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Molecular Cloning and Functional Analysis of a Novel Tetracycline Resistance Determinant, *tet*(V), from *Mycobacterium smegmatis*

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The nucleotide sequence and mechanism of action of a tetracycline resistance gene from *Mycobacterium smegmatis* were determined. Analysis of a 2.2-kb sequence fragment showed the presence of one open reading frame, designated *tet*(V), encoding a 419-amino-acid protein (molecular weight, 44,610) with at least 10 transmembrane domains. A database search showed that the gene is homologous to membrane-associated antibiotic efflux pump proteins but not to any known tetracycline efflux pumps. The steady-state accumulation level of tetracycline by *M. smegmatis* harboring a plasmid carrying the *tet*(V) gene was about fourfold lower than that of the parental strain. Furthermore, the energy uncoupler carbonyl cyanide *m*-chlorophenylhydrazone blocked tetracycline efflux in deenergized cells. These results suggest that the *tet*(V) gene codes for a drug antiporter which uses the proton motive force for the active efflux of tetracycline. By primer-specific amplification the gene appears to be restricted to *M. smegmatis* and *M. fortuitum*.

Tetracyclines are a family of broad-spectrum antibiotics that are used for the treatment of infections caused by both gram-positive and gram-negative bacteria. Members of this family include tetracycline, chlortetracycline, doxycycline, and minocycline. These antibiotics inhibit protein synthesis by preventing the binding of aminoacyl-tRNA molecules to the 30S ribosomal subunit (41). As a consequence of their widespread therapeutic use in human and veterinary medicine and their use as growth promoters in animal feeds, resistance to tetracyclines is found in almost all bacterial genera. The resistance determinants may be located on the chromosome, on either nonconjugative or conjugative plasmids, or on transposons, several of which are conjugative (36, 37, 44).

Bacterial resistance to the tetracyclines is mediated by two major mechanisms, which can be further subdivided into several classes based on DNA hybridization (25). In several bacterial genera, a mechanism protects ribosomes from the action of the antibiotic (6, 11, 35, 37, 41). The process is mediated by a related group of resistance determinants belonging to the TetM, TetO to TetQ, TetS to TetU, or OtrA class. Most of these resistance genes have been sequenced and have been shown to encode large cytoplasmic proteins (ca. 72.5 kDa) which have N-terminal amino acid sequences similar to those of elongation factors Tu and G (40, 43, 46), which are GTP-binding proteins involved in the chain elongation step of protein synthesis.

Active transport of tetracycline out of the cell by means of a transmembrane transport protein is found in gram-negative bacteria (TetA-E to TetG and H) and gram-positive bacteria (TetK, TetL, TetP, and OtrB) (37, 41). This energy-dependent efflux of tetracycline appears to involve the exchange of a proton with a tetracycline-cation complex (50). Therefore, it can be considered an antiport system that requires the proton motive force as an energy source and is inhibited by compounds that block the electrochemical proton gradient (24). The resistance determinants that mediate active efflux of tetracycline encode related transmembrane proteins of approximately 46 kDa which have 12 (gram-negative bacteria) or 14 (gram-positive bacteria) hydrophobic membrane-spanning regions (α-helices spanning the inner membrane) separated by short central hydrophilic regions of amino acids (9).

Tetracycline resistance can also be conferred by some multidrug resistance genes, encoding efflux pumps with low specificities. A chromosomal efflux system associated with the *mar* locus in *Escherichia coli* has been described to confer resistance to tetracycline and other antibiotics (24), whereas three multidrug resistance operons, *mexAB-oprM*, *mexCD-oprJ*, and *mexEF-oprN*, have been identified in *Pseudomonas aeruginosa* (23, 26, 34); these operons confer resistance to tetracycline, ciprofloxacin, and chloramphenicol. Spontaneous multidrug-resistant mutants of *Klebsiella pneumoniae*, which have an increased level of resistance to a range of unrelated antibiotics including tetracycline, have also been described (17). Similar mutants have been found in *Serratia marcescens*, *Enterobacter* spp., and *Campylobacter jejuni* (7).

Since efflux proteins are responsible for resistance to a variety of unrelated antibacterial compounds in both gram-negative and gram-positive bacteria (24, 30, 32), they may be involved in the intrinsically low level of susceptibility of mycobacteria to a wide range of compounds. The LfrA protein was identified a few years ago in a ciprofloxacin-resistant mutant of *Mycobacterium smegmatis* and confers resistance to fluoroquinolones as well as acriflavine and ethidium bromide (45). We also recently identified a mycobacterial multidrug resistance efflux protein, TapA, which is present in *Mycobacterium tuberculosis* and *Mycobacterium fortuitum* and which confers low-level resistance to tetracycline and some aminoglycosides (1).

