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Mycobacterium tuberculosis efpA Encodes an Efflux Protein of the QacA Transporter Family

JAMES L. DORAN,[1,2]* YIJUN PANG,[3]† KHIZIMUZI E. MDLULI,[1,2]‡ ALISON J. MORAN,[1,2] TOMMIE C. VICTOR,[4] RICHARD W. STOKES,[2,5,6] ESHWAR MAHENTHIRALINGAM,[2,5,6] BARRY N. KREISWIRTH,[7] JENNIFER L. BUTT,[4] GERALD S. BARON,[1] JANICE D. TREIT,[1,2] VALERIE J. KERR,[1,2] PAUL D. VAN HELDEN,[4] MARILYN C. ROBERTS,[3] AND FRANCIS E. NANO[1,2]

Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 3P6,[1] Canadian Bacterial Diseases Network,[2] and Division of Infectious and Immunological Diseases, British Columbia's Children's Hospital,[5] and Department of Paediatrics, University of British Columbia,[6] Vancouver, British Columbia V5Z 4H4, Canada; Department of Pathobiology, University of Washington, Seattle, Washington 98195[3]; Department of Medical Biochemistry, University of Stellenbosch, Tygerberg, South Africa[4]; and Public Health Research Institute Tuberculosis Center, New York, New York 10016[7]

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The *Mycobacterium tuberculosis* H37Rv *efpA* gene encodes a putative efflux protein, EfpA, of 55,670 Da. The deduced EfpA protein was similar in secondary structure to Pur8, MmrA, TcmA, LfrA, EmrB, and other members of the QacA transporter family (QacA TF) which mediate antibiotic and chemical resistance in bacteria and yeast. The predicted EfpA sequence possessed all transporter motifs characteristic of the QacA TF, including those associated with proton-antiport function and the motif considered to be specific to exporters. The 1,590-bp *efpA* open reading frame was G+C rich (65%), whereas the 40-bp region immediately upstream had an A+T bias (35% G+C). Reverse transcriptase-PCR assays indicated that *efpA* was expressed in vitro and in situ. Putative promoter sequences were partially overlapped by the A+T-rich region and by a region capable of forming alternative secondary structures indicative of transcriptional regulation in analogous systems. PCR single-stranded conformational polymorphism analysis demonstrated that these upstream flanking sequences and the 231-bp, 5′ coding region are highly conserved among both drug-sensitive and multiply-drug-resistant isolates of *M. tuberculosis*. The *efpA* gene was present in the slow-growing human pathogens *M. tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium bovis* and in the opportunistic human pathogens *Mycobacterium avium* and *Mycobacterium intracellular*. However, *efpA* was not present in 17 other opportunistically pathogenic or nonpathogenic mycobacterial species.

Tuberculosis is the single leading cause of human mortality due to infectious disease. Annually, there are over 8 million new active cases and 3 million deaths from tuberculosis (53). There has been an unexpected and dramatic rise in tuberculosis cases in the United States since 1989 (52). This increase has occurred primarily among inner-city homeless people, people living in closed communities such as correctional facilities and hospitals, and among human immunodeficiency virus-infected individuals. While the incidence of tuberculosis in developing countries is linked in large part to the severity of the human immunodeficiency virus pandemic (53), the emergence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* has greatly heightened the probability of tuberculosis epidemics in industrialized countries (7, 8, 13). In 1991, 26% of *M. tuberculosis* isolates in the United States were found to be resistant to at least one antibiotic, 9.5% were resistant to isoniazid or rifampin, and 3.5% were MDR (defined by resistance to both isoniazid and rifampin) (8). Of these MDR strains, 61.4% were isolated in New York City. One New York City strain, referred to as the W strain, is resistant to all of the front-line antibiotics, namely, isoniazid, rifampin, ethambutol, streptomycin, ethionamide, kanamycin, and rifabutin (7).

While the genetic or biochemical changes that contribute to resistance to isoniazid, ethionamide, rifampin, ethambutol, or streptomycin are often understood (4, 10, 19, 20, 34, 36, 49, 50, 70), in a proportion of *M. tuberculosis* MDR strains, the basis of multiple drug resistance has not been fully defined (26, 45, 61). Since efflux proteins are known to have important roles in resistance to a variety of unrelated antibacterial compounds in many gram-positive and gram-negative bacteria (28, 39, 42), it is of interest to discover and characterize efflux proteins of *M. tuberculosis*. This study describes the first identification and characterization of an *M. tuberculosis* gene that encodes a putative efflux protein. EfpA is predicted to be highly related to members of the QacA transporter family (QacA TF) (15), which is also known as the drug resistance transporter family (32), that is a part of the large, superfamily of non-ABC transporters possessing 12 to 14 transmembrane helices (15, 18). Although all other members of the QacA TF transport antibacterial compounds, no evidence was found linking *efpA* with drug resistance in *M. tuberculosis.*

* Corresponding author. Mailing address: Department of Biochemistry and Microbiology, Petch Building, P.O. Box 3055, University of Victoria, Victoria, British Columbia V8W 3P6, Canada. Phone: (250) 472-4168. Fax: (250) 721-8885. E-mail: jdoran@uvic.ca.

† Present address: Department of Medical and Research Technology, University of Maryland Medical School, 400E 100 Penn St., Baltimore, MD 21201.

‡ Present address: National Institutes of Health, National Institute of Allergy and Infectious Disease, Rocky Mountain Laboratories, Hamilton, MT 59840.

MATERIALS AND METHODS

Bacterial strains and media. Isolates of slow-growing mycobacterial species and isolates of some fast-growing species were incubated at 37°C on Middle-

