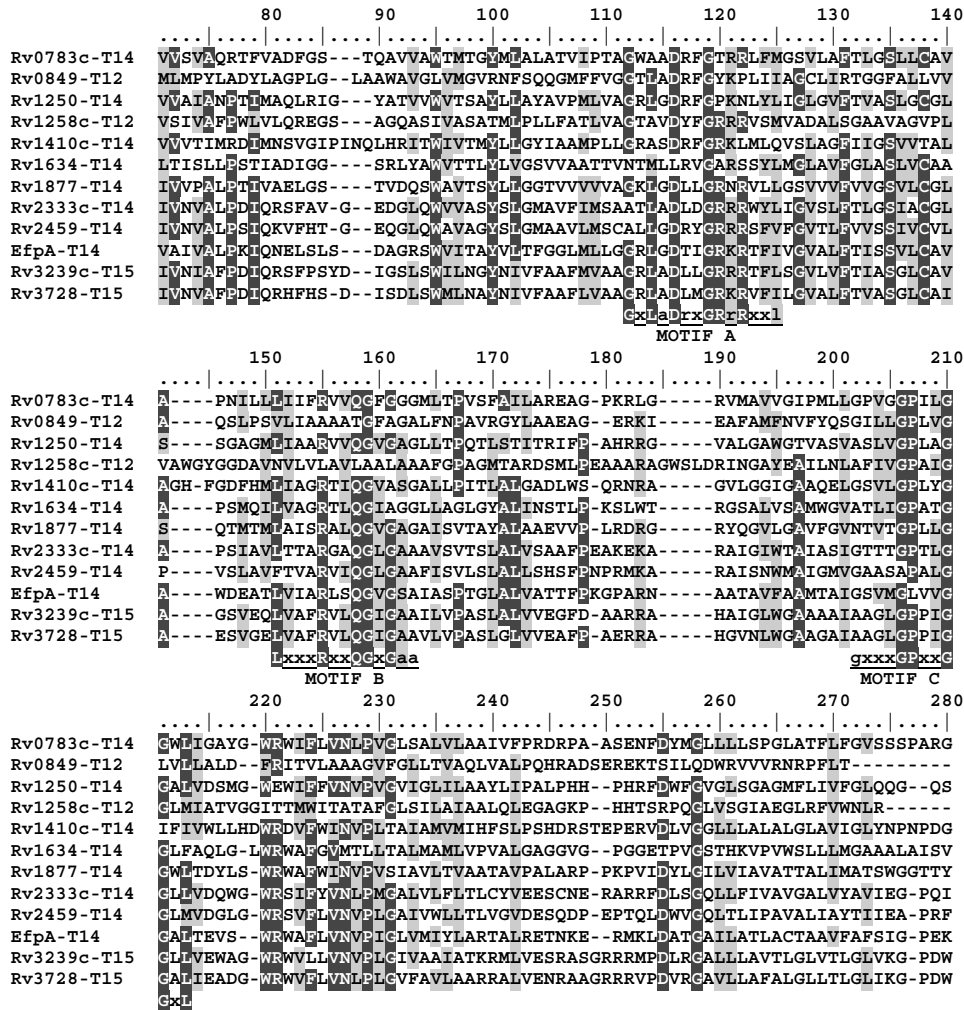
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              80        90       100       110       120       130       140
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Rv0783c-T14  VVSVAQRTFVADFGS---TQAVVAXTMTGXMLALATVIPTAGWAADRFGTRRLFMGSVLAFTLGSLLCAV
Rv0849-T12   MLMPYLADYLAGPLG--LAAWAVGLVMGVRNFSQQGMFFVGCTLADRFCYKPLIIACCLIRTGGFALLVV
Rv1250-T14   VVAIANPTLMAQLRIG---YATVVXVTSAXLLAYAVPMLVACRLGDRFGPKNLYLICLGVFTVASLGCGL
Rv1258c-T12  VSIVAFPWLVLQREGS---AGQASIVASATMLPLLFATLVACTAVGYFGRRRVSMVADALSGAAVAGVPL
Rv1410c-T14  VVVTIMRDLMNSVGIPINQLHRITWIVTMXLLGYIAAMPLLGRASDRFGRKLMLQVSLAGGIIGSVVTAL
Rv1634-T14   LTISLLPSTIADIGG----SRLYAXVTTLXLVGSVVAATTVNTMLLRVCARSSYLMCLAVFGLASLVCAA
Rv1877-T14   IVVPALPTLVAELGS----TVDQSXAVTSXLLGGTVVVVVACKLGDLLGRNRVLLGSVVVFVVGSVLCGL
Rv2333c-T14  IVNVALPDIQRSFAV-G--EDGLQXVVASXSLGMAVFIMSAATLADLDGRRRWYLICVSLFTLGSIACGL
Rv2459-T14   IVNVALPSIQKVFHT-G--EQGLQXAVAGXSLGMAAVLMSCALIGDRYGRRRSFVFGVTLFVVSSIVCVL
EfpA-T14     VAIVALPKIQNELSLS---DAGRSWVITAXVLTFGGLMLLGGRLGDTIGRKRTFIVCVALFTISSVLCAV
Rv3239c-T15  IVNIAFPDIQRSFPSYD--IGSLSWILNGXNIVFAAFMVAACRLADLLGRRRTFLSCVLVFTIASGLCAV
Rv3728-T15   IVNVAFPDIQRHFHS-D--ISDLSXMLNAXNIVFAAFLVAACRLADLMGRRRVFILCVALFTVASGLCAI
                                               GxLaDrxGRrRxxl
                                                 MOTIF A