We report here the identification, molecular cloning, and characterization of a novel tetracycline resistance gene from *M. smegmatis*. This gene encodes a putative hydrophobic 44-kDa protein which confers resistance only to tetracycline. The

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derived amino acid sequence and level of [^{3}H]tetracycline accumulation in whole cells suggest that this tetracycline resistance gene codes for an energy-dependent efflux pump which keeps the intracellular levels of tetracycline lower in *M. smegmatis* containing the gene on a multicopy vector than in an isogenic strain containing only the cloning vector. In keeping with this function, this tetracycline resistance gene was named *tet*(V).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. smegmatis* mc^{2}155 and mc^{2}6 were grown in Middlebrook 7H9 broth and Middlebrook 7H11 agar (Difco) supplemented with 10% Middlebrook OADC enrichment (Difco) and 0.2% glycerol. Spontaneous drug-resistant mutants were isolated by plating 10^7 cells of *M. smegmatis* mc^{2}155 on 7H11 agar containing 50 μg of doxorubicin per ml. Resistance to doxorubicin and other anthracyclines is often associated with a multiple-drug resistance phenotype (18). This phenotype was assessed by plating some of the mutants on media containing the antibiotics reported in Table 1, and multidrug-resistant mutant mc^{2}11 was selected for further analysis.

For cloning and preparation of sequencing templates, *E. coli* DH5α was grown in Luria-Bertani broth and agar medium. All of the cultures were incubated at 37°C. Kanamycin was added, when required, at final concentrations of 25 μg/ml for *M. smegmatis* and 50 μg/ml for *E. coli*.

Cloning procedures. A cosmid library of *M. smegmatis* mc^{2}6 was constructed by standard procedures in the vector Tropist4 (13). Approximately 800 *E. coli* recombinants were cultured individually in microtiter plate wells and pooled, and cosmid DNA was extracted. This was electroporated into *M. smegmatis* mc^{2}6, and recombinant colonies were selected on 25 μg of kanamycin per ml. About 1,000 colonies were scraped off, pooled, aliquoted, and stored in 25% glycerol at −80°C. The library was plated out on 0.2 μg of tetracycline per ml, and the colonies that were able to grow were selected. Genomic DNA was isolated from multidrug-resistant strain mc^{2}11 as described previously (48). After a partial digestion with *Sau*3AI, 35- to 40-kb fragments were ligated into the *Bam*HI site of the shuttle cosmid pUYB18 (21); the ligation mixture was packaged in vitro (Gigapack-III Gold; Stratagene) according to the manufacturer's instructions, and the resulting phage particles were transduced into *E. coli* HB101. The colonies were pooled, and cosmid DNA was isolated (39) and electroporated into *M. smegmatis* mc^{2}155 (21), which was then plated onto 7H11 plates with kanamycin at 25 μg/ml and doxorubicin at 50 μg/ml or tetracycline at 0.2 μg/ml.

Plasmid DNA was isolated from *E. coli* by alkaline lysis (39) or with Qiagen columns and was characterized by restriction analysis prior to transformation of *M. smegmatis* by electroporation. Plasmids were recovered from *M. smegmatis* transformants by electroduction into *E. coli* (3) or by isolating plasmid DNA via a modified alkaline lysis method (21).

Subcloning and sequencing of *tet*(V). Clone pTet35, which confers tetracycline resistance to *M. smegmatis*, was cut with various enzymes to generate a restriction map, and fragments were subcloned into vector pMD31 (14). Miniprep DNA of the subclones was electroporated into mc^{2}155, and kanamycin-resistant colonies were tested for tetracycline resistance. The insert from pTetKE1, which confers tetracycline resistance, was sequenced with universal and reverse primers and with custom-designed internal primers. Sequencing of both strands was performed with Sequenase, version 2.0 (U.S. Biochemical Corporation), according to the supplier's instructions, and with [α-^{35}S]dATP (1,000 Ci/mmol; Amersham International). The DNA sequences were processed and analyzed with the PC/GENE program (Intelligenetics Inc.). Databases were searched with the programs BLASTN, BLASTX, and BLASTP (2). The predicted amino acid sequence of Tet(V) was analyzed for potential transmembrane domains with the TMpred program (20). Sequence alignments were performed with the CLUSTAL program (19).

MIC determination. *M. smegmatis* strains were cultured in Luria-Bertani broth–0.05% tyloxapol until an optical density at 600 nm of 1.0 was reached and was diluted to 10^6 CFU/ml in fresh medium, and 150 μl was added to the wells of a microtiter plate. A total of 150 μl of antibiotic at an appropriate concentration was added to the first well, and the antibiotic was serially diluted and dilutions were added to all other wells. The plate was incubated at 37°C for 3 to 4 days. The MIC was defined as the lowest concentration of antimicrobial agent that inhibited visible growth.