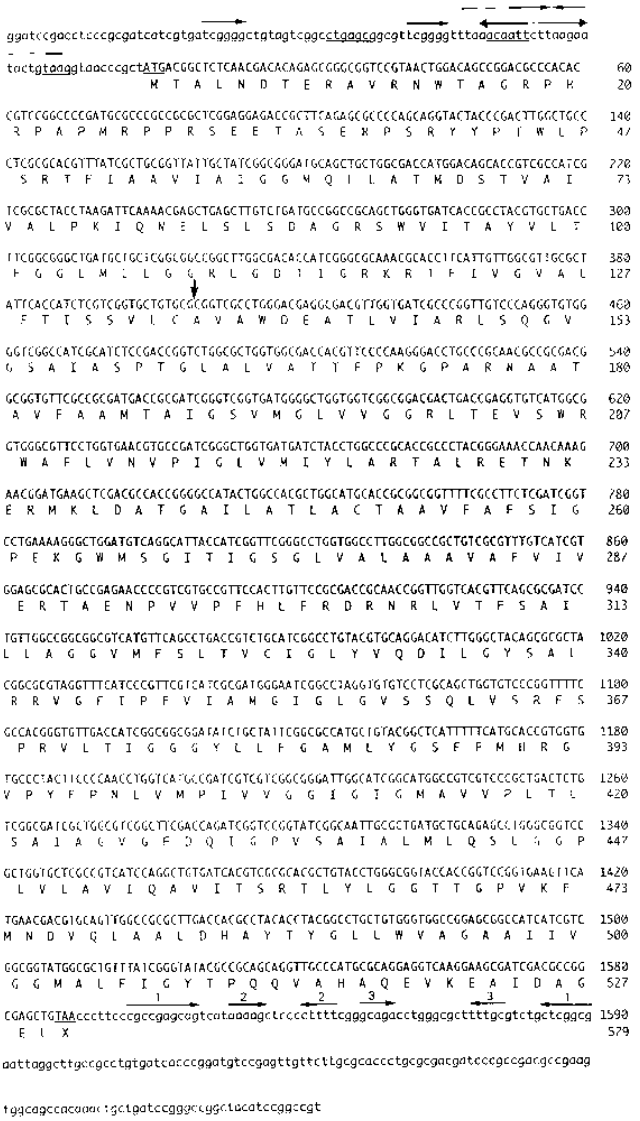

FIG. 1. Nucleotide sequence of *efpA* (uppercase letters) and flanking regions (lowercase letters) and the predicted amino acid sequence of EfpA. The numbering of the nucleotide and amino acid sequences refers only to the *efpA* ORF and the predicted protein product. The positions of sequences encoding the presumed AUG initiation codon and UAA termination codon are indicated by the underlying EfpA amino acid sequence. A putative Shine-Dalgarno ribosome binding motif (UAAGG), located 9 bp upstream of the start codon, and potential −10 (ACAATT) and −35 (CTGAGC) promoter sequences recognized in the upstream sequence are underlined. Directly repeated sequences and inverted repeat sequences in the upstream and downstream flanking regions and a near-palindromic sequence in the upstream region are indicated by overlying horizontal arrows. The numbers accompanying the arrows, overlying the sequence immediately 3′ to the *efpA* ORF, denote complementary regions capable of contributing to a stable mRNA secondary structure. The position in the *efpA* gene at which the fusion was created in pJDT1H87 is indicated by a vertical arrow. The GenBank/EMBL accession number for the *efpA* sequence is L39922.

brook 7H10 or 7H11 agar containing ADC enrichment (Difco Laboratories, Detroit, Mich.) or in Middlebrook 7H9 broth (Difco) containing ADC and 0.02% Tween 80. For the growth of cultures of *M. tuberculosis* or *Mycobacterium bovis*, bovine serum albumin (fraction V) was added to the medium to a final concentration of 5 mg liter^{-1}. In some instances, the cultures were grown from a large inoculum for 5 days, at which time glycine was added and the cultures were incubated for an additional 2 days. Most cultures of fast-growing mycobacteria and all *Streptomyces* and *Nocardia* strains were grown in Mueller-Hinton broth (Difco) containing 0.02% Tween 80 at 35°C for 2 days, and then glycine was added to 1% prior to an additional 24 h of incubation.

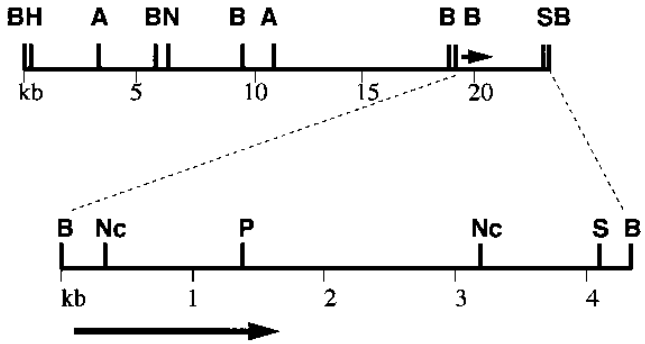

FIG. 2. Physical maps of regions of the cosmid pJD5C4 (upper) and the plasmid pKEM10 (lower) encoding *efpA*. Restriction site abbreviations: A, *Ase*I; B, *Bam*HI; H, *Hin*dIII; Nc, *Nco*I; N, *Nde*I; S, *Spe*I. The arrows indicate the position and orientation of *efpA*.

Construction of *phoA* fusion libraries. Recombinant DNA clone banks of the virulent *M. tuberculosis* strain H37Rv were constructed with 1- to 3-kb fragments generated by partial digestion (51) with *Hin*PI, *Hpa*II, *Taq*I, or *Aci*I and inserted into the *Bst*BI sites of one of three pJDT, frame series, *phoA*-fusion vectors (33). The recombinant plasmids were transformed into *Escherichia coli* DH5α (51) and then isolated and transformed into *E. coli* KS330, which lacks a periplasmic protease that often degrades periplasmic fusion proteins (55). Colonies expressing enzymatically active alkaline phosphatase fusion proteins were identified by their ability to hydrolyze the chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate (14).

Genomic library screening by DNA sequence analysis. *phoA* fusions were partially characterized by DNA sequence analysis conducted with the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals, Cleveland, Ohio), the *Taq*-Track sequencing system (Promega Corp., Madison, Wis.), and/or the Ampli-Cycle manual sequencing kit (Perkin-Elmer Applied Biosystems Canada Inc., Mississauga, Ontario) and the Perkin-Elmer GeneAmp 9600 thermal cycler. These reactions were directed by the oligodeoxynucleotide primer ′CACGCAG AGCGGCAGTCTGATC′, which is complementary to a region of the *phoA* gene located 48 bp upstream of the junction with cloned *M. tuberculosis* DNA. In an attempt to rapidly determine the nature of the *M. tuberculosis* genes from which partial DNA sequence data was obtained, potential translations were compared by alignment to known and predicted protein sequences using the BLASTX program (1) available at the National Center for Biotechnology Information at the National Library of Medicine (Bethesda, Md.).

***efpA* cloning and sequencing.** A 4.3-kb *Bam*HI fragment containing the *efpA* gene was identified in genomic digests of *M. tuberculosis* H37Rv DNA by Southern hybridization (51) using a fluorescein-labeled probe (enhanced chemiluminescence random primer labeling system; Amersham Canada Ltd., Oakville, Ontario) prepared from the approximately 425-bp *efpA* fragment encoded on pJDT1H87. The hybridizing 4.3-kb *Bam*HI fragment was gel purified (51) and subcloned into pRL498 (11) to form pKEM10.

The sequence of the *efpA* gene was determined with a series of small, overlapping restriction fragments subcloned in pUC18 (68) or pTZ18U (Bio-Rad Laboratories, Richmond, Calif.) and harbored in *E. coli* DH5α (51). DNA sequencing was conducted with the universal forward or reverse sequencing primers (68) or with one of several oligonucleotide primers specific to *efpA*.

The sequence of *efpA* was analyzed with programs available in the GeneWorks and PCGENE suites (Intelligenetics, Mountain View, Calif.) and the Lasergene biocomputing package (DNASTAR, Madison, Wis.). Confirmation of the extent of the *efpA* open reading frame (ORF) by analysis of the G+C content by codon position was conducted with the FRAME program of Bibb et al. (6). The stability of secondary structures was determined as described by Tinoco et al. (60) or with the GeneWorks RNAFOLD program. The BLASTX, BLITZ (57), and TMAP (43) programs were used to search existing data banks for proteins which were related to EfpA by primary sequence and to estimate the degree of similarity on the basis of primary sequence and predicted secondary structure. The predicted amino acid sequence of EfpA was analyzed for potential transmembrane domains with the TMAP and PHD-PredictProtein (24) programs available at the websites http://www.embl-heidelberg.de/tmap/tmap_sin.html and http://www.public.iastate.edu/~pedro/pprotein_query.html, respectively, and by use of the Kyte-Doolittle algorithm (27) available in the GeneWorks suite. Multiple sequence alignments were performed with the CLUSTAL program available in the Lasergene suite and by use of the TMAP program.