             150       160       170       180       190       200       210
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Rv0783c-T14  A----PNILLLIIFRVVQGFGGGMLTPVSFAILAREAG-PKRLG-----RVMAVVGIPMLLGPVGGPILG
Rv0849-T12   A----QSLPSVLIAAAATGFAGALFNPAVRGYLAAEAG--ERKI-----EAFAMFNVFYQSGILLGPLVG
Rv1250-T14   S----SGAGMLIAARVVQGVGAGLLTPQTLSTITRIFP-AHRRG-----VALGAWGTVASVASLVGPLAG
Rv1258c-T12  VAWGYGGDAVNVLVLAVLAALAAAFGPAGMTARDSMLPEAAARAGWSLDRINGAYEAILNLAFIVGPAIG
Rv1410c-T14  AGH-FGDFHMLIAGRTIQGVASGALLPITLALGADLWS-QRNRA-----GVLGGIGAQELGSVLGPLYG
Rv1634-T14   A----PSMQILVAGRTLQGIAGGLLAGLGYALINSTLP-KSLWT-----RGSALVSAMWGVATLIGPATG
Rv1877-T14   S----QTMTMLAISRALQGVGAGAISVTAYALAAEVVP-LRDRG-----RYQGVLGAVFGVNTVTGPLLG
Rv2333c-T14  A----PSIAVLTTARGAQGLGAAAVSVTSLALVSAAFPEAKEKA-----RAISNWMAIASIGTTTGETLG
Rv2459-T14   P----VSLAVFTVARVIQGLGAAFISVLSLALLSHSFPNPRMKA-----RAISNWMAIGMVGAASAPALG
EfpA-T14     A----WDEATLVIARLSQGVGSAIASPTGLALVATTFPKGPARN-----AATAVFAAMTAIGSVMGLVVG
Rv3239c-T15  A----GSVEQLVAFRVLQGIGAAILVPASLALVVEGFD-AARRA-----HAIGLWGAAAAIAAGLGPPIG
Rv3728-T15   A----ESVGELVAFRVLQGLGAAVLVPASLGLVVEAFP-AERRA-----HGVNLWGAGAIAAGLGPPIG
                    LxxxRxxqGxGaa                              gxxxGPxxx
                      MOTIF B                                     MOTIF C

             220       230       240       250       260       270       280
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Rv0783c-T14  GWLIGAYG-WRWIFLVNLPVGLSALVLAAIVFPRDRPA-ASENFDYMGLLLLSPGLATFLFGVSSSPARG
Rv0849-T12   LVLLALD--FRITVLAAAGVFGLLTVAQLVALPQHRADSEREKTSILQDWRVVVRNRPFLT---------
Rv1250-T14   GALVDSMG-WEWIFVNVPVGVIGLILAAYLIPALPHH--PHRFDWFGVGLSGAGMFLIVFGLQQG--QS
Rv1258c-T12  GLMIATVGGITTMWITATAFCLSILAIAALQLEGAGKP--HHTSRPQCLVSGIAEGLRFVWNLR------
Rv1410c-T14  IFIVWLLHDWRDLVWINVELLTAIAMVMIHFSLPSHDRSTEPERVDLVGGLLLALALGLAVIGLYNPNPDG
Rv1634-T14   GLFAQLG-LWRWAFGVMTLLTALMAMLVPVALGAGGVG--PGGETPVGSTHKVPVWSLLLMGAAALAISV
Rv1877-T14   GWLTDYLS-WRWAFWINVEVSIAVLVTVAATAVPALARP-PKPVIDYLGILVIAVATTALIMATSWGGTTY
Rv2333c-T14  GLLVDQWG-WRSIFYVNLPMGALVLFLTLCYVEESCNE-RARRFDLSGQLLFIVAVGALVYAVIEG-PQI
Rv2459-T14   GLMVDGLG-WRSVLLVNVLGAIVWLLTLVGVDESQDP-EPTQLDWVGQLTLIPAVALIAYTIIEA-PRF
EfpA-T14     GALTEVS--WRWAFLVNVPIGLVMIYLARTALRETNKE--RMKLDATGAILATLACTAAVFAFSIG-PEK
Rv3239c-T15  GLLVEWAG-WRWVLLVNVPLGIVAAAIATKRMLVESRASGRRRMPDLRGALLLAVTLGLVTLGLVKG-PDW
Rv3728-T15   GALIEADG-WRWVFLVNLPLGVFAVLAARRALVENRAAGRRRVPDVRGAVLLAFALGLLTLGLIKG-PDW
             GxL
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Fig. 1. **Multiple-sequence alignment for 12 members of the hypothetical mycobacterial drug efflux proteins.** Highly conserved motifs are displayed below the alignment; the *consensus* sequences of the motifs are displayed as follows: x, any amino acids; capital letters, the frequency of occurrence of the amino acids is greater that 70%; lower case letters, the frequency of occurrence of the amino acids is greater than 40%. Motifs A, B, and C correspond to the motifs described by Paulsen et al. (16). The alignment shows only the region containing the above-cited motifs. Identical amino acids are highlighted in black boxes; conserved amino acid substitutions are shown in shaded boxes.

identified by using the SEARCH PATTERN program, but they were not considered for further analysis because their TMS numbers were 11, 10, and 9, respectively. This could be indicate that transporters with a number of TMS smaller than 12 may be also involved in drug transport. Rv0191 is predicted to contain 12 TMS; however, this protein was not selected because of the high number of mismatches between its motifs A and B and those used for the screening.