Uptake and efflux of tetracycline. Uptake experiments were performed essentially as described previously (28). All such experiments were repeated three times. *M. smegmatis* mc^{2}155 cells, bearing plasmid pMD31 or pTetKE1, grown to the exponential phase of growth were harvested by centrifugation at room temperature, washed twice in 0.1 M potassium phosphate (pH 7.0), and resuspended in prewarmed assay buffer (0.1 M potassium phosphate [pH 7.0], 1 mM $MgSO_4$). Aliquots of 1 or 1.5 ml were preincubated for 5 to 10 min at 37°C with vigorous aeration by shaking, and the assay was started by the addition of [^{3}H]tetracycline (0.76 Ci/mmol; New England Nuclear) to a final concentration of 5 μM. At various time intervals thereafter, 50 μl of the suspension was removed, diluted in 1 ml of ice-cold 0.1 M potassium phosphate (pH 7.0) buffer containing 0.1 M LiCl, and immediately filtered through a 0.45-μm-pore-size filter (Millipore). The filter was rapidly washed twice with 4 ml of the same buffer and dried, and the radioactivity was then determined in a Beckman LS 7000 liquid scintillation counter by using Ecolume scintillation cocktail (ICN Biomedicals). To analyze the energy dependence of the accumulation process, aliquots of cells incubated with [^{3}H]tetracycline were transferred to a new tube containing 0.2 mM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and were treated as described above. In the assay with deenergized cultures, cell suspensions were preincubated with 0.2 mM CCCP in phosphate buffer with gentle shaking at 37°C for 30 min so that the endogenous energy reserve of the cells was exhausted. The starved cells were then washed three or more times with phosphate buffer at room temperature to remove the CCCP. The uptake assay was started by adding [^{3}H]tetracycline to give a final concentration of 5 μM as described above. After 15 min of incubation to allow tetracycline uptake by the cells, these were reenergized by adding 200 mM succinate, and the intracellular level of tetracycline in 50-μl aliquots removed at different time intervals was determined.

TABLE 1. MICs for *M. smegmatis* wild-type mc^{2}155 and the resistant mutant mc^{2}11

Drug	MIC (μg/ml)	
	mc^{2}155	mc^{2}11
Ciprofloxacin	0.2	1.5
Daunomycin	1	>50
Doxorubicin	4	>100
Ethidium bromide	8	30
Rhodamine 123	5	75
Tetracycline	0.06	0.7

PCR amplification and Southern blotting of mycobacterial genomic DNA. DNAs from several mycobacterial species were extracted by the following procedure. A loopful of organisms from a colony was suspended in 1 ml of distilled water and boiled for 10 min. Samples (10 μl) were then used directly in the PCRs. PCR amplification was performed with the primers ForRG142 (5′-GAC AACGGCATGAAC-3′) and RevRG144 (5′-GTTCGCGAGCATGTTC-3′). As a template, genomic or plasmid DNAs were heat denatured (3 min at 94°C) prior to the amplification. PCR was performed with a 40-μl mixture of 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 200 mM (each) dGTP, dATP, dCTP, and dTTP; 100 pmol of each primer; 1 to 2% dimethyl sulfoxide; template DNAs; and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus). The temperature profile was 30 s at 94°C, 1 min at 44°C, and 2 min at 72°C for 30 cycles, followed by a further incubation at 72°C for 10 min. PCR products were analyzed by electrophoresis through a 1.5% (wt/vol) agarose gel in TAE (Tris-acetate-EDTA) buffer.

Genomic DNA was purified from *Mycobacterium* spp. as described previously (48). For Southern blot analysis (39), 5 μg of mycobacterial DNA that had been double digested with *Kpn*I and *Eco*RV was transferred onto nylon filters (Nytran-N; Schleicher & Schuell) according to the supplier's recommendations. The 2.2-kb *Kpn*I-*Eco*RV probe was labelled by random primer labelling (Rediprime; Amersham) with [α-^{32}P]dCTP (Redivue; Amersham). Prehybridization and hybridization were carried out in 35% formamide at 37°C for 2 and 20 h, respectively. The filters were washed at 50°C twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and were exposed to X-ray film (Hyperfilm MP; Amersham).

Nucleotide sequence accession number. The sequence of *tet*(V) has been deposited in the GenBank database under accession no. AF030344.

RESULTS

Identification of tetracycline resistance determinants in *M. smegmatis*. Two approaches were taken to identify the genes involved in the inherent resistance to tetracycline. (i) A cosmid library of *M. smegmatis* mc^{2}6, a strain with a wild-type level of tetracycline resistance (MIC, 0.078 μg/ml), was produced in the vector Tropist4 and was electroporated into *M. smegmatis* mc^{2}6. This library was screened on plates containing 0.2 μg of tetracycline per ml. It was reasoned that an increase in the level of resistance would be due to the increase in the copy number of a Tet^r determinant. A clone for which the MIC was 0.31 μg/ml was isolated and the cosmid was named pTet1. (ii) A multidrug-resistant derivative, mc^{2}11, of *M. smegmatis* mc^{2}155 was obtained by plating on doxorubicin. The multidrug resistance profile of this strain (Table 1) includes a 12-fold increase in the level of resistance to tetracycline. Susceptibility to isoniazid, streptomycin, rifampin, and erythromycin was not af-