Mapping *efpA* in plasmid and cosmid clones. A cosmid, pJD5C4, encoding *efpA* was identified by hybridization to a genomic library of *M. tuberculosis* H37Rv DNA prepared in the cosmid pYUB328 (3) and harbored in *E. coli* LE392. pKEM10 and pJD5C4 were purified with the Nucleobond-AX plasmid purification system (Macherey-Nagel, Düren, Germany). The restriction maps of

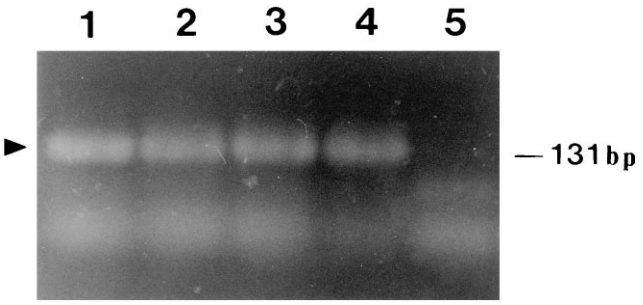

FIG. 3. RT-PCR amplification of a 131-bp fragment of *efpA* mRNA. Lanes: 1, PCR fragment amplified from pKEM10 (positive control); 2, RT-PCR fragment generated with mRNA prepared from *M. tuberculosis* H37Rv grown in broth culture for 1 day; 3, RT-PCR fragment generated with mRNA prepared from *M. tuberculosis* H37Rv grown in peritoneal macrophage culture for 1 day; 4, RT-PCR fragment generated with mRNA prepared from *M. tuberculosis* H37Rv isolated from a lysed (7-day) peritoneal macrophage culture; 5, the results of a PCR using a sample of mRNA prepared from *M. tuberculosis* H37Rv grown in peritoneal macrophage culture for 1 day (no-RT control).

pKEM10 and pJD5C4 were determined by analyzing the results of a series of single and double restriction endonuclease digests and with Southern hybridizations (51).

Growth of *M. tuberculosis* in macrophages. Cells of *M. tuberculosis* H37Rv were grown in murine peritoneal macrophages, in three-well tissue culture plates, as described previously (54). Approximately 10^6 peritoneal exudate cells were added per well to provide about 5×10^5 adherent macrophages. The macrophages were maintained in complete medium (RPMI medium plus 10% fetal calf serum, 10 mM L-glutamine, and 10 mM sodium pyruvate) at 37°C in a 5% CO_2 environment. After 4 h of incubation, nonadherent cells were removed by washing, fresh medium was added, and the macrophage cultures were incubated overnight. Macrophages were infected by the addition of 5×10^6 cells of *M. tuberculosis* H37Rv to each well (a multiplicity of infection of approximately 10:1). After 3 h of incubation, uningested *M. tuberculosis* H37Rv cells were washed off and fresh medium was added to cultures prior to incubation for 1 to 7 days. Simultaneously, static broth cultures of strain H37Rv growing anexically in Middlebrook 7H9 medium or RPMI medium were set up at an initial concentration of 6.8×10^6 cells ml^{-1} for comparison with the mycobacteria grown in macrophage tissue culture.

For each time point following infection with *M. tuberculosis* H37Rv (3 h and 1, 3, and 7 days), three infected resident mouse macrophage monolayers and *M. tuberculosis* cells grown in broth culture and in tissue culture medium were used for mRNA extraction as described below. Two monolayers that were grown on glass coverslips were processed to monitor infection. One coverslip was fixed (Formol-ethanol), stained (Ziehl-Neelsen), and examined microscopically to assess the distribution of mycobacteria within the macrophage population. A second coverslip was processed to assess the numbers of viable CFU within the macrophage population. It was placed in 500 μl of 1% Tween 80 in saline and sonicated for 10 s to release the cells of *M. tuberculosis* from the macrophages. Tenfold serial dilutions of the sonicate were plated on Middlebrook 7H10 agar, and the resultant colonies were counted after 3 to 4 weeks of incubation at 37°C.

mRNA purification. The extraction of intact mRNA from mycobacteria was conducted by use of a novel, simple, and rapid method. *M. tuberculosis* H37Rv was grown in vitro in Middlebrook 7H9 medium containing 0.05% Tween 80, in RPMI medium, or in macrophages as described above. Cells of strain H37Rv grown alone in culture were harvested by centrifugation and resuspended in 1 ml of guanidinium isothiocyanate-based extraction buffer (5 M guanidinium isothiocyanate, 25 mM sodium acetate [pH 5.2], 0.1 mM dithiothreitol, and 1% lauryl sarcosine in diethylpyrocarbonate-treated, deionized water) to prevent RNA degradation. To the sample contained in a 2-ml screw-cap microcentrifuge tube (Sarstedt, St. Laurent, Quebec, Canada), approximately 1 ml of heat-sterilized, 0.1-mm-diameter zirconium beads (Biospec Products, Bartlesville, Okla.) was added. The cells were disrupted by 3 min of high-speed reciprocation on a Mini Beadbeater cell disrupter (Biospec Products). Beads and cellular debris were sedimented by centrifugation (17,000 × *g*, 3 min, 4°C), and the cleared lysate (500 to 700 μl) was retained. To improve the yield of nucleic acid, a small volume of chloroform (700 μl) was added to the beads, and the tube was spun briefly to displace the aqueous volume above the beads. The lysates were pooled and extracted with an equal volume of neutral phenol-chloroform (1:1) and then extracted with an equal volume of chloroform. Following the addition of 0.05 volume of 5 M NaCl to the remaining extract, the RNA and DNA were precipitated by the addition of 2.5 volumes of ethanol (−70°C, 1 h). The nucleic acids were collected by centrifugation, washed with 70% ethanol, dried, and dissolved in 500 μl of fresh guanidinium extraction buffer. Phenol-chloroform extraction, followed by chloroform extraction, was repeated to remove residual RNase activity, and the nucleic acids were collected by ethanol precipitation. DNA was digested by dissolving the nucleic acid pellet in 100 μl of 10 mM $MgCl_2$–20 mM Tris-HCl [pH 8] and incubating the sample with 5 U of RNase-free DNase