In summary, from our analysis, we retrieved a total of 16 hypothetical efflux proteins, whose characteristics are described in Table 1. In these 16 protein sequences, motifs A, B, and C are highly conserved, independent of the number of TMS. This has allowed us to define more precisely the sequence motifs A, B, and C, specifically for the *M. tuberculosis* drug efflux proteins (Tables 2A, 2B, and 2C, respectively). In this way, motif A in *M. tuberculosis* can be regarded as GrLaDrfGrRRv[l,f]l (versus general consensus GxLaDrxGrkxxxl); motif B would be LvaaRvlQGxGA[g,a] (versus general consensus lxxxRxxqGxgaa); finally, motif C, which is highly conserved in the majority of drug efflux proteins, can be defined as gxvxGPllGGlL (versus general consensus gxxxGPxxGGxl). These signatures will be very useful in identifying and characterizing new members of the drug efflux family from other mycobacterial species. For example, in *M. smegmatis* two drug transporters of the MFS superfamily have been identified, LfrA and Tet(V), which do not seem to have any homologue in the *M. tuberculosis* chromosome. This indicates that *M. smegmatis* (for which it has been estimated a genome size of 7.5 Mb versus the 4.4 Mb of *M. tuberculosis*) and also other mycobacterial species will contain many other MFS transporters, different from those identified in *M. tuberculosis*.