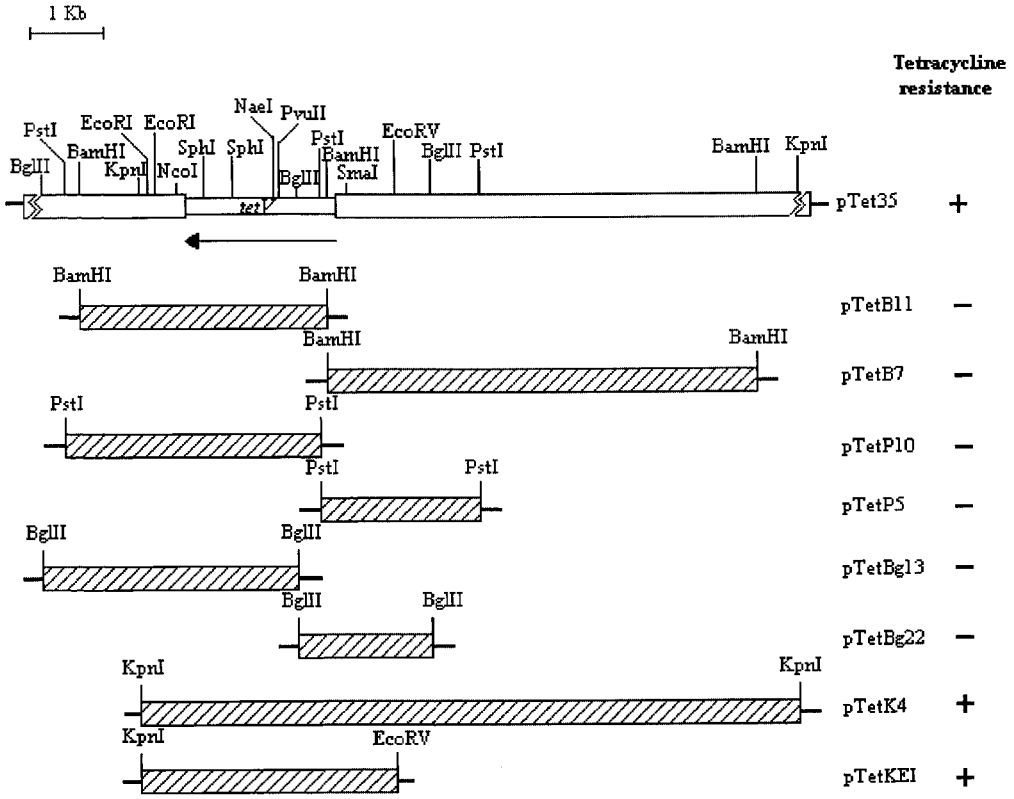

FIG. 1. Schematic representation of cosmid pTet35 and the deletion derivatives used to identify the sequence encoding the tetracycline resistance gene. Thin bars at both ends represent vector sequences. The thicker regions represent *M. smegmatis* mc^{2}11 insert DNA. The white area identifies the location of the ORF encoding the tetracycline resistance gene. The hatched boxes represent pTet35 sequences retained in the respective deletion derivatives. Plus and minus signs indicate the tetracycline resistance and sensitivity, respectively, of *M. smegmatis* mc^{2}155 transformed with these plasmids.

fected (data not shown). A cosmid library was constructed and was screened on plates containing 25 μg of doxorubicin per ml. Five clones were found to be capable of conferring resistance to doxorubicin. Subcloning of the common fragments, followed by transformation of *M. smegmatis*, allowed us to isolate a 3.2-kb *Pst*I fragment that conferred resistance to doxorubicin. This fragment likewise rendered *M. smegmatis* resistant to other anthracyclines, ciprofloxacin, rhodamine 123, and ethidium bromide but not to tetracycline. The fragment was sequenced, and it proved to contain a 1,565-bp coding sequence identical to that of the *lfrA* gene, which is responsible for resistance to fluoroquinolones in *M. smegmatis* (45). Upstream of the *lfrA* gene, we identified a putative *lfrR* transcriptional regulator. We extended this study by measuring the accumulation of doxorubicin in strain mc^{2}155 with and without plasmid carrying the *lfrA* gene (unpublished data).

To identify the gene that confers tetracycline resistance, we screened the same cosmid library on plates containing 0.2 μg of tetracycline per ml. Clone pTet35 had a 10-fold increased level of resistance to tetracycline but not to any of the other antibiotics to which the original mutant mc^{2}11 was resistant. That the tetracycline resistance was linked to a pump was confirmed indirectly by the finding that CCCP, an energy uncoupler that has been shown to inhibit the actions of other efflux pumps (24), reduced the level of antibiotic resistance (data not shown). A similar reduction in the level of resistance was observed in an *M. smegmatis* strain carrying the tetracycline efflux protein from *E. coli* vector pBR322 (28) (data not shown).

Cloning and sequencing of the tetracycline resistance determinant. Retransformation of cosmids pTet1 or pTet35 into susceptible strain mc^{2}155 conferred tetracycline resistance to the host, confirming that resistance was due to the cosmid. Restriction mapping of both cosmids showed that they had overlapping inserts of 37 and 35 kb, respectively.