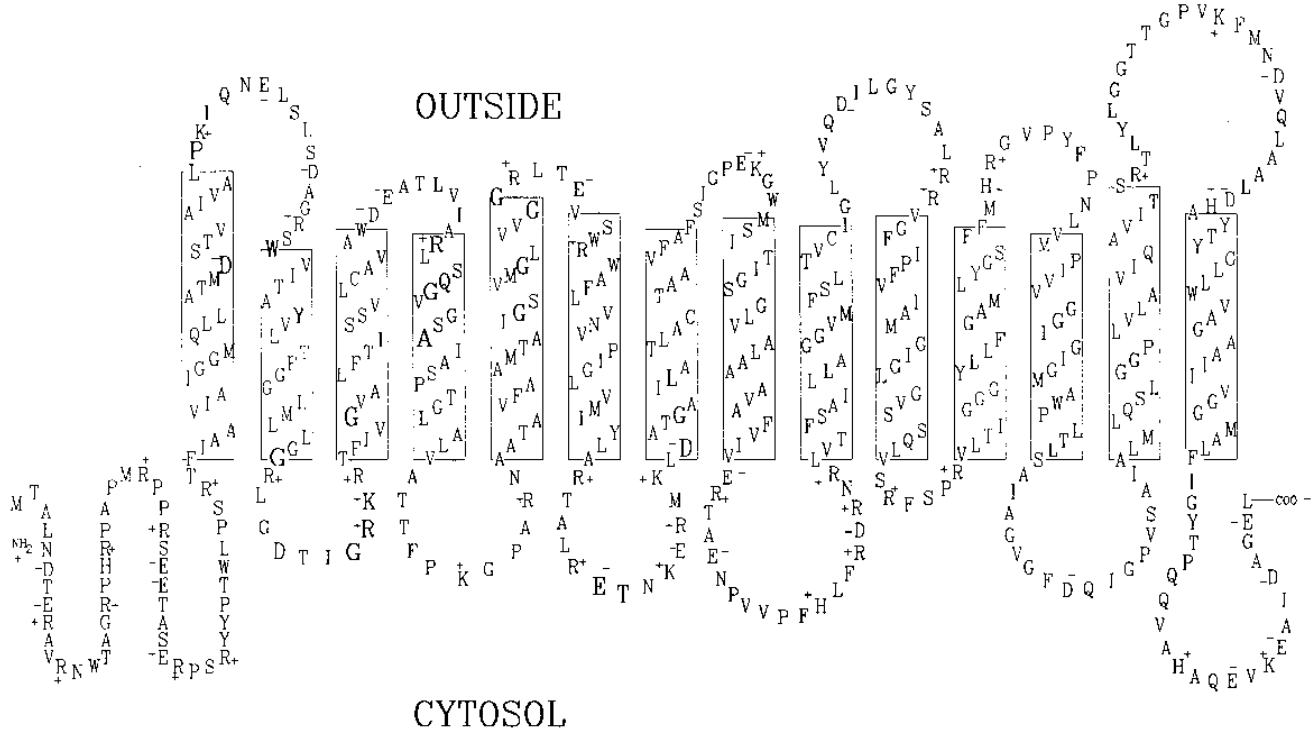

FIG. 4. Schematic representation of the predicted secondary structure of EfpA. The 14 potential TMHs are boxed. Residues conserved among the QacA TF appear in boldface type.

TABLE 1. Degree of sequence similarity between EfpA and related prokaryotic and eukaryotic transporters

Transporter family and protein	Function	Bacterial species	% Similarity[a]	Similarity score by:	
				BLAST[b]	BLITZ[c]
Family III—QacA TF (13 to 14 transmembrane domains)[d]					
Pur8	Puromycin resistance	*Streptomyces alboniger*	37	337	603
CmcT	Cephamycin export	*Nocardia lactamdurans*	35	292	553
LmrA	Lincomycin transporter	*Streptomyces lincolnenesis*	34	225	427
MmrA	Methylenomycin A resistance	*Streptomyces coelicolor*	33	243	448
MmrA	Methylenomycin A resistance	*Bacillus subtilis*	31	284	455
TcmA	Tetracenomycin C resistance	*Streptomyces glaucescens*	26	257	427
LfrA	Fluoroquinolone resistance	*Mycobacterium smegmatis*	25	223	ND[e]
QacA	Multiple antiseptic resistance	*Staphylococcus aureus*	24	209	335
ActII-2	Putative actinorhodin transporter	*Streptomyces coelicolor*	25	223	397
EmrB	Multidrug resistance	*Escherichia coli*	19	130	378
SmvA	Methyl viologen resistance	*Salmonella typhimurium*	ND	187	315
EmrB	Multidrug resistance	*Haemophilus influenza*	ND	153	367
Atr1	Aminotriazole resistance	*Saccharomyces cerevisiae*	ND	110	264
Sge1	Crystal violet resistance	*Saccharomyces cerevisiae*	ND	160	198
TetL	Tetracycline resistance	*Bacillus stearothermophilus*	ND	144	198
TetK	Tetracycline resistance	*Staphylococcus aureus*	ND	137	188
Family II (12 transmembrane domains)[d]					
Bmr	Multidrug efflux	*Bacillus subtilis*	ND	110	152
NorA	Quinolone export	*Staphylococcus aureus*	ND	114	150

[a] Sequence pair distances of 11 aligned transporters, including EfpA, were determined with CLUSTAL with a PAM250 residue weight table.
[b] BLASTX similarity scores were determined as described by Altschul et al. (1). The seven highest-scoring segment pairs as well as lower-scoring representative matches are shown. BLASTX scores greater than 90 are considered indicative of some homology between high G+C sequences.
[c] BLITZ similarity scores were determined as described by Sturrock and Collins (57). The 14 highest scores as well as lower-scoring, representative matches are shown.
[d] Family II and family III designate related families of transport proteins sharing a common mechanism of action (15). The QacA transporter family is named by the method of Henderson (18).
[e] ND, not determined.

(Boehringer Mannheim, Laval, Quebec, Canada) for 1 h at 37°C. DNase was removed by phenol-chloroform extraction, and the RNA was collected by ethanol precipitation, dissolved in water, and stored at −70°C. The method yielded approximately 100 μg of RNA per 100 mg (wet weight) of mycobacteria.

To extract mRNA from macrophages infected with *M. tuberculosis* H37Rv, infected macrophages were washed extensively with phosphate-buffered saline to remove extracellular bacteria. The monolayers were then lysed by the addition of 300 μl of guanidium extraction buffer to each well. The pooled lysates (approximately 1 ml) were added to an equivalent amount of heat-sterilized, 0.1-mm-diameter zirconium beads in a 2-ml screw-cap tube. The cells of *M. tuberculosis* were disrupted with a Mini Beadbeater, and mRNA was purified as described above.

RT-PCR. cDNA was prepared from *M. tuberculosis* mRNA with random hexamer primers and the Superscript II reverse transcriptase (RT) kit (Gibco BRL, Gaithersburg, Md.). Following RNase H digestion, a unique 131-bp fragment of *efpA* (encompassing base pair positions 241 to 371 [see Fig. 2]) was amplified from the cDNA pool by PCR using the primers AACGAGCTGAGC TTGTCTGATGCC and CCAACAATGAAGGTGCGTTTGCG. PCR was conducted for 30 cycles of denaturation (95°C, 1 min), annealing (61°C, 1 min), and extension (72°C, 3 min) with a Perkin-Elmer 9600 thermal cycler. Control reactions were conducted with mRNA samples that had not been subjected to RT. The PCR products were analyzed by electrophoresis in an agarose (2.0%) gel with a TAE (40 mM Tris-acetate, 1 mM EDTA) buffer system (51).