From the CLUSTALW alignment of these transporters, a phylogenetic tree showing the relationships among the proteins is shown in Figure 3. It appears that the proteins potentially involved in *M. tuberculosis* drug efflux are scattered in three different groups. Two of the three groups include proteins with 14–15 transmembrane segments; the third group is composed by a subgroup of proteins
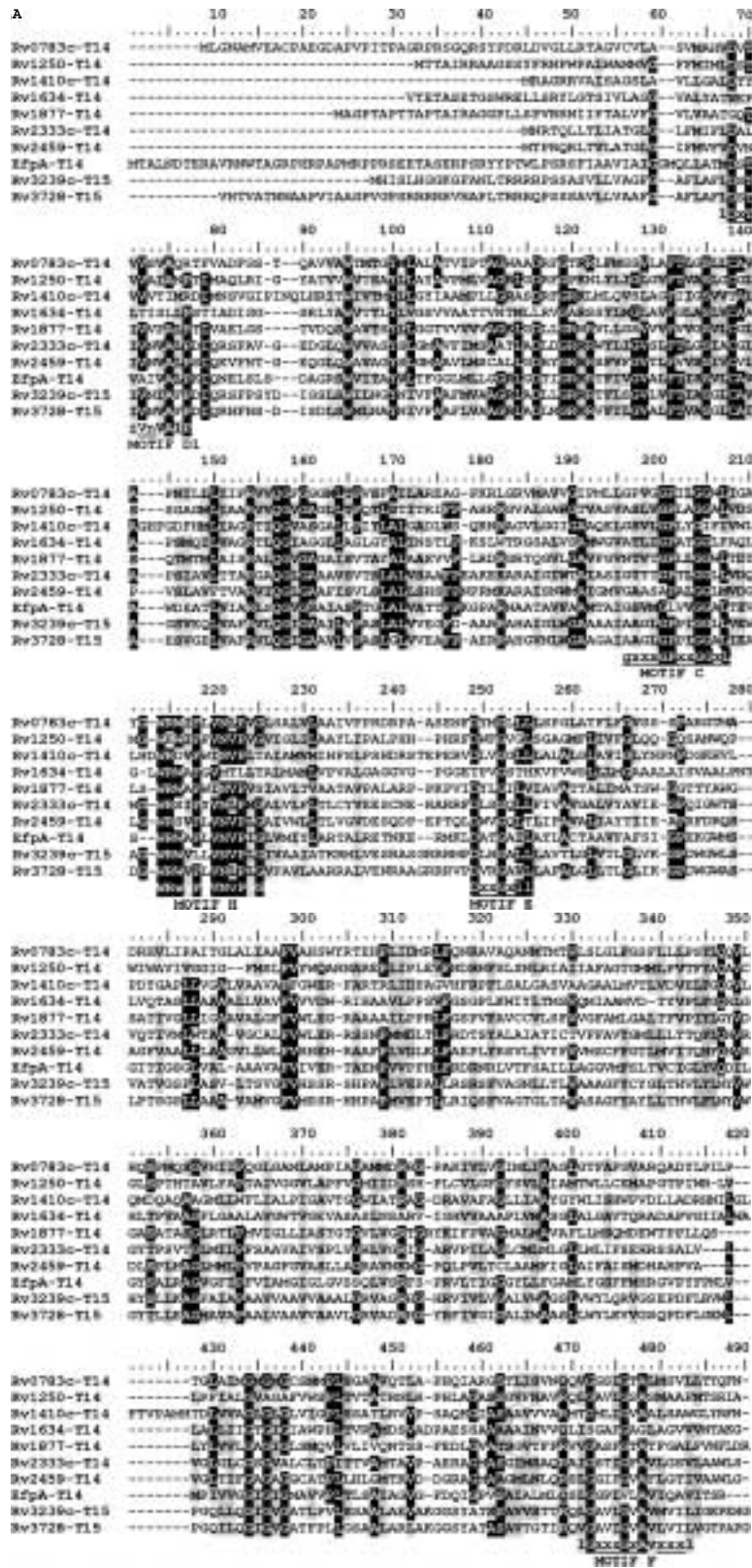
Fig. 2.   Multiple-sequence alignments for members of the mycobacterial 14- and 15-TMS (A), and 12-TMS drug efflux transporters (B), respectively. Details on these alignments can be found in the legend for Figure 1. Motifs D1, H, E, F, and G correspond to the motifs described by Paulsen et al. (16). Motif C, described as characteristic of drug export proteins, is indicated. The alignment shows only the region containing the above-cited motifs. Identical amino acids are highlighted in black boxes; conserved amino acid substitutions are shown in shaded boxes.
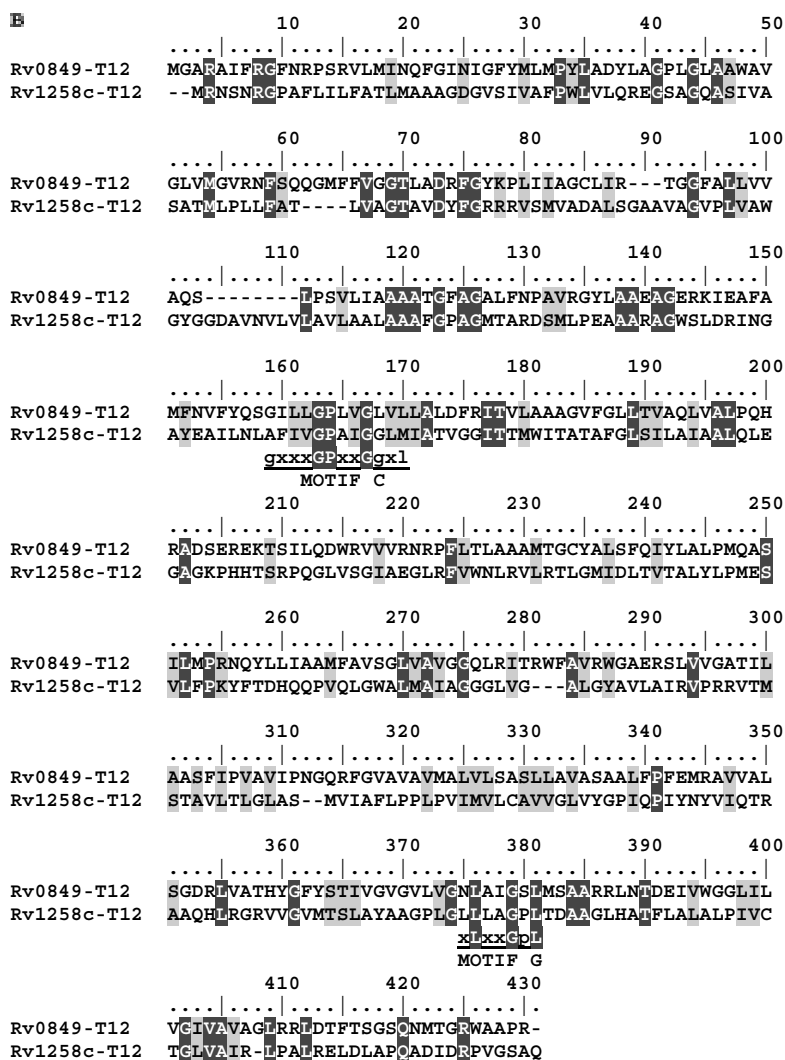
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B                    10        20        30        40        50
             ....|....|....|....|....|....|....|....|....|....|
Rv0849-T12   MGARAIFRGFNRPSRVLMINQFGINIGFYMLMPYLADYLACPLGLAAWAV
Rv1258c-T12  --MRNSNRGPAFLILFATLMAAAGDGVSIVAFPWLVLQRECSAGQASIVA

                     60        70        80        90       100
             ....|....|....|....|....|....|....|....|....|....|
Rv0849-T12   GLVMGVRNFSQQGMFFVGGTLADRFGYKPLIIAGCLIR---TGGFALVV
Rv1258c-T12  SATMLPLLEAT----LVAGTAVDYFGRRRVSMVADALSGAAVAGVPLVAW

                    110       120       130       140       150
             ....|....|....|....|....|....|....|....|....|....|
Rv0849-T12   AQS--------LPSVLIAAAATGFACALFNPAVRGYLAAEAGERKIEAFA
Rv1258c-T12  GYGGDAVNVLVLAVLAALAAAFGPAGMTARDSMLPEAAARAGWSLDRING

                    160       170       180       190       200
             ....|....|....|....|....|....|....|....|....|....|
Rv0849-T12   MFNVFYQSGILLGPLVGLVLLALDFRITVLAAAGVFGLLTVAQLVALPQH
Rv1258c-T12  AYEAILNLAFIVGPAIGGLMIATVGGITTMWITATAFGLSILAIAALQLE
                             gxxxGPxxCgxl
                               MOTIF C