M. smegmatis strains containing subclones of pTet35 in pMD31 were tested for tetracycline resistance, revealing that the region required for the expression of this phenotype was present only on plasmids pTetK4 and pTetKE1 (Fig. 1). The latter contained a 2.2-kb *Kpn*I-*Eco*RV fragment. Analysis of the 2,224-bp nucleotide sequence revealed that the largest open reading frame (ORF), ORF1, corresponded to the region identified by deletion analysis as that which encodes the tetracycline determinant (Fig. 1). This gene was designated *tet*(V).

The same 2.2-kb *Kpn*I-*Eco*RV fragment was isolated and cloned from cosmid pTet1. Both nucleotide sequences were identical, leading us to conclude that in spite of the multidrug resistance phenotype, strain mc^{2}11 contains the *tet*(V) wild-type gene.

Sequence analysis of *tet*(V). There are two putative start codons for the *tet*(V) ORF, either at nucleotide 474 or at nucleotide 504, with a stop codon at position 1760, on the complementary strand. The first is preceded by a putative ribosome-binding site, AGGTTGG, and the second is preceded by AAGAA. The gene product would encode a protein of 419 or 429 amino acids with a deduced molecular weight of either 44,610 or 45,746. Both possible ORFs were amplified by PCR, cloned in the mycobacterial expression vector pSODIT-2

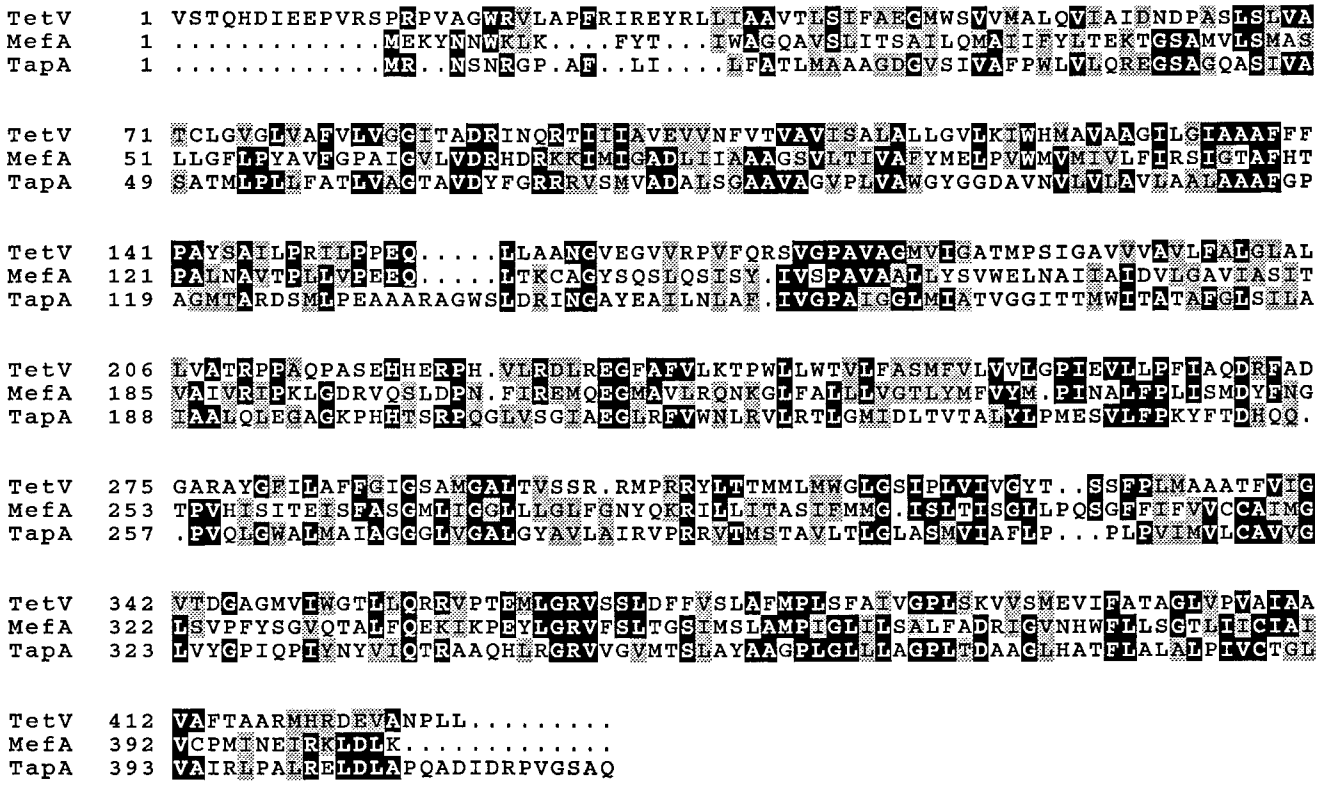

FIG. 2. Sequence alignments of *M. smegmatis* Tet(V), *M. tuberculosis* TapA, and *S. pyogenes* MefA. Identical amino acids are highlighted in black boxes. Conserved amino acid substitutions are shown in shaded boxes. To take into account the two putative start codons, the first 10 amino acids of Tet(V) are given.