Determination of the distribution of *efpA*. Two DNA probes were used to determine the distribution of *efpA* among *Mycobacterium* spp. by hybridization. One probe was produced by PCR amplification of a 724-bp region from base pair positions 560 to 1284 (see Fig. 2) of the *M. tuberculosis* H37Rv *efpA* gene. PCR amplification was conducted with the primers GCATCTCCGACCGGTCTGG and GTAGGGCACACCACGGTGC and 30 cycles of denaturation (95°C, 1 min), annealing (65°C, 1 min), and extension (72°C, 3 min). The 724-bp probe was isolated by agarose-TAE gel electrophoresis and purified with the Nucleotrap extraction kit (Macherey-Nagel). A second DNA probe was prepared by the isolation of a 478-bp *Bam*HI DNA fragment from pJDT1-H87 which encoded the region described below (see Fig. 2) from base pair positions −99 to 379. Nonradioactive DNA probes were prepared with the Genius system (Boehringer Mannheim, Indianapolis, Ind.) or enhanced chemiluminescence labeling kit (Amersham Life Sciences, Inc., Arlington Heights, Ill.). ^{32}P-labeled probes were prepared by random-hexamer primer extension (51).

Genomic DNA was purified from *Mycobacterium* spp. or from *Streptomyces* or *Nocardia* spp. as described previously (21, 22). All isolates were examined by Southern hybridization (51) to genomic digests prepared with *Bam*HI, *Eco*RI, or *Hin*dIII except for certain isolates of *M. bovis* which were analyzed for the presence of *efpA* by dot blot analysis (MegaGraph membranes; Micron Separations Inc.) (see Table 2). Hybridization was conducted at 61 or 68°C under stringent conditions (40, 51). In some instances, the distribution of *efpA* was confirmed by PCR amplification of the 724-bp fragment from genomic DNA samples as described above.

Determination of the distribution of *tetK* and *tetL*. The distributions of *tetK* and *tetL* among various *Mycobacterium*, *Nocardia*, and *Streptomyces* spp. were determined by hybridization and PCR as described previously (40, 41).

PCR-SSCP analysis of clinical isolates. DNA samples from 40 MDR and 10 drug-susceptible clinical isolates of *M. tuberculosis* originating in South Africa were isolated as described previously (63). Overlapping primer sets efpa151 (dGATCCGACCTCCCGCGATCA)-efpa131 (dGCGCTCTGAAGCGGTCTC CT) and efpa251 (dCCGTAACTGGACAGCCGGAC)-efpa231 (dTAGGTAG CGCGACGATGGCG) were designed to amplify 209- and 200-bp regions located immediately upstream of *efpA* or at the 5′ end of *efpA*, respectively. Amplifications were done in 100-μl reaction volumes and conducted for 30 cycles at 93°C for 3 min, 66°C for 1 min, and 72°C for 2 min with a Medtech Multigene thermal cycler. Single-stranded, conformational polymorphism (SSCP) analysis was performed under the conditions described previously (45).

RESULTS

Cloning and sequencing of *efpA*. Approximately 300 recombinant clones of *M. tuberculosis* DNA fragments encoding membrane or secreted proteins were screened by DNA sequence analysis. By comparison of potential translations of the *M. tuberculosis* DNA sequences with known or predicted protein sequences, the fragment cloned in pJDT1H87 (Fig. 1) was found to encode a polypeptide similar to bacterial efflux proteins responsible for drug or antiseptic resistance. Hence, the corresponding gene was designated *efpA* (efflux protein A).

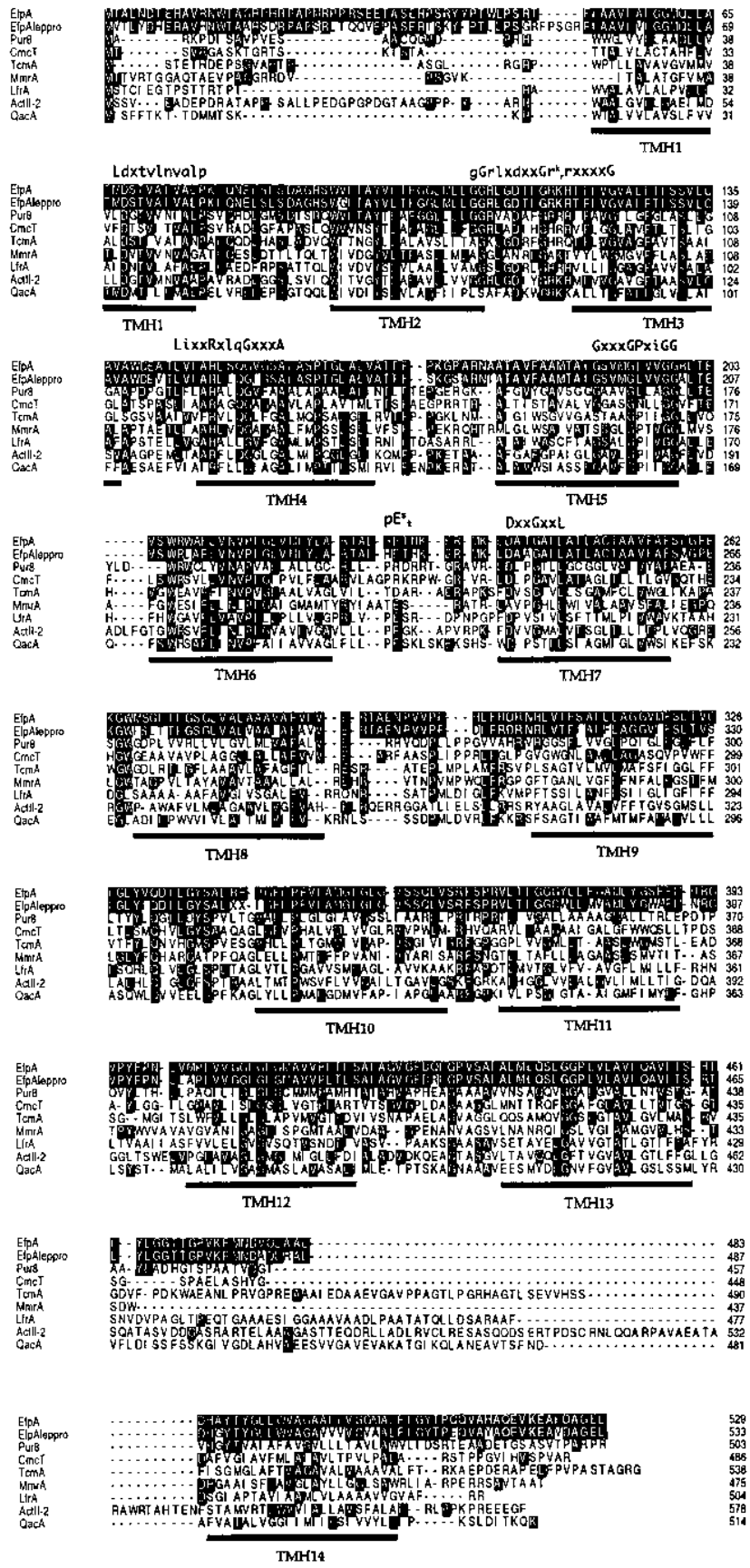

FIG. 5. Sequence similarity alignments of *M. tuberculosis* EfpA, the *M. leprae* EfpA homolog, *Streptomyces alboniger* Pur8 (59), *N. lactamdurans* CmcT (9), *Streptomyces glaucescens* TcmA (16), *Streptomyces coelicolor* MmrA (37), *M. smegmatis* LfrA (58), *Streptomyces coelicolor* ActII-2 (12), and *S. aureus* QacA (48). Amino acid identities with reference to EfpA are highlighted. These alignments were determined by combining the results of an analysis using CLUSTAL, available in the Lasergene suite, and one using TMAP to predict transmembrane domains. The conserved motifs of the QacA TF are indicated above the aligned amino acid sequences. The positions of the potential 14 TMHs of EfpA, which were closely aligned with those of other members of the QacA TF, are indicated by the labeled underlying lines.