                    210       220       230       240       250
             ....|....|....|....|....|....|....|....|....|....|
Rv0849-T12   RADSEREKTSILQDWRVVVRNRPFLTLAAAMTGCYALSFQIYLALPMQAS
Rv1258c-T12  GAGKPHHTSRPQGLVSGIAEGLRFVWNLRVLRTLGMIDLTVTALYLPMES

                    260       270       280       290       300
             ....|....|....|....|....|....|....|....|....|....|
Rv0849-T12   ILMPRNQYLLIAAMFAVSGLVAVGCQLRITRWFAVRWGAERSLVVGATIL
Rv1258c-T12  VLFPKYFTDHQQPVQLGWALMAIACGGLVG---ALGYAVLAIRVPRRVTM

                    310       320       330       340       350
             ....|....|....|....|....|....|....|....|....|....|
Rv0849-T12   AASFIPVAVIPNGQRFGVAVAVMALVLSASLLAVASAALFPFEMRAVVAL
Rv1258c-T12  STAVLTLGLAS--MVIAFLPPLPVIMVLCAVVGLVYGPIQPIYNYVIQTR

                    360       370       380       390       400
             ....|....|....|....|....|....|....|....|....|....|
Rv0849-T12   SGDRLVATHYCFYSTIVGVGVLVGNIAIGSLMSAARRLNTDEIVWGGLIL
Rv1258c-T12  AAQHLRGRVVCVMTSLAYAAGPLGLLLAGPLTDAAGLHATFLALALPIVC
                                       xLxxGPL
                                        MOTIF G

                    410       420       430
             ....|....|....|....|....|....|.
Rv0849-T12   VGIVAVAGLRRLDTFTSGSQNMTGRWAAPR-
Rv1258c-T12  TGLVAIR-LPALRELDLAPQADIDRPVGSAQ
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Fig. 2. (Continued).

with 14 transmembrane segments (Rv1410c and Rv1877) and by a subgroup of proteins with 12 or less than 12 transmembrane segments, with the exception of Rv1634.

Cloning and Phenotypic Analysis of Hypothetical Drug Transporters

Although most of the proteins identified in this analysis remain uncharacterized, a few of them have been previously shown to function as multidrug transporters; Rv1410c protein was demonstrated to be an efflux pumps, conferring resistance to tetracycline and aminoglycosides (8), similarly to the *M. fortuitum* homolog of Rv1258c (7). In the latter report, the expression of the *M. tuberculosis* Rv1258c gene under the control of the *hsp60* promoter conferred resistance to only tetracycline (but see below for a revision on this phenotype). In the case of Rv2846c, it has been published that this protein does not confer any resistant phenotype to *M. smegmatis* (9).

We decided to further characterize some of the *M. tuberculosis* H37Rv putative drug transporters of the MFS identified in this report.

Plasmids containing ORF Rv1634 conferred resistance to different fluoroquinolones (ciprofloxacin, norfloxacin, ofloxacin, and lomefloxacin) in *M. smegmatis* (Table 3A), demonstrating that a novel fluoroquinolone-resistant determinant is present in *M. tuberculosis*. The first efflux pump described in mycobacteria, LfrA, was identified in *M. smegmatis* because its overexpression conferred resistance to ciprofloxacin. LfrA belongs to the family of proton antiporters and, in addition to the hydrophilic fluoroquinolones, its overexpression also confers resistance to acriflavine and ethidium bromide (5). Disruption of the *lfrA* gene in *M. smegmatis* caused an increased sensitivity to ethidium bromide and acriflavine, but did not significantly affect resistance to ciprofloxacin (28), arguing against a major role of the LfrA in the innate resistance of *M. smegmatis* to

Table 1. List of *M. tuberculosis* ORFs described as hypothetical drug efflux proteins belonging to MFS.

Sequence	Length	Hypothetical Function*	TMS (*N*)	Relative Position of Motif A	Motif B	Motif C
Rv0037c	441	Probable membrane protein	11	89–101	124–136	169–180
Rv0191	413	Probable chloramphenicol resistance protein	12	77–89	111–123	157–168
Rv0783c	540	Multidrug resistance protein	14	99–111	134–146	179–190
Rv0849	419	Possible quinolone efflux pump	12	69–81	104–116	148–159
Rv1250	579	Probable drug efflux protein	14	75–87	110–122	152–163
Rv1258c	419	Probable multidrug resistance pump	12	63–75	102–114	153–164
Rv1410c	518	Probable drug efflux protein	14	66–78	104–116	149–160
Rv1634	471	Probable drug efflux protein	14	75–87	110–122	155–166
Rv1877	687	Probable drug efflux protein	14	83–95	108–120	163–174
Rv2333c	537	Probable tetracenomycin C resistance protein	14	63–75	98–110	145–156
Rv2456c	418	Probable transmembrane transport protein	10	68–80	104–116	149–160
Rv2459	508	Probable drug efflux protein	14	63–75	98–110	145–156
Rv2846c (EfpA)	530	EfpA putative efflux protein	14	109–121	144–156	190–201
Rv2994	445	Probable fluoroquinolone efflux protein	9	69–81	104–116	149–160
Rv3239c	1048	Probable drug efflux protein	15	81–93	116–128	161–172
Rv3728	1065	Possible sugar transporter	15	97–109	132–144	177–188

*As reported by Cole et al. (18).