(1), and electroporated into *M. smegmatis*. Both constructs gave identical levels of resistance, showing that the region between positions 474 and 504 does not have to be included in the functional protein (data not shown).

The *tet*(V) sequence predicts that, if translated, its product would be a hydrophobic protein with an index of hydrophobicity of 0.93 and an isoelectric point of 10.34. Prediction of its topology by the TMpred computer program (20) led to the prediction of a highly hydrophobic protein with at least 10 transmembrane-spanning α-helical segments (data not shown).

Screening of the EMBL and GenBank databases with the BLAST and BEAUTY program of the National Center for Biotechnology Information revealed that the Tet(V) protein shows the highest degrees of homology to the multidrug resistance proteins TapA of *M. fortuitum* and *M. tuberculosis* (1) (accession no. AJ000283 and Z77137, respectively), a putative transporter from *M. smegmatis* (designated ORF4) (accession no. U46844) (47), and the macrolide-efflux protein MefA from *Streptococcus pyogenes* (9). Pairwise alignment of amino acid sequences showed that Tet(V) was 25, 24, 24, and 23% identical (67, 69, 68, and 65% similarity) to TapA from *M. fortuitum* and *M. tuberculosis*, ORF4, and MefA, respectively (Fig. 2). Optimal alignment was obtained by using the *tet*(V) translation starting at nucleotide 504. Homologies were also found with the *E. coli* membrane protein P43 (23% identity and 67.8% similarity) (8, 42), *Rhizobium* sp. Y4rN protein (accession no. AE000095; 25% identity and 67.4% similarity) (16), and an ORF from a *Synechocystis* sp. (accession no. D90899; 18.7% identity and 62.7% similarity) (22), all of which have unknown functions.

TapA and MefA are members of the major facilitator superfamily (MFS) of antibiotic efflux proteins. This group of proteins contains several motifs (33), some of which can be recognized in the Tet(V) sequence. Motif A (GxLaDrxGrkxxl) is represented by GITADRINQRTII; motif C (gxxxGPxxGGxl) is represented by QRSVGPAVAGMV, and motif G (GxxxGPL) is represented by FAIVGPL. The consensus sequences of the motifs are displayed as follows: x, any amino acid; capital letters, most frequently observed amino acids; lowercase letters, frequently observed letters. Other motifs could not be identified. Motif A is found in all MFS proteins, motif C is found in those containing 12 or 14 transmembrane segments, and motif G is found only in those with 12 transmembrane segments.

Specificity of Tet(V). Determination of the MICs of 30 compounds for the *M. smegmatis* clone containing cosmid pTet1 was performed to determine the specificity of cosmid pTet1. The *tet*(V) gene conferred a two- to fourfold increase in the level of resistance to tetracycline and a twofold increase in the level of resistance to chlortetracycline but not to the tetracycline derivatives doxycycline or minocycline or to acridine orange, amikacin, ciprofloxacin, clarithromycin, crystal violet, cycloserine, daunomycin, doxorubicin, erythromycin, ethambutol, ethionamide, gentamicin, isoniazid, kanamycin, lincomycin, ofloxacin, *p*-aminosalicylic acid, phosphomycin, puromycin, pyrazinamide, rhodamine 123, rifampin, streptomycin, thiacetazone, or vancomycin.

Distribution of *tet*(V) among other mycobacteria. The distribution of the *tet*(V) gene among *Mycobacterium* spp. was examined by PCR. No *tet*(V) fragments could be amplified from DNA of *M. simiae*, *M. chelonae*, *M. gordonae*, *M. marinum*, *M. bovis*, *M. flavescens*, *M. kansasii*, *M. xenopi*, *M. terrae*, *M. abcessus*, *M. avium*, *M. vaccae*, and *M. paratuberculosis* (data not shown). Amplification of *M. fortuitum* DNA produced the

expected 400-bp fragment that hybridized to the *tet*(V) probe. The nucleotide sequence confirmed that this fragment is part of a *tet*(V) gene. Furthermore, except for *M. fortuitum* and *M. smegmatis*, Southern hybridization experiments with DNA from *M. avium*, *M. vaccae*, *M. paratuberculosis*, *M. tuberculosis*, and *M. aurum* gave negative results (data not shown). Finally, a search of the *M. tuberculosis* and *M. leprae* genomic sequence data available in MycDB (4) did not identify a sequence homologous to that of *tet*(V).