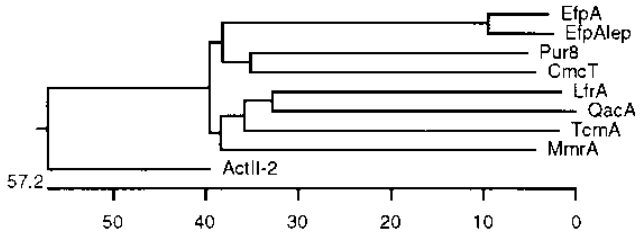

FIG. 6. Dendrogram indicating the phylogenetic relationships between the efflux proteins *M. tuberculosis* EfpA, the *M. leprae* EfpA homolog, *Streptomyces alboniger* Pur8, *N. lactamdurans* CmcT, *Streptomyces glaucescens* TcmA (17), *Streptomyces coelicolor* MmrA, *M. smegmatis* LfrA, *Streptomyces coelicolor* ActII-2, and *S. aureus* QacA, all of which are members of the QacA TF. Pairwise comparisons were conducted by use of a PAM250 residue mutational matrix and the sequence alignments represented in Fig. 5. The length of each pair of branches corresponds to the degree of sequence divergence. The scale at the bottom indicates the distance between sequences measured as the number of substitution events.

A 4.3-kb *Bam*HI fragment containing the *efpA* gene (Fig. 2) was identified in genomic digests of *M. tuberculosis* H37Rv DNA by Southern hybridization and cloned into pRL498 to form pKEM10. DNA sequence analysis revealed that the *efpA* gene encoded an ORF of 1,590 bp (GenBank accession number L39922) (Fig. 1), the extent of which was confirmed by analysis of the G+C content as a function of codon position (data not shown). The *efpA* ORF had a G+C composition of 65%, whereas the 40-bp region immediately upstream of the *efpA* ORF was A+T rich (35% G+C) (Fig. 1). A Shine-Dalgarno motif (UAAGG) was located 9 bp upstream of the putative *efpA* start codon and had a predicted binding strength of -9.4 kcal $\cdot$ mol^{-1}. Potential -10 (ACAATT) and -35 (CTGAGC) σ^{70}-type promoter sequences located 16 bp apart were recognized by comparison with putative σ^{70}-type *Mycobacterium* and *Streptomyces* promoters (25, 56). Overlapping the apparent -10 promoter box was a punctuated, inverted-repeat sequence of 26 bp and a 15-bp near-palindromic sequence (AACAATT:C:TTAAGAA) (Fig. 1). The 74-bp region distal to *efpA* encoded mRNA sequences capable of assuming a stable, complex, secondary structure ($\Delta G^\circ = -19$ kcal $\cdot$ mol^{-1}) reminiscent of an attenuated transcription terminator.

***efpA* expression in vitro and in situ.** RT-PCR assays demonstrated that *efpA* mRNA was produced by *M. tuberculosis* H37Rv growing in peritoneal macrophage tissue culture for 3 h to 3 days (Fig. 3). During this time period, the level of infection of the macrophages increased from 56 to 78% as the numbers of *M. tuberculosis* cells per macrophage culture increased from 3.5×10^4 to 9.5×10^4. *efpA* mRNA was also detected in cells of strain H37Rv obtained following destruction of the macrophage cultures. Over the same 3-day period, *efpA* mRNA was detected in Middlebrook 7H9 broth cultures grown from 6.8×10^6 to 3.3×10^7 cells ml^{-1} (Fig. 3). Therefore, *efpA* was expressed in situ and in vitro.

Predicted secondary structure of EfpA. *efpA* encoded a protein (EfpA) of 529 amino acids (Fig. 1) with a molecular mass of 55,670 Da. Codon usage in *efpA* was characteristic of *M. tuberculosis* genes (data not shown) (2). Kyte-Doolittle analysis and comparative sequence analyses (see below) indicated that EfpA contained 14 transmembrane helices (TMHs) (Fig. 4), although secondary-structure analysis using the PHD-PredictProtein algorithm suggested only 13 TMHs. The TMHs were predicted to be oriented within the membrane with consideration to the asymmetrical distribution of positively charged amino acids (62) between the cytosolic (18%) and external loops (11%) and by comparison with closely related members of the QacA TF (Fig. 4). The truncated EfpA-PhoA fusion protein expressed from pJDT1H87 involved a fusion of PhoA just prior to the TMH3 to TMH4 loop (Fig. 1 and 4). TMH1, TMH4, TMH6, and TMH7 were predicted to be amphipathic. The identification of a Shine-Dalgarno box and a highly A+T-rich region proximal to the presumed initiation codon indicated that EfpA possessed a long, positively charged, N-terminal leader sequence unless processed. This region and a short charged C-terminal sequence were predicted to exist on the cytoplasmic side of the membrane (Fig. 4).

Relatedness of EfpA to prokaryotic and eukaryotic efflux proteins. Comparison of the EfpA primary sequence with known and predicted protein sequences by use of the BLASTX, BLITZ, and TMAP programs revealed a high degree of similarity to sequences of members of the QacA TF, each possessing 13 or 14 TMHs and each responsible for antibiotic or antiseptic efflux (Table 1, and Fig. 5 and 6). Pairwise comparisons conducted with the CLUSTAL program and a PAM250 residue mutational matrix provided significant alignment scores in the range of 18.5 to 35.4% amino acid sequence similarity (Table 1). EfpA was most closely related to *Streptomyces alboniger* Pur8 (59), *Nocardia lactamdurans* CmcT (9), *Streptomyces glaucescens* TcmA (16), *Streptomyces coelicolor* and *Bacillus subtilis* MmrA (37, 46), and *Streptomyces lincolnenesis* LmrA (69) (Table 1). EfpA was related to LfrA (Fig. 6), the *Mycobacterium smegmatis* fluoroquinolone efflux protein (30, 58), and was similar to *Staphylococcus aureus* QacA (Table 1 and Fig. 5) and *E. coli* EmrB, which provide resistance to several structurally unrelated, lipophilic antibacterial agents (31, 48). EfpA was also similar to *Saccharomyces cerevisiae* Atr (23), *Staphylococcus aureus* TetK, and *Bacillus stearothermophilus* TetL (15). The 14 predicted TMHs of EfpA aligned with those of members of the QacA TF (Fig. 5), as evident by the conservation of hydropathy profiles (data not shown). EfpA was most dissimilar to these transporters in the C-terminal region. Similarity between EfpA and members of the non-ABC transporter superfamily of efflux proteins possessing 12 transmembrane domains, including the *B. subtilis* multidrug efflux transporter Bmr (38) (Table 1), was notable in the N-terminal halves of the protein sequences.