Table 2A. Common sequence motifs A localized in TMS2 and TMS3 in the mycobacterial drug efflux transporters.

Sequence	Predominant Residue at Position 1 G	2 X	3 L	4 A	5 D	6 R	7 X	8 G	9 R	10 K	11 X	12 X	13 L
Rv0037c	G	A	L	M	D	R	W	D	R	R	W	V	L
Rv0191	V	R	W	T	A	H	W	P	R	R	R	A	L
Rv0783c	G	W	A	A	D	R	F	G	T	R	R	L	F
Rv0849	G	T	L	A	D	R	F	G	Y	K	P	L	I
Rv1250	G	R	L	G	D	R	F	G	P	K	N	L	Y
Rv1258c	G	T	A	V	D	Y	F	G	R	R	R	V	S
Rv1410c	G	R	A	S	D	R	F	G	R	K	L	M	L
Rv1634	N	T	M	L	L	R	V	G	A	R	S	S	Y
Rv1877	G	K	L	G	D	L	L	G	R	N	R	V	L
Rv2333c	A	T	L	A	D	L	D	G	R	R	R	W	Y
Rv2456c	G	A	L	V	D	R	T	T	A	K	R	A	L
Rv2459	A	L	L	G	D	R	Y	G	R	R	R	S	F
Rv2846c	G	R	L	G	D	T	I	G	R	K	R	T	F
Rv2994	G	Y	L	L	D	H	V	G	E	R	M	V	M
Rv3239c	G	R	L	A	D	L	L	G	R	R	R	T	F
Rv3728	G	R	L	A	D	L	M	G	R	K	R	V	F
Consensus	G	R	L	[GA]	D	R	F	G	R	[RK]	R	V	[LF]

Table 2B.   Common sequence motifs B localized in TMS4 in the mycobacterial drug efflux transporters.

	Predominant Residue at Position												
Sequence	**1** **L**	**2** **X**	**3** **X**	**4** **X**	**5** **R**	**6** **X**	**7** **X**	**8** **Q**	**9** **G**	**10** **X**	**11** **G**	**12** **A**	**13** **A**
Rv0037c	G	D	V	P	L	L	V	G	A	L	V	A	N
Rv0191	L	A	A	G	R	V	L	C	A	V	T	H	G
Rv0783c	L	I	I	F	R	V	V	Q	G	F	G	G	G
Rv0849	V	L	I	A	A	A	A	T	G	F	A	G	A
Rv1250	L	I	A	A	R	V	V	Q	G	V	G	A	G
Rv1258c	N	V	L	V	L	A	V	L	A	A	L	A	A
Rv1410c	L	I	A	G	R	T	I	Q	G	V	A	S	G
Rv1634	L	V	A	G	R	T	L	Q	G	I	A	G	G
Rv1877	L	A	I	S	R	A	L	Q	G	V	G	A	G
Rv2333c	L	T	T	A	R	G	A	Q	G	L	G	A	A
Rv2456c	I	S	V	L	Q	A	V	T	G	I	A	S	S
Rv2459	F	T	V	A	R	V	I	Q	G	L	G	A	A
Rv2846c	L	V	I	A	R	L	S	Q	G	V	G	S	A
Rv2994	I	G	V	F	L	F	L	G	G	M	A	A	G
Rv3239c	L	V	A	F	R	V	L	Q	G	I	G	A	A
Rv3728	L	V	A	F	R	V	L	Q	G	I	G	A	A
Consensus	L	V	A	A	R	[VA]	[LV]	Q	G	[VI]	G	A	[GA]

Table 2C.   Common sequence motifs C localized in TMS5 in the mycobacterial drug efflux transporters.