[^{3}H]tetracycline accumulation by *M. smegmatis* cells. Tetracycline uptake experiments were performed to determine whether *M. smegmatis* cells carrying pTetKE1 were more resistant to tetracycline due to an active drug efflux mechanism. As shown in Fig. 3A, cells harboring the cloning vector pMD31 take up [^{3}H]tetracycline rapidly and achieve a steady-state level of accumulation within about 10 to 15 min of incubation. This accumulation is approximately fourfold lower when cells harbor plasmid pTetKE1. A reduced level of accumulation of the drug may be caused either by a decreased level of drug permeation or by active drug extrusion through the cytoplasmic membrane. To study the effect of membrane deenergization on the uptake of tetracycline, the protonophore CCCP was added to cells containing [^{3}H]tetracycline. Upon the addition of CCCP, the level of tetracycline accumulation increased in the case of the pTetKE1-harboring strain and reached a level almost equal to that observed in the case of the strain containing only the cloning vector pMD31 (Fig. 3A). On the contrary, under our conditions, CCCP had no significant effect on the level of tetracycline accumulation in the strain carrying the cloning vector (Fig. 3A). These data indicate that Tet(V) pumped out tetracycline in an energy-dependent process, presumably by using proton motive force. A second experiment was designed to see if energization of deenergized cells could lead to tetracycline efflux from resistant cells. As shown in Fig. 3B, energy-starved cells of the parental and resistant strains accumulated almost equal amounts of tetracycline. However, upon the addition of succinate as an energy source, sensitive cells did not extrude significant amounts of tetracycline, whereas the resistant ones rapidly eliminated the drug (Fig. 3B). Together with the results obtained from the sequence analysis, these results strongly indicate that the *tet*(V) gene encodes a novel tetracycline efflux system.

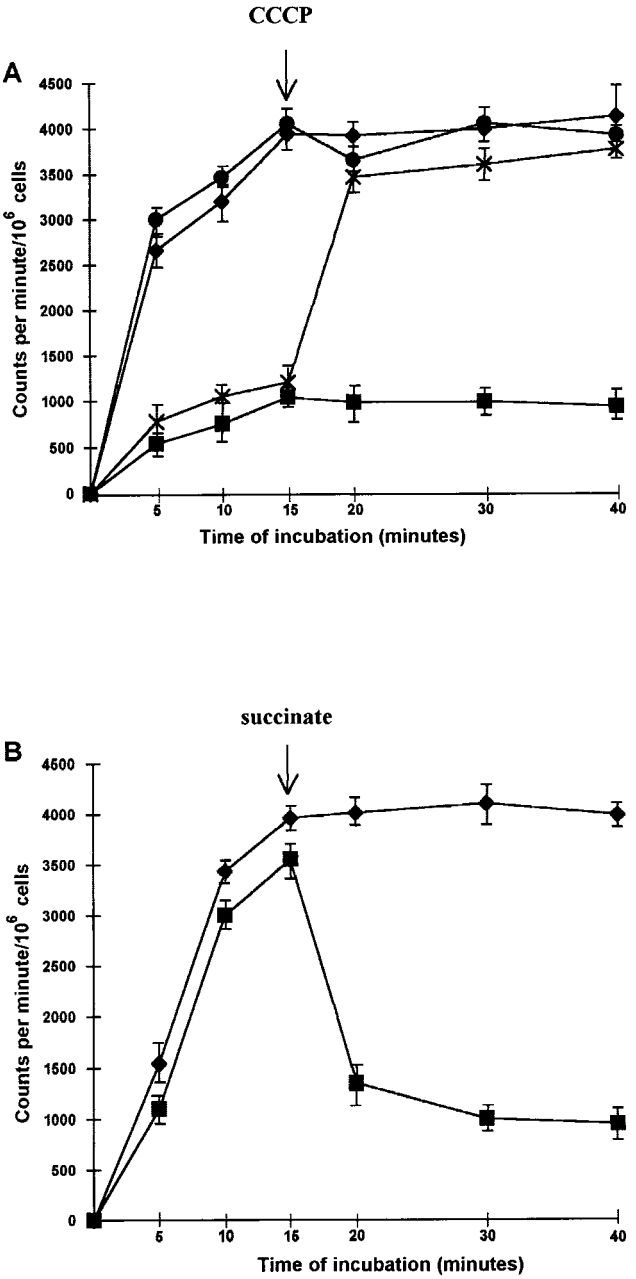

FIG. 3. Effect of the addition of CCCP on the accumulation and efflux of [^{3}H]tetracycline by *M. smegmatis* carrying the *tet*(V)-containing plasmid (■, no CCCP addition; ×, addition of CCCP) or cloning vector pMD31 (◆, no CCCP addition; ●, addition of CCCP). (A) [^{3}H]tetracycline was added to the cells at time zero, and the energy uncoupler CCCP was added 15 min later. (B) The cells were deenergized with CCCP for 30 min prior to the addition of [^{3}H]tetracycline. At the time indicated by the arrow, energy was supplied by adding succinate. The addition of succinate had no effect on the level of tetracycline accumulation in the case of cells not treated with CCCP (data not shown).