EfpA motifs characteristic of the QacA TF. The EfpA sequence contained all motifs associated with members of the QacA TF (Fig. 4 and 5). Motif D1 (LDXTVLNVALP [42]), including the conserved LP residues (48) and an aspartyl residue (18, 64), was present in TMH1 as MDSTVAIVALP. TMH2 contained the conserved tryptophan on the external face of the helix and a conserved internal tyrosine residue appropriately positioned 12 residues proximal to the extended polycationic motif GGRLXDXXGRK/RRXXXXG (35, 42, 66) that was highly conserved in EfpA as GGRLGDTIG RKRTFIVG (Fig. 4 and 5). A diffuse motif B (LIXXRXLQG XXXA [42]) was represented in EfpA as LVIARLSQGVGSA and supported the existence of an amphipathic TMH4, which was not predicted by the PHD PredictProtein program (Fig. 4 and 5). Motif C, ascribed to TMH5 (GXXXGPXIGG′ [42, 48]), was present in EfpA as GSVMGLVVGG. In the amphipathic TMH7, the motif DXXGXXL (motif E [42]) was represented in EfpA as DATGAIL.

Limited distribution of *efpA* among clinically significant mycobacteria. The distribution of *efpA* among *Mycobacterium* spp. and related gram-positive bacteria with high G+C genomes was examined by genomic hybridization and confirmed by PCR (Table 2). *efpA* probes hybridized under stringent conditions to genomic fragments of *M. tuberculosis* H37Rv, *M. tuberculosis* Erdman, and *M. bovis* ATCC 19210 and to strains

TABLE 2. Distribution of *efpA* among mycobacteria and some other gram-positive species possessing high G+C genomes

Bacterial species	Strain	*efpA*[a]	*tetK*[a]	*tetL*[a]	Tc[rb]
M. tuberculosis	H37Rv	+[c,d]	−	−	ND[j]
	Erdman	+[c,d]	−	−	ND
	W strain	+[c,d]	ND	ND	ND
M. bovis	ATCC 19120	+[d]	−	−	ND
M. bovis BCG	Connaught, ATCC 35745	+[d]	−	−	ND
	Pasteur, ATCC 35734	+[c]	ND	ND	ND
M. avium	ATCC 35712	+[e]	+	−	r
	ATCC 35718	+[e]	+	−	r
	MA1071#3[i]	+[f]	+	−	ND
	ATCC 25291	+[c]	ND	ND	ND
M. intracellulare	MA531#5[i]	+[f]	+	−	r
	MA577#2[i]	−[f]	+	−	r
	MA682#5[i]	+[f]	−	−	r
	MA968[i]	+[f]	+	−	r
	MA1070#2[i]	+[f]	−	−	r
	MA1074[i]	+[f]	+	−	r
	MA1088[i]	+[f]	−	−	r
	MA1120[i]	+[f]	−	−	r
	ATCC 13950	+[c]	ND	ND	ND
M. chelonae	ATCC 14470	−[c]	ND	ND	ND
M. chitae	ATCC 19627	−[c]	ND	ND	ND
M. fortuitum	ATCC 6841	−[e]	−	−	s
	Mf89[i]	−[e]	+	+	r
	Mf307[i]	−[e]	−	−	r
	Mf414[i]	−[e]	−	−	r
M. gordonae	MO16[h]	−[g]	−	−	r
	ATCC 35752	−[c]	ND	ND	ND
M. heidelbergense	2554/91[i]	−[g]	−	+	r
M. kansasii	ATCC 12478	−[g]	−	−	r
	ATCC 35775	−[g]	−	−	r
M. lentiflavum	E162[h]	−[g]	−	−	ND
	2186/92[h]	−[g]	−	−	ND
	89-446[h]	−[g]	−	−	ND
	89-313[h]	−[g]	+	−	ND
M. marinum	ATCC 927	−[g]	ND	ND	ND
M. microti	ATCC 35781	−[c]	ND	ND	ND
M. nonchromogenicum	M0100[i]	−[g]	+	−	ND
M. peregrinum	ATCC 14467	−[e]	+	−	r
M. porciferae	ATCC 35087	−[c]	ND	ND	ND
M. pulveris	ATCC 35154	−[c]	ND	ND	ND
M. scrofulaceum	ATCC 35785	−[g]	−	−	r
	ATCC 35793	−[g]	+	−	r
M. simiae	ATCC 25275	−[g]	−	−	r
M. smegmatis	155	−[g]	−	−	s
	155mc^2	−[c]	ND	ND	ND
M. terrae-triviale complex	M0107[h]	−[g]	−	−	ND
Mycobacterium spp.	88-885[h]	−[g]	+	−	ND
	89-564[h]	−[g]	−	−	ND
	89-738[h]	−[g]	−	−	ND
	W58[h]	−[g]	−	+	ND
N. asteroides	ATCC 19247	−[f]	+	−	r
	N394[i]	−[f]	−	−	r
	N410[i]	−[f]	+	−	r
N. brasiliensis	ATCC 19296	−[f]	−	−	r
N. farcinica	N3318[i]	−[f]	+	−	r
N. nova	ATCC 33726	−[f]	−	−	r
	ATCC 33727	−[f]	+	−	r
	N3[i]	−[f]	+	−	r
	N38[i]	−[f]	+	−	r
N. transvalensis	N44[i]	−[f]	−	−	r
Streptomyces spp.	As5[i]	−[e]	+	+	r
	As32[i]	−[e]	+	−	r
	As41[i]	−[e]	+	−	r
	As43[i]	−[e]	+	+	r
	As44[i]	−[e]	+	−	r
	As256[i]	−[f]	−	−	s

[a] *tetK* and *tetL* were detected by hybridization and verified by PCR as described previously (40, 41). +, present; −, absent.

[b] Resistance is defined as an MIC equal to or higher than 8 μg/ml for tetracycline and/or doxycycline and/or minocycline (41). r, resistant; s, susceptible.

[c] *efpA* was detected by Southern hybridization of a 724-bp probe to genomic digests prepared with *Bam*HI and confirmed by PCR amplification of this 724-bp region from base pair positions 560 to 1284 of the *M. tuberculosis* H37Rv *efpA* gene as shown in Fig. 2.

[d] *efpA* was detected by dot blot hybridization using a probe prepared from the 478-bp *Bam*HI DNA fragment from pJDT1-H87 which encoded the region described in Fig. 2 from base pair positions −99 to 379.

[e] *efpA* was detected by Southern hybridization of a 478-bp *Bam*HI fragment to genomic digests prepared with *Eco*RI.

[f] *efpA* was detected by Southern hybridization of a 478-bp *Bam*HI fragment to genomic digests prepared with *Hin*dIII.

[g] *efpA* was detected by Southern hybridization of a 478-bp *Bam*HI fragment to chromosomal DNA.

[h] Clinical isolate from AIDS patient at Harborview Hospital, Seattle, Wash.

[i] Clinical isolate from Richard J. Wallace, Jr., University of Texas Health Center at Tyler.

[j] ND, not determined.