	Predominant Residue at Position											
Sequence	**1** **G**	**2** **X**	**3** **X**	**4** **X**	**5** **G**	**6** **P**	**7** **X**	**8** **X**	**9** **G**	**10** **G**	**11** **X**	**12** **L**
Rv0037c	S	G	A	V	S	A	F	L	G	A	N	F
Rv0191	A	L	V	V	G	S	P	L	T	A	A	M
Rv0783c	G	P	V	G	G	P	I	L	G	G	W	L
Rv0849	G	I	L	L	G	P	L	V	G	L	V	L
Rv1250	A	S	L	V	G	P	L	A	G	G	A	L
Rv1258c	A	F	I	V	G	P	A	I	G	G	L	M
Rv1410c	G	S	V	L	G	P	L	Y	G	I	F	I
Rv1634	A	T	L	I	G	P	A	T	G	G	L	F
Rv1877	N	T	V	T	G	P	L	L	G	G	W	L
Rv2333c	G	T	T	T	G	P	T	L	G	G	L	L
Rv2456c	G	N	A	S	A	A	G	A	T	G	A	L
Rv2459	G	A	A	S	A	P	A	L	G	G	L	M
Rv2846c	G	S	V	M	G	L	V	V	G	G	A	L
Rv2994	G	I	A	S	G	A	L	V	I	P	E	L
Rv3239c	A	A	G	L	G	P	P	I	G	G	L	L
Rv3728	A	A	G	L	G	P	P	I	G	G	A	L
Consensus	[GA]	[TA]	V	[VL]	G	P	L	L	G	G	[AL]	L
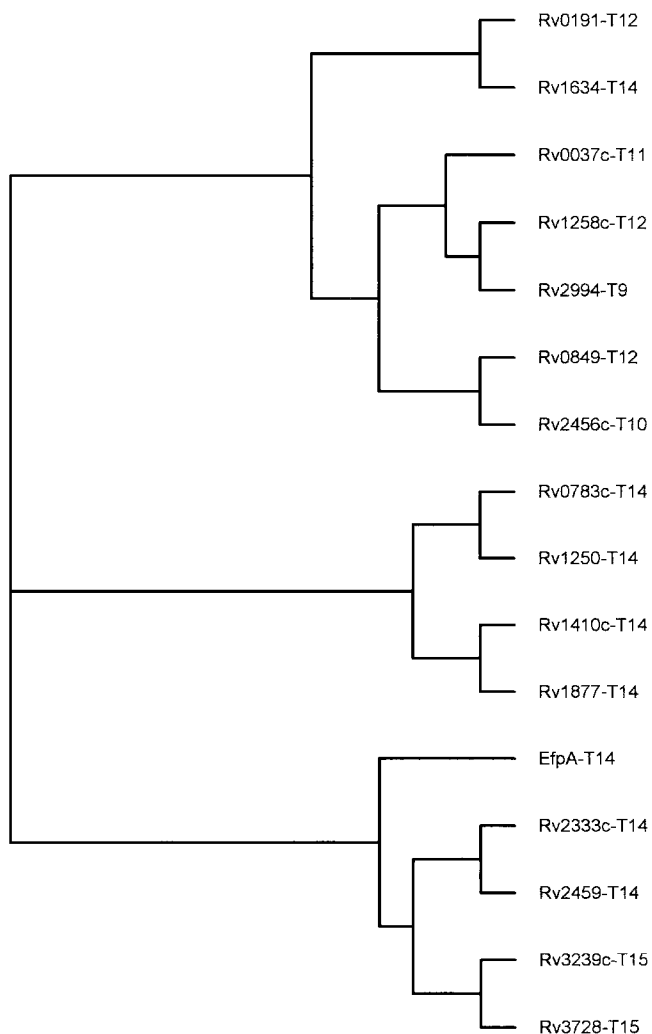
Fig. 3. Phylogenetic tree showing the relationships among the drug transporters subfamily of *M. tuberculosis* belonging to the MFS.

this drug, and suggesting the involvement of additional quinolone transporters in this species. As mentioned, homologs of the *M. smegmatis* *lfrA* gene could not be detected in the *M. tuberculosis* genome; however, we have detected a gene highly homologous to Rv1634 on the *M. smegmatis* chromosome

(data not shown). Consequently, the *M. smegmatis* homologue of Rv1634 could be the real transporter responsible for fluoroquinolones efflux. The *M. leprae* genome contains a nucleotide sequence similar to ORF1634, but no corresponding protein was found in the Leproma website (http://genolist.pasteur.fr/Leproma), suggesting that it may be a pseudogene.

As mentioned, ORF Rv1258c expressed from the *hsp60* promoter conferred resistance to only tetracycline, whereas its *M. fortuitum* homolog also conferred resistance to several aminoglycosides (7). As a part of this work, we decided to make a new construct containing the *M. tuberculosis* Rv1258c and the region upstream of it, where its own promoter should be located. The new plasmid was introduced in *M. smegmatis* and it conferred detectable levels of resistance to tetracycline and aminoglycosides (Table 3B). The *M. tuberculosis* putative efflux pump Rv1258c transports the same antibiotics that its *M. fortuitum* homolog, although the levels of resistance conferred by the *M. tuberculosis* protein are notably lower.

ORFs Rv0037c, Rv0783c, Rv0849, Rv1250, Rv1877, Rv2333c, Rv2459, Rv2994, and Rv3239c were amplified by PCR, cloned into different vectors, and transformed into *M. smegmatis*. MICs of a broad range of compounds (including representative cationic dyes, antimicrobial agents, antiseptics, anticancer drugs, uncouplers, and detergents) were determined for *M. smegmatis* cells harboring these plasmids.

Apparently, none of these genes confer resistance to any of the compounds tested at detectable levels. It is conceivable that either we have not tested the real substrates of these efflux pumps or that the resistance levels are so low that we are unable to detect them using our methodology. Another possibility is that the clones we analyzed do not contain the sequences necessary for its expression. Also, we cannot rule out the possibility that *M. smegmatis* transcription machinery is unable to express some *M. tuberculosis* genes. Further experiments are being carried out to elucidate the substrate profile and the role of these putative efflux pumps.

Table 3A. Drug resistance of *M. smegmatis* cells harboring plasmids carrying putative drug transporter Rv1634.

mc²155 Transformed with	Antibiotics (µg/ml)			
	Ciprofloxacin	Lomefloxacin	Norfloxacin	Ofloxacin
pMD31	0.12	0.06	1.25	0.125
pMD31/Rv1634	0.48	0.24	5.0	0.25
pSUM38	0.12	0.06	1.25	0.125
pSUM38/Rv1634	0.24	0.12	5.0	0.25

Table 3B. Drug resistance of *M. smegmatis* cells harboring plasmids carrying putative drug transporter Rv1258c.

mc²155 Transformed with	Antibiotics (µg/ml)				
	Tetracycline	Streptomycin	Gentamicin	2′-N-Ethylnetilmicin	6′-N-Ethylnetilmicin
pSUM36	0.5	2	2	4	4
pSUM36/Rv1258c	4	4	8	32	16

Accumulation of Norfloxacin

The resistance levels conferred to *M. smegmatis* by Rv1634, cloned into two different vectors, were 2- to 4-fold the MIC value for fluoroquinolones (Table 3A). In general, efflux pumps confer low levels of resistance, which contrast with the high levels conferred by mutations in genes encoding the primary targets of these agents (DNA topoisomerase and DNA gyrase) (29).