DISCUSSION

Tetracycline resistance is common among commensal and clinical isolates of bacteria (9, 44). Two major resistance mechanisms of clinical relevance have been identified: active efflux and ribosomal protection (36). Among the active efflux group of tetracycline resistance determinants are classes A through E, G, and H among gram-negative bacteria and classes K, L, and P among gram-positive bacteria (37, 41). The tetracycline susceptibility of pathogenic, rapidly growing mycobacteria is highly variable. Approximately 50% of isolates of *M. fortuitum*, *M. peregrinum*, and *M. mucogenicum*, 25% of isolates of *M. chelonae* and *M. abcessus*, as well as all isolates of the nonpathogenic species *M. smegmatis* are highly susceptible (MICs, less than 1 μg/ml (49). Recent studies suggest that for some isolates this variable resistance may be correlated to the presence of one or more tetracycline resistance genes similar to the *tetK* and *tetL* genes seen in gram-positive species such as the streptococci (31). The strain used in this study, *M. smegmatis* mc^{2}155, has been shown not to have a homolog of the *tetK* and *tetL* genes (15).

In this report we have described the isolation of an *M. smegmatis* mutant that is resistant to several anthracyclines, ciprofloxacin, ethidium bromide, rhodamine 123, and tetracycline. From this strain, we cloned two genes, *lfrA* and *tet*(V), which encode efflux pumps. These pumps confer resistance either to anthracyclines, fluoroquinolones, rhodamine 123, and ethidium bromide (LfrA) or to tetracycline only (TetV). Obviously, the mutation conferring the complete multidrug resistance phenotype to *M. smegmatis* mc^{2}11 was never cloned. Indeed, comparison of the *lfrA* nucleotide sequences of sensi-

tive and resistant cells revealed no differences; the same was true for *tet*(V) nucleotide sequences. It is possible to hypothesize that the mutation is located in a regulatory sequence outside the coding region. However, the promoter regions upstream of the *tet*(V) gene were identical, and this phenomenon has also been described for other efflux pumps, such as LmrP of *Lactococcus lactis* (5) and EmrB of *E. coli* (27). It is therefore likely that mc^{2}11 has a mutation in another gene involved in drug resistance. This could be either in an unrelated efflux protein or in a regulatory locus that increases the level of resistance to several drugs by increasing the levels of expression of *tet*(V) and other efflux pumps. The increased level of expression of the latter has been encountered in *E. coli* in which mutations in the regulators *mar* (12) or *sox* (29) increase the level of expression of efflux pumps and thus increase the level of resistance. In our experiments, extended antibiotic susceptibility tests show that the *tet*(V) gene conferred resistance only to tetracycline. The level of resistance in the recombinant is probably related to the level of combined expression of the *tet*(V) gene from the chromosomal gene and those present on the multicopy cosmid. However, the amount of Tet(V) protein in *M. smegmatis* cells harboring plasmid pTetKE1 is probably not very high, since we have not been able to identify the corresponding protein in membranes prepared from these cells and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

It is noteworthy that the *tet*(V) gene had no homology at the DNA or protein level to tetracycline antiporters from gram-positive microorganisms, which are known to be transmembrane polypeptides conferring resistance through an active efflux of tetracycline. Homology was observed between Tet(V), the MefA protein from *S. pyogenes* (10), and TapA from *M. tuberculosis* and *M. fortuitum* (1). TapA and MefA are multidrug resistance proteins and members of the major facilitator class (MFS) of efflux proteins (33). Members of this class can show little sequence homology, but they are all strongly hydrophobic, lack a signal sequence at the N terminus (as is typical of integral membrane proteins), display 12 to 14 transmembrane segments that alternate with stretches of hydrophilic amino acids, and contain specific sequence motifs. Tet(V) showed little homology to other Tet proteins in this group, but it is highly hydrophobic and contains motifs typical of those of the MFS efflux proteins, so it can be concluded that Tet(V) belongs to this group. Similar to the Tet proteins and other MFS proteins, the *tet*(V) gene can be separated into two domains whose nucleotide sequences and hydropathy plots are similar to one another, suggesting, as hypothesized in the case of the Tet proteins, that the two domains arose from a duplication of a single smaller gene (38).

The mechanism of resistance conferred by the Tet(V) protein is the active extrusion of tetracycline. This was substantiated by the fact that the level of [^{3}H]tetracycline accumulation by *M. smegmatis* cells harboring plasmid pTetKE1 is significantly lower than that by the isogenic strain harboring the cloning vector. In addition, when the energy uncoupler CCCP was added, tetracycline accumulation reached almost identical levels in both strains. Furthermore, the addition of an energy source to energy-depleted cells of strain mc^{2}155/pTetKE1 resulted in the rapid efflux of [^{3}H]tetracycline.

Of all the mycobacteria tested, only *M. smegmatis* and *M. fortuitum* appear to have a *tet*(V) gene. However, the limitations of the PCR amplification approach used does not exclude the presence of a *tet*(V) gene in other mycobacteria. The primers may simply have been too divergent to anneal to DNA of other species. It is noteworthy, however, that no homologs have been identified in the genome sequence of *M. tuberculosis* or *M. leprae*.

In summary, we characterized Tet(V), a tetracycline efflux protein, and the corresponding gene in *M. smegmatis*. Tet(V) shows no sequence homology to other tetracycline resistance determinants but belongs to the MFS of efflux proteins. Preliminary evidence suggests that a homologous gene is present in *M. fortuitum* but not in other mycobacteria.

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