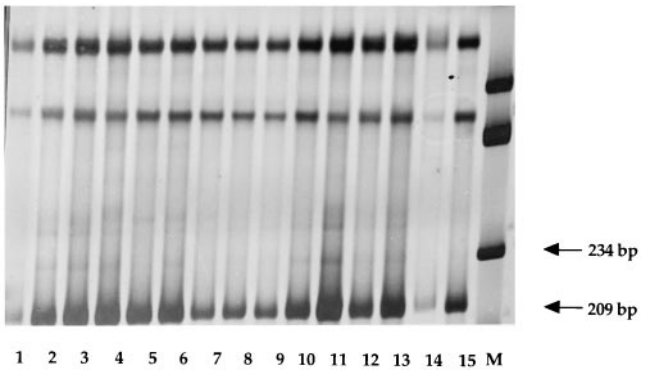

FIG. 7. PCR-SSCP analysis of the promoter region of *efpA*. DNA from MDR clinical isolates (lanes 1 to 15) was amplified with primer set efpa151-efpa131 and analyzed by SSCP. No mobility shifts were observed, indicating homogeneity. The molecular weight marker ϕx174/*Hae*III was loaded in lane M, and the arrows indicate the double-stranded 209-bp amplified product and a relevant marker band.

of *M. bovis* BCG. The 724-bp *efpA* probe identified a conserved hybridization fragment among a genetically characterized collection of 35 New York City clinical isolates. Twenty-three of the isolates were MDR, as defined as resistance to at least rifampin and isoniazid, as were representatives of eight different IS*6110* subtypes (26). The *efpA* probes hybridized to four isolates of *Mycobacterium avium* and to eight of nine strains of *Mycobacterium intracellulare* (Table 2). DNA from clinical or environmental isolates of the slow-growing mycobacterial species *M. chelonae*, *M. fortuitum*, *M. kansasii*, *M. marinum*, *M. scrofulaceum*, and *M. simiae* did not hybridize to the *efpA* probes nor could *efpA* fragments be amplified from these strains by PCR. No hybridization of *efpA* probes to DNA was detected in 30 strains of fast-growing mycobacteria, including clinical isolates of *M. chitae*, *M. gordonae*, *M. heidelbergense*, *M. lentiflavum*, *M. microti*, *M. nonchromogenicum*, *M. porciferae*, *M. pulveris*, *M. smegmatis*, and *M. terrae-triviale* complex. The majority of these isolates were resistant to tetracycline, minocycline, or doxycycline and carried genes for the TetK and/or TetL efflux proteins of the QacA TF (Table 2). The *efpA* gene was not encoded by tetracycline-resistant clinical isolates of *Nocardia asteroides*, *Nocardia brasiliensis*, *Nocardia farcinica*, *Nocardia nova*, *Nocardia transvalensis*, or *Streptomyces* spp. (Table 2). There was no correlation between resistance to one of the tetracyclines and the presence of *tetK*, *tetL*, or *efpA*.

A search of the *Mycobacterium leprae* genomic sequence data available in MycDB (5) revealed a homolog of *efpA* on cosmid B1529. The predicted amino acid sequence encoded by *M. leprae* had 88% sequence similarity with the *M. tuberculosis* EfpA (Fig. 5) but spanned two reading frames according to the *M. leprae* DNA sequence. It is unclear whether this is the result of a sequencing error or whether *M. leprae* carries an *efpA* gene with a frame-shift mutation located 1,035 bp from the start of the ORF. The DNA sequence in the 40-bp A+T-rich region upstream of *efpA* was 78% conserved between *M. tuberculosis* and *M. leprae.*

PCR-SSCP analysis of clinical isolates. PCR-SSCP analysis was used to search for aberrations in the 330-bp region from 98 bp upstream of the initiation codon to just beyond the sequence encoding motif D1. Clinical isolates of *M. tuberculosis* were examined, 10 of which were drug susceptible, 40 of which were MDR, and each of which had a unique IS*6110* DNA fingerprint (63). No sequence variations were observed among any of the isolates (Fig. 7), including six MDR isolates which did not have any mutations in the known drug resistance genes, such as *katG*, *inhA*, *rpoB*, *rrs*, or *rpsL* (44, 45, 61).

DISCUSSION

EfpA is the first *M. tuberculosis* efflux protein to be described. Analyses of the deduced amino acid sequence demonstrate that EfpA is homologous to members of the QacA TF produced by several genera. EfpA of *M. tuberculosis*, and its *M. leprae* homolog, were more closely related to Pur8 and CmcT of *Streptomyces* spp. than to *M. smegmatis* LfrA. Comparative analyses of the primary sequence of EfpA indicated conservation of an aspartyl residue in TMH1, a highly polycationic motif extending from TMH2 through the cytosolic loop into TMH3, and an arginine residue in motif B that may reflect a proton-mediated, energy-coupling mechanism (29, 35, 48, 64–66). Motif C in EfpA TMH5 may be involved in linking proton translocation with export since it is lacking in sugar uptake efflux proteins (42).

Much of the current interest in efflux proteins is centered on drug resistance. Efflux proteins are active as drug pumps in antibiotic-producing *Streptomyces* and *Nocardia* spp. (9, 12, 15, 16, 37, 47, 59, 69) and provide export-mediated resistance in many other gram-positive and gram-negative bacteria (15, 28–32, 38, 39, 42, 47, 48, 64). Antibiotic resistance characteristics have facilitated the identification of efflux proteins; however, *efpA* was discovered fortuitously during the screening of genes for novel *M. tuberculosis* membrane proteins. Although all other members of the QacA TF mediate resistance to antibiotics or antiseptics (15, 18, 30, 32), no evidence for a similar function for EfpA has been ascertained from this study. *efpA* was detected in all drug-susceptible and drug-resistant *M. tuberculosis* isolates examined. No *M. tuberculosis* MDR strains from New York City possessed wild-type loci for both *rpoB* and *katG*; therefore, it was unlikely that an efflux pump would be wholly responsible for the MDR phenotype defined as resistance to rifampin and isoniazid. It is conceivable that genetic alterations leading to the production of variants of EfpA with an altered spectrum of efflux activity might contribute to drug resistance. However, analysis of 5′ coding regions by PCR-SSCP revealed conservation of *efpA* in drug-susceptible and drug-resistant *M. tuberculosis* isolates. Although A+T-rich conserved regions upstream of *efpA* in *M. tuberculosis* and *M. leprae* possessed features associated with negative transcriptional regulation in analogous systems (e.g., *tcmA* and *tcmR*) (17), there was no evidence of mutations in these sequences in drug-resistant or drug-susceptible isolates.

Whereas the tetracycline efflux protein genes *tetL* and *tetK* were dispersed among clinical isolates of fast-growing and slow-growing mycobacterial species (40, 41, 47), sequences homologous to the *efpA* sequence were restricted to the slow-growing, human pathogens *M. tuberculosis*, *M. leprae*, and *M. bovis* and to the opportunistic pathogens *M. avium* and *M. intracellulare*, from among 22 *Mycobacterium* spp. examined. Although the *M. leprae efpA* homolog may not be expressed as a full-length protein, this limited distribution of *efpA* among important pathogens is indicative of the need for further studies to determine whether EfpA, or variants of it, have the capacity to affect the outcome of pathogenesis or to transport antibacterial agents, such as those produced by the macrophage, those used in clinical application, or agents under consideration as new antimycobacterial drugs.

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