Norfloxacin uptake experiments were performed to determine whether *M. smegmatis* cells, carrying the recombinant plasmid pMD31/Rv1634, were more resistant to this drug due to an active drug efflux mechanism. The accumulation of norfloxacin was determined by fluorimetry, as described in Material and Methods.

As shown in Figure 4A, cells harboring the cloning vector pMD31 take up norfloxacin rapidly and achieve a steady-state level of accumulation within about 2–3 min of incubation. This accumu-

lation is approximately 25% lower with cells containing the pMD31/Rv1634 recombinant plasmid. The reduction of norfloxacin uptake was repeatedly observed in several experiments. Analogous results were obtained with ciprofloxacin (data not shown). These accumulation data suggest that Rv1634 could be involved in fluoroquinolones efflux.

Accumulation of Tetracycline

M. smegmatis transformed with a plasmid-borne Rv1258c gene showed an 8-fold increase in the resistance levels to tetracycline (4 µg/ml) in comparison with the same strain transformed with the vector alone (0.4 µg/ml) (Table 3B). We used radiolabeled tetracycline to measure whether this increase of resistance was due to a lower accumulation of this drug.

Accumulation assays showed that the presence of the plasmid encoded Rv1258c gene consistently reduces the levels of tetracycline accumulation by
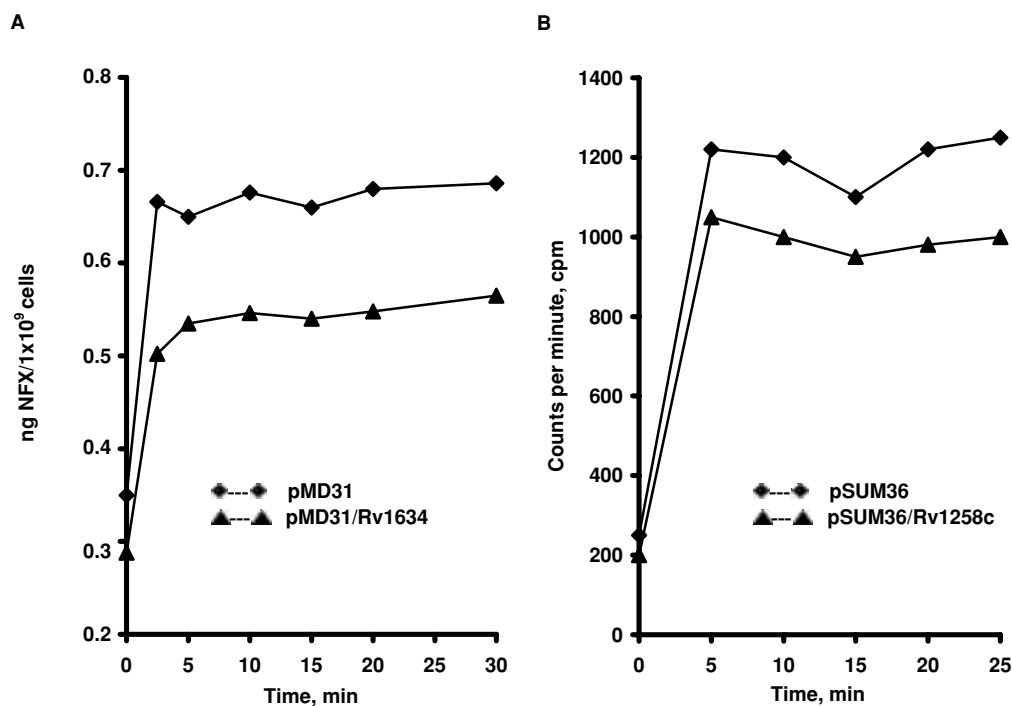

Fig. 4. (A) Accumulation of norfloxacin by *M. smegmatis* cells transformed with pMD31 and pMD31(Rv1634), respectively. (B) Accumulation of titriated tetracycline by *M. smegmatis* cells transformed with pSUM36 and pSUM36(Rv1258c).

10–15% (Fig. 4B), in comparison to the levels of tetracycline accumulation of cells containing only the cloning vector. This result supports the implication of the Rv1258c protein in the efflux of this antibiotic.

Conclusions

The clinical impact of antibiotic efflux pumps on resistance in MTB clinical isolates remains difficult to establish because we lack large-scale and international statistics comparing their prevalence with that of the other resistance mechanisms. This raises the question of the adequacy of the routine procedures used to detect these strains, which most often may be classified as moderately resistant and erroneously assigned to a conventional mechanism of resistance in the absence of further detailed investigation.

Further, the existence of antibiotic efflux pumps and their impact on therapy must now be taken fully into account for the selection of novel antimicrobials. The design of specific, potent inhibitors appears to be an important goal for the improved control of infectious diseases in the near future.

Acknowledgments

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